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MICROSTRUCTURE OF PURIFIED RUBBER PARTICLES

Delilah F. Wood[1] and Katrina Cornish

USDA-ARS, Western Regional Research Center, 800 Buchanan Street, Albany, California 94710, U.S.A.

Purified rubber particles from *Hevea brasiliensis* (Brazilian rubber tree), *Parthenium argentatum* (guayule), *Ficus elastica* (Indian rubber tree), and *Euphorbia lactiflua* were examined and compared using conventional scanning electron microscopy (SEM), field-emission SEM, cryo-SEM, and transmission electron microscopy (TEM). Rubber particles of all four species were spherical; they varied in size and had a uniform homogeneous material, the rubber core, surrounded by a contiguous monolayer (half-unit) membrane. Frozen-hydrated and/or untreated particles from *H. brasiliensis* and *P. argentatum* deformed and fused readily, whereas those from *F. elastica* and *E. lactiflua* retained their spherical shapes. These results indicate that the surface components of the *H. brasiliensis* and *P. argentatum* particles are more fluid than those of *F. elastica* or *E. lactiflua*. When fixed in aldehyde, *F. elastica* particles retained their spherical exterior shapes but had hollow centers, whereas *H. brasiliensis* and *P. argentatum* particles completely collapsed. In aldehyde–osmium tetroxide–fixed material, the rubber core of *F. elastica* was poorly preserved in some particles in which only a small amount of the rubber core remained adhering to the monolayer membrane, leaving a hollow center. *Euphorbia lactiflua* particles were well preserved in terms of retaining the rubber core; however, the membrane was not as easily discernible as it was in the other three species. Both *H. brasiliensis* and *P. argentatum* were well preserved following fixation; their cores remained filled with rubber, and their monolayer membranes were defined. The addition of potassium permanganate to the fixation-staining regime resulted in higher-contrast micrographs and more well defined monolayer membranes.

Keywords: microstructure, rubber particles, microscopy, high-resolution SEM, field-emission SEM, cryo-SEM, TEM.

Introduction

Currently, all commercial rubber is derived from *Hevea brasiliensis*, which produces primarily long-chain, high-molecular-weight, high-quality rubber. Economically and ecologically, a single-species source of any commodity is inadvisable because of the limited options available should any problem arise with that species. The major concerns with *H. brasiliensis* are that it grows only in tropical regions, it is genetically extremely narrow, and some of the proteins associated with its rubber are highly allergenic to certain individuals when they are exposed to such products as latex gloves (Siler and Cornish 1994; Siler et al. 1996). Thus, there is a strong incentive to develop an alternative source of commercial rubber.

Natural rubber is produced in numerous plant species, but not all rubber is of commercial-grade quality. Plants produce rubber in compartmentalized, enzymatically active microscopic particles (Backhaus and Walsh 1983; de Faÿ and Jacob 1989; Dennis and Light 1989; Goss 1991). Biochemical studies have shown that rubber transferase, the enzyme that catalyzes isoprene into rubber, is particle bound (Archer et al. 1963; Archer and Audley 1987; Madhavan et al. 1989; Cornish and Backhaus 1990; Cornish 1993; Cornish and Siler 1996). Because the particles are enzymatically active and because they synthesize and compartmentalize the same secondary product, we hypothesize similarities in microstructure among rubber particles isolated from vastly different species. Also, enzymatic activity is typically associated with a biomembrane. Thus, clarification of the presence and type of membrane or exterior covering is essential to understanding rubber biosynthesis and rubber particle ontogeny. Knowing the composition of covering/membrane may indicate mechanisms by which natural rubber from other sources can be engineered or modified.

By biochemical quantification of nonrubber constituents in rubber particles, Siler et al. (1997) found insufficient conventional membrane material to form a bilayer membrane surrounding the particles. In addition, in rubber particles from two species, Siler et al. (1997) reported insufficient membrane lipid (phospholipids, glycolipids, and sterols) to form a complete monolayer around the particle. Thus, it seemed possible that the rubber particles were bounded by an incomplete or patchy membrane. Further observations using electron paramagnetic resonance have provided direct evidence for a monolayer membrane, but these observations had to be combined with transmission electron microscopy (TEM) to demonstrate that no gaps occurred in the membrane (Cornish et al. 1999).

In this study, we provide further evidence for the presence of an exterior membrane that forms the surface of the rubber particle. We also compare the microstructures and the physical behaviors of purified rubber particles from four species—*Ficus elastica* (Indian rubber tree), *H. brasiliensis* (Brazilian rubber tree), *Parthenium argentatum* (guayule), and *Euphorbia lactiflua*—and their reactions under different conditions of specimen preparation.

[1] Author for correspondence; e-mail wood@pw.usda.gov. Formerly published as Delilah W. Irving.

