

Micelles of pulmonary surfactant in human
amniotic fluid at term

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Abstract: Studies using in vitro analysis have shown that the interaction between pulmonary surfactant and vernix caseosa could explain the appearance of amniotic fluid turbidity. That phenomenon is interpreted based on the "roll-up" hypothesis. We tested the roll-up hypothesis by examining the presence of micelles of pulmonary surfactant in human amniotic fluid at term. Amniotic fluid samples were collected from each of six healthy pregnant women at term and at 16 weeks of gestation. These samples were stained negatively and analyzed using an electron microscope. Ultrastructures present in amniotic fluid were compared with the structure of micelles derived from suspended surfactant TA isolated from bovine lung. Surfactant TA formed spheroidal and rod-shaped micelles 10-70 nm in diameter above the critical micelle concentration. Identical micelle particles were described in human amniotic fluid at term. In addition, surfactant protein B was identified in the micelle fraction of amniotic fluid. However, no micelles were found in human amniotic fluid taken at 16 weeks of gestation. Our results support the view that pulmonary surfactant could induce the detachment of vernix caseosa and increase the turbidity of the amniotic fluid.

9 May 2006

Dr. Sherin U. Devaskar, Editor in Chief
Dr. Jacques Bourbon, Consulting Editor
Pediatric Research

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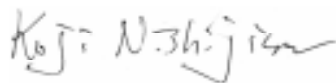
Dear Dr. Devaskar and Dr. Bourbon,

Please find enclosed our manuscript entitled "Micelles of pulmonary surfactant in human amniotic fluid at term," by Koji Nishijima *et al.*, for your consideration for publication as a regular article in *Pediatric Research*.

By submitting the manuscript to *Pediatric Research*, the authors affirm that the material presented in this paper has neither been published before, nor has it been submitted for publication to another scientific journal. I attest that this work has been approved by all co-authors. The authors also understand that should the submitted material be accepted for publication in the journal, they will automatically transfer its copyright to the publisher.

We attempted the requested additional experiments. During this process, we made several modifications to the manuscript based on comments raised by the editors. The comments allowed us to identify areas in our manuscript that required modification and clarification. We believe our new findings are of particular interest to the readers of *Pediatric Research*. We hope that the revised manuscript is now acceptable for publication in *Pediatric Research*.

Sincerely yours,



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RE: MS# PR-D-05-00455R1-Micelles of pulmonary surfactant in human amniotic fluid at term

Response to Editorial comments:

We gratefully acknowledge your review of our manuscript. We fully recognize our good fortune at receiving your review because we know your fine work well. We modified the original manuscript based on your comments. Your e-mail of February 16: "In terms of incremental knowledge, your manuscript in its present form does not reach the standard of the journal. However, it would if the additional study was performed. Final editorial decision could be made readily after resubmission and will not require further reevaluation by the reviewers." encouraged me greatly.

Major criticisms:

1) As suggested by the editor, we omitted the descriptions regarding the turbidity assay of vernix caseosa-surfactant TA mixture. Figure 3 and the Legend to Fig. 3 were completely omitted. Accordingly, the following figure was renumbered.

2) We thank the editor for the kind suggestion. We performed the additional experiment to show that micelles derived from human amniotic fluid induce the detachment of vernix caseosa. The request from Dr. Sherin Devaskar has been solved during revision process.

The last sentence of the Introduction section was rewritten as following: "We also performed experiments to show that a specific component of the surfactant; surfactant protein B (SP-B), is associated with the micelles, and that micelles derived from human amniotic fluid induce the detachment of vernix caseosa under *in vitro* condition." (page 6, line 7 to 10). In the Materials and Methods section, the sentences "Amniotic fluid samples of 15 ml were obtained by amniocenteses from four healthy Japanese pregnant women at term. The samples were

methodically fractioned by differential centrifugation (3, 15). The 22,500 x g sediments (micelle fractions) were dispersed with 1 ml of saline and subsequently added to each 1.5-ml polypropylene microfuge tube, interiorly coated with 10 mg of vernix caseosa (5). The samples from the vernix caseosa-saline mixtures (n = 4) were used as controls. Each sample was analyzed spectrophotometrically at 650 nm before and after overnight incubation at 37°C (5). The optical density correlated with solution turbidity that resulted from detachment of vernix from the walls of the microfuge tubes (5). Changes in absorbance values of micelle samples during overnight incubation were compared with those of control samples.” were added (page 9, 10). The Results section (page 11, line 1 to 5) and the Discussion section (page 11, line 14, 15) were modified. Figure 4 and the Legend to Fig. 4 were added.

