รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาพฤติกรรมความเป็นคอลลอยด์และการเกิดฟิล์มของยางธรรมชาติ

(Elucidating the colloidal behavior and film formation of natural rubber latex)

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มีนาคม พ.ศ.2555

สัญญาเลขที่ **RSA5280009**

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มหาวิทยาลัยมหิดล ถนนพระราม 6

แขวงทุ่งพญาไท กรุงเทพ ฯ 10400

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

คณะผู้วิจัยฯ ขอกราบขอบพระคุณ สำนักงานกองทุนสนับสนุนการวิจัย (สกว.) ที่ให้การสนับสนุนทุนวิจัยตามสัญญาโครงการเลขที่ RSA 5280009 รวมทั้งคณะกรรมการและผู้ทรงคุณวุฒิที่ได้คัดเลือกให้ได้รับทุนพัฒนานักวิจัยประจำปี 2552 นี้ และสุดท้ายขอขอบคุณ ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล และสถาบันวิจัยชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล ที่เอื้อเฟื้อสถานที่ในการทำการวิจัย และทุกท่านที่มีส่วนร่วมในความสำเร็จของโครงการวิจัยนี้

คณะผู้วิจัยขอกราบขอบพระคุณเป็นอย่างสูงมา ณ ที่นี้

รศ.ดร.จิตต์ลัดดา ศักดาภิพาณิชย์
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Abstract

Natural rubber (NR) from the *Hevea brasiliensis* tree is the main commercial feedstock for rubber and latex dipping industries. The particle of NR latex is presumed to be covered with some proteins and phospholipids deriving colloidal stability of NR latex. Therefore, the colloidal stability of the latex is believed to be extremely sensitive to pH as well as to the ionic environment of the dispersing medium. Previous work on particle microelectrophoresis has shown that the charge on the particle surface is derived mainly from carboxylic groups of long chain fatty acids (*ca.* 86%). On the other hand, freshly tapped field NR (FNR) latex from the tree is believed to be stabilized by proteins and phospholipids before hydrolysis sets in upon leaving the tree. However, an exact arrangement of the adsorbed proteins and phospholipids and the thickness of this adsorbed layer have yet to be successfully determined. Owing to this assumption, the proteins and phospholipids layer presenting on the surface of freshly tapped FNR particle is also presumed to mainly cause the irregular film formation behavior of the freshly tapped FNR latex. Therefore, to clarify these assumptions, firstly this work attempted to study and characterize the proteins and phospholipids in NR latex involving the colloidal stability by using scanning electron microscope (SEM), zeta potential analyzer and ^{1}H-NMR. Secondly, the arrangement of these proteins and phospholipids on the particle surface of a freshly tapped NR latex was studied by using phase contrast atomic force microscopy (AFM). Furthermore, in order to get a deep understanding on the effect of proteins and phospholipids layer presenting on the surface of FNR particle on film formation behavior of the FNR latex, film formation of deproteinized FNR (DP-FNR) and saponified FNR (SP-FNR) latices were investigated and compared with that of untreated FNR latex by using AFM. Based on the results, the highest stability of rubber particles was found at pH 9 and dominated the lowest zeta potential value. In addition, ^{1}H-NMR spectrum of saponified phospholipids removed from rubber particles revealed major signals, which is the important evidence to indicate that phospholipids associate to the rubber chain. Moreover, it was clearly observed a gray ring near the particle surface corresponding to the membrane layer derived from protein-lipid. The surface of the particles in freshly tapped FNR latex was proved to be distinctly different from that of a matured latex concentrate. Moreover, AFM images also show that rate of the freshly tapped FNR latex film formation was proposed to increase when the mixed protein and lipid layers were removed.

Keywords: natural rubber, proteins, phospholipids, field natural rubber latex

บทคัดย่อ

ยางธรรมชาติที่ได้จากต้นยางพาราพันธุ์ *Hevea brasiliensis* เป็นวัตถุดิบเชิงพาณิชย์สำหรับอุตสาหกรรมยางและการจุ่มน้ำยางที่ใช้กันอย่างแพร่หลาย และนับจนปัจจุบันนักวิจัยหลายท่านเชื่อกันว่าอนุภาคของยางธรรมชาตินั้นถูกห่อหุ้มด้วยชั้นของโปรตีนและฟอสโฟไลปิดที่ช่วยในเรื่องของความเสถียรภาพของน้ำยาง ดังนั้น ความเสถียรภาพของน้ำยางนั้นจึงน่าจะมีความไวต่อค่าความเป็นกรดด่างและสภาพความเป็นไอออนของตัวกลางอย่างมาก จากงานวิจัยที่เกี่ยวกับ micro electrophoresis ที่ผ่านมา พบว่าประจุที่อยู่บนผิวของอนุภาคส่วนใหญ่มาจากหมู่คาร์บอกซิลิคของกรดไขมันอิ่มตัวสายโซ่ยาว แต่อย่างไรก็ตาม ในกรณีของน้ำยางธรรมชาติที่กรีดใหม่ๆจากต้นนั้น ถูกเชื่อว่า อนุภาคของน้ำยางธรรมชาตินั้นถูกทำให้เสถียรด้วยชั้นของโปรตีนและฟอสโฟไลปิดตั้งแต่ก่อนที่จะถูกกรีดออกมาจากต้น แต่อย่างไรก็ตาม จนกระทั่งตอนนี้ การเรียงตัวของโปรตีนและฟอสโฟไลปิดที่ถูกดูดซับอยู่ที่ผิวและความหนาของชั้นโปรตีนและฟอสโฟไลปิดที่อยู่ที่ผิวของอนุภาคของยางธรรมชาตินั้น ยังไม่มีนักวิจัยท่านใดทำการศึกษาได้อย่างถ่องแท้ และเนื่องด้วยสมมติฐานนี้ จึงทำให้เราเชื่อว่าชั้นของโปรตีนและฟอสโฟไลปิดที่อยู่ที่ผิวของอนุภาคของน้ำยางที่กรีดใหม่ๆน่าจะเป็นสาเหตุสำคัญของการเกิดฟิล์มที่ไม่ปกติเหมือนยางสังเคราะห์อื่นๆ ดังนั้น เพื่อที่จะอธิบายสมมติฐานต่างๆนี้ให้ชัดเจน งานวิจัยนี้จึงพยายามที่จะศึกษาเกี่ยวกับโปรตีนและฟอสโฟไลปิดที่เกี่ยวข้องกับความเสถียรภาพของน้ำยางที่อยู่ในน้ำยางโดยใช้เทคนิค scanning electron microscope หรือ SEM และ zeta potential analyzer และ ^{1}H-NMR หลังจากนั้น ก็ได้ทำการศึกษาการเรียงตัวของโปรตีนและฟอสโฟไลปิดที่อยู่บนผิวของน้ำยางที่ถูกกรีดใหม่ๆ โดยใช้เทคนิค atomic force microscopy หรือ AFM นอกจากนั้นงานวิจัยนี้ได้ทำการศึกษาเกี่ยวกับผลกระทบของโปรตีนปละฟอสโฟไลปิดที่อยู่บนผิวของอนุภาคของน้ำยางที่ถูกกรีดใหม่ๆต่อการเกิดฟิล์ม โดยเปรียบเทียบการเกิดฟิล์มของน้ำยางที่ถูกกรีดใหม่ๆที่ไม่ได้ผ่านกระบวนการใดๆกับการเกิดฟิล์มของน้ำยางที่ถูกกรีดใหม่แต่ผ่านกระบวนการการแยกโปรตีน และฟอสโฟไลปิดออกแล้ว ด้วยเทคนิค AFM จากผลการทดลองที่ได้ พบว่า อนุภาคของน้ำยางธรรมชาตินั้นมีความเสถียรภาพมากที่สุดที่ค่าความเป็นกรดด่างประมาณ 9 และทำให้ค่า zeta potential นั้นมีค่าน้อยที่สุด นอกจากนั้น สเปกตรัม ^{1}H-NMR ของฟอสโฟไลปิดหลังจากถูกตัดและแยกออกมาจากอนุภาคของยางแสดงให้เห็นสัญญาณที่สำคัญที่บ่งบอกว่าฟอสโฟไลปิดเหล่านั้นมีการเชื่อมโยงกับสายโซ่ของยางธรรมชาติ ยิ่งไปกว่านั้น ภาพจาก SEM ยังแสดงให้เห็นวงแหวนสีเทาๆอยู่บริเวณใกล้ๆกับผิวของอนุภาคของยางธรรมชาติ ที่น่าจะสัมพันธ์กับชั้นเมมเบรนของโปรตีนและฟอสโฟไลปิด ผิวของอนุภาคของยางธรรมชาติที่ถูกกรีดใหม่ๆก็ยังถูกพิสูจน์ให้เห็นว่ามีความแตกต่างอย่างชัดเจนกับผิวของอนุภาคของยางธรรมชาติที่เสร็จสมบูรณ์นานแล้ว นอกจากนั้น ภาพจาก AFM ก็แสดงให้เห็นว่า อัตราความเร็วในการเกิดฟิล์มของน้ำยางที่ถูกกรีดใหม่จะเร็วขึ้นเมื่อชั้นของโปรตีนและฟอสโฟไลปิดนั้นถูกขจัดออกไป

คำสำคัญ: ยางธรรมชาติ โปรตีน ฟอสโฟไลปิด น้ำยางธรรมชาติที่ถูกกรีดใหม่

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(Elucidating the colloidal behavior and film formation of natural rubber latex)

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แขวงทุ่งพญาไท กรุงเทพ ฯ 10400

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PART 1 CHARACTERIZATION OF ASSOCIATED PROTEINS AND PHOSPHOLIPIDS IN NATURAL RUBBER LATEX

ABSTRACT

Natural rubber latex is presumed to be covered with some proteins and phospholipids, deriving colloidal stability of natural rubber (NR) latex. Therefore, this work attempts to study and characterize the components in NR latex involving the colloidal stability. Proteins in cream fraction of NR latex were removed by proteolysis reaction. Proteins from both of cream and serum fractions were characterized by SDS-polyacrylamide gel electrophoresis. Then, the latex was further treated with NaOH to decompose phospholipids. Phospholipids were analyzed by ^{1}H-NMR spectroscopy. The colloidal behavior of rubber latices before and after removal of proteins and phospholipids was studied by scanning electron microscope and zeta potential analyzer. In this study, the highest stability of rubber particles was found at pH 9 and dominated the lowest zeta potential value. Moreover, the rubber particles in the cream fraction showed different protein compositions from that of serum phase. The major proteins in rubber particles and serum phase were 14.5, 25 and 27 kDa. In addition, the ^{1}H-NMR spectrum of saponified phospholipids removed from rubber particles revealed major signals, which were assignable to -$(CH_2)_n$-, -CH_2OP, -CH_2OC=O and -OCH_2CH_2NH-. The appearance of these signals is the important evidence to indicate that phospholipids associate to the rubber chain. In SEM analysis, it was clearly observed a gray ring near the particle surface corresponding to the membrane layer derived from protein-lipid.

Key words: natural rubber latex, protein, phospholipid and colloidal stability

INTRODUCTION

The latex from *Hevea brasiliensis*, the commercial source of natural rubber, contains about 36% of rubber fraction, 5% of non-rubbers such as protein, lipid and sugar, as well as around 59% of water. Many kinds of proteins causing an allergic reaction are claimed to contaminate in natural rubber products such as gloves and condom. The rubber particles are

generally spherical, although medium-sized and large ones in latex from certain mature tree may be pear shaped (1). In the previous study, it was proposed that the natural rubber particles are protected by complex film containing proteins and lipids (2). The lipid and proteins layer was inside and on the outside of a rubber particle, respectively (3).

The total protein content of fresh latex is approximately 1-1.5% of which about 20% adsorbed on the rubber particles and the similar proportion is associated with the bottom fraction (4). The residue is dissolved in the serum phase. The major component of the adsorbed proteins is probably identical with the α-globulin which its molecular weight is in the order of 200 kDa and it is insoluble in distilled water (5, 6). Hevein, the second principle protein and its molecular weight around 5 kDa, is water-soluble protein and generally found in the bottom fraction of the latex (7). This adsorbed protein layer represents as electrophoresis characteristic that possessing electrical charge on their structure. The proteins adsorbed on the rubber particles have not been studied in much detail due to the difficulties in removing them unchanged from the particle surface. Therefore, particles electrophoresis shows that the fresh latex particles have isoelectric points ranging from pH 4.0 to 4.6, depending on the rubber clonals (8). The variety of isoelectric points perhaps indicates that more than one kind of proteins are adsorbed on the rubber particle and that the relative proportions of the adsorbed proteins on the rubber particle are clone characteristics.

In the case of lipids, they are comprised mainly of neutral lipids and phospholipids (9). The principle phospholipids of the rubber particles are α-lecithin. Other lipids are sterol esters, fats and waxes (Tangpakdee, J., Ph.D. thesis, Tokyo university and technology, Tokyo, 1998). The presence of the phosphatidyl choline and small amounts of phosphatidyl ethanolamine in the lipids associated with the rubber particles has been reported (10). Recently, NR was reported to contain phospholipid molecules at the α-termini of rubber chain associated via mono- and diphosphate groups at the chain-end (11). Moreover, it was also observed that phosphate groups of phospholipids are presumed to originate the branching formation in DPNR (12). For clarity, the proteins and phospholipids associated to the rubber chain-end were referred to associated proteins and phospholipids, respectively.

The surface surrounding the rubber particles has been investigated by many techniques such as phase transfer which is migration of a reactant from one phase into another using counter ion (Baimark, Y., Master thesis, Mahidol university, Thailand, 1998), scanning electron

microscopic (SEM) (13) and transmission electron microscopic (TEM) techniques. It was shown that found the particles are surrounded by film of phospholipids-proteins layer 100 Å in thickness (14). The electrophoresis mobility technique is also used and the isoelectric point of *Hevea* latex is reported to be within the pH range of 3.0-5.0, corresponding to the characteristics of many proteins (15). The isoelectric point, NR particles are stabilized by negative charge of adsorbed long-chain fatty acid and hydrolysis products of phospholipids (16).

However, the proteins and lipids adsorbed on the rubber particles have not been studied in much detail due to the difficulties in removing them unchanged from the particle surface. Therefore, this study attempt to analyze the phospholipids and proteins associated to rubber surface and other components in serum. These properties are compared with those of cream rubber particles before and after removal of proteins and ester components by centrifugation, deproteinization and saponification reaction.

MATERIALS AND METHOD

Plant materials and chemicals

Fresh latex was obtained from Thai Rubber Latex Co., Ltd., Chonburi, Thailand. Sodium dodecylsulfate (SDS), Triton®X-100 and sodium hydroxide (NaOH) were purchased from BDH Company. Proteolytic enzyme (KP 3939) was received from KaO Corporation and it was used in deproteinization reaction. The received proteins from the deproteinization process were precipitated with acetone, trichoroacetic acid (TCA) and 2-mercaptoethanol obtained from Lab scan, Sigma-Aldrich chemical and Amersham Company, respectively. The pellet proteins were dissolved in the mixture solution of urea, CHAP, dithiothreitol (DTT) and Pharmalyte™ purchased from Amersham Company. Acrylamide and coomassie blue R-250 used in SDS-PAGE process were also purchased from Amersham Company. Chloroform, methanol and hydrochloric acid (HCl) were obtained from Lab scan. Acetic acid and methyl stearate were received from Fluka. Synthetic *cis*-1,4-polyisoprene was obtained from KaO company and proteins marker was purchased from Bio Rad company.

Extraction of proteins in rubber latex

Preparation of large and small rubber particles

The material used for sample preparation is freshly tapped natural rubber latex of the RRIM 600 clone of *Hevea brasiliensis,* which was collected and stabilized with 0.2% (w/v) of

Triton®X-100 and centrifuged at 19,000 rpm for 1 h at 20°C (17). The top creamy fraction of rubber particles was collected and re-suspended in 0.2% (w/v) Triton®X-100 solution. The serum fraction was then recentrifuged with the speed of 19,000 rpm for 1 h at 20°C to collect small rubber particles (SRP).

