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Spontaneous Formation of Bilayers and Vesicles in Mixtures of Single-Chain Alkyl Carboxylates: Effect of pH and Aging and Cytotoxicity Studies

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We report the observation of bilayer fragments, some of which close to form vesicles, over a large range of pH at room temperature from mixtures of single-chain biocompatible commercially available nontoxic alkyl carboxylic surfactants after neutralization with HCl. The pH at which the morphological transitions occur is varied only by changing the ratio between two surfactants: the alkyloligoethyleneoxide carboxylate and sodium laurate. The effect of aging of the mixed surfactant systems in the pH region desired for dermatologic application ($4.5 < pH < 7$) is also studied. Finally, we show results of cytotoxicity studies on the surfactant mixtures.

Introduction

There is growing interest in the field of synthetic surfactants used for dermatologic purposes. These surfactants exhibit a wide range of structures: particularly useful is the formation of vesicles, which can be used as delivery systems. For cosmetic reasons, the surfactants forming vesicles must be skin compatible (nonirritant), easy to manufacture, and the vesicle region must be stable within the range of physiological pH at room temperature.

A simple geometrical characterization of chain packing can be used to analyze trends in surfactant phase behavior.[1,2] The geometric properties of surfactants depend on the ratio between the cross-sectional area of the hydrocarbon part and that of the headgroup. Low packing parameters (around 1/3) are found for single-chain surfactants with a strongly polar headgroup. These systems tend to form spherical micelles, whereas a packing parameter value above 0.5 favors the formation of vesicles. When the packing parameter is increased even further ($P \approx 1$) lamellar plates form. An increase in the packing parameter can be obtained by adding a second chain, which doubles the hydrocarbon volume. Double-chain surfactants,[3,4] two surfactants of opposite charge,[5−8] or the association of a surfactant and a cosurfactant,[9−15] can be used. In the latter two cases, a pseudo-double-chain surfactant is obtained by either an ion-pair formation between the anionic and cationic surfactant or association of the two different molecules via hydrogen bonds.

As cationic surfactants and short alcohols are undesirable in cosmetic formulations due to their toxicity, and because long-chain alcohols ($>C_{12}$) exhibit high melting points, monoalkyl carboxylates were chosen for our study. Fatty acids form a range of aggregates depending on the acid concentration and the ionization degree of the terminal carboxylic group.[16,17] The formation of vesicles from monocarboxylic acids has long been known. Gebicki and Hicks[18] first observed the formation of vesicles from unsaturated, long-chain fatty acids. Later, Hargreaves and Deamer also showed that saturated fatty acids can form vesicles.[19] A vesicle phase is spontaneously formed when short- or middle-chain ($<C_{12}$) fatty acids are neutralized with HCl;[19,20] two types of amphiphiles are then present in solution: the protonated and the ionized forms. The ratio between the two determines the aggregation morphology. Despite the simplicity of the mechanism of vesicle formation, possible applications of fatty acid vesicles in cosmetics remain largely unexplored.[20] This may be a consequence of two obstacles: (1) the high solubility temperature of the long-chain carboxylates and (2) the generally too basic pH necessary for the solubilization of the carboxylate. The problem of the solubility temperature of the alkyl carboxylates has been discussed by Hargreaves et al.[19] Below 25 °C, only alkyl carboxylates with short alkyl chains are water-soluble. However, these are inappropriate due to their skin irritating properties. The solubility temperature of sodium laurate is reported to be equal to or above the room temperature, depending on the

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concentration.[16] With longer alkyl chains ($n > 12$), the solubility temperature becomes even higher.

It has previously been reported that the formation of vesicles occurs at pH values at or near the pK_a, where approximately half of the carboxylic groups are ionized.[16,19,21] For this reason, fatty acid vesicles are present only over a narrow pH range. Designing vesicles that become unstable at an easily tuned pH value is of great interest for targeted drug delivery. It is known that, for example, tumors and inflamed tissues exhibit a decreased extracellular pH.[22−26] For this reason, a large number of groups have focused their attention on the preparation of pH-sensitive liposomes[27−36] as possible drug carrier systems.