Additional points:

- 1) The additional research, for which you asked us, needed an expert person. Our study has been accomplished by his joining. Accordingly, in the revised manuscript, his name, “Makoto Orisaka“, should be added to the author of our manuscript. All authors have agreed to that.

- 2) We realize the limitation of our English. You can rewrite our original sentences for the readers of your journal, if necessary. All authors have agreed to that.

Running title: Surfactant micelles in amniotic fluid

Micelles of pulmonary surfactant in human amniotic fluid at term

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writing of the report.

Abstract

Studies using *in vitro* analysis have shown that the interaction between pulmonary surfactant and vernix caseosa could explain the appearance of amniotic fluid turbidity. That phenomenon is interpreted based on the “roll-up” hypothesis. We tested the roll-up hypothesis by examining the presence of micelles of pulmonary surfactant in human amniotic fluid at term. Amniotic fluid samples were collected from each of six healthy pregnant women at term and at 16 weeks of gestation. These samples were stained negatively and analyzed using an electron microscope. Ultrastructures present in amniotic fluid were compared with the structure of micelles derived from suspended surfactant TA isolated from bovine lung. Surfactant TA formed spheroidal and rod-shaped micelles 10–70 nm in diameter above the critical micelle concentration. Identical micelle particles were described in human amniotic fluid at term. In addition, surfactant protein B was identified in the micelle fraction of amniotic fluid. However, no micelles were found in human amniotic fluid taken at 16 weeks of gestation. Our results support the view that pulmonary surfactant could induce the detachment of vernix caseosa and increase the turbidity of the amniotic fluid.

Abbreviations:

Critical micelle concentration (CMC);

Surfactant protein B (SP-B)

Introduction

The turbidity of the amniotic fluid increases with gestational age during the third trimester (1–5). Studies using *in vitro* analysis have shown that the interaction between pulmonary surfactant and vernix caseosa could explain the appearance of amniotic fluid turbidity (5). Narendran *et al.* (5) speculated that pulmonary surfactant induces a “roll-up” phenomenon that leads to detachment of vernix caseosa from the fetal skin surface. Droplets of oil rise from the oil/substrate interface when the surfactant displaces the oily masses from the substrate in aqueous media. This displacement is called “roll-up” and is strictly controlled by micellization process kinetics (6, 7).

Surfactants are chemical amphipathic compounds; they contain both hydrophobic and hydrophilic ends (8). They form micelles according to their critical micelle concentration (CMC) in an aqueous environment (8). Pulmonary surfactant, a mixture of specific phospholipids and apoproteins, is a natural surface-active agent (1, 2). Pulmonary surfactant could thereby form micelles in human amniotic fluid. Numerous studies using electron microscopy have revealed pulmonary surfactant in amniotic fluid as a storage medium: lamellar body (2, 3). However, to our knowledge, no studies have investigated micelles of pulmonary surfactant.

To test the roll-up hypothesis proposed by Narendran *et al.* (5), we used electron microscopy to determine the presence of micelles of alveolar surfactant in human amniotic fluid at term. Since impurities in amniotic fluid disturb ultrastructural analysis, we first analyzed micelles derived from surfactant TA (Surfacten[®]; Mitsubishi Pharma Corp., Osaka, Japan) for pattern recognition. We then analyzed mixtures of surfactant

TA and vernix caseosa and samples of amniotic fluid collected at term or at 16 weeks of gestation. We also performed experiments to show that a specific component of the surfactant; surfactant protein B (SP-B), is associated with the micelles, and that micelles derived from human amniotic fluid induce the detachment of vernix caseosa under *in vitro* condition.