Extraction of proteins from rubber particle by SDS solution

The large and small rubber particles obtained from the centrifugation were dispersed in 2% (w/v) SDS and stirred overnight in the cold room (4°C). Each of the mixture was then centrifuged at 19,000 rpm for 1 h at 20°C to remove rubber particles from the proteins dissolved in SDS solution. Then the proteins dissolved in SDS solution were obtained.

Extraction of proteins from rubber particles by enzymatic deproteinization of latex

The centrifuged natural rubber latex of large rubber particle fraction (30% dry rubber content (DRC)) stabilized with 0.2% (w/v) Triton®X-100 was incubated with 0.04% (w/v) proteolytic enzyme (KP 3939) at 37°C for 16 h followed by centrifugation at 14,000 rpm for 1 h (11). The cream of deproteinized natural rubber (DPNR) was redispersed with 0.2% (w/v) Triton®X-100 aqueous solution and re-centrifuged with the same condition, as well as the obtained cream fraction was then dispersed in 0.2% (w/v) Triton®X-100 aqueous solution .

Extraction of proteins from rubber particles by saponification reaction

Deproteinized natural rubber latex was treated with 1% (w/v) NaOH to decompose phospholipids at 70°C for 3 h in saponification reaction (18) and centrifuged at 19,000 rpm for 1 h at 20°C to separate the serum fraction.

Proteins precipitation with TCA in acetone

Serum fraction was carried out in the presence of 10% (w/v) trichoroacetic acid (TCA) in acetone with 0.07% (v/v) 2-mercaptoethanol. Proteins were subjected to precipitate for 45 min at –20°C. Pellet proteins was collected by centrifugation and further washed with cold acetone containing 0.07% (v/v) 2-mercaptoethanol. Residual acetone was removed by air and the proteins were redissolved in lysis buffer, as following; 8 M Urea, 4% (v/v) CHAP, 60 mM DTT and 2% (v/v) Pharmalyte™.

Proteins analysis by SDS-PAGE

Proteins were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a separating gel of 15% acrylamide and a stacking gel of 3% acrylamide at 50

mA for 1 h (19) and 4 μg protein sample per lane was loaded onto SDS-PAGE gel (17). The gel was subsequently stained with a solution of Coomassie Blue R-250 staining system for 20 min and de-stained for several times with the mixture solution of 125 ml of methanol, 37.5 ml of acetic acid and 337.5 of distilled water.

Determination of nitrogen content

A nitrogen analyzer (LECO FP-258) was used for nitrogen analysis based on the combustion by oxygen gas. 0.25 g of rubber sample was weighed and subjected to the nitrogen analysis. The combustion of rubber sample converts the nitrogen compound to nitrogen gas, which is detected as nitrogen content (% w/w). In this experiment, EDTA was used as a standard with an accuracy of ± 0.02% (w/w). The results were obtained from triplicate analysis.

Determination of particle size

Particle size distribution was determined using Malvern 2000 laser particle analyzer. About 5-10% (v/v) of rubber latex was dispersed in distilled water before the analysis.

Determination of zeta-potential

The stock buffer solution is freshly prepared in the pH range of 4 to 12 for dispersing the natural rubber latex. The sample was prepared by adding one drop of latex to the 20 ml of buffer solution. Zeta-potential value of natural rubber particle was determined using zeta potential instrument (MALVERN S4700 version 1.27). The rubber latex was dropped into de-ionized water and then injected into the sample handling for re-flush and measurement, respectively. Zeta-potential was observed by the conversion of the frequency and scattering intensity function.

Fourier transform infrared (FT-IR) analysis

The rubber samples for FT-IR analysis were prepared by casting 1% (w/v) rubber solutions in chloroform on a KBr disk under a stream of nitrogen. The rubber film was scanned with an FT-IR spectrophotometer (JASCO: FT-IR SA 450 plus) at a resolution of 2 cm^{-1} with 100 scans. The content of long chain fatty-acid ester group was obtained from the intensity ratio of carbonyl group at 1,739 cm^{-1} (C=O) and unsaturated carbon absorbance at 1,664 cm^{-1} (C=C) according to the calibration curve obtained from a mixture of methyl stearate and synthetic *cis*-1,4-polypolyisoprene mixture.

Determination of morphology of natural rubber by scanning electron microscopy (SEM)

The morphology of natural rubber surface was analyzed using a scanning electron microscope (SEM S-2500). The samples were secured onto aluminium stub and coated with

platinum/paradium in a Polaron high resolution sputter coated to obtain enough contrast in the SEM micrographs and the energy of electron beam was 20 kV.

Associated phospholipids extraction

Dry rubber preparation

Fresh NR latex in 0.5% (w/v) SDS solution was centrifuged and cream fraction was dried at 60°C for at least 3 days. Dry cream NR was extracted with acetone in a Soxhlet apparatus for 16 h in nitrogen atmosphere and dried under vacuum oven at 40°C for 3 days. Dry acetone-extracted natural rubber (AENR) was then extracted once again with chloroform-methanol (2:1) mixture in a Soxhlet apparatus under the same condition. The $CHCl_3$-MeOH extracted AENR was obtained and dried in vacuum oven at 40°C for 3 days.

Extraction of linked phospholipids in dry natural rubber after saponification in solution form

Saponification of $CHCl_3$-MeOH extracted AENR 5 g was carried out in 500 ml toluene solution by reacting with 1% (w/v) potassium hydroxide at 70°C for 2 h. The toluene fraction containing rubber solution and phospholipids cleaved from the rubber was neutralized with 6% (v/v) acetic acid and followed by removal of water fraction with separatory funnel.

Rubber fraction in toluene solution was precipitated in excess methanol. The toluene-methanol mixture solution containing associated phospholipids was evaporated and the obtained product was characterized by NMR spectroscopy. Furthermore, acyl chains of phospholipids cleaved from the rubber were converted to fatty acids methyl esters before it was subjected to the GC-MS analysis.

Fatty acids methyl esters preparation

Fatty acids methyl esters were prepared as follows: 2 ml of 1% solution of sodium methylate in methanol were added to 10 mg of total lipids (20). The mixture was kept at 65°C for 2 h, then 2 ml of 5 % solution of HCl in methanol were added and the mixture was kept at 60°C for 0.5 h. After cooling at room temperature, fatty acids methyl esters were stored at -18°C.

Characterization of extracted substances

Determination of fatty acid ester molecule with nuclear magnetic resonance spectrometry (NMR)

The ^{1}H-NMR analyses of extracted lipid in $CDCl_3$ solution were performed at 300 MHz and 50°C. Chemical shifts were determined using Tetramethylsilane (TMS) was used as an internal standard.

Gas chromatography-mass spectrometry (GC-MS)

The volatile components of fatty acid methyl esters were collected and analyzed by GC-MS machine equipped with Innowax™ column (30m x 0.25mm *i.d.*). The carrier gas was helium with a constant pressure of 10.7 psi. The injection split was 1:60. Fatty acids methyl esters were analyzed under the column temperature of 210°C and the temperature of the injector was 250°C. The oven temperature was maintained at 150°C for 1 min, raised at 15°C min^{-1} to 200°C, raised at 2°C min^{-1} to 250°C and then kept at the final temperature of 5 min. The mass spectrometer was operated in electron impact ionization mode (70 eV). The temperature of transfer line and ion source was 280°C. The structure of fatty acids adducts was elucidated from their mass spectra.

RESULTS AND DISCUSSION

Study of basic properties of NR latex

Hevea latex is originally composed of spherical particles. Some of these particles become attached and form group or chains. Table 1 shows that the particle size of rubber particles from whole fresh NR latex is relatively smaller than that form centrifuged latex, deproteinized latex and saponified latex. The size of the small rubber particle from the serum phase is smallest as 189 nm. As expected, the natural rubber particles rapidly combined together due to their protein and phospholipid layer were removed.

The protein contents of small rubber particles and fresh latex were found to be high, while those of centrifuged latex, deproteinized latex and saponified latex were less than the obtained former cases. This indicates that the centrifugation of natural rubber latex in the presence of Triton®X-100 could significantly reduce soluble proteins but it allowed the tightly bound of some proteins to the natural rubber particles. Therefore, the most effective method to remove the proteins from the natural rubber is the treatment of natural rubber latex with proteolytic enzyme in deproteinization, followed by the centrifugation for removal of the decomposed proteins. In addition, the almost of all proteins associated rubber particles were also reduced by strong alkali hydrolyzes in the saponification reaction. As expected, the natural rubber particles became unstable after removal of protein associated rubber particles. The fatty

acid ester content involves both free- and associated-phospholipid as well as fatty acid esters presenting in the natural rubber. Table 1 shows fatty acid ester content in dry rubber samples, which remains abnormal groups such as hydroxyl group, aldehyde group and lactone group that can be detected by FTIR spectroscopy. It was clarified that the deproteinized latex composed of the low associated-fatty acid ester content compared to that obtained from centrifuged latex, while the associated fatty acid ester value of the saponified latex became to zero due to the removal of phospholipid groups. In addition, natural rubber was reported to contain saturated and unsaturated fatty acids (21). Therefore, the signal overlapping of unsaturated fatty acids and polyisoprenoid could have an effect on the determination of fatty acid ester content in natural rubber.

Table 1 The mean diameter, protein content and linked fatty acid ester content of fresh natural rubber latex, centrifuged latex, deproteinized latex, saponified latex and small rubber

Sample	Diameter (nm)	Protein content (%w/w of dry rubber)	Ester content (mmol/kg rubber)
Fresh latex	636 ± 2	4.30 ± 0.02	28.1 ± 0.2
Centrifuged latex	655 ± 1	0.96 ± 0.03	38.5 ± 0.5
Deproteinized latex	664 ± 3	0.07 ± 0.02	30.9 ± 0.7
Saponified latex	678 ± 1	0.05 ± 0.02	~0
Small rubber particles	189 ± 2	1.95 ± 0.03	~0

Characterization of extracted proteins in natural rubber latex

Proteins of natural rubber latex were distributed into two fractions, which are the rubber and serum fractions. For the characterization of proteins in two fractions, the obtained proteins were extracted after the process of many chemical reactions and washing with surfactant solution. It was noted that the removal of protein in rubber was confirmed with the decrease of protein content. These removed proteins were then analyzed using SDS-PAGE technique, as shown in Figure 1. SDS-PAGE gel image shows the standard proteins (lane A), soluble proteins

(lane B), decomposed proteins after deproteinization reaction (lane C), decomposed proteins after saponification reaction (lane D), SDS-extracted proteins from large rubber particles (lane E) and SDS-extracted proteins from small rubber particles (lane F).

In this result, SDS-PAGE of lane B revealed the protein band at the region of 6 to more than 200 kDa. The observed major bands were reported that they were derived from B- and C-sera of fresh latex, as well as rubber particles. The B-serum was incidentally released from the lutoids, which it can be burst by adding the ammonia. Molecular weights of proteins upper 30 kDa were mostly originated from the serum fraction, while protein molecular weight of 33 kDa and that of lower than 30 kDa were generated from the rubber fraction. Therefore, it can be assumed that these proteins are polar protein because they can reside in the water phase (17).

In lane C and D, they were found to contain low molecular weight of proteins, which were called as decomposed proteins due to the hydrolyzes of proteolytic enzyme and strong alkali, as well as short peptide chains were produced. In lane E and F, they were proteins removed from large and small rubber particles by SDS extraction. It was noted that SDS could extract the proteins associated with the rubber particles after the isolation of serum fraction. Although the received protein solution was contaminated with some SDS molecules, this surfactant could be removed using dialysis (18). SDS-PAGE of lane D revealed a prominent protein band at about 14.5 kDa and two less prominent bands at 25 and 27 kDa. In the case of small rubber (lane E), the protein bands at 14 and 27 kDa were also observed, but the intensity band at 27 kDa was higher than that of 14 kDa. It was found that some molecular weights of proteins were similar to Rubber Elongation Factor or REF (14.5 kDa) and Hevamines (29 kDa), which were known as allergic protein (22, 23).

Based on the study of the biosynthesis of natural rubber, it was believed that proteins consisting in natural rubber are the important factor concerning the initiation of natural rubber (24). Thus, the differentiation of the proteins band intensity indicates that proteins at the initiating end of small and large rubber particles are different owing to rubber molecules of large rubber particle are extended more than that of small rubber particle. During the chain extension process, the initiating end might be modified and gave the modified proteins, which is unidentified yet.

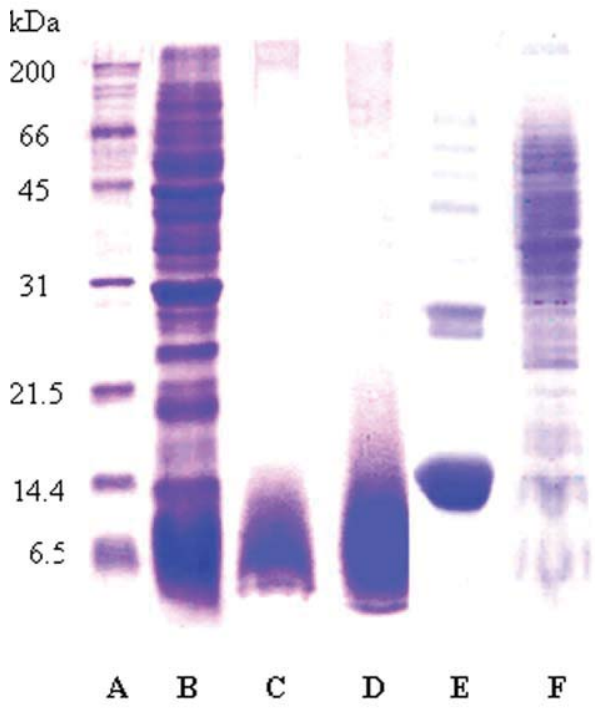

Figure 1 SDS-PAGE images (15% gel) of proteins from the rubber and serum fractions; (A) standard protein, (B) soluble protein, (C) decomposed protein after deproteinization, (D) serum protein after saponification and (E) SDS-extracted proteins from large rubber particles (F) SDS-extracted proteins from small rubber particles**.**

Characterization of extracted associated phospholipids in natural rubber

FTIR spectroscopy

In this study, it was also focused on the extraction of phospholipids associated to the natural rubber particle from dry rubber. As described previously that free phospholipids in natural rubber were concerned with non-rubber components, which might disturb the analysis of associated phospholipids structure. First, we have tried to decrease this side effect by preparing the dry rubber without free phospholipids. It was prepared from fresh natural rubber latex, which was subjected into the centrifugation and dry. Secondly, the obtained dry rubber was washed by acetone extraction to remove free fatty acid following by the chloroform-methanol extraction to remove free phospholipids and we can receive the dry rubber without free phospholipids in this step. Third, the obtained dry rubber was treated with KOH to decompose phospholipids in saponification reaction and neutralized with acetic acid. It was then

precipitated with methanol to separate coagulated rubber fraction and supernatant. The received rubber fraction was characterized by FTIR and the associated phospholipids presented in the supernatant, can be extracted with chloroform and analyzed by ^{1}H-NMR.

