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LIPID CONTENT IN SMALL AND LARGE NATURAL RUBBER PARTICLES

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ABSTRACT

Knowledge of the surface composition of natural rubber (NR) latex is essential to manufacturers of latex goods. Films made from only small rubber particles (SRPs) and a mix of SRPs and large rubber particles (LRPs) differ in mechanical properties. The reason for this difference, which is still under debate, is hypothesized to be linked with biomolecules (proteins and lipids) present in the NR particle surface. In this study, we characterize the surface chemistry, particularly lipid content of the SRP and LRP, by performing investigations directly on these particles in aqueous conditions. Fluorescent probes were used to display protein and lipid affinity and analyze them in situ with steady-state fluorescence spectroscopy, fluorescence correlation spectroscopy, and fluorescence lifetime measurements. Results are atypical in showing that lipids are more abundant in LRPs than in SRPs, suggesting thicker and/or denser membranes in LRPs. The degree of membrane compacity affects rigidity, influences biomolecular interactions, and might impact natural rubber coagulation. These results provide additional insights into colloidal behavior of NR for more efficient industrial applications. [doi:10.5254/rtc.15.85938]

INTRODUCTION

Natural rubber field latex harvested from the *Hevea* tree is a colloidal dispersion of ~35 wt% rubber and ~5 wt% non-rubber compounds (mainly proteins, lipids, and sugars) in aqueous phase (serum).¹ These non-rubber compounds are either free in the serum or form the natural rubber particles' membrane that maintains the colloidal suspension stability.^{2,3} Furthermore, these non-rubber components are believed to play a significant role in the improvement of the mechanical properties of natural rubber (NR) products.⁴

When submitted to centrifugation, field latex yields a concentrate (~60 wt% rubber) and a by-product (~10 wt% rubber).^{5,6} The by-product, also known as skim latex, presents a unimodal distribution of small rubber particles (SRPs, size <200 nm in diameter), while the concentrate has a bimodal distribution of SRPs and large rubber particles (LRPs, size ~200–1000 nm in diameter).^{7–9} Several studies have already been performed on the mechanical properties of solid NR prepared from the different types of latex solution. It has been observed that films made from the skim (SRPs) are more elastic and permeable⁹ and display smoother surfaces¹⁰ than films prepared from the concentrate (LRPs + SRPs). Although the reasons still remain under debate, both studies hypothesized that the different properties between the concentrate and the by-product are associated with the natural rubber particle membrane composition and/or their size distribution.

A natural rubber particle is described to exhibit a core-shell structure with polyisoprene as the core surrounded by a complex lipo-protein membrane.¹¹ From this point of view, studying the surface composition of the skim and concentrate particles is the key to better understanding the aforementioned differences. Recent studies on NR membrane models have already shown not only that LRPs and SRPs contain different proteins, but that the proteins in LRPs and SRPs are arranged differently and could have unique roles.^{12,13} The result on proteins clearly demonstrates inhomogeneities in latex composition relative to the size of the particles. It would then be equally interesting to understand the lipid composition in both sizes. Currently, the role of lipids is highly

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discussed, especially in NR branching through the creation of cross-links via ionic linkage (lipid–protein, lipid–polymer, and/or lipid–protein–polymer).^{14–16} These ionic linkages are recognized to be very important in understanding the mechanical properties of NR. However, no detailed study has yet been reported directly on natural rubber latex in solution to describe the potential relationship between the size and surface lipid composition as far as we know.

To address this question we use an original approach combining multimodal fluorescence methods (steady-state fluorescence spectroscopy, fluorescence correlation spectroscopy, fluorescence lifetime measurements, and fluorescence intensity imaging) and fluorescent probes with high affinity for the non-rubber compounds. In contrast to most studies performed on dried latex or on model membranes, the originality of our approach is to allow analysis in aqueous solution, thereby preserving the membrane structure of the native NR particles. Our converging results conclude that the lipid content is dependent on the size of the natural rubber particle. From an application point of view, variations in surface composition could lead to differences in the coagulation process of natural rubber.

EXPERIMENTAL

SAMPLE PREPARATION AND MATERIALS

To avoid possible artifacts due to the natural impurities in the field latex, we used an industrial high ammonia natural rubber latex concentrate (~60%, polydisperse in size) purchased from Trang Latex Co. Ltd., Thailand. The skim and cream fractions from the latex concentrate were separated by centrifugation method at 4000 g for 30 min at 25 °C. They were used without further chemical treatment.