Materials and Methods

Preparation of surfactant TA

The composition of surfactant TA, which is isolated from bovine lung and used clinically (9), is similar to Survanta[®] (Ross Laboratories, Columbus, OH). It comprises 83.5% phospholipids, 7.0% free fatty acids, 7.0% triglycerides, 1.0% hydrophobic surfactant-associated protein, and other components (10). First, we determined the CMC of surfactant TA in a physiological saline solution by measuring its surface tension with a Wilhelmy-type surface-tension meter (CBVP A-1; Kyowa Interface Science Co., Tokyo, Japan). A stock solution of surfactant TA was prepared by dissolving 108 mg of surfactant TA into saline and diluting it to 50 ml with saline solution. The stock solution was diluted accurately to determine the surface tension of surfactant TA. The value of the CMC of surfactant TA was obtained from the concentration/surface-tension curve (11, 12). Surfactant TA was suspended in a physiological saline solution at 37°C to analyze micelles. The surfactant TA solution was adjusted with a phospholipid concentration of 25, 50, 75, 100, 150, 200, or 500 µg/ml. Samples were maintained at 37°C until electron microscopic analysis.

Preparation of vernix caseosa-surfactant TA mixture

Samples were prepared according to the procedure of Narendran *et al.* (5). Briefly, vernix caseosa was harvested from full-term infants who were free of any antenatal complications, and stored in sterile vials at the time of delivery. Samples were refrigerated at -80°C until experiments. We applied 10 mg of vernix caseosa pooled from three term infants to the interior walls of 1.5-ml polypropylene microfuge tubes and spread it on the wall using a glass pestle to form an even coating. Then 1 ml of normal saline with 150 µg phospholipid derived from surfactant TA was added to the tubes. The mixture was agitated gently by slow rotation on a rotisserie/shaker (Labquake[®]; Barnstead/ThermoLyne Corp., Dubuque, IA). The rotisserie was placed overnight in a thermally equilibrated room at 37°C. The liquid was decanted and subsequently analyzed electron microscopically.

Preparation of amniotic fluid samples

Amniotic fluid samples were collected from six healthy Japanese women at term (≥ 37 -wk gestation) singleton pregnancies. All six had been scheduled for elective cesarean delivery. In addition, we studied six healthy pregnant women at 16 weeks of gestation who had been scheduled for transabdominal amniocentesis for fetal chromosome analysis. Patients were excluded if they had a chronic or acute physical illness, or if they were taking medications at the time of enrollment. Informed consent was obtained from each subject before this study. The Institutional Review Board of the University of Fukui approved this study.

Details of the procedure are the following: amniotic fluid samples of 15 ml were obtained using amniocenteses; the samples were fractioned by differential centrifugation according to the methods described by Hook *et al.* (3). Each amniotic fluid sample was centrifuged at 370 x g for 10 min to remove the sediment; which consisted mainly of cells. Subsequently, the supernatant was centrifuged at 9,200 x g for 20 min. The 9,200 x g supernatants were maintained at 37°C until analysis of micelles.

Analysis of micelles

All samples for electron microscopy were stained negatively using 2% phosphotungstic acid at pH 7.2 (13, 14) and analyzed within 1 h after sample preparation. Electron micrographs were viewed at 75 kV using an electron microscope (H-7100 FA; Hitachi, Tokyo).

Identification of SP-B in the micelle fraction of term amniotic fluid

The presence of SP-B in the micelle fraction was examined by using Western blot analysis (15). Briefly, 15 ml of the 9,200 x g supernatant sample retrieved from term pregnancy was centrifuged at 22,500 x g for 30 min (4°C). Half of the resultant pellet was dispersed with 100µl of saline and maintained at 4°C until analysis of micelles. The remaining pellet was used for Western blot analysis: solubilized in 60 µl of gel sample buffer, boiled for 5 min, and separated using a 15% acrylamide SDS-PAGE gel.