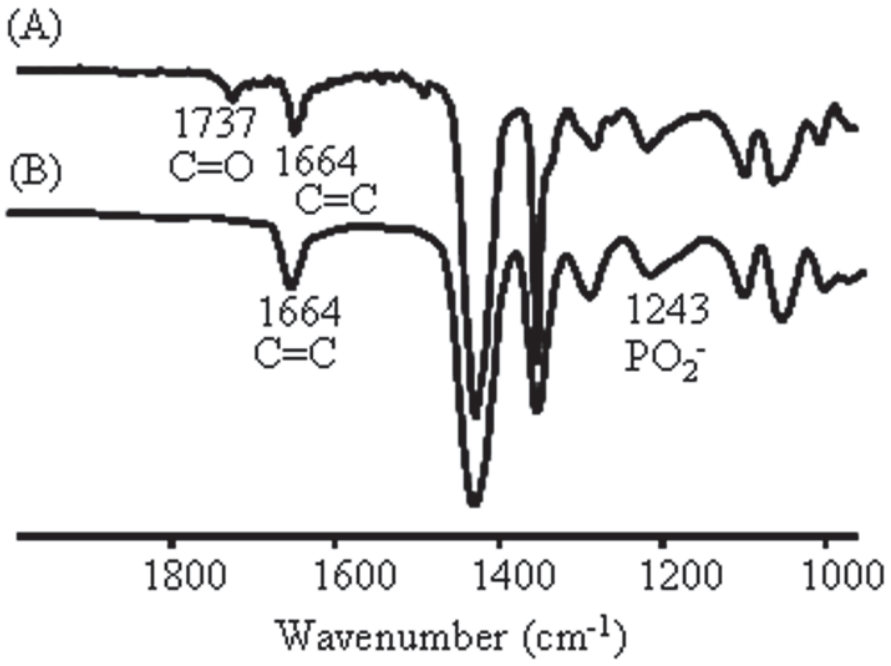

Figure 2 FTIR spectra of (A) NR and (B) phospholipid removed NR.

FTIR spectra of natural rubber before and after removal of associated phospholipids are shown in the Figure 2. It has been reported that a phospholipid shows the C=O stretching band of associated fatty acids ester at the range of 1738 - 1742 cm^{-1} (11). Moreover the absorption peak at 1240 cm^{-1} was assigned to O-P-O asymmetric stretching of phospholipid (25). This assignment of FT-IR spectrum corresponded to the structural analysis of phospholipids at the rubber chain-end by Tarachiwin *et al*. (12). In this study, the FTIR spectrum was utilized to confirm the remaining of associated phospholipids in dry natural rubber. Although the dry natural rubber sample was prepared using ultracentrifugation, the transmittance peak of carbonyl group in associated fatty acids at 1737 cm^{-1} and that of phosphate group at 1243 cm^{-1} were still observed in this FT-IR spectrum. It is the cause from phospholipid molecule linked to the rubber chain, while free fatty acids and free phospholipids were removed by centrifugation.

In another case, the transmittance peak of carbonyl group at 1737 cm^{-1} in the dry saponified NR sample disappeared, which it means that the associated phospholipids were removed. Therefore, it can be noted that the saponification reaction has high efficiency for the removal of associated phospholipids. Moreover, these associated phospholipids were then characterized and confirmed the chemical structure using ^{1}H-NMR spectroscopy.

^{1}H-NMR spectroscopy

The ^{1}H-NMR spectra of phospholipids standard of L-α-phosphatidylcholine (PC) and extracted associated phospholipids are given in Figures 3a and 3b, respectively. It was revealed that all signal positions on the spectrum of extracted phospholipids were not significantly shifted when they were compared to that of standard phospholipids. Characteristic signals of methylene and long chain methylene protons in fatty acid ester group were clearly observed at the chemical shift of 0.96 and 1.24 ppm, as the resonances around 2.05 and 2.82 ppm were assigned to the methylene protons next to the unsaturated ($C\underline{H}_2CH=CH$) and allylic ($C\underline{H}_2(C=C)_2$) bonds of fatty acid ester group, respectively. Moreover, the vinyl protons were found at 5.48 ppm. Methylene protons near the carboxyl group, $C\underline{H}_2$-OC=O, of PC gave the signal at 5.18 ppm, while those of the extracted associated phospholipids also showed a singlet signal at 5.18 ppm. In addition, two methylene protons linked to phosphate group of phospholipid, $C\underline{H}_2OP$, and the signal of the $C\underline{H}OC=O$ protons in glyceride backbone of the extracted phospholipids appeared at 4.29 and 5.89 ppm. The polar head group showed a unique resonance characteristic of each phospholipid. The resonance at 4.40 ppm of PC was assigned to the $C\underline{H}_2$-OP of choline group overlapping with $C\underline{H}_2OC=O$ protons of glyceride backbone. The signal of $C\underline{H}_2$-OP protons in ^{1}H-NMR spectrum of extracted associated phospholipids was appeared at 4.39 ppm and the resonance of $-N^{+}(C\underline{H}_3)_3$ protons was clearly observed at 3.54 ppm. The appearance of these signals clearly indicates that this fraction contains phospholipids associated to the rubber chain. The assignment of phospholipids associated to rubber chain is in good agreement with that by Tarachiwin *et al*. (11).

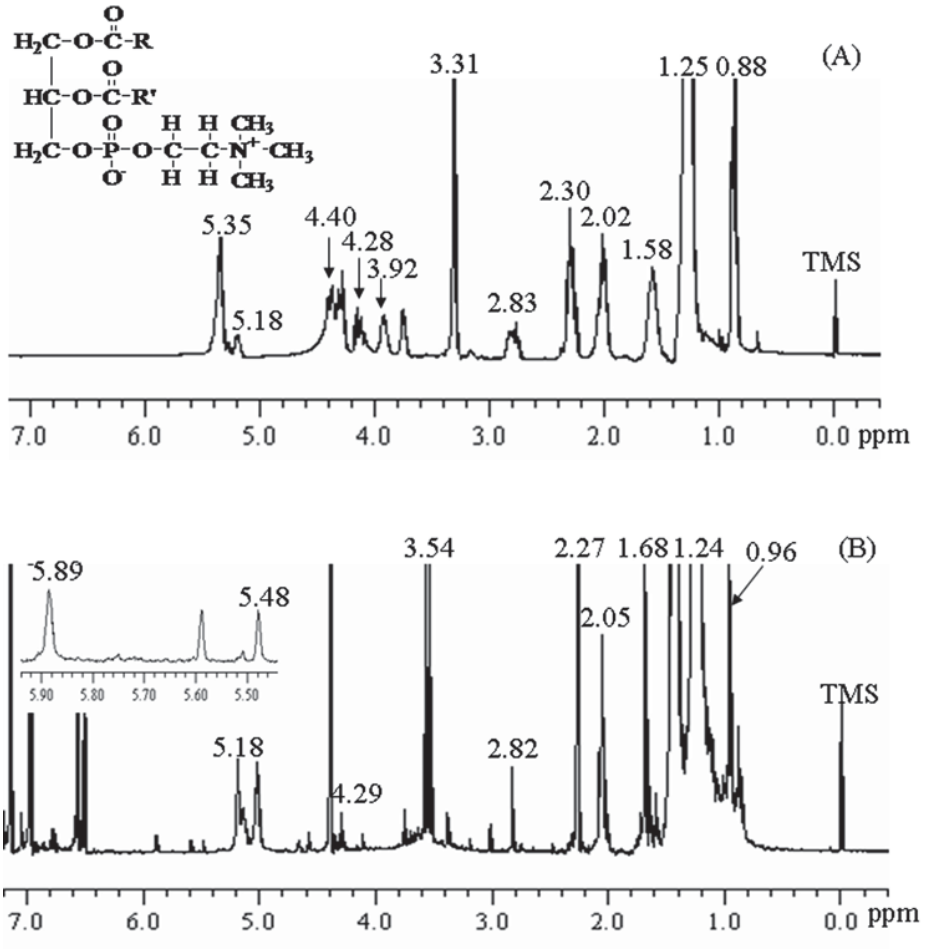

Figure 3 ^{1}H-NMR spectra of (A) L-α-phosphatidylcholine and (B) the extracted phospholipids in natural rubber.

The natural rubber latex, *H. brasiliensis* (RRIM 600), is generally known that it contains phospholipids. Owing to the phospholipids have the large molecular structure, they are hardly identified using gas chromatography-mass spectrometry (GC/MS) technique which was utilized for small molecule detection. To perform this analysis, the obtained lipids from rubber particle were formed fatty acid methyl esters by transesterification reaction. This technique is the efficient step to change a complex lipid mixture to simple molecular species (26). The obtained fatty acid methyl esters were characterized by GC-MS. All reference databases of fatty acids were then used to identify these fatty acid methyl esters. Therefore, the identification of phospholipids associated to rubber particles can be revealed by GC-MS chromatogram.

Characterization of fatty acid of associated phospholipids in natural rubber by GC-MS

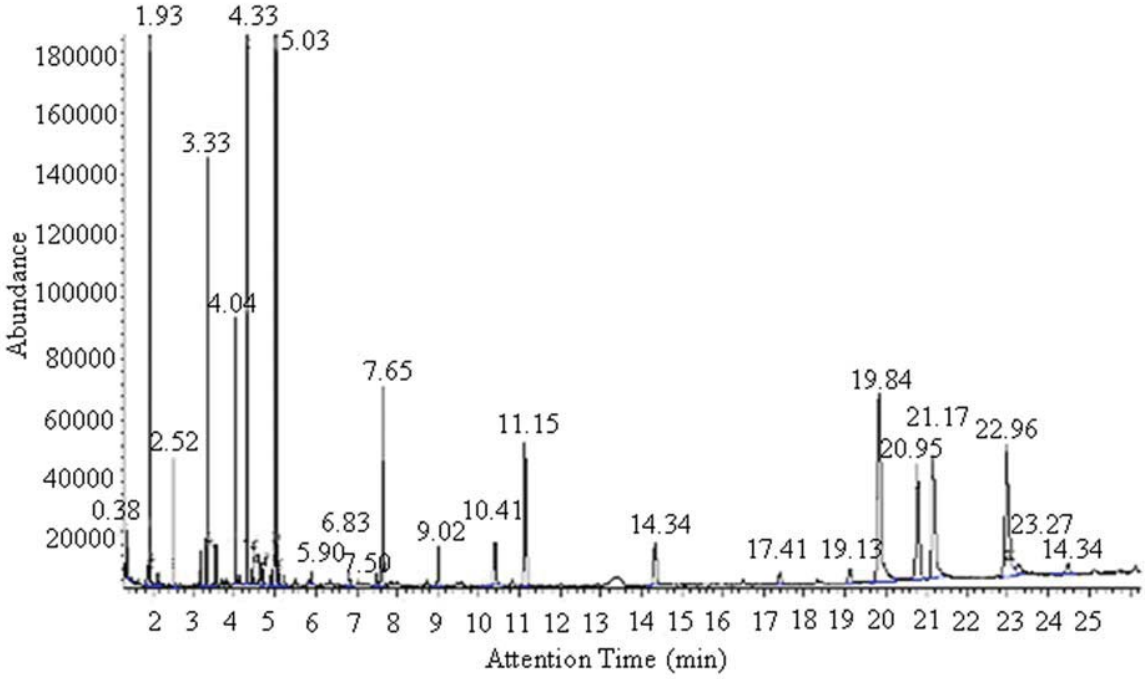

Figure 4 Gas chromatogram of fatty acid methyl esters from the extracts. The chromatogram was obtained on Innowax^{TM} capillary column at 210°C.

Figure 4 shows the GC-MS chromatogram of the extracted phospholipids obtained after saponification reaction of $CHCl_3$-MeOH extracted AENR. It can be observed the chromatogram that many kinds of fatty acids were liberated from this extract. It was expected that these fatty acids were obtained from the part of fatty acids associated to phospholipids. In other words, this may be due to these fatty acids were derived from hydrolysis of phospholipids during saponification reaction. In addition, retention time at around 4.63 min showed the peak related to 2, 6-Di-tert-butyl-p-cresol (BHT), which was used to be an antioxidant in the saponification reaction. It was implied that it remained the residual BHT in the extracts. The obtained result revealed that some components in the extract were fatty acids, which have been reported as the associated fatty acids in natural rubber latex. The data (Figure 4) indicated that fatty acid components associated phospholipids were capable of palmitic acid, stearic acid, lauric acid, myristic acid, linoleic acid and linolenic acid. These peaks palmitic acid (RT 5.09), stearic acid

(RT 6.84), lauric acid (RT 7.51), myristic acid (RT 10.41), linoleic acid (RT 21.17) and linolenic acid (RT 22.99), can be attributed to 16:0, 18:0, 12:0, 14:0, 18:2 and 18:3 esters, respectively. These peaks confirmed the presence of associated fatty acid in phospholipids. High abundance of the fatty acids was found that they consist of linoleic acid and linolenic acid. This may be due to these fatty acids were derived by hydrolysis of phospholipids during saponification reaction.

Effect of pH and ionic strength

The latex from *Hevea brasiliensis* was stabilized by Triton®X-100. It was then analyzed as a function of pH by zeta potential measurement. Figure 5 shows the zeta potential values of colloidal natural rubber which was evaluated in the medium with various pHs between 4-12 at an ionic strength constant of 0.015 M NaCl. The negative zeta potential value of rubber latex presented the negative charge on rubber particle surface. The almost linearly decrease of negative zeta potential values was found as the pH value of medium solution decrease and they became constant above pH 10 in all samples. This behavior was similar to the previous study (27), which was reported that negative zeta potential value would decrease with the decrease of pH value until it reached the isoelectric point (IEP). The negative charges were attributed to ionization of carboxylic acid groups surrounding on rubber particle surface. It is observed that the negative zeta potential value of the small rubber particles is lower than that of the fresh NR latex sample in all pH values. As pointed out above, these results were revealed that the stability of fresh NR latex is higher than that of small rubber particles. It could be explained that the increase of the amount of adsorbed long chain fatty acids and proteins leads to the increase of charge on the rubber particle surface. The distinction in the chemical nature of the adsorbed layer was reflected in the difference in the stability of two lattices. The more amounts of long-chain fatty acids and proteins adsorbed on

rubber surface, the higher negative zeta potential value of the NR particles was appeared.

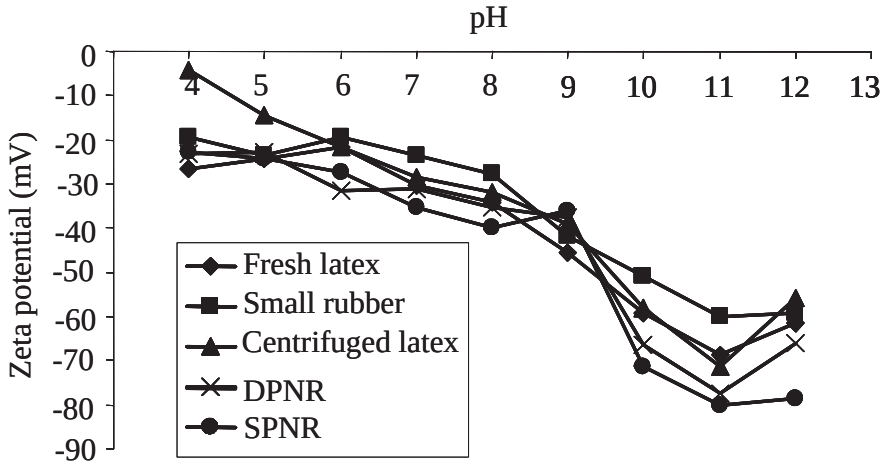

Figure 5 Zeta potential value of sample with ionic strength 0.015 M NaCl.

Table 2 shows the study of zeta potential value of natural rubber latex at pH 9 with ionic strength 0.015 M NaCl condition. It was revealed that the zeta potential value of natural rubber latex is always negative value due to the negative charge of proteins and carboxylic groups surrounding the rubber particles. It was reveal that the zeta potential value of fresh latex and small rubber were higher than that of the centrifuge latex, deproteinized latex and saponified. It can be explained that the negative charge on the rubber particle surface decreased due to the proteins and phospholipids were removed

.