A range of sugar-based gemini surfactants has been recently studied, because they exhibit pH-dependent aggregation behavior. However, these surfactants are cationic, and therefore, vesicles are observed only at neutral and high pH values.[37,38] The same problem occurs also in solutions of bola-amphiphiles.[39] Systems composed of mixed single- and double-short-tailed PEO ether phosphate esters might be promising for the formation of pH-sensitive vesicles. In that case, a vesicle phase was observed at higher concentrations. The pH effect, however, was only studied on vesicular solutions, so one cannot confirm a micelle-to-vesicle transition as a consequence of protonation.[40]

For carboxylates with longer alkyl chains, desirable for cosmetic formulations (i.e., lauric acid), the pH of vesicle formation is also generally too basic. The addition of medium- and long-chain alcohols has been proven to expand the pH region of vesicle formation,[19,21] but toward even higher pH values, which is undesirable. One way to lower the pH range of vesicle formation is to introduce another carboxylic group to the fatty acid chain, thus lowering the pK_a of the acid. This has been previously done by de Groot et al. by the formation of 2-(4-butyloctyl) malonic acid.[41] However, to the best of our knowledge, no one has tried to use a mixture of single-chain fatty acid soaps yet.

Recently, spontaneous formation of vesicles below room temperature, at acidic pH (between 2 and 4), by neutralization of a particular industrial single-chain alkyl carboxylate surfactant was reported.[42] Our intent was to obtain spontaneously formed vesicles over a wide range of physiological pH at room temperature, by changing the ratio of two surfactants: Akypo Soft 45NV (an alkyloligoethyleneoxide carboxylate already used in cosmetic formulations; AS) and sodium laurate (SL). The effect of pH and aging on vesicle formation was studied by visual observation, dynamic light scattering, and transmission electron microscopy. The biocompatibility of the surfactant mixture was checked by measuring cell viability. On the basis of our results, we present a method to obtain bilayers, some of which form vesicles, in a large range of physiological pH using only nontoxic alkyl carboxylic acids.

Experimental Section

Chemicals. Sodium laurate (SL) was purchased from Sigma-Aldrich at a purity of 99%. Sodium dodecylethercarboxylate (Akypo Soft 45NV; $Na^+C_{12}(EO)_{4-5}OCH_2COO^-$) was a gift from Kao Chemicals (Germany). According to the information given by Kao Chemicals, Akypo Soft 45NV (further denoted as AS) contains molecules with roughly a Gaussian distribution of the number of EO groups, with an average value of 4.5 (mass spectroscopy results show an EO population ranging from 3 to 9). The surfactant was thoroughly studied and reported in a previous publication.[42] Akypo Soft 45NV is supplied as an approximately 20 wt % aqueous solution. Stocks solutions of 1 wt % were prepared by dissolving weighed amounts of sodium laurate in deionized water and by dilution of the original Akypo Soft 45NV solution. Different solutions were then prepared by mixing different ratios of the two stock solutions.

Phase Diagrams. The phase behavior as a function of temperature was determined by visual observation. The samples were cooled down and left to equilibrate at 0 °C, then the temperature was raised by approximately 0.5 °C per minute. The measured solubility temperatures correspond to the transition from precipitate to isotropic phase, i.e., to a micellar or vesicular solution. The samples were titrated with 0.1 M HCl at 25 °C. A Consort type C831 pH meter, with a Bioblock Scientific glass electrode (Consort ref number SP02N), was used.

Dynamic Light Scattering. Particle size analysis was performed by a Zetasizer 3000 PCS (Malvern Instruments Ltd., England), equipped with a 5 mW helium−neon laser with a wavelength output of 633 nm. The scattering angle was 90°, and the intensity autocorrelation functions were analyzed using the CONTIN software. All measurements were performed at 25 °C.