Electrophoresed samples were transferred to a nitrocellulose membrane (Trans-Blot[®] Transfer Medium; Bio-Rad Laboratories, Inc, Hercules, CA) following the instructions

provided by the manufacturer. Immunoblotting was performed using a horseradish peroxidase system (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) and bands were visualized by enhanced chemiluminescence according to the ECL Western Blotting Detection System (Amersham, Buckinghamshire, UK). A 1:5,000 dilution of mouse anti-human SP-B monoclonal antibody (clone 1BQ, subclass IgG_{2b}; Chemicon International, Inc, Temecula, CA) was used as the primary antibody. The secondary antibody consisted of a 1:2,000 dilution of horseradish peroxidase–conjugated goat antimouse IgG (Santa Cruz Biotechnology).

Turbidity assay of mixtures of vernix caseosa and amniotic fluid micelles

Amniotic fluid samples of 15 ml were obtained by amniocenteses from four healthy Japanese pregnant women at term. The samples were methodically fractioned by differential centrifugation (3, 15). The 22,500 x g sediments (micelle fractions) were dispersed with 1 ml of saline and subsequently added to each 1.5-ml polypropylene microfuge tube, interiorly coated with 10 mg of vernix caseosa (5). The samples from the vernix caseosa-saline mixtures (n = 4) were used as controls.

Each sample was analyzed spectrophotometrically at 650 nm before and after overnight incubation at 37°C (5). The optical density correlated with solution turbidity that resulted from detachment of vernix from the walls of the microfuge tubes (5). Changes in absorbance values of micelle samples during overnight incubation were compared with those of control samples.

Statistical analysis

Data are expressed as mean \pm standard deviation. Statistical analysis was performed using StatView software, version 5.0 (SAS Institute Inc., Cary, NC). The Student *t*-test was used for continuous variables. All statistical tests were two-sided and significance was defined as $P < 0.05$.

Results

The CMC value of surfactant TA obtained from the concentration/surface-tension curve was 80 $\mu\text{g/ml}$ (Fig. 1). The value is converted to approximately 67 $\mu\text{g/ml}$ in phospholipid concentration (10). Surfactant TA formed micelles above its CMC at phospholipid concentrations of 75, 100, 150 (Fig. 2A), 200, and 500 $\mu\text{g/ml}$.

Vernix detached from the tube wall above the CMC of surfactant TA. Surfactant TA interacted with vernix caseosa and subsequently formed micelles (Fig. 2B).

Micelles were also detected in all but one 9,200 $\times g$ supernatants of human full-term amniotic fluid samples (Fig. 2C). In addition, Western blot analysis revealed the presence of SP-B in the micelle fraction of term amniotic fluid (Fig. 3A and B). However, no micelles were identified in human amniotic fluid samples harvested at 16 weeks of gestation.

Amniotic fluid micelles interacted with vernix caseosa and subsequently induced vernix detachment from the tube wall (Fig. 4). In contrast, for control samples, the mixtures of vernix caseosa and physiological saline remained clear after overnight incubation. The increasing absorbance values at 650 nm for micelle samples and control samples were 0.496 ± 0.178 and 0.055 ± 0.017 , respectively ($P = 0.0027$).

Discussion

The major finding of our study was the presence of micelles in human full-term amniotic fluid. Narendran *et al.* (5) speculated that amniotic fluid turbidity is secondary to roll-up detachment of vernix caseosa induced by pulmonary surfactant. The roll-up phenomenon is intrinsically controlled by micellization process kinetics; the detergent aqueous solution starts to remove contaminants at or near the CMC and reach maximum detergency at concentrations greater than the CMC (6, 7). Moreover, micelle induces the dissolution of the mass contaminant from surfaces directly into the aqueous phase (6). The present study also showed that human amniotic fluid micelles induce detachment of vernix caseosa under *in vitro* condition. Our results provide a strong support for the hypothesis proposed by Narendran *et al.* (5).