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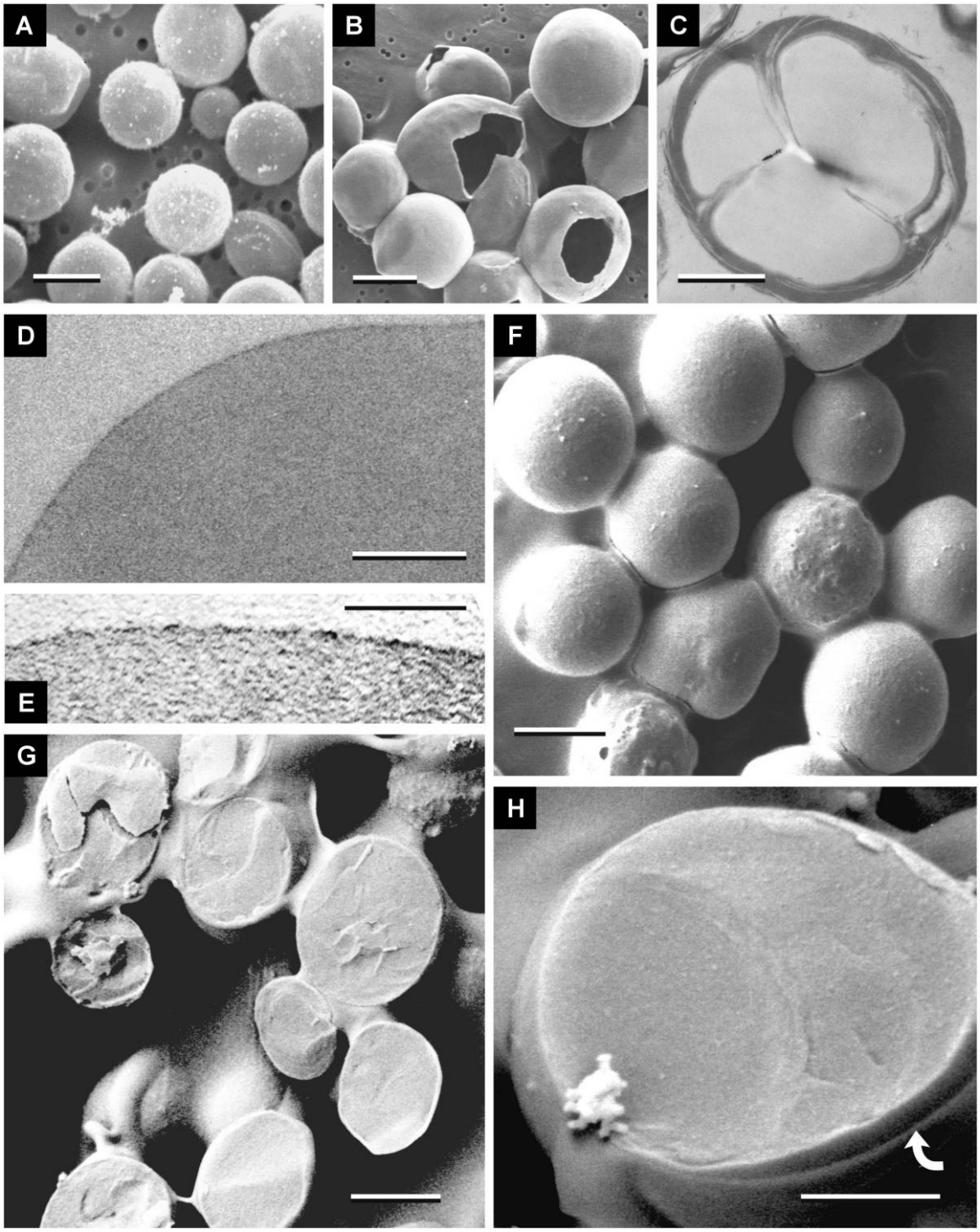


Fig. 1 *Ficus elastica* rubber particles. *A*, Conventional scanning electron micrograph (SEM). Sample was fixed in 3% glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate buffer, postfixed in 1% aqueous OsO_4, and critical-point dried. Magnification bar = 2.0 μm. *B*, Field-emission SEM at ambient temperature. Sample was fixed in 3% glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate buffer and critical-point dried. Note that the particles maintained their spherical shapes, indicating that the membrane material is highly proteinaceous. Large holes are apparent, which probably resulted from the liquid rubber core forcing its way out through areas of least resistance during specimen preparation. Magnification bar = 2.0 μm. *C*, Transmission electron micrograph (TEM) of a single large rubber particle in which the interior rubber core appears to have condensed along the outside of the membrane, thereby creating an empty-appearing particle. Material was fixed in buffered (0.1 M sodium cacodylate and 1 mM $CaCl_2$ [pH 8.0]) 3% glutaraldehyde and 2% formaldehyde; fixed in buffered 1% OsO_4; and fixed in buffered 1% $KMnO_4$. Sections were left unstained. Magnification bar = 1 μm. *D*, TEM of a rubber particle showing a single-track membrane. Material was fixed in 6% glutaraldehyde, 0.05 M sodium cacodylate (pH 7.2); fixed in 1% aqueous OsO_4. Sections were stained

Material and Methods

Rubber Particles

Rubber particles were purified from latex of *Euphorbia lactiflua* (Concepcion, Chile); latex of *Ficus elastica* (obtained from greenhouse-grown trees in Albany, Calif.); latex of *Hevea brasiliensis* (line PB 260, obtained from plantation-grown trees in Sumatra); and stem bark of *Parthenium argentatum* (line 11591, harvested from field-grown shrubs in Phoenix, Ariz.). Rubber particles were purified (as described) from *E. lactiflua* and *H. brasiliensis* latex samples (Cornish et al. 1993), from *F. elastica* latex (as described for buoyant particles; Cornish and Siler 1996), and from *P. argentatum* bark (Cornish and Backhaus 1990). Purified rubber particles were stored in buffered 10% or 30% glycerol at −20°C until prepared for microscopy.

Ambient-Temperature SEM

A drop of a suspension of rubber particles in glycerol was fixed in 3% glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate and 2 mM calcium dichloride ($CaCl_2$; pH 8.0). The suspensions were fixed overnight at 4°C and then filtered through a 13-mm-diameter (0.1, 0.2, or 0.4 μm) membrane filter (Nuclepore, Cambridge, Mass.), using a glass syringe. Each membrane filter was encased between two hard plastic mesh holders, which were subsequently tied together using thin nylon thread. The filters were encased so that they remained flat throughout processing and in order to maintain orientation. The encased filters (specimens) were then treated as a unit until they were mounted for SEM. The specimens were postfixed in 1% osmium tetroxide (OsO_4) in the above buffer for 2 h at 22°C and rinsed twice (20 min per rinse) in buffer. The specimens were dehydrated in a graded ethanol series and critical-point dried (Polaron E3000 Critical Point Dryer, Polaron Equipment Limited, Watford). Alternatively, specimens were treated as above but we omitted fixation in the OsO_4.

A suspension of *H. brasiliensis* was fixed in glutaraldehyde-formaldehyde, as previously described, and was then treated with 1% sodium dodecyl sulphate (SDS) to solubilize any non-rubber particles. The suspensions were postfixed in 1% aqueous OsO_4, dehydrated, and critical-point dried.