Cytotoxicity Tests. HeLa cells were provided by the ATCC (American type Culture Collection). The passage numbers of the HeLa cells used in the project varied from 25 to 30. Cells were cultured in Earle's minimum essential medium (MEM) containing 0.85 g/L $NaHCO_3$ supplemented with FCS (10%), L-glutamine (2 mM), NEA (1%), Amphotercin B (0.4 μg/mL), and Penicillin G/Streptomycin sulfate (100 u/mL). Keratynocytes (SK-Mel-28) were provided by the ATCC. The passage numbers varied from 15 to 20. Cells were cultured in Dulbecco's MEM containing 3.7 g/L $NaHCO_3$ supplemented with FCS (10%), L-glutamine (2 mM), NEA (1%), Amphotercin B (0.4 μg/mL), and Penicillin G/Streptomycin sulfate (100 u/mL).

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay procedure was prepared according to Mosman[43] in 96-well microplates using the doubling dilution method. First, the wells of the first column were filled with 135 μL of MEM medium. All subsequent columns were filled with 75 μL of medium. Then, 15 μL of 1 wt % test solutions was added to each well in the first column and the solutions were thoroughly mixed with a six-channel pipet.

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75 μL of the test substance/medium mixture was transferred from the wells in the first column to those in the second. Again, the solutions were mixed and the procedure was repeated for the whole plate. Then, 75 μL aliquots of cells were seeded (2.5×10^3 cells/well). After a 68 h incubation at 37 °C, 15 μL aliquots of MTT (5 mg /mL) were added to each well. After a 4 h incubation, the medium containing MTT was removed and 150 μL of 10% SDS solution was added. The microplates were placed in laminar flow overnight, then the optical density of each plate was measured with a microplate reader at 560 nm. The IC_{50} value (μg /mL), which represents the concentration of test substance that lowers MTT reduction by 50% compared with the untreated control, was calculated for each substance from the concentration−response curve. Experiments were repeated four times ($n = 5$), and the average IC_{50} is reported. Maximal observed (absolute) standard deviation was about 15%. Positive control measurements were performed with xanthohumol (HeLa cells: $IC_{50} \sim 7$ μg/mL).

Cryo-TEM. We prepared vitrified cryo-TEM specimens in a controlled environment vitrification system (CEVS), at a controlled temperature of 25 °C and fixed 100% relative humidity, followed by quenching into liquid ethane at its freezing point.[44] We examined the specimens, kept below −178 °C, by an FEI T12 G^2 transmission electron microscope, operated at 120 kV, using a Gatan 626 cryo-holder system. Images were recorded digitally in the minimal electron-dose mode by a Gatan US1000 high-resolution cooled-CCD camera with the DigitalMicrograph software package.

Results and Discussion

Lowering of the Solubility Temperature of Fatty Acids. To form vesicles at room temperature, the solubility temperature (T_{sol}) of the surfactant mixture has to be equal to or below this temperature. The use of sodium laurate is therefore limited, because it is insoluble in water at temperatures below 24 °C (for solutions of 1 wt %). We are able to lower the solubility temperature by mixing sodium laurate with a surfactant that is soluble at lower temperatures. AS was chosen because its T_{sol} = 5 °C. Figure S1 (Supporting Information) shows that the solubility temperature decreases linearly with increasing amounts of AS for a fixed total surfactant concentration of 1% (wt) and can be described by the equation T_{sol} [°C] $= -19.9 \times A + 23.9$, where T_{sol} represents the solubility temperature of the mixture and A the amount of AS, expressed in wt %.