Table 2 Zeta potential value of natural rubber latex at pH 9 with ionic strength 0.015 M NaCl

Sample	Zeta potential (mV)
Fresh latex	-45.7 ± 0.5
Centrifuged latex	-39.3 ± 0.1
Deproteinized latex	-38.7 ± 0.3
Saponified latex	-35.9 ± 0.1
Small rubber particles	-41.7 ± 0.5

The fresh latex mainly compose of phospholipids, carbohydrates, proteins and metal ion which effect to zeta potential value and it leads to the highest of zeta potential value. Since any attempt to remove the serum (water phase) by centrifugation, it is reasonable to assume that the soluble protein and metal ion are lost and it causes the lower negative zeta potential value in centrifuged latex sample. Moreover, the removal of the phospholipids and proteins results in the exchange of the chemical composition of the rubber/water interface in NR latex. It was found that the zeta potential values of both deproteinized latex and saponified latex samples are in the vicinity. Deproteinization of fresh latex leads to the low nitrogen content, as seen in Table 1. It may be due to long peptide chain was decomposed by proteolytic enzyme in the presence of surfactant, and it causes the negative zeta potential value become lower. The existence of the residual phospholipids and short peptide chain on the rubber particle surface could be removed by saponification with NaOH/Triton®X-100 in the solution. It is reasonable to assume that the decrease in the negative zeta potential value of deproteinized latex was lower than that of the saponified latex.

Study of morphology of natural rubber particles by scanning electron microscope (SEM)

Electron microscopic examination of rubber particles (28) was indicated that NR particle consists of hydrophobic rubber polymers enclosed by a monolayer membrane. This membrane consists of lipids, proteins and other components. Furthermore, these components also associate with the good mechanical properties of NR. Therefore, the natural rubber particles were subjected to study their physical and chemical characteristics. Many suitable methods were rapidly developed to clarify the rubber particle membrane. The method, developed for determining the size and shape of rubber particles using SEM, involved with air-drying of a particle suspension after fixation in osmium tetroxide.

The scanning electron micrographs of fresh latex, centrifuged latex, deproteinized latex, saponified latex and small rubber are shown in Figure 6. In the micrographic image, small features can be seen on the rubber particle surface. In comparison, in air-dried preparation particles of freshly tapped latex from mature *Hevea* tree remained their natural shape and size. *Hevea* rubber particles were spherical, occurring predominantly as discrete individual particles in a range of sizes. Size measurements for *Hevea* particles placed the particles in 0.10-0.75 μm range, which agrees with previously reported values for particle size in this species (28, 29).

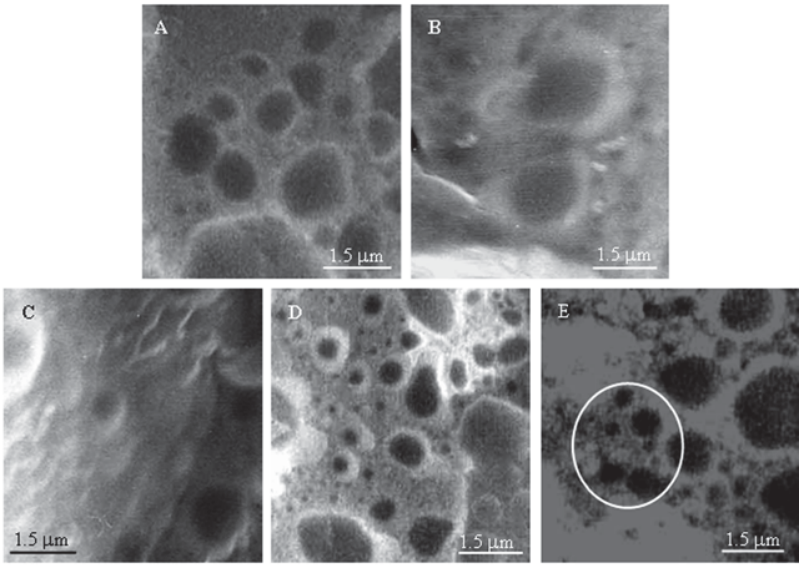

Figure 6 Scanning electron micrograph of the rubber particles; (A) fresh latex, (B) centrifuged latex, (C) deproteinized latex, (D) saponified latex and (E) small rubber particles showed in closed circle.

SEM analysis reveals that the features of rubber particles surface are most probably proteins and phospholipid. In the case of fresh latex (Figure 6A), a continuous electron-rich membrane was clearly observed around the rubber particle surface. Inside the rubber particle, in most cases, a homogenous electron density distribution was observed: a less-dense region (dark gray) and a denser region (light gray). An enlargement of the centrifuged rubber particles (Figure 6B), the gray ring was clearly observed, which is probably the phospholipids-proteins layer. In the case of deproteinized (Figure 6C) and saponified rubber particle of cream rubber (Figure 6D), the gray rings were also observed. This is probably the surfactant layer. It could be explained that the rubber particle surface would be replaced by surfactant layer for stabilization of rubber particles after the removal of phospholipids-proteins layer by deproteinization and saponification treatments. While the small rubber particle (Figure 6E) could not be observed its gray ring, this might correspond to the results on the lower content of proteins and fatty acid esters, as given in Table 1.

In this study, the serum phase of natural rubber latex showed the molecular weight of proteins different from those of rubber particles. The proteins of serum phase were in regions of 6 to more than 200 kDa, while the major proteins of rubber particle were 14.5 and 29 kDa, which were similar to Rubber elongation factor and Hevamines, respectively. It was also found that enzymatic deproteinization could breaks down the protein linkages selectively and remained oligopeptide groups associated to rubber molecules. The saponification can remove both of protein and phospholipid linkages. Characterization of the extracted associated phospholipids obtained from saponification treatment of dry AENR, were carried out by ^{1}H-NMR spectroscopy and GC-MS, compare with database. The ^{1}H-NMR spectrum of the extracts revealed major signals, which are assignable to -($\underline{C}H_2$)$_n$-, -CH_2OP, -$\underline{C}H_2OC$=O and -O$\underline{C}H_2CH_2NH$-. The appearance of these signals indicates that this serum contain phospholipids associated to rubber chain. Based on GC-MS chromatogram, the data signified that the composition of fatty acid ester groups associated to NR molecule at the terminal was identified to be saturated fatty acids contained palmitic acid, stearic acid, lauric acid, myristic acid, and unsaturated fatty acids such as linoleic acid and linoleinic acid, which evidenced the presence of

associated fatty acid in phospholipids. Based on SEM and zeta potential analyses, it was revealed that there are phospholipid-protein layers surrounded on surface of rubber particles to stabilize colloidal of NR latex. After removal of phospholipid-protein layers by deproteinization and saponification, surfactant layer replaced the surface of rubber particles.

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PART 2 SURFACE NANOSTRUCTURE OF *HEVEA BRASILIENSIS* NATURAL RUBBER LATEX PARTICLES

ABSTRACT

Natural rubber (NR) from the *Hevea brasiliensis* tree is the main commercial feedstock for rubber and latex dipping industries. NR latex particles in a matured commercial latex concentrate are stabilized by charged groups derived from proteins, long-chain fatty acid soaps and polypeptides adsorbed on the particle surface. The colloidal stability of the latex is extremely sensitive to pH as well as to the ionic environment of the dispersing medium. Previous work on particle microelectrophoresis has shown that the charge on the particle surface is derived mainly from carboxylic groups of long chain fatty acids (*ca.* 86%). Freshly collected field latex from the tree, on the other hand, is believed to be stabilized by proteins and phospholipids before hydrolysis sets in upon leaving the tree. The exact arrangement of the adsorbed proteins and phospholipids and the thickness of this adsorbed layer have yet to be successfully determined. The present study has been focused on unraveling the arrangement of these proteins and phospholipids on the particle surface of a freshly tapped NR latex. In order to visualize these molecules, they were imaged using phase contrast atomic force microscopy (AFM). Moreover, the molecules in the particle were labeled with fluorescent Rhodamine B (RB) and uncharged fluorescein-5(6)-isothiocyanate (FITC), and monitored by confocal laser scanning microscopy (CLSM) thereby permitting an *in situ* observation of their locations on and inside the particles. The selective labeling of the proteins and phospholipids with RB and FITC in protein-free as well as in protein-and-lipid-free latex particles permitted an unequivocal determination of their presence both on the surface and in the interior of the particles. An indentation study of the surface layer of the original untreated NR particle was also carried out to confirm the hypothesis of a core-shell structure with a mixed layer of proteins and phospholipids surrounding the hydrophobic core of polyisoprene particles. According to the obtained results, the surface of the particles in freshly tapped latex is distinctly different from that of a matured latex concentrate.

KEYWORDS: Natural rubber latex particle, surface structure, atomic force microscopy, latex proteins, lipids

Introduction

Natural rubber (NR) from *Hevea brasiliensis*, composed primarily of *cis*-polyisoprene, is an important source of natural rubber due to its excellent physical properties. Solid NR consists of approximately 94% rubber hydrocarbon and 6% non-rubber components such as lipids, proteins carbohydrates, etc.[1] These non-rubber components play an important role in stabilizing the latex particles and in contributing to the outstanding properties of NR.[2] Recently, the molecular structure of the NR molecules has been found to comprise 2 *trans*-isoprene units connected to long-chain *cis*-isoprene units. Moreover, two terminal groups, referred to as ω and α, have been postulated to link with mono- and di-phosphate groups associated with phospholipids by H-bonding at the α-terminal, whereas the ω-terminal is a dimethylallyl group linking to the protein by H-bonding.[3] From ^{13}C-NMR analysis, the NR molecule has been found to be functionalized with phospholipid-comprising branch-points.[4] Phospholipids in NR latex have been identified as mainly L, α-phosphatidylcholine and phosphatidylethanolamine.[5] The molecular structures of these phospholipids are shown in Figure 1.

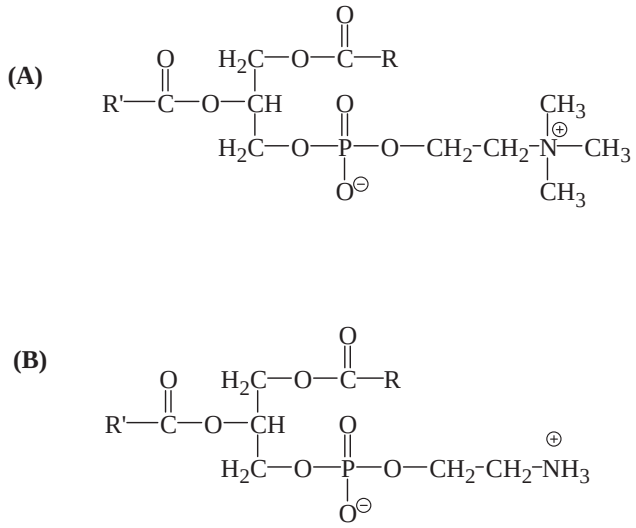


Figure 1 The chemical structures of (A) L, α-phosphatidyl choline, and (B) phosphatidyl ethanolamine where R and R' are long-chain alkyl groups.

Numerous studies on the structure of the NR particle surface have been published,[6-10] two possible scenarios have emerged on the arrangement of proteins and phospholipids on the NR particle surface. These include a double-layer in which an inner layer of phospholipids resides beneath a protein layer on the outside of a rubber particle[6,7] or a mixed monolayer of proteins and phospholipids.[8,10] Based on transmission electron microscopy (TEM) analysis, Gomez *et. al*. estimated the surface structure to *ca.* 10 nm in thickness.[9] Although numerous reports have reported on the protein-lipid layer surrounding the rubber particle, the detailed structure of this layer has yet elucidated in terms of the spatial arrangement of the proteins and phospholipids. The reason for this is that there is so far no direct experimental technique permitting the *in situ* and intact study of these molecules on the latex particle surface. The film formation process of NR latex is found to be strongly influenced by the presence of the non-rubbers - in particular, the proteins and phospholipids.[11]

With recent rapid advances in instrumental analysis and visualization techniques such as atomic force microscopy (AFM), it is now possible to visualize surface structures at the nano-scale.[12] AFM has been also used to investigate nano-mechanical properties of surfaces and interfaces, e.g., friction and adhesion forces, indentation moduli, surface viscoelasticity, *etc*.[13-17] Employed in combination with other techniques, e.g., confocal laser scanning microscopy in conjunction with selective fluorescence labeling of specific constituents in composite systems, AFM has become a powerful tool for studying complex multi-component systems.[18-19]

The present paper describes the application of these combined techniques to unravel the surface morphology of freshly tapped NR latex particles. Fluorescence labeling would provide information on the distribution of proteins and phospholipids on the latex particle surface, whereas AFM with an indentation study should yield important information on the properties of the mixed layer of proteins and phospholipids on the latex particle surface.

Experimental Section

Materials. NR latex, obtained from *Hevea* trees of the *RRIM 600* clone, was collected in an ice-cooled cup. This freshly tapped NR latex, called fresh latex (FL-latex), was filtered with muslin cloth to remove any impurities and then preserved by adding ammonia (0.6% v/v). Protein-free latex or deproteinized natural rubber (DPNR) latex was prepared by treating FL-latex with 0.04% w/v proteolytic enzyme (KP-3939, Kao Co. Japan) in the presence of 1% v/v of sodium dodecyl sulphate (SDS) solution followed by incubation at 37°C for 12 h under

stirring. The resulting latex was then centrifuged twice at 19,000 rpm (43,300 *g*) for 40 min. The collected cream fraction was diluted with distilled water to obtain a 30% w/w DPNR latex. Lipid-free latex was prepared by subjecting a 15% w/w DPNR latex to saponification using 2% w/v NaOH solution followed by incubation at 70°C for 3 h and then cleaned by centrifugation as outlined above.

Preparation of an NR film and surface analysis by atomic force microscopy (AFM)

An NR film was obtained by dip-coating freshly tapped, lightly ammoniated NR latex on a cleaned glass substrate with a dipping speed of 0.25 mm/sec. Visualization and force measurements of this latex were obtained using a NanoScope IIIa atomic force microscope from Digital Instruments (Santa Barbara, CA) equipped with a J scanner for a maximum scan area of 150 μm. Images were recorded in a tapping mode using silicon tips (Nanosensors, Germany). The Z scan rate was 1 Hz. Moreover, force curves were obtained in contact mode with silicon nitride cantilevers having a 0.12-0.18 N/m spring constant (Nanosensors, Germany), and were recorded at random locations on the particle surface to obtain reproducible results.

Data analysis according to the *Hertzian* model

Assuming that both the AFM tip and the NR particle were spherical in shape, the *Hertzian* model was selected to fit the approaching force-distance data.[20-21] The indentation represented by the difference between the piezo movement ($z-z_0$) and the cantilever deflection ($d-d_0$) is given in Equation (1). In this study, the NR particle was assumed to behave like a vesicle surrounded by a protein-lipid surface layer with rubber hydrocarbon as the polymer core.

$$\left|Z - Z_0\right| - \left(d - d_0\right) = \delta = A\left(d - d_0\right)^{2/3} = 0.825\left[\frac{k^2\left(R_{tip} + R_{ves}\right)\left(1 - \nu^2{}_{ves}\right)^2}{E^2{}_{ves} R_{tip} R_{ves}}\right]^{1/3}\left(d - d_0\right)^{2/3} \qquad (1)$$

Here, δ is the indentation, E_{ves} is Young's modulus of the vesicle, R_{tip} and R_{ves} are the radii of the tip and vesicle, respectively, ν_{ves} is Poisson's ratio of the vesicle, and k is the cantilever spring constant. Theoretically, a Poisson ratio of 0.5 the vesicle should be employed

to calculate Young's modulus.[20,22-23] Moreover, a tip radius of 50 nm were used. E_{ves} could be calculated by measuring the slope of the plot between the cantilever deflection ($d-d_0$) and $\delta^{3/2}$.