Following critical-point drying, the membrane filters were removed from the plastic mesh holders by cutting the nylon thread. Filters were mounted onto aluminum specimen stubs using carbon conductive tabs (Ted Pella, Redding, Calif.), gold coated in a Polaron E5100 sputter-coating unit (Polaron), and viewed at 10 kV in a Hitachi S-530 SEM or at 2 kV in a Hitachi S-4200 field-emission SEM.

Low-Temperature SEM

Glycerol suspensions of *H. brasiliensis*, *P. argentatum*, and *F. elastica* rubber particles were diluted with distilled water (which had been adjusted to pH 8 with NH_4OH) and centrifuged (flotation for *H. brasiliensis* and *P. argentatum*, precipitation for *F. elastica*) to remove the glycerol and to concentrate the particles. Solution exchanges were made by inserting a drawn-out Pasteur pipette below the concentrated floating particles or above the precipitated particles and then adding fresh solution. A drop of washed rubber particle suspension was placed on a brass specimen holder, and a thin sheet of roughened copper (ca. 5 × 10 mm) was placed on the surface of the suspension. The holder was plunged into a Styrofoam cup containing liquid nitrogen, where the sample was fractured as we forced off the copper sheet (using forceps). Other particles were prepared by diluting with distilled water (at pH 8), filtered through a 0.4-μm Nuclepore filter, and then frozen by plunging into liquid nitrogen. Frozen-hydrated samples were transferred under vacuum to the preparation chamber of an Oxford CT1500-HF cryopreparation system (Oxford), sublimated at −80°C for 10 min, sputter-coated with gold-palladium, transferred to the cold stage of a Hitachi S-4100 field-emission SEM, and viewed and photographed at 1.5–5 kV.

Rubber particles prepared for cryo-SEM were rinsed and reconcentrated to remove the electron-opaque glycerol. The stability of the rubber suspension was, therefore, compromised as a result of dilution of glycerol, the centrifugation steps, and the exposure to water. As rubber particle suspensions were more stable above pH 7.0, the pH of the distilled water used in sample preparation was adjusted to pH 8.0 using NH_4OH. The use of a standard buffer (e.g., sodium phosphate or sodium cacodylate) was avoided in order to prevent the possibility of a salt residue after sublimation of water during cryopreparation. The entire preparation of rubber particles from either *P. argentatum* or *H. brasiliensis* congealed when the particles were washed at an acid pH.

Euphorbia lactiflua rubber particles were treated slightly differently as a result of the difficulty associated with concentrating the particles by centrifugation, since they had densities very close to that of water. A few drops of suspended *E. lactiflua* rubber particles in 30% glycerol were fixed for 10 min in a fixative containing 1 mL of 3% glutaraldehyde, 2% formaldehyde, 0.1 M sodium cacodylate, 2 mM $CaCl_2$, and two drops of 2% aqueous OsO_4. The OsO_4 served to increase the density of the particles so that they pelleted following centri-

using uranyl acetate–lead citrate. Magnification bar = 250 nm. *E*, TEM of part of a rubber particle showing the single-track membrane. Material was fixed in buffered 3% glutaraldehyde and 2% formaldehyde, fixed in buffered 1% OsO_4, and fixed in buffered 1% $KMnO_4$. Sections were left unstained. Magnification bar = 100 nm. *F*, Low-temperature field-emission SEM (<−130°C; at 2 kV) of whole frozen-hydrated rubber particles. Note the smooth spherical particles. Magnification bar = 2.0 μm. *G*, Low-temperature field-emission SEM (at 2 kV) of frozen-hydrated and fractured rubber particles showing the homogeneous rubber core. The observed texture is the result of the stress of fracturing, which is obvious because there is no particular pattern in the various particles. Magnification bar = 2.0 μm. *H*, Low-temperature field-emission SEM of frozen-hydrated and fractured rubber particle. Note the homogeneous rubber core in the center of the particle and the membrane, which has fractured on a slightly different plane (arrow). Magnification bar = 1.0 μm.