Effect of pH on Vesicle Formation. The titration of alkaline soaps with HCl has been described by Rosano et al.,[45] when investigating the effects of surface charge on lipid−water interfaces. In these experiments, the appearance of a plateau (buffering capacity) at a precise pH during titration coincided with the formation of lipid liquid crystals. We observed this plateau in solutions of dominating quantities of sodium laureate. As more SL is replaced by AS, the plateau region becomes smaller, disappearing below 0.5% SL (mass ratio 1:1). By reducing the pH of the samples, we observed a succession of two (or three) phases, depending on the mass ratio of the two surfactants (Figure 1). At high pH, all solutions were isotropic and colorless, corresponding to the micellar region of the phase diagram. By decreasing the pH, a bluish color appeared, attributed to formation of vesicular structures. In the presence of pure AS, only two phases appeared consecutively, while in SL/AS mixtures, a second phase transition to precipitation was observed at low pH values. In 1 wt % SL solutions, precipitation begins at values below 7, whereas the addition of AS lowers the precipitation boundary to pH = 3.5 (in solutions with SL/AS ratio 25/75). Precipitation when the surfactant ratio nears equimolarity had been observed

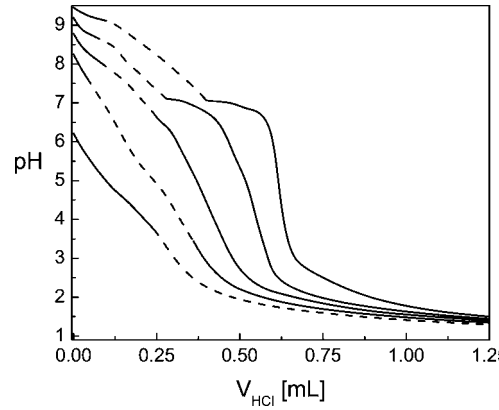

Figure 1. Different phase transitions observed by titration of mixed surfactant solutions (sodium dodecanoate and AS; $c_s^{tot} = 1$ wt %; $V_o =$ 15 mL) containing (from bottom to top) 1, 0.75, 0.5, 0.25, and 0 wt % of AS. The dashed lines represent a bluish solution (possibly vesicles); the solid lines represent the clear (micellar) solution (to the left) and the turbid phase (to the right of the vesicular phase).

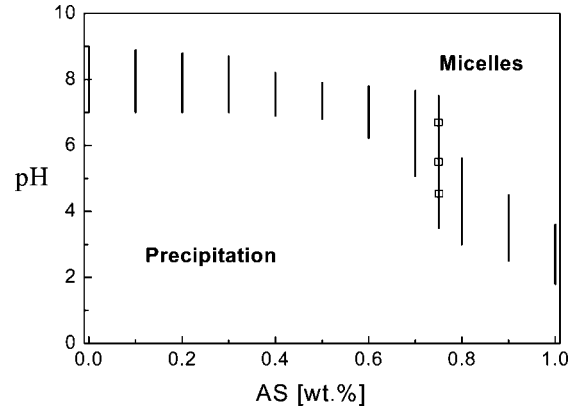

Figure 2. pH range where (after 3 weeks) the average hydrodynamic radius R_H is approximately 100 nm (possible region of vesicle formation) in mixed surfactant solutions (SL and AS) as a function of AS concentration ($c_s^{tot} = 1$ wt %).; the open squares (□) represent the solutions examined by cryo-TEM.

Table 1. Development of Various Surfactant Self-Assembled Aggregates with Time[a]

	no. of days after preparation				
pH	5	10	17	28	60
4.5		D, MP	M	D	MP
5.5	M, D		M, D	M, V	M, MP
6.7			MP, V	M, D, V (C)	M, MP, V (I)

[a] Legend: M, micelles; D, discs; MP, membrane pieces; V, vesicles; V (C/I), vesicles with either cusps, or incomplete vesicles.

in previous studies and was attributed to the increased concentration of the conjugated acid.[42] The titrations were performed one week after the preparation of the samples. Macroscopically, the pH values at which the phase transitions take place remain the same with respect to time. This is not true microscopically, as described below.