Micelle particles in term amniotic fluids are similar to the micelles derived from surfactant TA. We also demonstrated the association of SP-B with micelles of human amniotic fluid. SP-B is important for optimizing the surface active properties of pulmonary surfactant and is co-secreted with the surfactant (2). Accordingly, our results suggest that amniotic fluid micelles are associated with pulmonary surfactant.

Secretion of pulmonary surfactant begins at 25–30-wk gestation (1, 2). In addition, detachment of the vernix caseosa from fetal skin into the amniotic fluid commences at 34-wk gestation (4). These chronological events fit with the results of the present study of the absence of micelles in all amniotic fluid samples taken at 16 weeks of gestation. The absence of micelles in amniotic fluid from 16-wk pregnancies is a further indication that pulmonary surfactant is associated with the micelles in the

full-term amniotic fluid. Micelles were not detected at term in one of our six amniotic fluid samples. Thus, the latter case points to the limitation of our procedure.

Numerous studies have reported that the surface-active fractions in amniotic fluid originate from fetal lung (2, 3). Hook *et al.* (3) indicated that the lamellar bodies in amniotic fluid are pulmonary surfactants based on differential centrifugation of the amniotic fluid. Lamellar bodies containing concentric whorls of phospholipids are recognized as storage forms of pulmonary surfactant (2, 3, 16). However, Hook's procedure is unsuitable for analysis of small lipid vesicles. Furthermore, the lamellar bodies range in size from 1 to 5 μm (3); they are sufficiently large to obscure micelles (10–70 nm). On the other hand, a negative staining method is useful for examining suspensions of small particles, including micelles (13, 14). Using such technique, we were able to identify the presence of micelles in the 9,200 x *g* supernatant fraction of human full-term amniotic fluid, which differed from the fraction of lamellar bodies. This finding suggests that micelles are not derived from lamellar bodies during the preparation of amniotic fluid sample. Rather, micelles are naturally present in human amniotic fluid.

Micellization is an important step for postnatal lipid absorption because fat is insoluble in the aqueous environment of the small-intestinal lumen (1, 8). Micelles exist in amniotic fluid swallowed by the fetus and in human breast milk ingested by neonates. Numerous studies have shown that maturation of the fetal gastrointestinal tract is partially enhanced by swallowed amniotic fluid (1, 17). The fetal lung synthesizes and secretes much greater amounts of surfactant than are needed for air adaptation after

birth. The micelles in swallowed amniotic fluid might act as a promoter of fetal intestinal maturation.

Visualization of the micelle particles in human amniotic fluid has not been reported previously. Our results support the view that pulmonary surfactant could induce the detachment of vernix caseosa, thus increasing the turbidity of the amniotic fluid. Although further studies are needed, our findings enhance our understanding of the physiologic interaction between lung development and skin development.