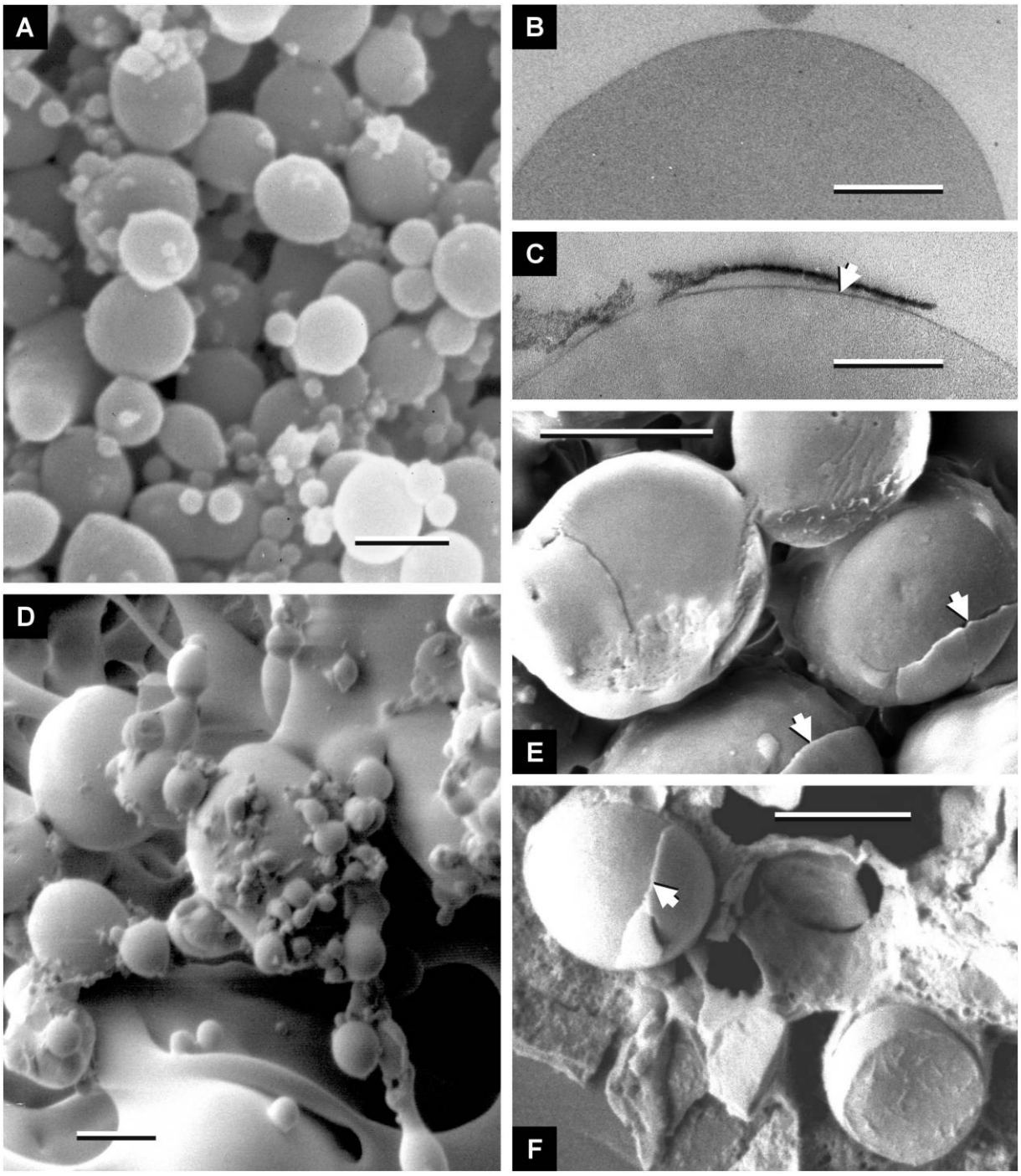


Fig. 2 *Hevea brasiliensis* rubber particles. *A*, Conventional scanning electron microscopy (SEM) at 10 kV. Sample was fixed in 3% glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate buffer, postfixed in 1% aqueous OsO_4, and critical-point dried. Magnification bar = 2 μm. *B*, Transmission electron micrograph (TEM) of a single rubber particle showing a single-track membrane (arrow). Material was fixed in 6% glutaraldehyde, 0.05 M sodium cacodylate (pH 7.2); fixed in 1% aqueous OsO_4. Sections were stained using uranyl acetate–lead citrate. Magnification bar = 500 nm. *C*, TEM of part of a rubber particle showing the single-track membrane. Material was fixed in buffered 3% glutaraldehyde and 2% formaldehyde, fixed in buffered 1% OsO_4, and fixed in buffered 1% $KMnO_4$. Sections were left unstained. The densely stained material exterior of the membrane at the top of the micrograph probably represents condensed portions of disrupted or broken rubber particles that have adhered to the sticky exterior membrane. Magnification bar = 250 nm. *D*, Low-temperature field-emission SEM (at 1.5 kV) of frozen-hydrated whole particles. Note again the variation in particle size. Magnification bar = 2 μm. *E*, Low-temperature field-emission SEM (at 4 kV) of frozen-hydrated fractured particles. Magnification bar = 2 μm. *F*, Low-temperature field-emission SEM (at 2 kV) of frozen-hydrated and fractured particles. Note in both *E* and *F* that the exterior membrane has fractured at a slightly different plane than the interior core material (arrows). Magnification bar = 2 μm.

Table 1

Rubber Particle Diameters

	Ficus elastica		*Hevea brasiliensis*		*Parthenium argentatum*		*Euphorbia lactiflua*	
	SEM	LLS	SEM	LLS	SEM	LLS	SEM[a]	LLS
n	27	...	11	...	21	...	66	...
Mean (μm)	2.92	3.80	1.34	0.96	2.73	1.41	0.208	0.42
Standard deviation (μm)	0.37	...	0.95	...	0.48	...	0.90	...

Note. *n* = number of particles measured from SEM micrographs; LLS = laser light scattering. SEM measurements were of frozen-hydrated particles. LLS measurements using laser light-scattering particle sizing (Cornish et al. 1993).

[a] Particles were fixed 10 min in glutaraldehyde-formaldehyde-OsO_4 and then frozen-hydrated.

fugation. The lightly fixed particles were rinsed in distilled water and centrifuged five times over a period of 20 min. The final pellet was resuspended in ca. 0.25 mL distilled water, and a drop of the suspension was placed on a brass specimen holder and was observed in whole or fractured form, as described above for the other rubber particles. Samples were sublimated at −80°C for 10 min and sputter-coated with gold-palladium in an Oxford Alto 2500 cryopreparation system. The frozen-hydrated preparations were observed and photographed in a Hitachi S4700 field-emission SEM.

TEM: Uranyl Acetate–Lead Citrate Staining

A suspension of rubber particles (in glycerol) was fixed for 6 h at 23°C in 6% glutaraldehyde buffered in 0.05 M sodium cacodylate (pH 7.2), rinsed in the same buffer, and postfixed in 1% aqueous OsO_4 for 12 h at 4°C, following the procedure of Gilliland and van Staden (1983), who described rubber particles *in situ* in *P. argentatum*. Suspensions were centrifuged between each treatment to concentrate the particles (flotation for *E. lactiflua*, *H. brasiliensis*, and *P. argentatum*; sedimentation for *F. elastica*), thereby allowing for solution exchanges. Sucrose (500 mM) was added to solutions containing *E. lactiflua* in order to create a density difference between the particles and the solutions so that the particles could be isolated. After *E. lactiflua* particles were treated with OsO_4, they became more dense and pelleted on centrifugation, as did all the other rubber particles; therefore, the addition of sucrose was not necessary in the OsO_4 or in any of the following solutions. Samples were dehydrated in a graded ethanol series, embedded in Spurr's resin (Spurr 1969), and sectioned. Sections were stained in uranyl acetate and lead citrate (Reynolds 1963), viewed, and photographed in a JEOL 100C TEM.