The formation of fatty acid vesicles is restricted to a narrow pH region close to the pK_a of the acidic components.[16,19,21] The apparent shift in the pK_a of fatty acid anions (from the $pK_a =$ 4.67 of carboxylic group[46] to the apparent $pK_a = 8$ of lauric acid[19]) has been attributed to the local decrease in pH at highly charged surfaces (as degree of ionization of the micellar aggregate

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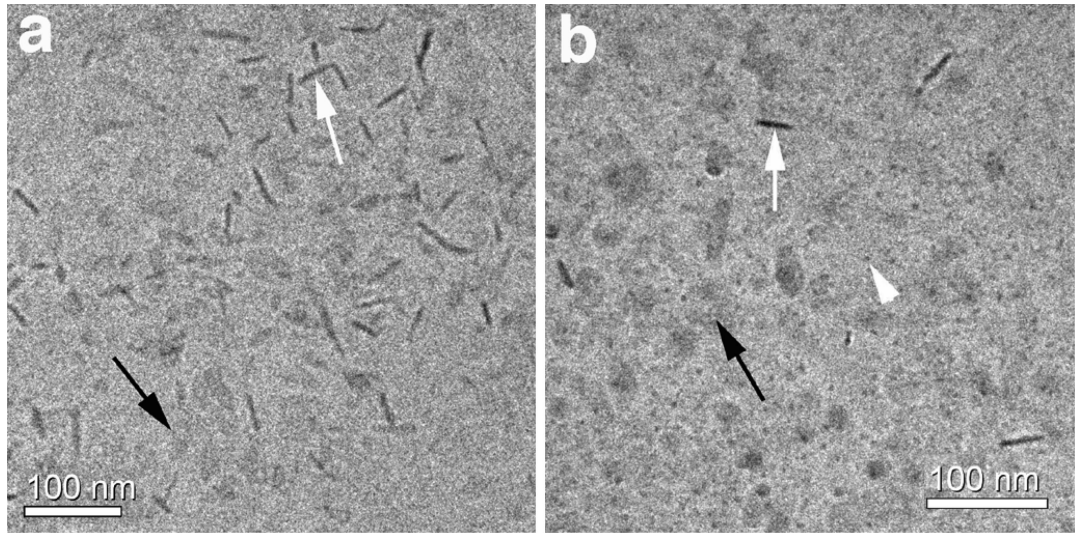

Figure 3. Cryo-TEM images of a SL/AS mixed surfactant solution at (a) pH = 4.5, 10 days after mixing, and (b) pH = 5.5, after 5 days of mixing. The black arrows in both images point to discs imaged face-on, whereas the white arrows to discs imaged edge-on. An arrowhead in (b) points to a globular aggregate, possibly a micelle.