Quant-iT™ protein (QP, Invitrogen, Life Technologies, Grand Island, NY, USA) and Bodipy® (Bpy, Invitrogen, Life Technologies) were used to probe their interaction with biomolecules (proteins, lipids) on natural rubber latex (NRL) concentrate, skim, cream, and deproteinized NRL (DPL, provided by Michelin, Clermont-Ferrand, France) prepared as detailed elsewhere.^{17,18} Such interactions were also probed with the individual representative molecules of NRL: *cis*-polyisoprene synthetic rubber (obtained from Michelin), casein (Sigma-Aldrich, St. Louis, MO, USA), the phospholipid P2767 (Sigma-Aldrich), and the surfactant triton X-100 (Sigma-Aldrich), which replaced the proteins in DPL. Milli-Q water was the solvent for all experiments except for solutions involving QP and *cis*-polyisoprene. The former was prepared as recommended by Life Technologies, while the latter was required to be first solubilized in methylcyclohexane and then diluted in 96% ethanol. These conditions permit control of the interaction between *cis*-polyisoprene and Bpy but preclude any experiments with QP.

DYNAMIC LIGHT SCATTERING

Dynamic light scattering (DLS) measurements were performed using a Malvern Zetasizer Nano ZS instrument equipped with a 633 nm He–Ne laser and a backscattering angle of 173°. The samples were pre-equilibrated for 1 min at 25 °C prior to measurement. Results were analyzed using the Zetasizer software 6.01. The reported sizes are based on the intensity distribution, while the populations of the particles are based on number distribution.

SCANNING ELECTRON MICROSCOPY

Scanning electron microscopy (SEM) was performed using FE-SEM Hitachi S4500 (Hitachi) at MIMA2 microscopy platform (Jouy-en-Josas, France). An alternative air-drying method to that

currently used for biological samples was optimized to preserve the three-dimensional (3D) ultrastructure of natural rubber particles. A drop of the concentrate was deposited on cleaned glass for 1 h 30 min and then fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at room temperature and overnight at 4 °C. The sample was washed at room temperature (three times, 10 min each) before post-fixing with 1% osmium tetroxide in 0.1 M sodium cacodylate for 1 h. Following washing in sterile water (three times, 10 min each), the sample was dehydrated at increasing ethanol concentration (50%–70%–90%–3 × 100%) for 10 min each and then air-dried. The glass was mounted on aluminum stubs before finally sputter coated in argon plasma with platinum (approx. 30 nm thick) in Polaron SC7640 device (Elexience, Verrières-le-Buisson, France). Observations were done in high vacuum with a sample holder tilted at 45° and using low secondary electron detector at 2 kV and 21 mm working distance.

FLUORESCENCE SPECTROSCOPY AND CONFOCAL MICROSCOPY

The fluorescence spectra were recorded using Cary Eclipse (Varian, Palo Alto, CA, USA) spectrofluorometer. QP was excited at 460 nm and Bpy at 350 nm—its second excited electronic state. Fluorescence images were acquired using Leica TCS SP5-AOBS confocal laser scanning microscope. Solutions of latex concentrate containing fluorescent probes were deposited on a coverslip for 30 min. The non-adhered particles were washed by a stream of solvent (3 mL) that gently flowed on the top border of the tilted coverslip. Afterward, the coverslip was placed in Attofluor® chamber, and 1 mL of solvent was added. The samples were imaged using 63 × 1.4 numerical aperture plan apochromat oil immersion objective. The size of the *xy* images was 512 × 512 pixels (image size ~17 × 17 μm) recorded on 8 bits. Argon visible laser (488 nm) was used as the excitation source regardless of the fluorescent probes. The corresponding fluorescence was collected in the 500–750 nm spectral range. Each image corresponds to an average of 4 to 16 frames.

FLUORESCENCE LIFETIME SPECTROSCOPY

Fluorescence lifetime measurement were obtained using Ti:Sa pulsed laser (150 fs, 80 MHz, Chameleon, Coherent, Santa Clara, CA, USA) coupled to the same confocal microscope Leica TCS SP5-AOBS as described in the previous part. Bpy and QP-labeled NRL were biphotonically excited at 950 and 860 nm, respectively. The PicoHarp 300 device (PicoQuant), based on time-correlated single photon counting, collected the fluorescence via an avalanche photodiode. A 800 nm short-pass emission filter was used to remove any residual laser light, and the emitted fluorescence was recorded within the range 500–650 nm for Bpy and 500–750 nm for QP. The recorded decay curves were fitted using single exponential function.