Fluorescence measurement by confocal fluorescence microscopy

A 5% w/w NR latex was labeled with a 0.01 mol dm^{-3} fluorescence dye solution, Rhodamine B (RB), by mixing at a 1:1 ratio. The mixture was gently stirred at room temperature for an hour, after which it was dialyzed three times against Milli-Q water to remove the unlabeled RB. A dialysis membrane with a 50-kDa molecular weight cut-off was utilized. RB molecules electrostatically bound to SDS were removed by dialysis three times against a 0.1-mol dm^{-3} NaCl solution. To verify the effect of RB, the above experiment was repeated with an uncharged fluorescence dye, fluorescein-5(6)-isothiocyanate (FITC). In this case, no dialysis with NaCl solution was required. The fluorescence emission of RB-labeled NR latex particles was determined with a Carl Zeiss LSM 510 laser scanning microscope equipped with a multi-line, 543-nm He Ne laser and Ar laser (458, 488, and 514 nm) as excitation light sources for RB and FITC, respectively. Moreover, a 63× oil-immersion objective was utilized.

RESULTS AND DISCUSSION

Morphology of NR latex particle and the protein-lipid layer

AFM images, obtained in tapping mode, of dip-coated slightly ammoniated NR latex particles, taken one day after dipping, revealed that the NR latex particles were predominantly spherical in shape and randomly packed. This was the result of the NR particles being very polydisperse in size. However, the non-rubber components on the rubber particle could not be clearly visualized from the morphology alone. Nevertheless, this issue was overcome by using a phase contrast technique that has been extensively utilized beyond simple topological imaging to detect variations in composition, adhesion, friction and other properties.[24-26] The phase images of the NR particle shown in Figure 2 (B) exhibited separate phases. The light and dark brown areas, were indicative of the various components on the NR particle surface. Based on the assumption that the NR particle was covered with proteins and phospholipids, the two areas with different phase contrasts were inferred to areas of the adsorbed proteins and phospholipids.

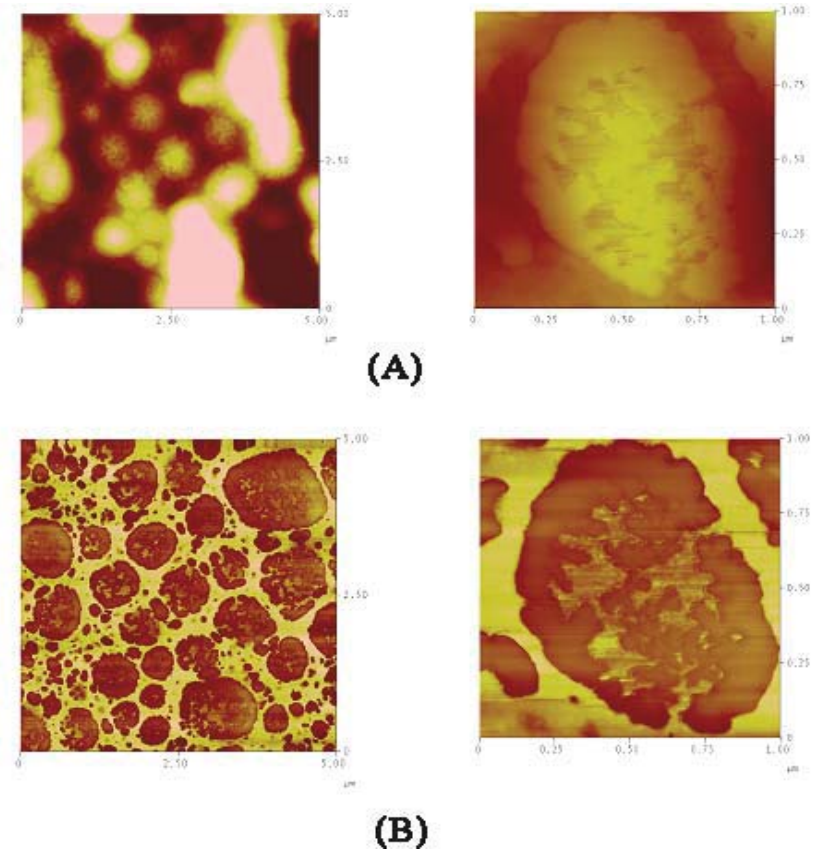


Figure 2 Micrographs from AFM in tapping mode of 20% (w/w) freshly tapped NR latex dip-coated on a glass substrate. The images were taken the day after preparation of the NR film. The Z scale for the height images is 0-200 nm from dark brown to yellow, whereas it is 0-180° for the phase images. The scan size for the images to the right is 5 μm, and 1 μm for those to the left. (A) Two-dimensional images and (B) phase contrast images of the same region.

The non-uniform phase contrast images suggested that the NR particle surface was surrounded by a layer made up of mixed domains of proteins and phospholipids (Fig 3B), in contrast to the previously perceived structure of a double-layer (Fig 3A).[6,8,27-29] Here, the NR molecule linked with proteins and lipids at respectively the ω- and α- terminal ends was believed to orientate themselves, so that these hydrophilic ends were located on the particle surface as a thin layer. The polyisoprene molecules should then form the hydrophobic core, in essence giving rise to a core-shell-like particle.

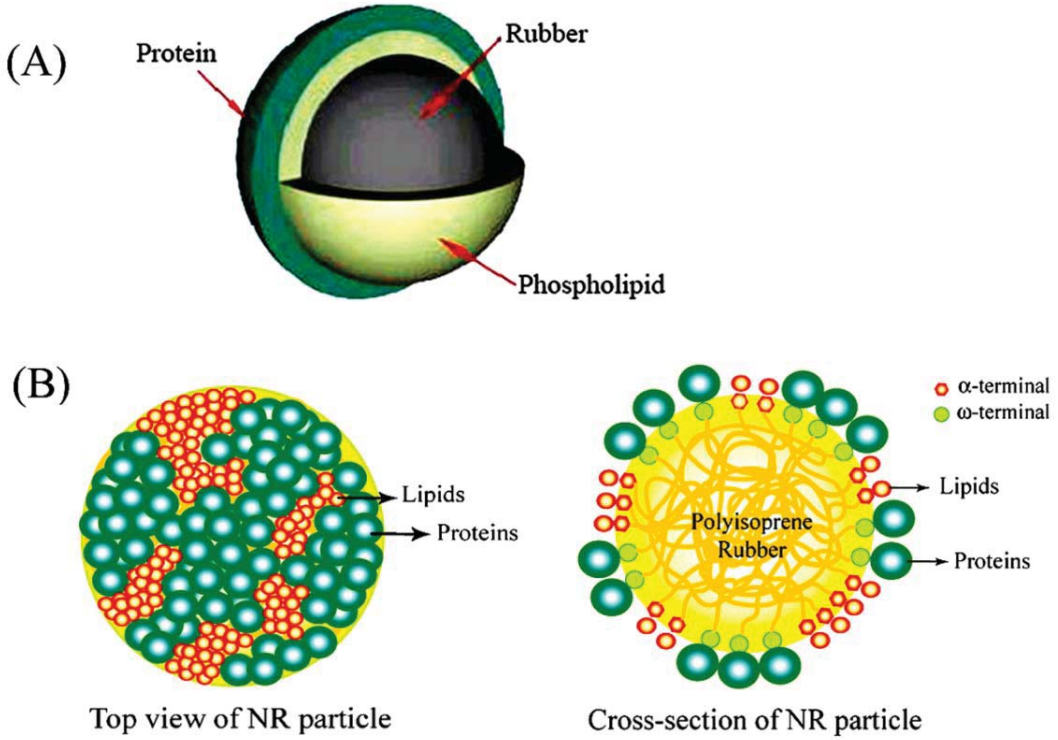
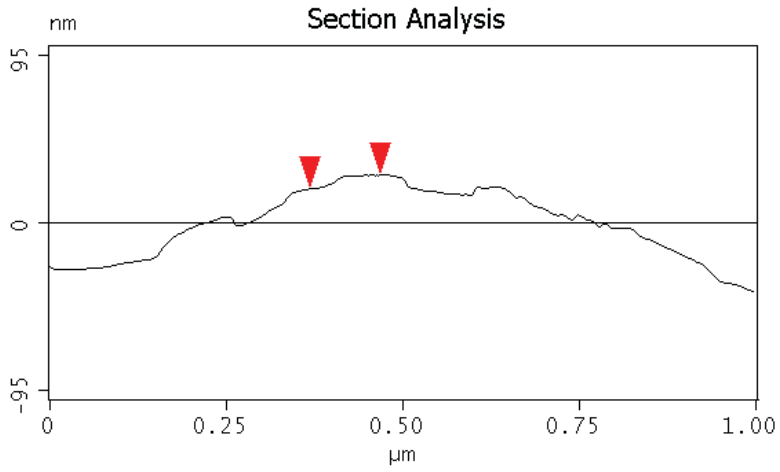


Figure 3 Two possible models for the structure of the rubber latex particle surface. (A) A current model of an NR latex particle surrounded by a double-layer of proteins and phospholipids, and (B) the proposed new model consisting of a mixed layer of proteins and lipids around the latex particle.

The proteins and phospholipids were phase-separated into domains on the surface of the NR particle. To obtain further detail of these domains, a cross-sectional profile of the surface was analyzed and the result is depicted in Figure 4. The average difference in vertical heights between the clusters was 5.4±2 nm. This value was taken to represent the size difference of the protein and phospholipid molecules on the NR particle surface. The proteins associated with the rubber particle have been reported to have molecular weights of about 14.5, and 24 kDa.[30-31] In contrast, phospholipids such as phosphatidyl choline and phosphatidyl ethanolamine, have approximate molecular weights of about 0.7-0.8 kDa. Hence, it was reasonable to assume the

higher clusters on the NR particle to be those of proteins, whereas the lower ones should correspond to the phospholipids. From surface integral analysis of the cross-sectional data, the average surface area covered by proteins was approximately 84%, whereas that for the phospholipids was about 16%. This suggests that the major surface constituents of freshly tapped latex particles were proteins and that their charges controlled the colloidal stability of the latex dispersion.



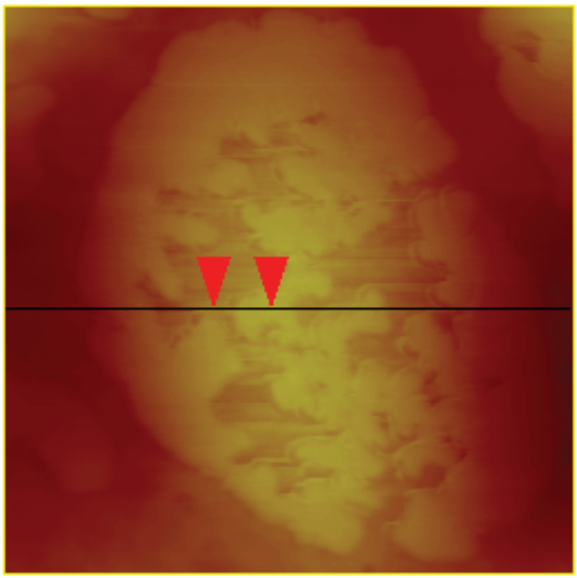


Figure 4 A cross-sectional profile of the rubber latex particle obtained by AFM (sample imaged one day after preparation).

The latex particle size was also estimated from the micrographs obtained for the dip-coated film and was found to be in the range of 0.1-2.0 μm, as shown in Figure 5. This was in close agreement with values obtained from a light-scattering method,[32] and the findings confirmed a previous notion that freshly tapped rubber latex particles are covered mostly by proteins and to a lesser extent by phospholipids. The findings from the present investigation complemented those obtained by particle microelectrophoretic studies on matured commercial latex in which the particles are stabilized by mostly long chain fatty acid soaps (86%), proteins and polypeptides.[33] The current results also corroborated with the previous findings involving the fact that the coalescence of the latex particles proceeded with time and that the presence of proteins and lipids somehow impeded and slowed down the coalescence rate.[11]

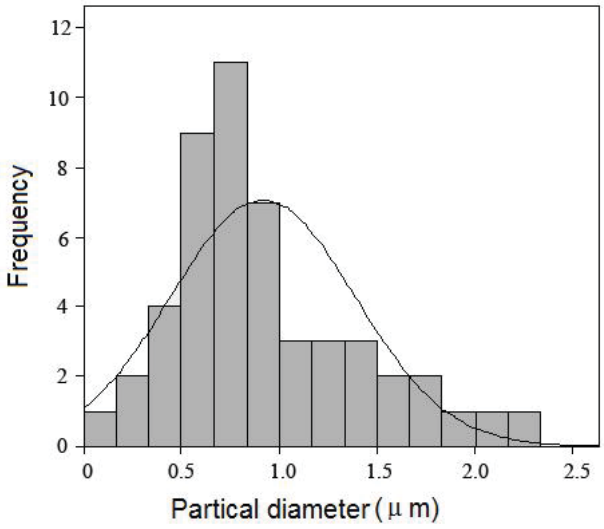


Figure 5 The particle size distribution of uncoalesced NR latex particles dip-coated on a glass substrate imaged one day after preparation.

Protein-lipid layer structure by confocal fluorescence microscopy

Confocal fluorescence microscopy was used to investigate the spatial distribution of proteins and phospholipids on the NR particle surface. This technique rendered it be possible to obtain information with respect to the location of fluorescence molecules inside a particle based

on the presence of fluorescent molecules within the sample. Rhodamine B (RB), an ionic red dye, was used to electrostatically bind the amine functional groups in proteins and phospholipids. Figure 6 illustrates the confocal laser scanning micrograph (CLSM) of RB-labeled NR latex films at various sample depths. As can be seen from Figure 6 (A), numerous bright spots emanated from the fluorescent RB molecules and corresponded to the transmission image of the sample shown to the right. This indicated the presence of amino-functional groups on the exterior of the NR particle. However, only dark spots were discernable in place of the bright red spots for the interior of the NR particle at the same position as illustrated in Figure 6 (B). This signified an absence of fluorescent molecules inside the NR particles. In other words, no amino groups were present within the latex particle, which should contain only polyisoprene hydrocarbon as the core. This result was in complete agreement with the proposed core-shell structure of the NR latex particle surrounded by a shell layer of hydrophilic proteins and phospholipids.

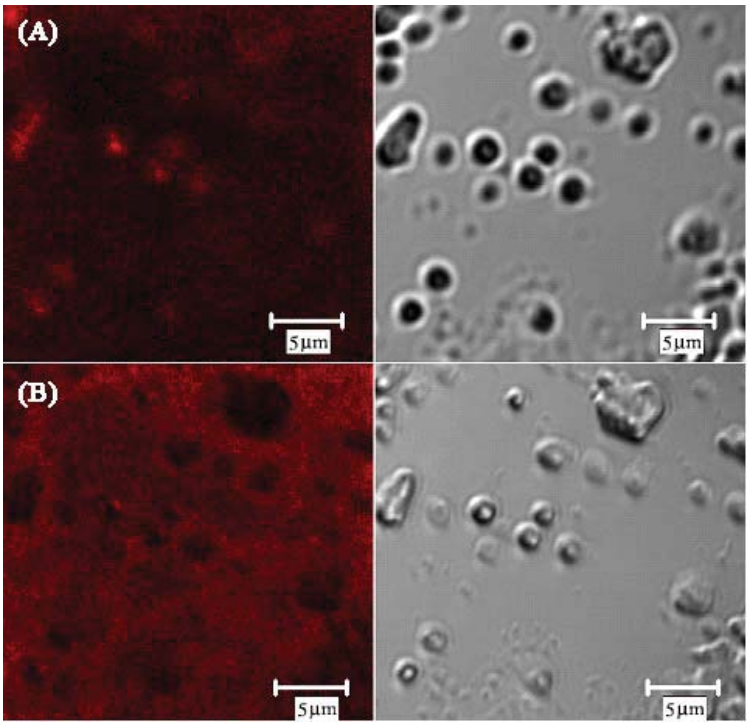


Figure 6 Confocal laser scanning micrographs (CLSM) of RB-labeled NR latex particles viewed (A) on the surface and (B) in the interior of sample.