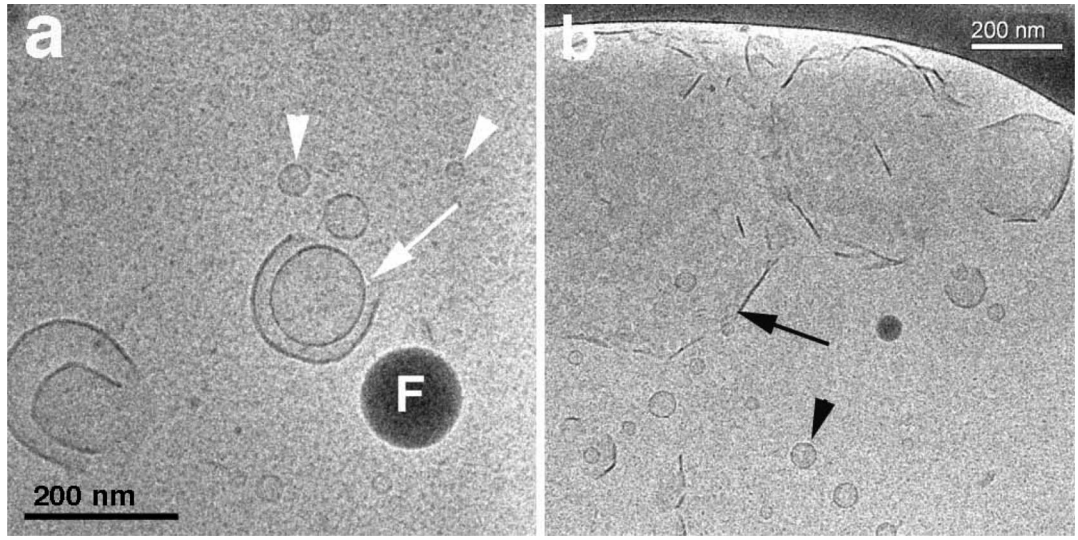

Figure 4. (a) The formation of small vesicles after 2 weeks in samples with pH = 6.7 and (b) large multilamellar vesicles after 4 weeks (pH = 5.5); arrowheads point to very small perfect vesicles, while the arrows points to an incomplete vesicles ("F" indicates a frost particle).

influences the effective pK_a of the neighboring surfactant molecules).[18] Since pure fatty acid/soap vesicles (without additional amphiphiles) contain an amphiphile that is not charged (the neutral form of fatty acid), the headgroups of the two bilayer-forming components are associated through hydrogen bonds instead of electrostatic interactions.[47] Such hydrogen bonds explain the formation of vesicles near the pK_a of the acid, where the ionized and neutral acid forms are present in comparable amounts.

It has been observed that the addition of alcohols to fatty acids causes an increase in the pH of the region of vesicle formation.[21] In that case, vesicles are formed due to stable hydrogen bonds between the alcohol headgroup and the ionized acid headgroup. The addition of the alcohol means a larger presence of the nonionized species in the solution (due to the high pK_a of the alcohols); a larger amount of carboxylic acid will be in the ionized form to achieve the same protonated/ionized headgroup ratio needed for vesicle formation (1: 1). An opposite effect can then be expected in the case where the added component has a much lower pK_a than the fatty acid, as is the case of AS. This will be completely ionized at a neutral pH; therefore, more protonated acid groups will be required for the formation of vesicles, resulting in a decrease of the pH of vesicle formation. This is confirmed by our titrations, where the pK_a (and corresponding bluish region) decreases with increasing amount of AS in the mixture.

The hydrodynamic radius of particles in solutions at different surfactant ratios was measured by dynamic light scattering. The results confirm the presence of large aggregates (possibly vesicles) over a large pH region (Figure 2) in mixed surfactant solutions. The regions where R_H was found to be 80−120 nm (the average radius of vesicles) are plotted in Figure 2 for different surfactant ratios. Increasing the amount of AS leads to a decrease of the pH, at which vesicles are formed. The width of the pH range depends strongly on the surfactant ratio. This range is the largest for the ratio AS/SL of 0.75/0.25, where it extends from pH 3.5 to 7.5. At this ratio, the complete range of skin pH or physiological

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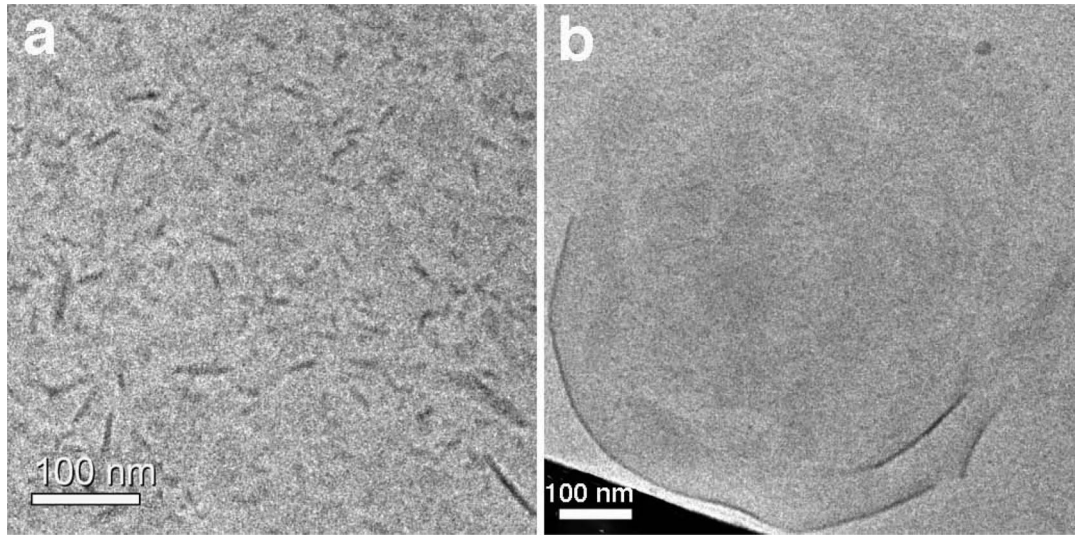