FLUORESCENCE CORRELATION SPECTROSCOPY

Fluorescence correlation spectroscopy (FCS) was performed using a two-photon excitation custom-built setup. A detailed explanation of the experimental setup and data analysis has been previously reported.^{19,20} Briefly, the samples were mounted under a 63 × 1.4 numerical aperture objective and then biphotonically excited at 860 nm by a Ti:Sa pulsed laser (150 fs, 76 MHz, MIRA 900, Coherent Inc.). The fluorescence was then collected through the same objective and separated from the excitation beam by a dichroic mirror before being focused on a photomultiplier. Fluorescence intensity fluctuations were recorded using a commercial correlator (Flex2kx2-12 Correlator, Correlator, Somerville, NJ, USA) and analyzed with homemade programs. The auto-correlated curves were fitted with two-component 3D-Brownian diffusion model:¹⁹

$$g(\tau) = 1 + \frac{1}{\sqrt{8N}} \left\{ \alpha \left[\frac{1}{1 + (\tau/\tau_1)} \right] \left[\frac{1}{1 + (\omega_0/z_0)^2 (\tau/\tau_1)} \right]^{1/2} + (1 - \alpha) \left[\frac{1}{1 + (\tau/\tau_2)} \right] \left[\frac{1}{1 + (\omega_0/z_0)^2 (\tau/\tau_2)} \right]^{1/2} \right\} \quad (1)$$

where α and $1 - \alpha$ are the fractions of the molar concentrations of the two diffusive species and τ_1 and τ_2 are their respective translational diffusion times, N is the number of fluorescent species inside the excitation volume, and ω_0/z_0 is the lateral/axial radii ratio of the laser beam ($\omega_0/z_0 = 0.30 \pm 0.03$, determined elsewhere).²⁰

RESULTS AND DISCUSSION

NRL SIZE DISTRIBUTION

DLS analysis confirmed the polydispersity of the latex concentrate (Figure 1a): 90% of the NR particles have sizes ranging from 80 to 250 nm in diameter with a maximum at ~ 120 nm (called small rubber particles, SRPs), while the rest of the particles have higher diameters centered at ~ 500 nm up to 1000 nm (called large rubber particles, LRPs), in agreement with literature.^{7,21} This size heterogeneity was imaged with SEM (Figure 1b). After latex concentrate centrifugation, the size distribution of each fraction (skim and cream) was also characterized. DLS analysis shows that the skim is only composed of SRPs with a diameter of 130 ± 20 nm, while in the cream fraction, the natural rubber particles show a size distribution ranging from ~ 100 nm to 900 nm close to the initial latex concentrate.

INTERACTION OF QP AND BPY WITH LATEX

We first identified the probe's specificity toward the NRL components (proteins, phospholipids, surfactant, and elastomer). The absorption and fluorescence emission maxima of QP and Bpy are shown in Figure 2a and 2b, respectively. The increase in QP fluorescence intensity in the presence of casein, phospholipids P2767, and Triton X-100 surfactant as well as the relative spectral shifts reveal an interaction between the fluorescent probe and these natural rubber membrane components. In contrast, Bpy interacts only with proteins and phospholipids, which is characterized by an intensity decrease in fluorescence spectra associated to a slight bathochromic shift for Bpy-P2767 interaction. No interaction was observed between Bpy and *cis*-polyisoprene (Figure 2b).

We then look into the interaction of both QP and Bpy with the latex concentrate. For QP, an increase in fluorescence intensity ($\sim 40\%$) is observed (Figure 2c), showing its interaction with the natural rubber particles. This was confirmed by fluorescence lifetime variation from 0.90 ± 0.20 ns (free QP) to 1.30 ± 0.17 ns (QP-NR complexes). Both fluorescence intensity and lifetime enhancements are characteristic of a reduced non-radiative deactivation due to the shielding of QP from the aqueous solvent. This was in agreement with the representation of the phospholipid/protein distribution of the NR shell interfacial structure postulating their presence in close vicinity to the polyisoprene NR core.⁷