Acknowledgments

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Figure legends

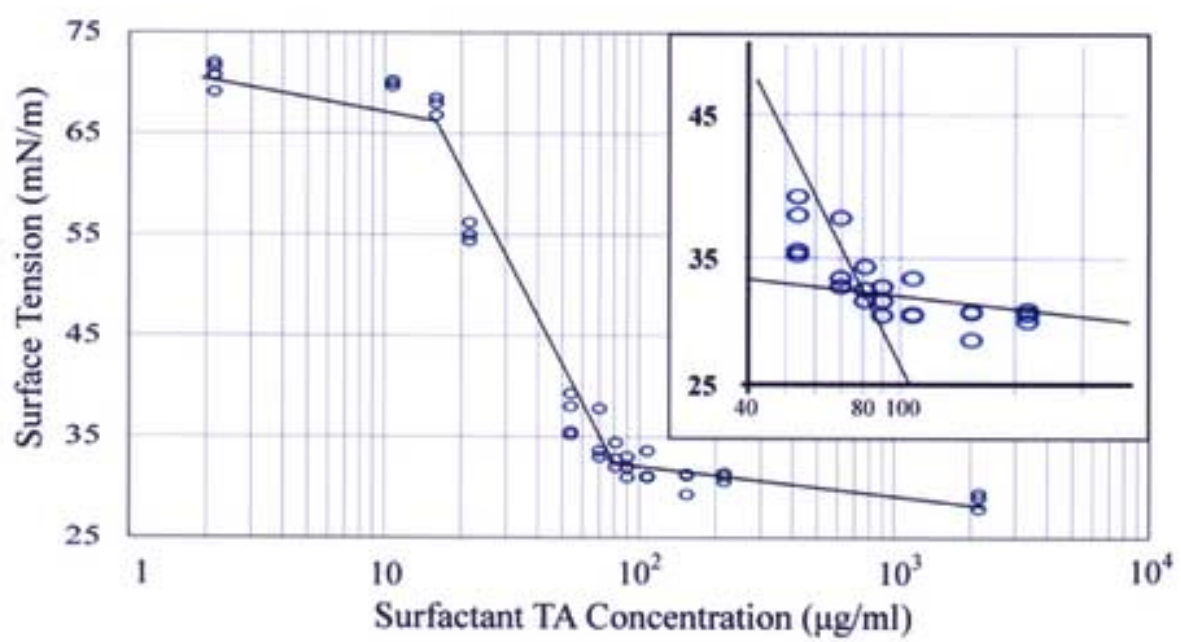
Figure 1. Surface tension behavior of surfactant TA solutions. The inset shows a magnified image of the region of interest. Surface tension decreased from 70.8 to 28.8 mN/m with increasing concentration of surfactant TA. The CMC represented the concentration at the intersection of the two lines of the concentration/surface-tension curve. The CMC value was 80 $\mu\text{g/ml}$.

Figure 2. Negative stain electron micrograph of micelles. (A) Micrograph of surfactant TA solution at phospholipid concentration of 150 $\mu\text{g/ml}$. Micelles are visible against the stained background. The diameter of spheroidal and rod-shaped micelles ranges from 10 to 70 nm. The average diameter of micelles derived from surfactant TA is 35.4 nm. (B) Micrograph of mixtures of vernix caseosa and surfactant TA. Micelles are visible similarly to surfactant TA solution (see Fig. 2A). The average diameter of micelles derived from the mixtures is 46.8 nm, which was not significantly different from the average diameter of surfactant TA micelles ($P > 0.05$). (C) Micrograph of human amniotic fluid at term. Despite their lower number, micelles are similar to surfactant TA solution (see Fig. 2A and 2B). Calibration bar: 100 nm. (original magnification: $\times 200,000$)

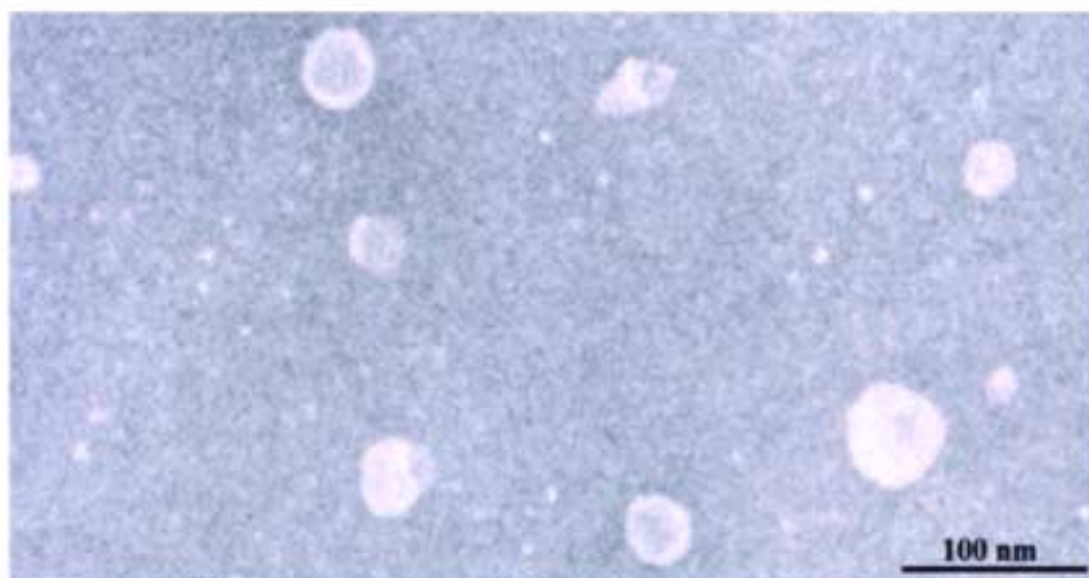
Figure 3. Identification of SP-B in the micelle fraction of full-term amniotic fluid. (A) Negative stain electron micrograph of 22,500 \times g sediments retrieved from human amniotic fluid at term. Micelle particles are visible (see Fig. 2). Calibration bar: 100 nm.