TEM: Potassium Permanganate ($KMnO_4$) Staining

Rubber particle suspensions in glycerol were fixed overnight in a solution containing 3% glutaraldehyde, 2% formaldehyde, 0.1 M sodium cacodylate, and 1 mM $CaCl_2$ (pH 8.0); rinsed in the same buffer; and centrifuged three times. Sucrose (500 mM) was added to the fixative and rinses for preparing *E. lactiflua* particles. The particles were treated overnight in 1% OsO_4 in 0.1 M sodium cacodylate and 1 mM $CaCl_2$ (pH 8.0) at 22°C. The particles were rinsed five times over several hours, reconcentrated, treated for 1 h in 1% $KMnO_4$ in the cacodylate buffer, and rinsed five times in buffer over several hours. Treated rubber particles were dehydrated in a graded ethanol series, infiltrated and embedded in Spurr's resin (Spurr 1969), and sectioned. Sections were cut and observed without further staining in a Philips CM12 TEM.

Rubber Particle Diameters

Measurements were made on whole particles from digitized SEM photomicrographs. Means and standard deviations were calculated, and sizes were compared with the measurements obtained from laser light scattering (Cornish et al. 1993).

Results

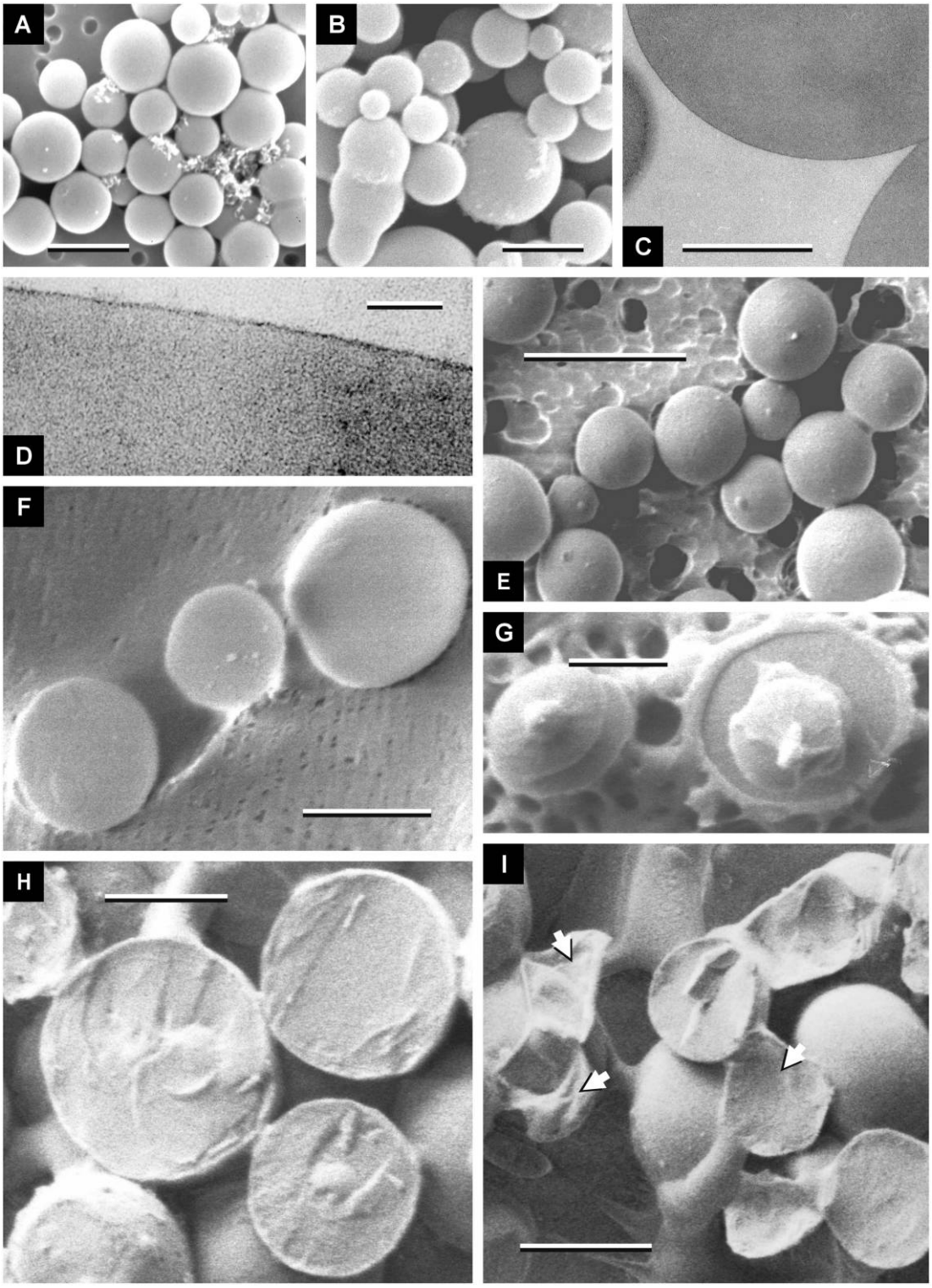
Rubber particles from all four species were spherical and varied in size. Particle diameters, determined from SEM, were compared with those obtained from laser light scattering (table 1). Fractures of frozen-hydrated rubber particles observed using cryo-field-emission SEM revealed that the interiors were entirely filled with uniform material, indicating a homogeneous rubber core.