To further confirm the presence of proteins and phospholipids on the surface of the NR particles, control experiments, using protein-free and protein-and-lipid-free latex particles, i.e.,

enzymatic deproteinized and saponified latices, were carried out. Figure 7 illustrates the CLSM of an RB-labeled NR latex. Again, the original untreated NR latex particles exhibited the RB-labeled bright spots very clearly. After removal of the proteins by enzymatic deproteinization, these bright spots were still present. Since the deproteinized latex contained only a very small amount of nitrogen (*ca.* 0.02% w/w) as compared to the original untreated NR (*ca.* 0.6% w/w), it was assumed that the bright spots observed in the deproteinized NR particle were derived from phospholipid molecules tagged with fluorescent RB. For the protein-and-lipid-free latex, there was no fluorescence emission at all, which was taken to correspond to the absence of amino groups since both proteins and lipids had been removed from the latex particles.

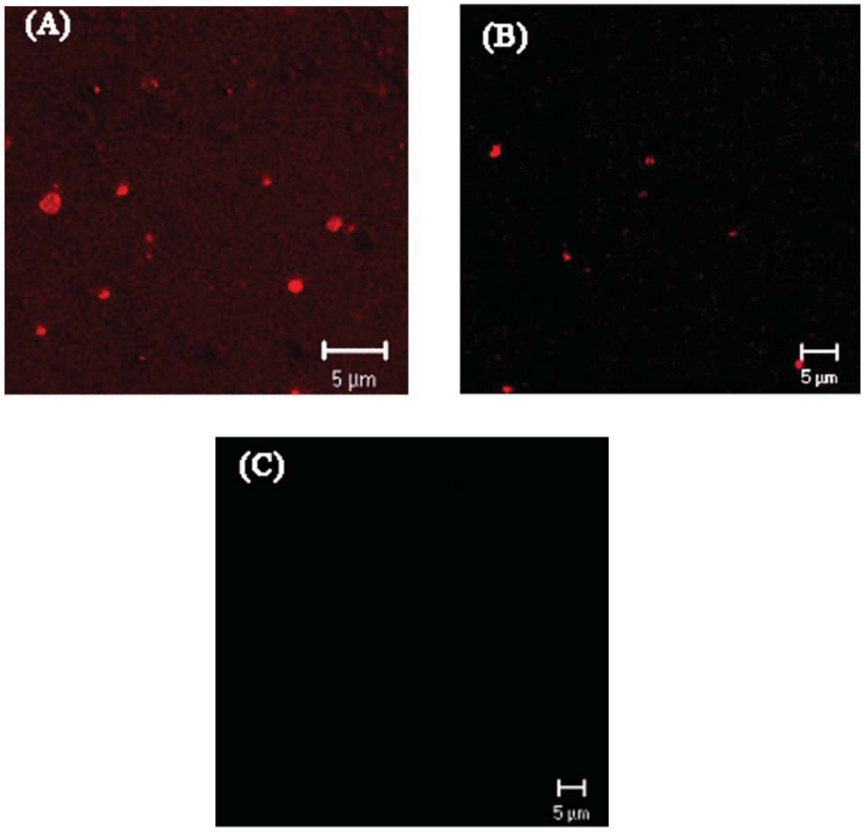


Figure 7 Confocal laser scanning micrographs (CLSM) of RB-labeled latex particles. (A) Original untreated NR latex, (B) protein-free NR latex, and (C) lipid-and-protein-free NR latex.

It should be noted that the positively charged RB molecules shown in Figure 8 might have become electrostatically attached to the SDS used in the deproteinization of the latex and

hence adsorbed onto the latex particle surface. For this reason, it was necessary to ascertain that the observed fluorescence was indeed derived from the RB molecules tagged to the proteins and phospholipids on the NR particle and not from the RB bound to the SDS. The experiment was thus repeated using an uncharged fluorescence dye, i.e., fluorescein 5(6)-isothiocyanate (FITC) to eliminate the effect of charge, and Figure 9 shows the results of CLSM of an NR latex labeled with this FITC dye. As can be seen, the protein-free particles fluoresced whereas the protein-and-lipid-free latex particle did not. This was in total agreement with findings from the RB-labeled system, and represented clear evidence that the surface of the NR particle was covered with a layer of proteins and phospholipids.

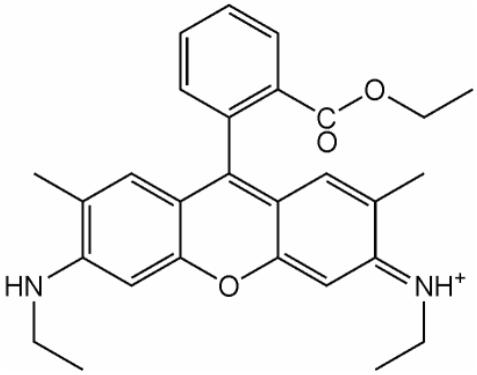


Figure 8 The molecular structure of Rhodamine B.

Analysis of elastic properties of the mixed protein-lipid layer on the particle surface

Recently, Liang *et al.* have shown that cholesterol-containing liposomes can be studied by AFM based on the *Hertzian* model.[34] Their results indicate that AFM can provide a direct method for unraveling the mechanical properties of liposomes. In the present study, force curves between an AFM tip and the NR particles were obtained in the contact mode and employed to elucidate the stepwise penetration of the AFM tip through the mixed protein-lipid layer on the surface of the NR particles. The indentation by the AFM tip was obtained in several places on the surface of the NR film by focusing on the top of the NR particle. Approximately 100 readings were recorded over the whole NR film.

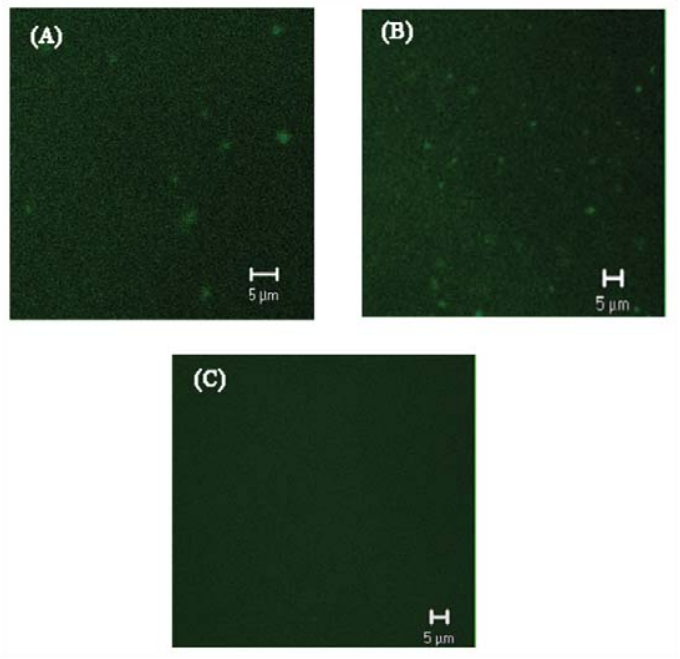


Figure 9 Confocal laser scanning micrographs (CLSM) of fluorescein 5(6)-isothiocyanate (FITC)-labeled latex particles. (A) Original untreated NR latex, (B) protein-free NR latex, and (C) lipid-and-protein-free NR latex.

Figure 10 shows a typical deflection curve of the AFM tip on the NR particle, while Figure 11 presents an indentation versus deflection curve calculated from Figure 10 after defining the zero force and zero separation point according to the *Hertzian* model described in Equation (1). Five distinct regions of the indentation process could be distinguished in these curves as illustrated in Figure 12. Region I corresponded to the non-contact region when the AFM tip was still far from the particle surface and the force between the tip and the particle was zero. At point II, the tip had just come into contact with the surface layer and immediately experienced an attractive pull of the surface. Region III illustrates the elastic deformation of the mixed protein-lipid layer on the particle surface as it was compressed by the tip. This region provides information on the elastic properties of the surface layer (Young's modulus). Region IV shows the indentation process at the stage when the tip has penetrated through the mixed protein-lipid layer and finally reached the NR polyisoprene core. In this region, the slope of the curve was negative indicating a higher adhesion between the soft rubber matrix and the AFM tip

due to the higher contact area. Region V presents a further compression of the tip through the matrix of the rubber core.

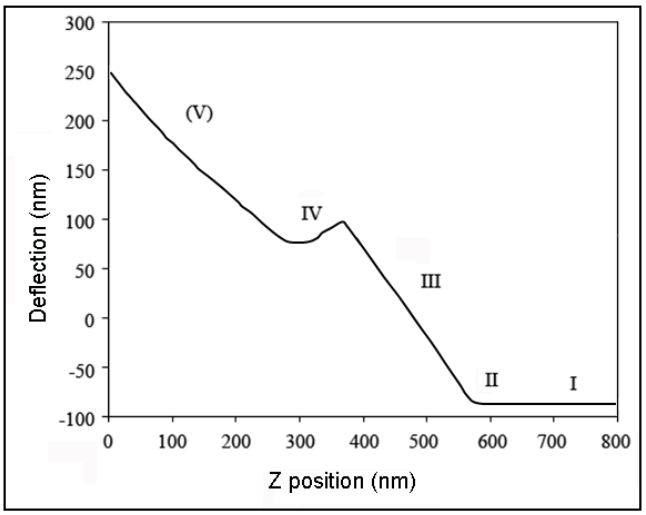


Figure 10 Indentation curve of the NR latex film plotted in terms of deflection versus the Z position. The x-axis represents the Z position (Z scan distance) and y-axis presents the cantilever deflection of the tip.

Since, in Region III, only a single distortion on the indentation curve was found, corresponding to a single-layer structure instead of a stepwise penetration for a double layer morphology, it was deduced that the proteins and phospholipids were indeed arranged in a single layer surrounding the rubber particle. From the indentation depth in Region III, the thickness of this mixed protein-lipid layer was deduced to be *ca.* 20 nm; a value larger than that estimated by Gomez[9] from TEM analysis. The thickness of a cell membrane composed of a bilayer of lipid molecules mixed with protein molecules is typically 7-10 nm.[35] The membrane thickness is often dependent on the make-up of its constituents, pH and temperature. One should note that the surface layer of long chain fatty acids, proteins and polypeptides on particles of a mature latex concentrate has been shown to be rather diffuse and extended from the surface.[10] It can thus be envisaged that a similar situation may exist also in the freshly tapped latex system.

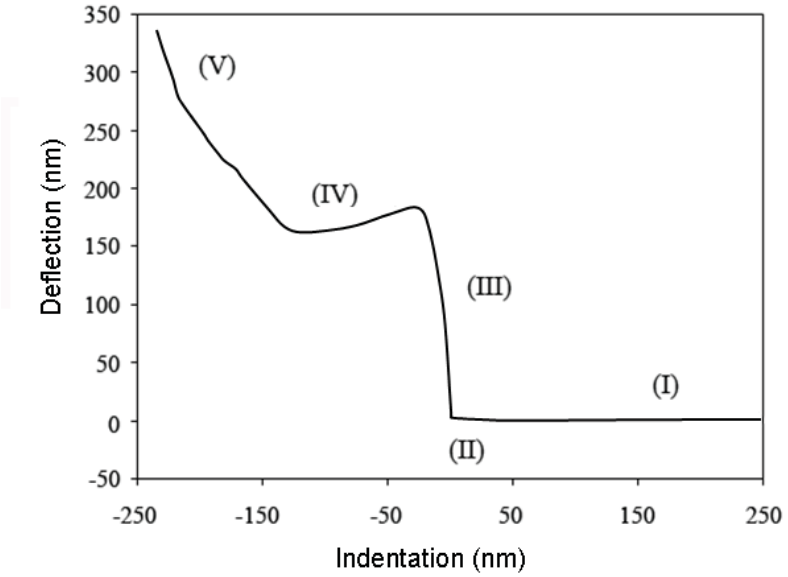


Figure 11 A typical deflection versus indentation curve of an NR latex particle.

Considering the difference in size of the protein and phospholipid molecules, this mixed surface layer can be depicted as shown in Figure 13. The Young modulus, E, of the mixed protein-lipid layer in region III was evaluated from the slope of a plot of $\delta^{3/2}$ against the deflection according to the *Hertzian* equation. Figure 14 shows the distribution of E (MPa) from the indentations on the protein-lipid layer of the NR film. The values of E ranged from 5 to 80 MPa, with an average of 30 ± 19 MPa. Values of Young's modulus ranging from 80 to 500 MPa for monolayers of globular proteins have previously been reported,[36-37] and the Young modulus for pure phosphatidylcholine has been found to be approximately 2 MPa (ranging up 10 MPa) according to the composition of and inside the lipid layer.[34] In the present study, the Young modulus of the mixed layer of proteins and lipids surrounding the latex particle was thus within the range for monolayers of proteins and lipids found in the literature. This reinforced the idea of the presence of a mixed layer of proteins and lipids on the surface of the NR latex particles.

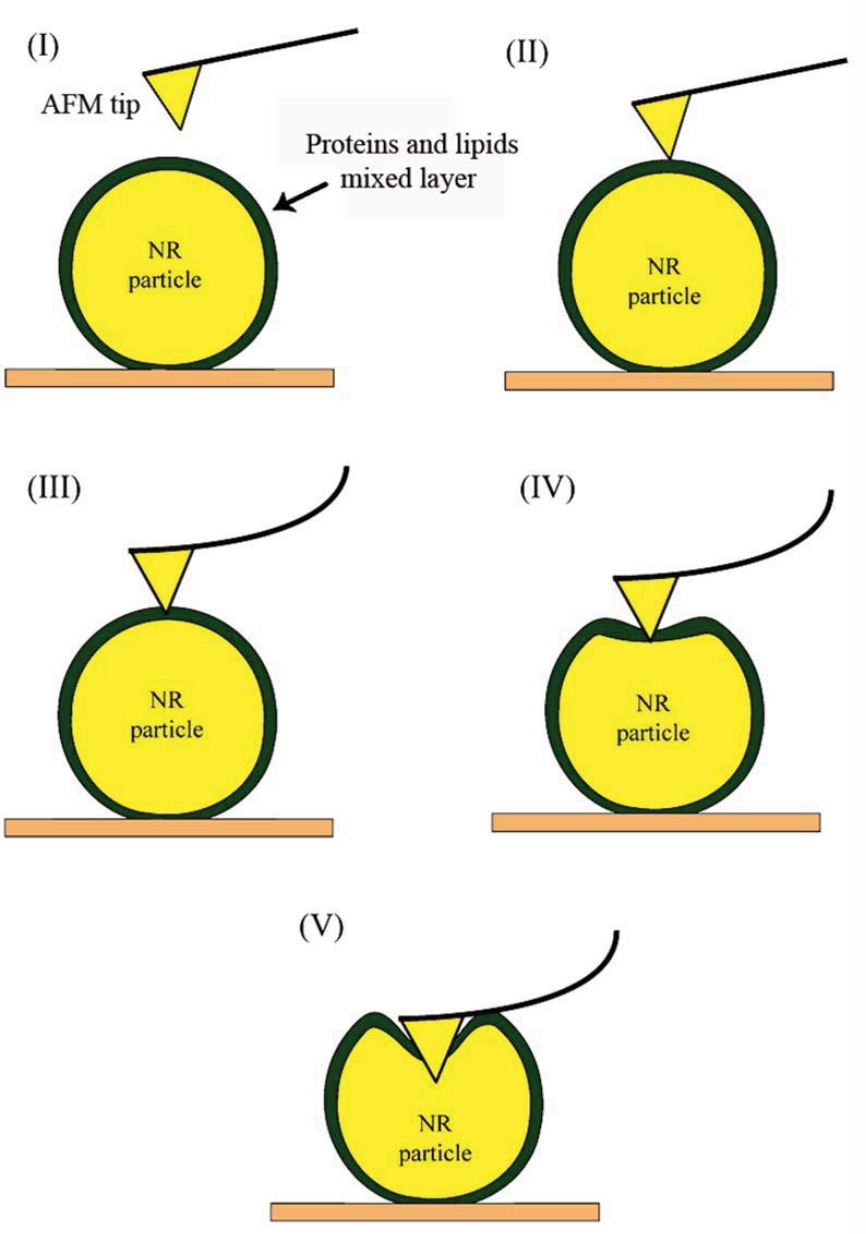


Figure 12 A schematic representation of the indentation process of the AFM tip through the surface of the NR latex particle.