Figure 5. Nanostructure after 2 months: (a) small membrane fragments at low pH of 4.5; (b) large destabilized vesicle at the higher pH 6.7.

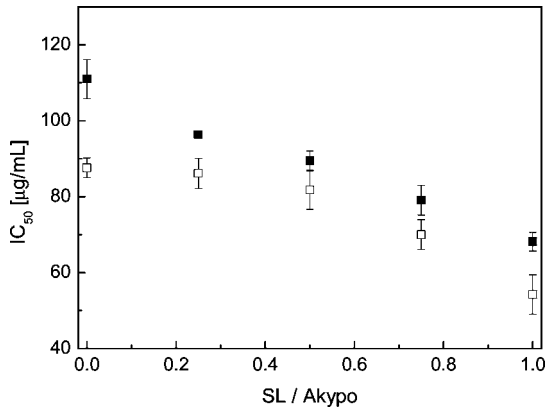

Figure 6. The mitochondrial reduction of MTT after 3 day incubation of SL/AS mixture at different surfactant ratios with HeLa cells (■) and Keratynocytes (□).

pH is covered. A reduction of this range is observed when the amount of Akypo Soft 45NV is either increased or decreased.

The (average) hydrodynamic radius (R_H) of the objects present in the vesicle containing solutions is shown in Figure S2 (Supporting Information) for different surfactant ratios as a function of the pH. The obtained values suggest the presence of vesicles, but large variations exist. In presence of 0.75 wt % of AS only small objects are measured with a small polydispersity index (around 0.2). At smaller ratios of AS, or in the presence of pure sodium laurate, bigger objects of higher polydispersity are observed. Because light scattering is insufficient to characterize systems of high polydispersity, cryo-TEM was used to analyze the system further.

Cryo-TEM Study of Time-Dependent Vesicle Formation. The AS/SL ratio (75/25), at which the largest pH interval of vesicle formation was measured, was investigated by cryo-transmission electron microscopy. The evolution of self-assembly aggregates was followed over a period of two months at three different pH values. The sequence of observed morphologies is summarized in Table 1.

We see from Table 1 that in the first weeks after preparation micelles and discs are the observed structures; these are presented in Figure 3. With time, membranes start forming, and eventually, vesicles appear (Figure 4). The higher the pH of the solution, the faster this transition takes place; in samples with pH = 6.7, vesicles are observed already after 2 weeks, whereas in solution with pH = 5.5, these are visible just after 4 weeks. After 2 months, vesicles were still not observed in solution with the lowest pH (cf. Figure 5a). After 2 months, the two samples at higher pH values already show incomplete vesicles and membrane pieces (cf. Figure 5b). It seems that, after a certain time, the vesicles in these systems begin to destabilize. We noted that, despite the rich morphological development that can be observed by microscopy, macroscopically the solutions appeared about the same during the period of observation. Furthermore, we can see that not all regions where big objects were detected by DLS contain vesicles. These were only found at higher pH values (above 5.5), whereas at low pH, discs and membrane pieces were the dominating forms.