As expected, the interaction of Bpy with latex concentrate results in a linear decrease in fluorescence intensity with increasing latex concentration (Figure 2d), while the fluorescence lifetime is unaffected (5.70 ± 0.03 ns). Consequently, when excited, the Bpy-NR complexes deactivate by a process faster than the excited singlet state lifetime of free Bpy, characterizing a static fluorescence quenching frequently reported for polycyclic planar molecules.^{22,23}

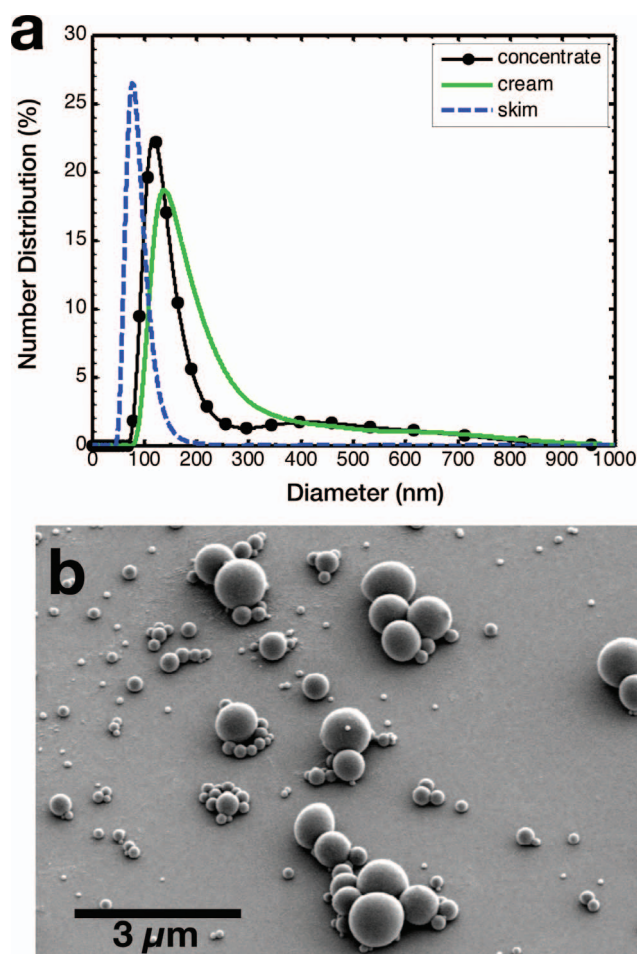


Fig 1. — (a) Number distribution (%) of natural rubber particles in the concentrate (•), skim (---), and cream (—) as obtained from DLS measurements. The concentrate, cream, and skim have the same final concentration 1.2×10^{-3} wt%. (b) SEM micrographs of the concentrate (1.7×10^{-2} wt%) shows the structure and size distribution of the natural rubber particles.

The interaction between concentrate and the fluorescent probes (QP and Bpy) was further studied by FCS. The normalized auto-correlated intensity curve of QP in the presence of natural rubber particles clearly shows a shift to longer mean diffusion times compared with that of free QP (Figure 3). The fits of these FCS curves using Eq. 1 give a single diffusion time of 4 ± 2 ms for free QP and an additional 84 ± 4 ms representing a population of 37% in the presence of natural rubber particles, revealing a complex formation. Since the Bpy–NRL particle complex is non-fluorescent, no difference was observed in their FCS curve by comparison with the free Bpy.

The interaction of QP and Bpy with LRPs in solution could also be imaged by fluorescence microscopy (Figure 4b,c). Since the size of the natural rubber particles is within the resolution limit of the confocal microscope, it should be kept in mind that these images also include diffracted light as observed on transmission (Figure 4a). The QP interaction with NRL (Figure 4b) displays a fluorescent ring with a dark interior and a dark background attributed to the phospholipid–protein–QP complexes formed in the shell surrounding the elastomeric core of latex particles. On the contrary, Bpy–latex interaction displays a non-fluorescent ring around a fluorescent sphere (Figure