Ficus elastica

The conventional SEM preparation technique of aldehyde and OsO_4 fixation followed by dehydration and critical-point drying showed particles that were spherical but slightly collapsed in some cases (fig. 1*A*). When OsO_4 was omitted during fixation, some of the particles had holes in the membranes and hollow interiors (fig. 1*B*). Transmission electron micrographs also revealed particles with hollow or non-electron-dense centers, in which core material had presumably condensed along the interior surface of the membranes, even with OsO_4 fixation (fig. 1*C*). Other particles, however, were completely filled with electron-dense material, and the single-track membrane was clearly visible (fig. 1*D*, 1*E*). Low-temperature SEM showed that whole rubber particles were spherical and not collapsed (fig. 1*F*). Fractures of the frozen-hydrated *F. elastica* rubber particles revealed a homogeneous interior in which the only apparent differences in the structure of the interiors were the result of fracture-strain artifacts (fig. 1*G*, 1*H*). The exterior membrane of the particles had slightly different fracture planes, in some cases, than did the interiors (fig. 1*H*), and it revealed a contiguous monolayer membrane (fig. 1*H*, arrow).

Hevea brasiliensis

Conventional SEM preparations of aldehyde- and OsO_4-fixed *H. brasiliensis* rubber particles yielded spherical particles



with no evidence of collapse (fig. 2*A*), regardless of the wide size range found. Fixation in aldehyde alone resulted in completely deformed and fused particles (not shown). In TEM sections, all particles were filled with electron-dense material surrounded by a slightly more electron-dense membrane (fig. 2*B*, 2*C*). Unfixed frozen-hydrated particles viewed under low-temperature conditions (fig. 2*D*) revealed an even greater size distribution than did those particles from fixed preparations. The fracture planes in frozen-hydrated particles of *H. brasiliensis* (fig. 2*E*, 2*F*) appeared to be smoother than those in *F. elastica* (fig. 1*G*, 1*H*). The particles of *H. brasiliensis* also showed differences in the fracture planes between the exterior membrane and the interior core material (fig. 2*E*, 2*F*); the membrane appeared like a broken shell in some of the fractures (fig. 2*E*, 2*F*, arrows).

Parthenium argentatum

Parthenium argentatum particles showed less variability in size than did *H. brasiliensis* particles (table 1). The rubber particles were well stabilized by fixation in aldehyde-OsO_4, regardless of whether the particles were critical-point dried (fig. 3*A*) or air-dried from ethanol (fig. 3*B*). Rubber particles not postfixed in OsO_4, however, were flattened and deformed on drying (not shown). The particles tended to fuse readily (fig. 3*B*, lower left corner), and TEM sections showed that the membranes of individual particles merged to form the exterior membrane of a new fused particle (not shown). Although not highly contrasted, the single-track membrane was evident in TEM sections (fig. 3C, 3*D*). Frozen-hydrated rubber particles were spherical with smooth surfaces (fig. 3*E*). Three types of fracture planes were noted in the rubber particles: completely smooth fractures (fig. 3*F*), fractures showing plastic deformation (i.e., particle interiors deformed during fracturing at liquid nitrogen temperatures of −190°C; fig. 3*G*), and fractures that were apparently attributable to fracture strain (fig. 3*H*, 3*I*), as was noted in both *F. elastica* (fig. 1*G*, 1*H*) and *H. brasiliensis* (fig. 2*E*, 2*F*). In some cryopreparations, the fracture plane occurred at the rubber particle membrane, pulling out the rubber core and leaving the membrane "ghosts" behind (fig. 3*I*, arrows).

Euphorbia lactiflua

Euphorbia lactiflua particles had the smallest average size of all the rubber particles studied, with a mean diameter of 0.208 μm (table 1). Only the smallest rubber particles from *H. brasiliensis* were smaller than the *E. lactiflua* particles. Frozen-hydrated rubber particles were lightly fixed in aldehyde-OsO_4 in order to facilitate isolation of the particles. The particles were spherical in shape (fig. 4*A*, 4*B*) and did not appear to be completely smooth (fig. 4*B*), which could be attributed to disruption by the fixatives, impurities sticking to the particle surface, or actual disruptions on the particle surface. The rubber particles remained spherical after aldehyde and OsO_4 fixation (not shown), but they deformed and coalesced following fixation with aldehyde alone (not shown). An electron-dense monolayer membrane (fig. 4*C*–4*E*) could be seen in some TEM of the particles. An electron-dense interior, the rubber core, was apparent in all TEM sections. Frozen-hydrated and fractured particles revealed smooth fractures of the interior rubber core (fig. 4*F*, 4*G*).

Discussion

The electron microscopy investigation of rubber particle structure in this article has demonstrated some structural commonalities among the four species. Native rubber particles were spherical, filled with homogeneous material (the rubber), and surrounded by discrete monolayer membranes, which confirms the conclusions of Cornish et al. (1999). In addition, the membrane was clearly a contiguous (not patchy) structure surrounding the particles of at least *Ficus elastica*, *Hevea brasiliensis*, and *Parthenium argentatum*. The sticky nature of rubber particles, particularly of *Euphorbia lactiflua*, makes it difficult to see the membrane in TEM sections because other substances stick to and obscure the membrane surface. In addition, fixation does not appear to adequately stabilize the particles for TEM preparations. Certainly, *E. lactiflua* particles have membranes, since the particles have more membrane components than any of the four species studied (Siler et al. 1997). Differences in rubber particle behavior and appearance were observed on freeze-fracturing for SEM and under different fixation regimes. The differences in behavior can be related to known characteristics of the four particle types.