The results suggest that it is possible to obtain vesicles, not all intact, in the region defined by the two pK_a. This is in accordance with results obtained by de Groot et al.[41] for a branched monoalkyl surfactant with a malonate headgroup. A bluish zone was observed between the two pK_a values (the pK_as of the two carboxylate groups on malonic acid are 2.85 and 5.70, respectively).[41] In that region, coexistence of small unilamellar (SUV) and multilamellar (MLV) vesicles was found. Above pH = 5.8, the solutions were clear and only SUV were observed by TEM.

Such a high polydispersity of morphologies, where flat discs coexist in equilibrium with spherical open and complete unilamellar vesicles, was previously observed in catanionic systems. A possible explanation for the coexistence of these structures was proposed by Jung et al. by evaluating the parameters contributing to the spontaneous curvature.[48] In some systems, however (namely, mixtures of anionic and zwitterionic surfactants), discoidal structures are only short-lived intermediates in the micelle-to-vesicle transitions.[49] The vesiculation depends on the balance between the unfavorable edge energy of the disks and the bending energy required to form spherical structures. In the case of catanionic surfactant mixtures, the edges of the discs are stabilized by the excess cationic surfactant. Further, one could imagine that the EO groups of the negative surfactants also shield the contact between the alkyl chain and water at the edges.

Cytotoxicity Potential of Surfactant Mixtures. Akypo Soft 45 NV is commonly used in cosmetic formulations, such as hair

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conditioners and hair dyes.[50−54] Improving the biocompatibility of products used in cosmetic formulation even further is always sought after. Cytotoxicity of our mixtures on HeLa cells and Keratynocytes was measured in order to identify their skin irritating properties for use in pharmaceutical and cosmetic applications. Cell viability was evaluated by the tetrazolium MTT reduction assay, based on the uptake and reduction of the soluble yellow MTT tetrazolium salt by mitochondrial dehydrogenases to a blue insoluble MTT formazan product. The IC_{50} value (concentration of test substance that lowers MTT reduction by 50% compared with the untreated control) was calculated from absorbance data. HeLa cells were used, because they reportedly show good reproducibility and a significant correlation with *in vivo* results,[55] whereas keratinocytes were chosen to check the skin compatibility of such surfactant mixtures.

IC_{50} values reported in Figure 6 show a dose-dependent decrease in toxicity when AS is exchanged for SL. Therefore, any surfactant mixture formed with SL is beneficial. The order of the IC_{50} values is the same for both cell lines; however, the absolute values are slightly lower in the case of the Keratynocytes. The lowering of the pH of the surfactant test solutions showed no pronounced effect. The volume of the added surfactant solution is probably too small to change the pH value of the cell medium significantly and the buffering capacity of the medium too large for any effects to be observed.

Conclusions

Two obstacles mentioned in the introduction, namely, the high solubility temperature of alkyl carboxylates and limited pH region of vesicle formation, have been overcome by using a mixture of two alkyl carboxylates with two very different pK_a values. The presented results show that it is then possible to spontaneously form bilayer structures, such as vesicles, in a pH range between the two considered pK_a. Sodium laurate (with a pK_a around 8.5) and Akypo Soft 45 NV (with a $pK_a = 4.67$) can be thus used at different mixing ratios to obtain vesicular solutions over the entire range of pH comparable to skin or physiological pH. Furthermore, we have used commercially available and biocompatible surfactants that are already used for cosmetic purposes and are inexpensive, which is of significant importance for application purposes.[56] However, further research is required to improve their application potential by increasing the colloidal stability of fatty acid vesicles and ensure that the vesicles are completely closed. Both are common problems in such systems.[20,57]

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Supporting Information Available: Solubility temperature of mixed surfactant solutions as a function of AS and the average hydrodynamic radius of the aggregates as a function of pH for different surfactant ratios. This material is available free of charge via the Internet at http://pubs.acs.org.

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