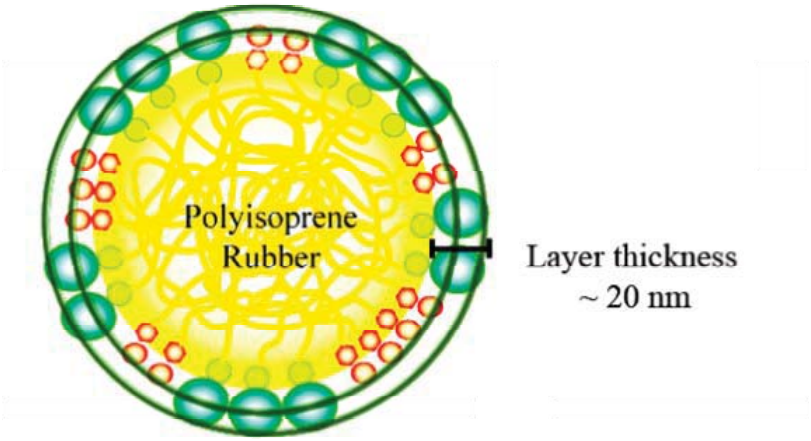


Figure 13 The proposed new model of the NR latex particle surrounded by a mixed layer of proteins and phospholipids (thickness of *ca.* 20 nm) with polyisoprene rubber as the hydrophobic core.

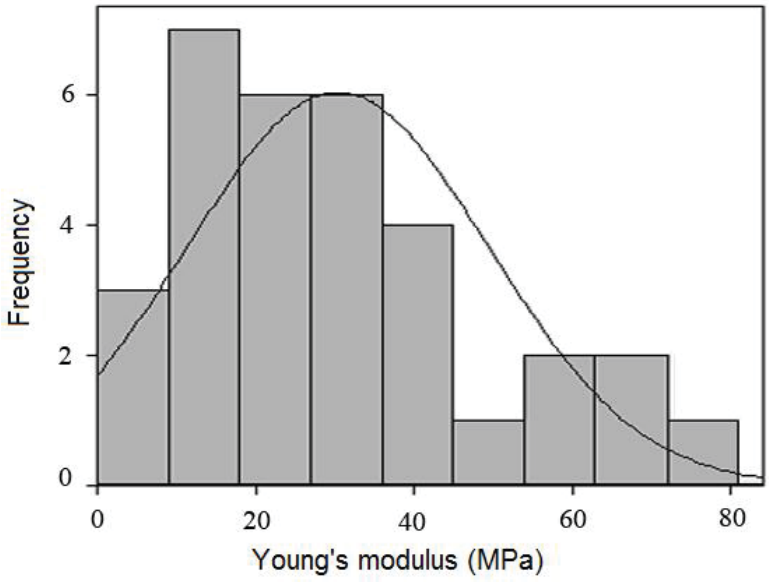


Figure 14 The distribution of the elastic Young modulus of the mixed layer of proteins and lipids on the NR latex particles.

Conclusions

Atomic force microscopy in conjunction with confocal fluorescence microscopy constitutes a powerful technique for elucidating the nanostructure of the surface layer of NR latex particles. The combination of these techniques permitted a direct determination of the intact surface structure without its prior removal. The experimental evidence supported the idea of a core-shell structure of the NR latex particle made up of a hydrophobic core of NR molecules surrounded be a mixed layer of proteins and phospholipids *ca.* 20 nm thick. All previous models of the surface structure of the rubber latex particle have been unable to provide any direct measurement of the thickness of this surface layer and the molecular arrangement of the proteins and phospholipids within it. The new model proposed herein suggested that the surface layer consisted mainly of proteins, which made up 84% of the surface with phospholipids constituting only 16%. Moreover, the constituents were found to be phase separated into distinct domains within the layer. This model was in complete agreement with the amphoteric nature of the NR latex and with the fact that charged proteins were in control of the colloidal stability of the freshly tapped latex. The Young modulus of the mixed protein-lipid layer was also estimated, and the present findings could thus provide information on the structure of freshly tapped NR latex particles which was hitherto unresolved until the advent of new instrumentations and analytical techniques.

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PART 3 UNIQUE FILM FORMATION BEHAVIOR OF NATURAL RUBBER LATEX AS A RESULT OF NON-RUBBER COMPONENTS

ABSTRACT

In order to get a deep understanding on the effect of proteins and phospholipids layer presenting on the surface of field natural rubber (FNR) particle on film formation behavior of the FNR latex, film formation of deproteinized FNR (DP-FNR) and saponified FNR (SP-FNR) latices were investigated and compared with that of untreated FNR latex. Atomic force microscopy (AFM) was used to distinguish behavior of FNR latex film formation by monitoring as a function of aging time. The flattening rate of the films was analyzed to investigate roughness of the films. The AFM images show clear spherical contour of FNR particle after film formation occurred. The flattening feature of the dip coated untreated FNR film was different from DP-FNR and SP-FNR films. Rate of FNR latex film formation was proposed to increase when the mixed protein and lipid layers were removed. The mechanism of film formation in NRs was aimed to be unveiled. The unveiling on film formation of NRs was established based on the obtained results.

Keywords: natural rubber, film formation, proteins, phospholipids

1. Introduction

Natural rubber (NR) latex obtained from *Hevea brasiliensis* is well known to be the largest source for rubber industries, such as NR latex dipping used for the production of gloves as well as condom. The glove made from NR latex is more excellent green strength, tensile strength and tear strength than that from synthetic rubber latex. However, the film formation of glove made from NR latex may be more irregular and unstable than that from synthetic rubber latex. It may cause from the polydispersity in NR particle size, gel content and presence of non-rubber components, i.e., proteins and phospholipids [1], as they do not exist in synthetic rubber latex [2]. In this regard, we focus on the effect of proteins and lipids, since almost all of proteins and lipids were proposed to present at the surface of NR particle [3-9]. For this reason, they may be main factors affecting on the irregular and unstable film formation of NR latex. Therefore, in

order to clarify the assumption, it is necessary to investigate the effect of the proteins and lipids on film formation behavior of NR latex.

The film formation of NR latex may be investigated by monitoring the change in surface morphology as the tops of the latex particles deform with time and measuring essentially the rate of flattening of the latex film surface [1, 10-15]. In this regard, AFM is considered to be extremely useful tool for studying behavior of NR film formation.

In the previous work [16], the film formation mechanism of an ideally stable dispersion of colloidal polymer particle was proposed through three main stages, i.e., the evaporating of water, the packing and deforming of the particles, and the aging or annealing for interdiffusion and coalescence across the particles, as shown in Figure 1. As water evaporates from the dispersion, the particles come into proximity. When the forces accompanying drying exceed the modulus of the particles, particle deformation occurs to yield a void-free film comprised of space filling polyhedral cells. At this stage, the film is still mechanically weak. In the final stage, film maturation involves polymer diffusion across the intercellular boundary to provide the entanglements that give strength to the film. These stages are not always well separated, and within each stage subtle features operate that often differ from system to system. The regular arrays of orderly arrange particles with sharp spherical contours are often seen in monodisperse synthetic latex films [12, 15]. However, the film formation of NR latex may be different from the common synthetic latex. Ho and Khew [1] investigated the film formation behavior of synthetic polyisoprene (IR) latex and high-ammonia NR (HANR) latex which having the same T_g and molecular weight. They found that the flattening rate of IR latex was very much faster than that given by the HANR latex. The different flattening rate was explained to be due to the presence of non-rubbers and high gel content of HANR latex.

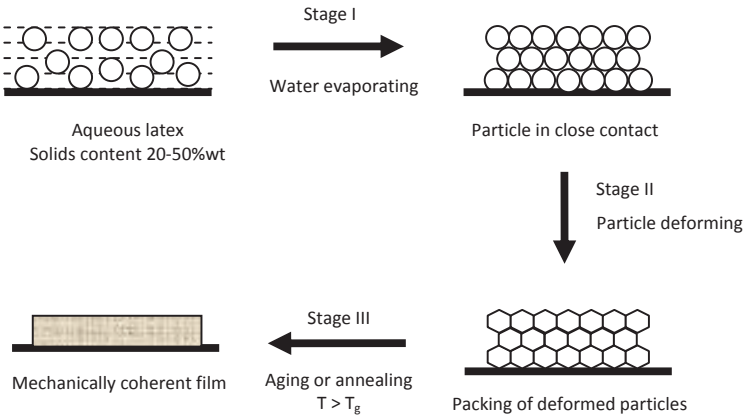

Figure 1 Schematic view of film formation mechanism for an ideally stable dispersion of colloidal polymer particle.

Besides, Joanicot and coworkers found that some factors (uniform particle size, low ionic strength) promote ordering of the dispersion in the liquid state [17]. Distler and Kanig [18] showed that a poly(butyl acrylate) (PBA) copolymer films prepared from a latex that contains 2% N-methylol-acrylamide (NMA) and 1% acrylic acid has a regular but heterogeneous structure, in which polar polymer forms a web-like interconnected membrane separating the individual cells. The boundary between the cells did not disappear even over long periods of aging (two years) at ambient temperature, even though the aging temperature was 50^{o} C greater than the T_g of the polymer. Therefore, it can be referred that a latex particle having polar or ionic groups at surface to provide colloidal stability may strongly affect the behavior of film formation.

As in the case of NR particle, since the NR particle was proposed to be a core-shell like structure, of which the core is dense with polyisoprene, the rubber main chain, and the shell is a mixed proteins and phospholipids layer, derived by the terminal ends of rubber chains, in which this direct evidence makes a good complement to the colloidal behavior of NR particle proposed so far [3-9]. The stabilization of NR particles was proved to be partially involved by the increment of negative charge from the proteins and phospholipids upon storage the NR latex in the presence of ammonia [1]. Therefore, the negative charges of proteins and phospholipids layer on the surface of NR particles may play an important role on the film formation of NR latex.

In the previous work, Ho and Khew [1] investigated the influence of non-rubber materials and additives on their behavior at HANR latex film surface during film formation. However due to the fact that some proteins and lipids presenting at the surface of NR particle and some proteins and lipids dissolving in water phase may be losed by centrifugation during the concentration process in the production of commercial latex concentrates, the achieved results may come to be indistinct.

Hence, to clarify the effect of proteins and phospholipids layers on the film formation behavior of NR latex, freshly-tapped FNR is used in the present work. The film formation of FNR latex after removing proteins and lipids are compared with that of untreated FNR latex. The proteins in FNR latex is removed by enzymatic deproteinization. Both of proteins and lipids are taken away from FNR latex by saponification with NaOH. AFM is used to imaging array of NR particles and observing roughness at the surface of film. Effect of aging time on the film formation behavior is studied. Moreover, film formation mechanism of FNR latex is also aimed to be established.

2. Experimental

2.1 Materials

FNR latex was achieved by collecting NR latex from RRIM 600 *Hevea* rubber tree. Some impurities consisting in FNR latex were removed by filtering the latex through a muslin cloth before collecting in a cleaned glass bottle. FNR latex was preserved by the addition of ammonia to make 0.6% v/v in latex.

DP-FNR latex was prepared by treating FNR latex with 0.04% (w/v) proteolytic enzyme (KP-3939, KaO Co. Ltd.) in the presence of 1% (v/v) of polyethylene glycol *p*-isooctylphenyl ether (Triton® X-100) by incubation at 37°C for 12 h with gently stirring. The resulting latex was centrifuged twice at 19,000 rpm (43,300 g) for 40 min each. The collected cream fraction was diluted with distilled water to make 30% DRC.

SP-FNR latex was obtained by diluting FNR latex with distilled water to make 15% (w/v) DRC latex in the presence of 0.5% (v/v) Triton® X-100 and saponified with 2% (w/v) NaOH at 70°C for 3 h, followed by neutralization with 2% (v/v) formic acid.

2.2 Latex film formation

NR film was prepared by dip coating technique. FNR latex was poured into a small container, and then a clean glass substrate was dipped into the latex with the speed of 0.25 mm/sec. After a further 5 min purging with N_2 (g) and 15 min drying at room temperature, the prepared film was then kept in desiccators until measurement.

2.3 Characterizations

2.3.1 Estimation of Nitrogen content

The nitrogen content in NR was analyzed by a LECO-FP258 Nitrogen Analyzer. The rubber sample of *ca.* 0.25 g was accurately weighed and subjected to the nitrogen analysis. The combustion of rubber sample converts the nitrogen compound to nitrogen gas, which is detected as nitrogen content (%w/w). In this experiment, EDTA was used as a standard with an accuracy of ±0.02 %w/w. The results are obtained from triplicate analysis.

2.3.2 Determination of Ester content

Methyl stearate was used as a model compound for Fourier Transform Infrared spectroscopy (FTIR) analysis of the long-chain ester group in NR by mixing synthetic *cis*-polyisoprene with various amounts of methyl stearate. FTIR measurements were carried out to get the relationship between the integrated intensity of carbonyl group at 1739 cm^{-1} (C=O) and unsaturated carbon (C=C) absorbance at 1664 cm^{-1}. The intensity ratio of two bands was plotted against the concentration of ester groups in the rubber to get a calibration curve, as mentioned in the previous work [4].

2.3.3 Atomic force microscope (AFM)

AFM imaging and force measurements were conducted using a NanoScope IIIa Atomic Force Microscope from Digital Instruments (Santa Barbara, CA). A J scanner with a maximum scan area of 150 μm was used to monitor the morphology of NR particle and film formation during aging.

The images were measured in tapping mode with silicon tips [Nanosensors, Germany]. Indentation of AFM tips was performed with contact mode by approaching a silicon nitride cantilevers having 0.12 and 0.6 N/m spring constant [Nanosensors, Germany]. Force curve from

the indentation were captured mostly on particle surfaces at random locations. 100 indentation curves were obtained at the same particle to get the reliable result.

3. Results and Discussion

3.1 Characterization of deproteinization and saponification

The amount of proteins and phospholipids were estimated by the nitrogen content and ester content, respectively. It was found that the total nitrogen contents of untreated FNR and DP-FNR were 0.82 and 0.02%w/w, respectively. The nitrogen content significantly reduced to be about 1/30 after enzyme-deproteinization. However, the ester content of DP-FNR was close to that of FNR before deproteinization. This demonstrates that the almost all of proteins was removed, whereas the lipids cannot be displaced after enzyme-deproteinization.

As for SP-FNR, the total nitrogen content was about 0.01%w/w. The nitrogen content steadily reduced to about 1/80 after saponification. The total ester content was also significantly decreased after saponification. This can be described that almost all of proteins and lipids presenting in FNR latex were removed. Therefore, it was proved that DP-FNR and SP-FNR were effective and can be used as samples for further experimental.

3.2 Film formation behavior of FNR, DP-FNR and SP-FNR

Figure 2 shows height images of dip coated films of FNR, DP-FNR and SP-FNR after aging time for 1 day at room temperature. AFM image of all films show the irregular packing of NR particles corresponding to the polydispersity of FNR particles, as mentioned in the previous work [1]. It can be also seen that the height image of FNR film is composed of aggregates of particles causing the uneven of the film surface. Whereas, in the case of DP-FNR and SP-FNR, their particles coalesce more readily and become rather smooth films than FLNR, in which features of individual particles are discernible.

Moreover, to describe obviously film formation behavior of the NR lattices, we take a notice of the surface mean roughness, R_a, of the NR film, which can be calculated by the Digital software as to the equation (2) shown below;

$$R_a^{\,2} = (1/N)\sum (Z_i - Z_0)^2 \qquad (2)$$

where $Z_0 = (1/N)\sum Z_i$ and where N is the number of Z values used and Z_i is the height of point i.