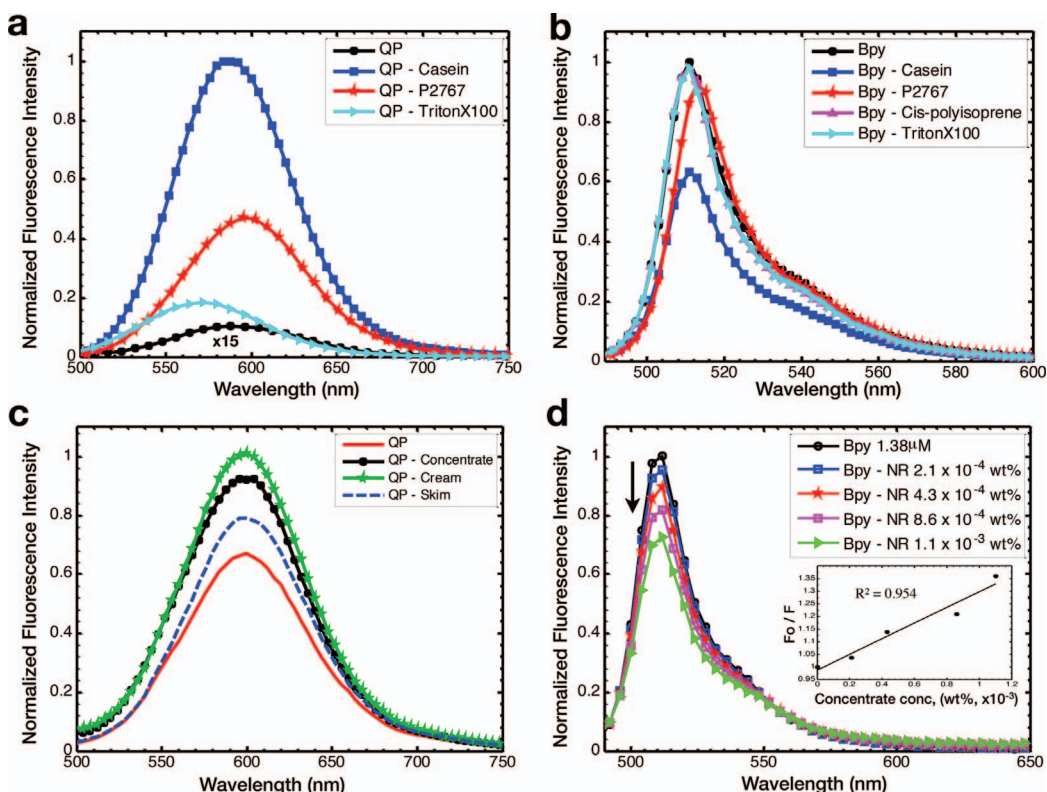


Fig 2. — Normalized fluorescence emission spectra of (a) QP (diluted 200 \times) and (b) Bpy (2 μ M) either free or in the presence of latex particle components: casein (2×10^{-2} wt%, \square), P2767 (250 μ M, \star), *cis*-polyisoprene (8×10^{-3} wt%, Δ), and triton X100 (100 μ M, \triangleright). The excitation wavelengths were fixed at 460 nm and 350 nm for QP and Bpy, respectively. For the sake of clarity, the emission spectra of free QP was multiplied 15 \times . (c) Normalized fluorescence emission spectra of free QP (straight line) and in the presence of concentrate (\circ), cream (\star), and skim (- -) in water. The concentrate, skim, and cream have the same final concentration 4.3×10^{-4} wt%. The excitation wavelength was fixed at 460 nm. (d) Normalized fluorescence emission spectra of Bpy in the presence of increasing concentrate concentration. The excitation wavelength was fixed at 350 nm. Inset: linear dependence between the ratio of the free Bpy fluorescence intensity (F_0) and in the presence of concentrate (F), as a function of concentrate concentration.

4c) in agreement with the fluorescence quenching previously described for phospholipid–protein–Bpy complexes. The observed fluorescent background (Figure 4c) is derived from noise and a few free Bpy molecules. The slightly higher intensity of the central sphere could be explained by the diffusion of free Bpy through the polyisoprene core of the natural rubber particle.

MEMBRANE COMPOSITION

In this section, we first focus on the interaction of QP and Bpy with the different colloidal suspensions: concentrate, skim, and cream. FCS attests to these interactions with a shift to longer diffusion times by comparison with free QP (Figure 3). The analysis of the curves also points out the differences in the latex size distribution between the different solutions. QP in interaction with skim particles can be fitted using a single component diffusion model, with $\tau_D = 8 \pm 2$ ms, which is ~ 2.7 times the diffusion of free QP ($\tau_D = 3 \pm 1$ ms). This increase in τ_D is in line with interaction of QP with SRPs population of skim suspension (~ 120 nm as