Fig. 3 *Parthenium argentatum* rubber particles. *A*, Conventional scanning electron microscopy (SEM) at 10 kV. Particles were fixed in 3% glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate buffer, postfixed in 1% aqueous OsO_4, and critical-point dried. Note that the particle size variability is not as great as that noted in *Hevea brasiliensis*. Magnification bar = 2 μm. *B*, Conventional SEM at 10 kV. Particles were fixed in 3% glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate buffer, fixed in 1% aqueous OsO_4, and air-dried. Note the fused particles in the lower left corner of the micrograph. Magnification bar = 2 μm. *C*, Transmission electron micrograph (TEM) of rubber particle showing a single-track membrane. Material was fixed in 6% glutaraldehyde, 0.05 M sodium cacodylate (pH 7.2); fixed in 1% aqueous OsO_4. Sections were stained using uranyl acetate–lead citrate. Magnification bar = 1 μm. *D*, TEM of part of a rubber particle showing the single-track membrane. Material was fixed in buffered 3% glutaraldehyde and 2% formaldehyde, fixed in buffered 1% OsO_4, and fixed in buffered 1% $KMnO_4$. Sections were left unstained. Magnification bar = 25 nm. *E*, Low-temperature field-emission SEM at 2 kV. Frozen-hydrated whole rubber particles showing smooth spherical particles. Magnification bar = 1 μm. *F–I*, Low-temperature field-emission SEM (at 2 kV) of frozen-hydrated and fractured particles. Three types of fracturing may be seen: *F*, smooth fractures, magnification bar = 1 μm; *G*, particles showing plastic deformation during fracturing at liquid nitrogen temperatures of −190°C, magnification bar = 1 μm; and *H*, fractures that appeared to result from fracture stress, magnification bar = 2 μm. *I*, Fractured particles showing areas in which the rubber core was differentially fractured away from the membrane, leaving portions of the membrane behind (arrows). Magnification bar = 2 μm.

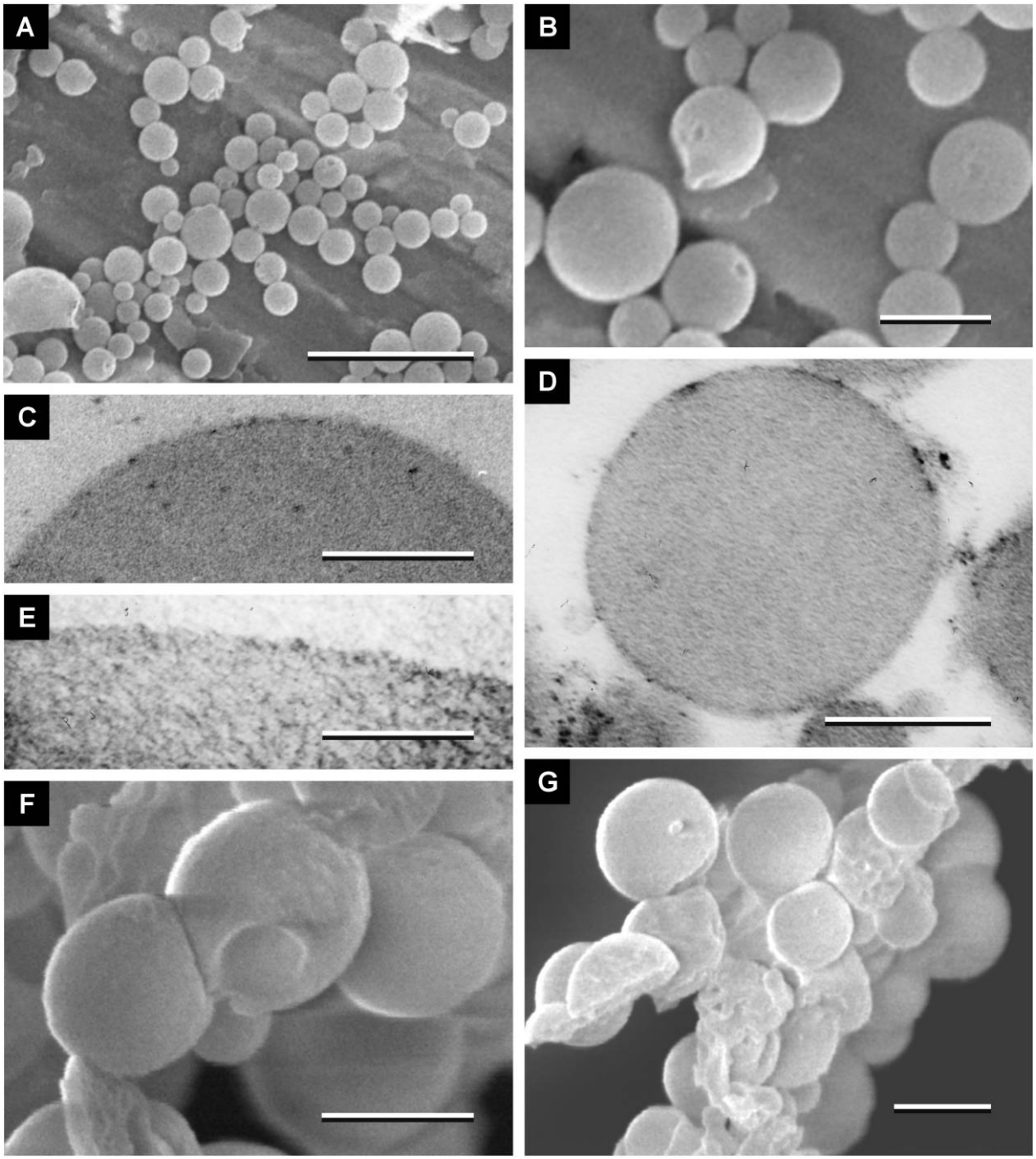


Fig. 4 *Euphorbia lactiflua* rubber particles. *A*, *B*, Field-emission cryo-scanning electron microscopy (SEM) of frozen-hydrated rubber particles at 4 kV. Material was lightly fixed in a mixture of glutaraldehyde and formaldehyde and OsO_4. *A*, Magnification bar = 1 μm. *B*, Magnification bar = 250 nm. *C*, Transmission electron micrograph (TEM) of a single rubber particle showing areas in which the membrane appears to be single track. Material was fixed in 6% glutaraldehyde, 0.05 M sodium cacodylate (pH 7.2); fixed in 1% aqueous OsO_4. Sections were stained using uranyl acetate-lead citrate. Magnification bar = 500 nm. *D*, *E*, TEM of rubber particles showing areas of single-track membrane. Material was fixed in buffered 3% glutaraldehyde and 2% formaldehyde; fixed in buffered 1% OsO_4; and fixed in buffered 1% $KMnO_4$. Sections were left unstained. *D*, Magnification bar = 200 nm. *E*, Magnification bar = 100 nm. *F*, *G*, Field-emission cryo-SEM showing fractured particles. Material was lightly fixed in a mixture of glutaraldehyde, formaldehyde, and OsO_4. *F*, Magnification bar = 250 nm. *G*, Magnification bar = 250 nm.