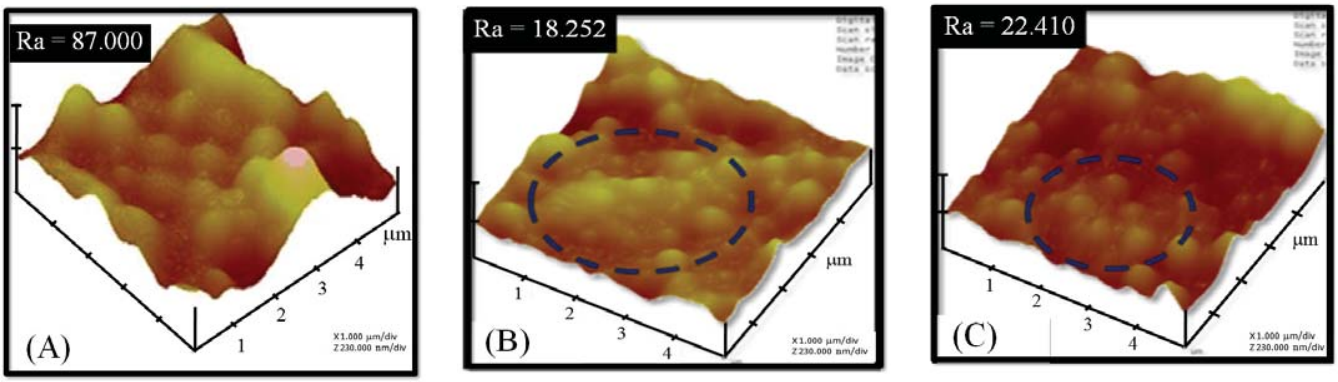

Figure 2 Height images of dip coated films of (A) FNR, (B) DP-FNR and (C) SP-FNR after aging time for 1 day at room temperature (Scan size = 5 μm).

It was found that the R_a of FNR film at the nascent stage was *ca*. 87 nm. However, the R_a of DP-FNR and SP-FNR films were *ca.* 18 and 22 nm, respectively. That is R_a of FNR film was numerously higher than that of DP-FNR and SP-FNR. This may be explained to be due to a complex protein and phospholipid layers presenting at the surface of FNR particle, that is, the complex layers may make FNR particle stable, according to the previous work [3-9]. Based on this reason, the packing and deforming of FNR particle may be blocked, the R_a of FNR film became much higher than that of DP-FNR and SP-FNR films. This demonstrated that film formation of FNR particle is different from that of DP-FNR and SP-FNR after aging for 1 day at room temperature.

3.3 Rate of film formation of FNR, DP-FNR and SP-FNR

Firstly, in order to make a reliable data, the flattening rates of the FNR film which can be calculated by the slope of linear fit curve of log(R_a) versus aging time were reproductively taken into account. Figure 3 represents the linear plot of log(R_a) as a function of aging time of NR film. It was very obviously that these two experiments gave relevant results with a small

deviation. The slopes of these two linear curves were exactly the same value, i.e. -3.0E-07 (nm/sec).

Therefore, to investigate the rate of film formation of FNR, DP-FNR and SP-FNR latices, this method was applied. The film formation behavior of FNR, DP-FNR and SP-FNR lattices was investigated as a function of aging time at room temperature.

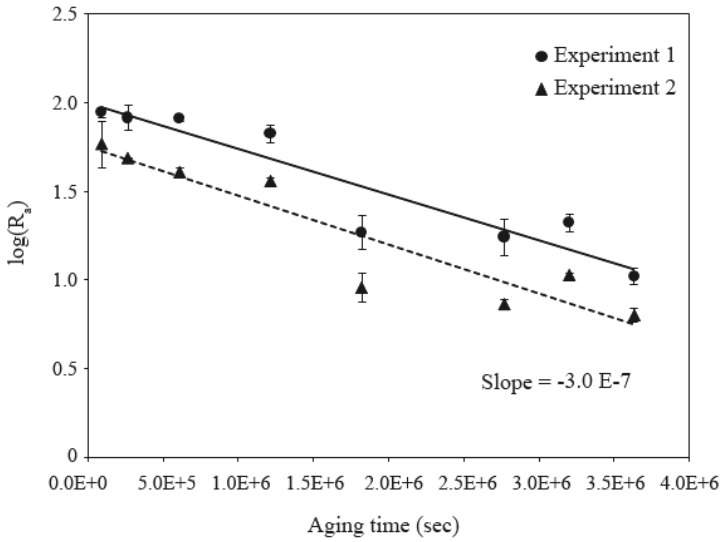

Figure 3 Linear plot of log(R_a) versus aging time for FNR film

Figure 4 shows height-mode images of dip coated FNR film as a function of aging time at room temperature. The similar pattern of surface morphology of NR film was observed until the aging time reached 14 days. It can be seen that some big aggregates of particles are also discernible. The particle contour starts to fade and the surface becomes rather smooth when aging time reached 21 days, as shown in Figure 4(D). Figure 5 shows the change of R_a of NR film surface as a function of aging time. It can be noteworthy to note that the R_a of NR film gradually decreased in the nascent stage of film formation at the beginning 14 days. After that stage, the R_a value was then drastically decreased and finally became in the range of 10-20 nm.

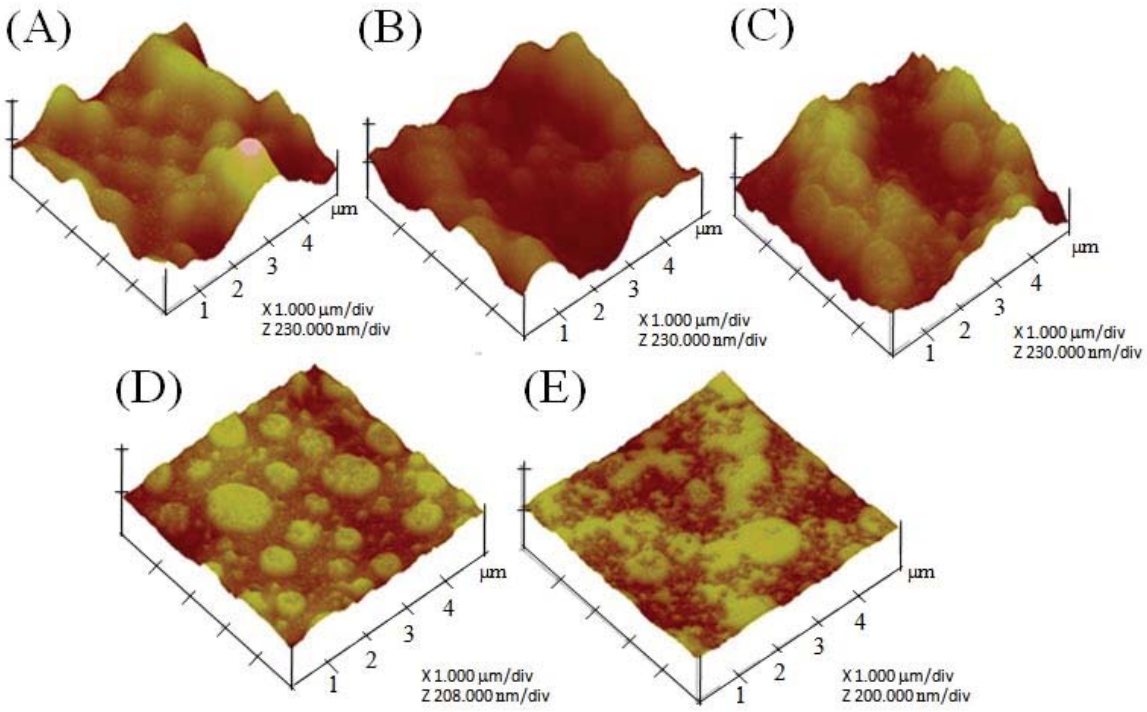

Figure 4 Surface morphologies of dip coated FNR film as a function of aging time at room temperature; (A) 1 day, (B) 7 days, (C) 14 days, (D) 21 days and (E) 42 days (Scan size = 5 µm).

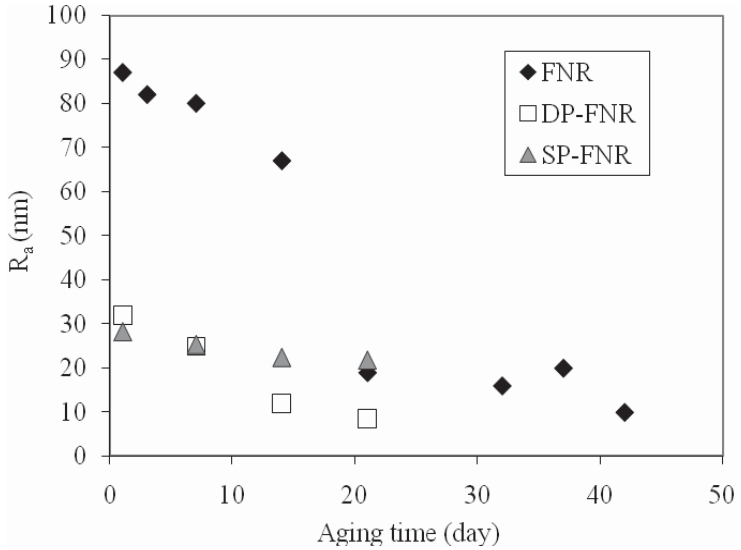

Figure 5 The plot of roughness, R_a (nm), of dip coated FNR, DP-FNR and SP-FNR films on glass substrate as a function of aging time.

Figure 6 shows surface morphologies of the dip coated DP-FNR film as a function of aging time at room temperature. The particle contour can be observed and the surface become flat and smooth after the film was formed since aging time reached 14 days. The R_a value of DP-FNR decrease rapidly, as shown in Figure 5. When it was compared with that of untreated FNR latex, the flattening rate of DP-FNR is very much faster than that of untreated FNR. This implied that the proteins presenting at the surface of NR particle play an important factor on film formation behavior of FNR latex.

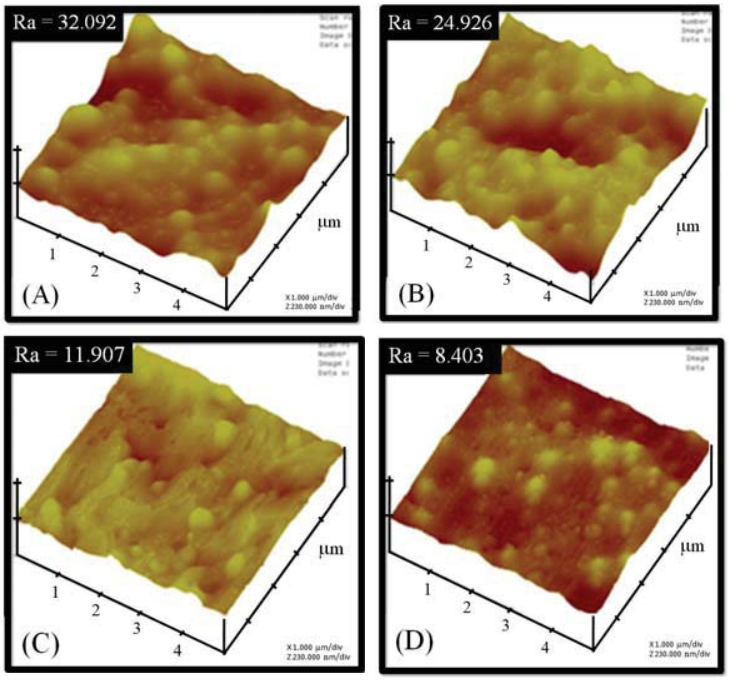

Figure 6 Surface morphologies of dip coated DP-FNR film as a function of aging time at room temperature; (A) 1 day, (B) 7 days, (C) 14 days and (D) 21 days (Scan size = 5 µm).

Figure 7 shows surface morphologies of dip coated SP-FNR film as a function of aging time at room temperature. It was found the spherical contour of particles can be observed at the nascent stage. However, the surface becomes rather flat when aging time reached 14 days. In addition, the R_a value of DP-FNR also decreased when the aging time increased. When it was compared with that of untreated FNR, the flattening rate of SP-FNR is quite faster than that of untreated FNR, as shown in Figure 5. This may be explained to be due to the low amount of

proteins and lipids presenting at the surface of NR particle after saponification. This demonstrates that the rate of NR film formation increase as the non-rubber components was removed: the more vigorously leaching to remove non-rubber components, the cleaner the surface and the fastest the rate, according to previous literature [1, 19-20]. Thus, it can be deduced that the presence of non-rubber components, particularly the proteins and lipids layer on the surface of NR particle is an influential parameter controlling the behavior of film formation, as summarized in Figure 8.

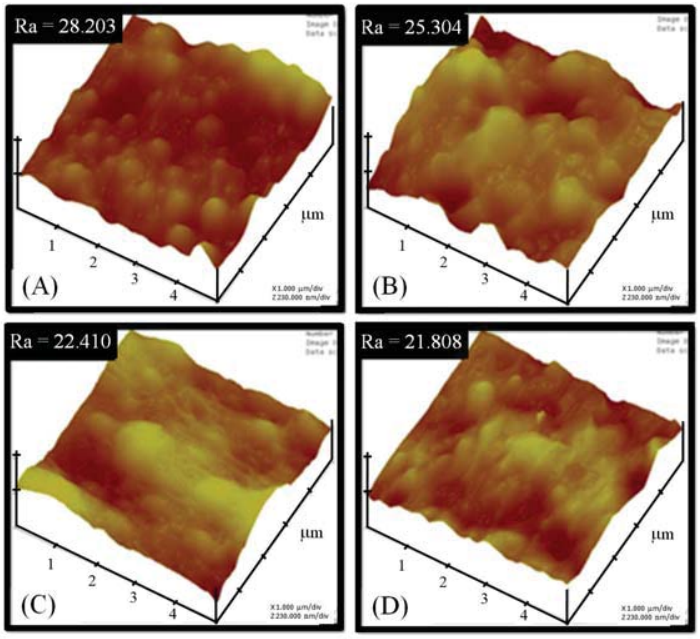

Figure 7 Surface morphologies of dip coated SP-FNR film as a function of aging time at room temperature; (A) 1 day, (B) 7 days, (C) 14 days and (D) 21 days (Scan size = 5 μm).

4. Conclusion

The proteins and lipids presenting on the surface of FNR particle was proved to be an important factor influencing on the film formation behavior of FNR latex. The flattening rate of FNR latex increased after the proteins and lipids presenting on the surface of FNR particle was removed. Surface images achieved by AFM show that the surface layer on the particle is critical in maintaining the spherical shape of the NR particle at the nascent stage of film formation. The polydispersity of the NR latex particle results in a film made up randomly packed particles. The interdiffusion of the polyisoprene molecules between the NR particles took placed after this surface layer was ruptured. Film formation proceeds through diffusion of the rubber molecules from the particle core across the particle surface after rupture of the surface layer.

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เอกสารแนบหมายเลข 3

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า)

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2. การนำผลงานวิจัยไปใช้ประโยชน์

- เชิงวิชาการ มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่

3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

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2. Sansatsadeekul, J. and Sakdapipanich, J.T. “Characterization of associated proteins and phospholipids in natural rubber latex” Asian Workshop on Polymer Processing 2010, Hanoi, Vietnam, December 7-10, 2010.
3. Sakdapipanich, J.T. “Recent study on the topology of colloidal particles and film formation of *Hevea brasiliensis* natural rubber” Pure and Applied Chemistry International Conference 2011, Miracle Grand Hotel, Bangkok, Thailand, January 5-7, 2011.

ลงนาม.....

(ดร.จิตต์ลัดดา ศักดาภิพาณิชย์)

(หัวหน้าโครงการ)

วันที่ 26 มีนาคม 2555