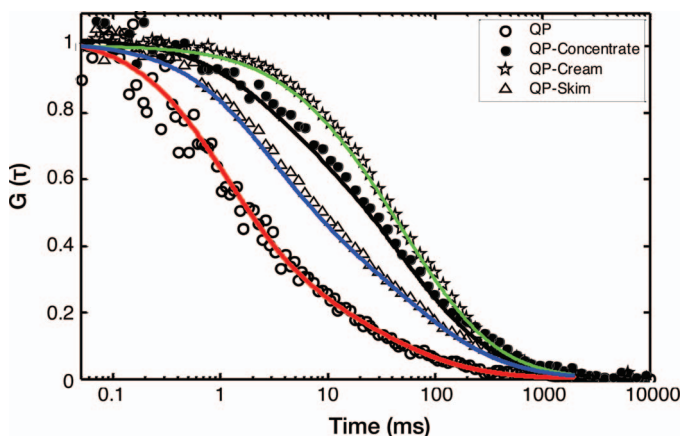


Fig 3. — Normalized auto-correlation curves of free QP (diluted 200 \times , \circ) and in the presence of concentrate (\bullet), cream (\star), and skim (\triangle). QP was biphotonically excited at 860 nm. All the latex suspension were diluted to the same concentration of 4.3×10^{-4} wt%.

revealed by DLS). As for QP–concentrate complexes, the FCS curves of QP–cream must be analyzed using Eq. 1, leading to diffusion times $\tau_{D1} = 6 \pm 1$ ms and $\tau_{D2} = 72 \pm 10$ ms, similar to that obtained for QP–concentrate suspension (4 ± 2 ms and 84 ± 4 ms, respectively). Nevertheless the population distribution was quite different with $\alpha_1 = 25 \pm 10\%$ and $\alpha_2 = 75 \pm 10\%$ for QP–cream complexes and $\alpha_1 = 63 \pm 4\%$ and $\alpha_2 = 37 \pm 4\%$ for QP–concentrate complexes: this confirms the DLS data, which reveals an enrichment of LRPs in cream.

However, the affinity of QP differs depending of the colloidal suspension. At identical natural rubber particle mass concentration, the increase in QP fluorescence intensity should be the same in the presence of skim, concentrate, and cream, but an increase of $\sim 25\%$, $\sim 40\%$, and $\sim 50\%$ were observed, respectively, in reference to free QP (Figure 2c). Similar conclusions can be drawn from the interaction of Bpy, with the separated natural rubber populations showing an increasing fluorescence quenching from skim ($\sim 4\%$) to concentrate and cream ($\sim 20\%$) by reference to free Bpy (Figure 5). Considering cream and concentrate composition (both SRPs and LRPs) by comparison with skim (only SRPs), these results bring to light that there are more proteins and/or phospholipids in LRPs than SRPs. To further investigate the importance of lipids in this quenching

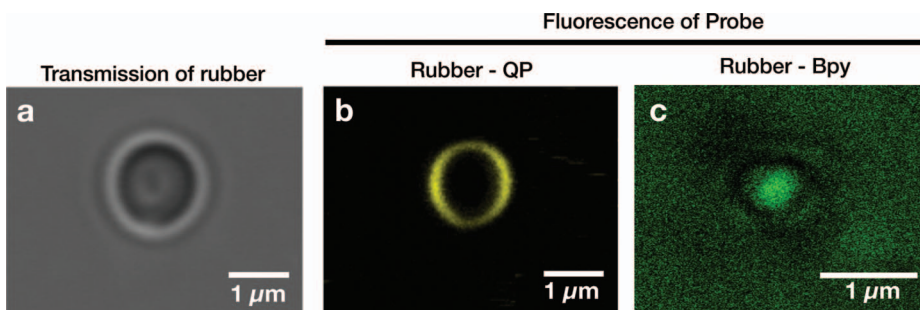


Fig 4. — Confocal micrographs of QP-labeled NRL and Bpy-labeled NRL excited at 488 nm. (a) The transmission image shows the NRL (central gray sphere) and the diffracted light (airy pattern or alternating dark and white spherical ring). This pattern is pronounced because the size of NR is just within the optical resolution limit of the confocal microscope.

Fluorescence images of (b) QP-NRL and (c) Bpy-NRL.