(original magnification: $\times 200,000$) (B) Western blot analysis revealed 42-kilodalton pro-SP-B and 8-kilodalton mature SP-B in the micelle fraction of term amniotic fluid. The fraction of the lamellar bodies, which is rich in SP-B, was used as positive control.

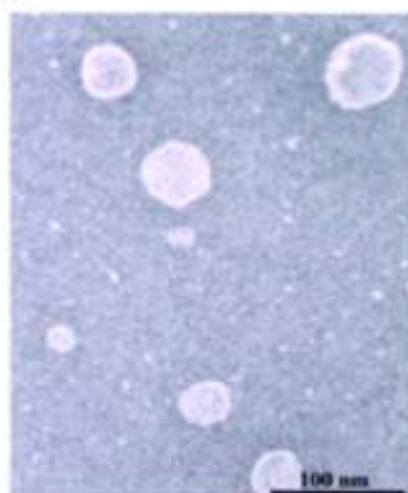
Figure 4. Series of images showing time-dependent detachment of vernix caseosa by amniotic fluid micelles (top) and physiological saline (bottom). The turbidity of vernix caseosa- amniotic fluid micelle samples increased significantly during overnight incubation at 37°C (top left, pre-incubation; top right, post-incubation). In contrast, the mixtures of vernix caseosa and physiological saline remained clear after overnight incubation (bottom left, pre-incubation; bottom right, post-incubation).



A.



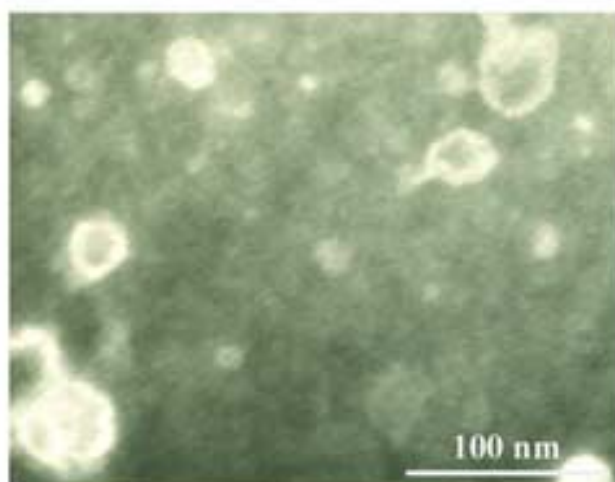
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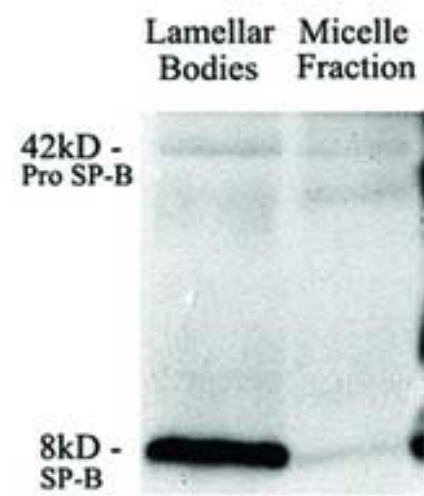
C.



A.



B.



Figures
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