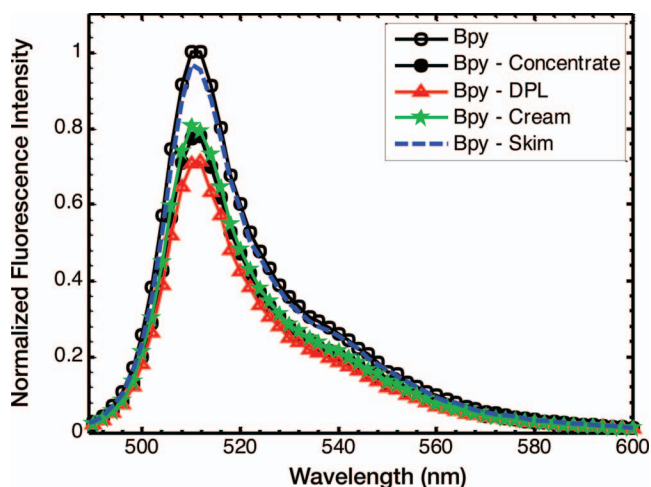


Fig 5. — Normalized fluorescence emission spectra of Bpy (○) free or in presence of concentrate (●), DPL (▷), cream (★), and skim (- -). The concentrate, skim, and cream have the same final concentration 3×10^{-3} wt%. The excitation wavelength was fixed at 350 nm.

effect, experiments were performed on DPL in the presence of Bpy (because unlike QP, Bpy does not interact with the surfactants). Steady-state fluorescence measurements display similar degrees of Bpy quenching ($\sim 30\%$) before and after protease treatment of natural rubber particles (Figure 5), signifying that quenching by the proteins appears insignificant compared with the lipids. This result supports the greater number of phospholipid molecules than proteins on a single NRL particle, as classically described.^{24,25}

All of the results indicate that there are more lipids in LRPs than SRPs in solution. This is atypical since at a given weight concentration, SRPs having higher surface area should contain more lipids than LRPs. Our result, therefore, can only be explained if the membrane of SRPs has a lower degree of compacity and/or is thinner compared with LRPs. It has also been reported, but on dry rubber, that the ester content (lipids) in SRPs is ~ 80 times less than in LRPs.²⁶ Such disparities between LRPs and SRPs is not unique to lipids; it occurs in proteins as well. Previous studies on various NR model membranes have shown some proteins specific only to either LRPs or SRPs.^{12,13} As our results demonstrate for the first time, similar observations can be obtained for the lipids that are directly in the native aqueous natural rubber itself.

The difference in lipid content in both SRP and LRP is an important parameter related to the NR's mechanical properties, notably tensile strength. It has been shown that rubber consists of 96% *cis*-polyisoprene and 4% non-rubber components, essentially lipids and proteins.⁴ As reported several times, the tensile strength of NR is higher compared with its synthetic counterpart ($\sim 98\%$ *cis*-polyisoprene), which does not contain any protein or phospholipid groups.

Sakdapipanich et al.^{14,15,27,28} highlighted the role of proteins and lipids in linking the isoprene chains to create a linear rubber chain network. When proteins were removed from NR, the tensile strength remained unchanged,²⁹ while trans-esterification significantly decreases the tensile strength of NR to a level comparable to synthetic rubber.³⁰ It is believed that such trans-esterification decomposes the network points of lipids, thus breaking up the network structure. This strongly suggests that in the naturally occurring network of NR, the lipids are responsible for the higher tensile strength.

Our findings therefore also indicate that SRPs having fewer lipids will have lower tensile strength compared with LRPs. This could be beneficial in rubber processing by taking advantage of low energy consumption.

CONCLUSION

SRPs and LRPs differ not only in particle size but also in surface composition. SRPs contain fewer lipids than LRPs. This by itself indicates that the membrane composition of NR is size dependent and that the membrane of SRPs might have lower degree of compacity than and/or be thinner than LRPs.

One of the important implications of membrane compacity and thickness would be the rigidity of the membrane, which impacts the fluidity of the rubber and flow of materials. Proteins and lipids are thought to generate cross-linking points and bridging with polyisoprene.^{14,15,31} Thus, the variation in the compacity and thickness of the membrane could also effectively influence the interaction of protein–lipid–polymer, and consequently the NR coagulation process, through interaction between either identical particles (NR–NR) or different particles (NR–fillers). Introduction of fillers to NR is a common practice in the rubber industry to improve the properties of the elastomeric compound. Because of the potential technological applications, the behavior of SRPs and LRPs with organic fillers deserves further investigation. This is currently under study in our laboratory.

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