Rubber Particle Size

Rubber particle diameters measured on SEM differed from those measured using laser light scattering (table 1; Cornish et al. 1993). Both *H. brasiliensis* and *P. argentatum* had larger diameters, whereas *E. lactiflua* and *F. elastica* had smaller diameters, respectively, than those measured by laser light scattering. Differences in chemical composition between the species could account for their observed differences in behavior. *Ficus elastica* and *E. lactiflua* contain mostly low-molecular-weight (fluid) rubber, which might shrink on freezing. Both *P. argentatum* and *H. brasiliensis* rubber particles are composed mainly of high-molecular-weight (semisolid) rubber (Cornish et al. 1993, 1999), which may expand on freezing. Also, the protein and lipid composition of the different rubber particles vary considerably (Siler et al. 1997), and there may be other physical properties of these rubber particles that account for the lack of discernible differences in shrinkage in particles of *P. argentatum*. Alternatively, the observed differences could partially reflect the limited number of samples investigated, or they could reflect an inadvertent preferential size exclusion during specimen preparation, which is unlikely because of the wide range of particle sizes observed in *H. brasiliensis*.

Chemical Preservation of Particle Structure in Aldehydes

Aldehyde fixation of the protein component effectively prevented particle fusion, a fact that apparently contradicts the results of Condon and Fineran (1989). They reported fusion of latex particles of *Calystegia silvatica* following different fixation regimes and concluded that fixatives did not stabilize the latex particles either *in situ* or in latex exudate. However, the latex particles in *C. silvatica* contain lipid and may not display behavior similar to that associated with rubber latex. In addition, the latex in this study was stored in glycerol, which served as an effective rubber particle anticoagulant (Cornish and Bartlett 1997). Aldehyde fixative was added directly to the glycerol latex suspensions and was used at a basic pH, which maintained the negative surface charge on the particles, further preventing particle interaction during fixation. The particles did not fuse during subsequent steps following aldehyde fixation. Since glutaraldehyde is highly effective in cross-linking proteins, the nonfusion of the particles indicates that sufficient protein surrounded individual rubber particles to form a cross-linked network across the monolayer rubber particle membrane.

The *F. elastica* particles that were fixed with aldehyde alone and viewed by SEM showed ruptured particles with cavities surrounded by apparently intact "shells." The holes created by the rupture indicate that lines of relative weakness may exist in the rubber particle membrane that gave way during sample preparation, thus allowing for the extraction of the fluid rubber interior. Alternatively, the missing pieces could represent areas or lines that are not well preserved by aldehyde fixation and that are subsequently disrupted by solvents. Fixation of *H. brasiliensis*, *P. argentatum*, and *E. lactiflua* particles in aldehyde alone followed by dehydration and critical-point drying resulted in particles that had collapsed completely onto the filter substrate (not shown). When the suspension was sufficiently dilute, the particles remained distinct; thus, the aldehyde fixation prevented coalescence of particles but indicated that, unlike *F. elastica*, the cross-linked proteins did not provide sufficient rigidity to allow the particles to retain their spherical shapes.

Chemical Preservation of Particle Structure in OsO_4

Subsets of *F. elastica* rubber particle cores were not well fixed following both primary aldehyde and secondary OsO_4 chemical processing for microscopy, as was evident in TEM preparations of *F. elastica*. *Ficus elastica* contains short-chain rubber, which may not be completely stabilized by OsO_4. Alternatively, it is possible that neither long- nor short-chain rubber is preserved by treatment with OsO_4. The short-chain rubber may be more mobile than long-chain rubber and, therefore, may be more likely to leak through holes in particle membranes as a result of damage that occurs during sample preparation. Rubber from the centers of the particles dissolved in the solvents following OsO_4 treatment, leaving behind a layer of material against the exterior membrane of the *F. elastica* rubber particle (fig. 1*C*). The particles remain distinct once the proteins have been cross-linked with glutaraldehyde, even without the core. The shape retention of the particle still supports the notion that the particles are surrounded by a membrane, even though we cannot now see it (fig. 1*C*). In the absence of a membrane, dissolving the rubber would eliminate the entire particle, and no hollow particles would remain.

The TEM sections revealed that the remains of the hollow particles of *F. elastica*, the "shells" shown by SEM, were composed of core material that adhered to the interior surface of the membrane of the particle, which indicated retention of the higher-molecular-weight component of the rubber particles, condensation of core material, or limited penetration of OsO_4 to the depth of the electron-dense material. The subset of entirely electron-dense *F. elastica* rubber particles may represent the small amount of high-molecular-weight rubber that *F. elastica* is known to contain (Cornish et al. 1993).

The *E. lactiflua* particle did not appear to be well preserved following fixation with aldehydes, OsO_4, or $KMnO_4$. The rubber cores remained intact, but the outer membrane was sometimes indistinct and somewhat thickened in areas. The fact that the membrane does not appear to be continuous in TEM may be attributed to the nature of the particles themselves. Impurities in the preparations or other damaged particles may have stuck to the particle, thereby obscuring the surface details. Transmission electron micrographs in which $KMnO_4$ was used to prepare rubber particles of *H. brasiliensis* show adhesion of impurities to the surface of the membrane (fig. 2*C*). In addition, latex was shown to be difficult to prepare for TEM observation by Condon and Fineran (1989), who described the effects of chemical preparation of latex from *C. silvatica*. Even though *C. silvatica* was not shown to contain rubber, the latex particles are very likely to have the same sort of membrane system as rubber particles since both types of particles are hydrophobic units existing in aqueous environments. At this point, further research on fixatives would be necessary to obtain better electron micrographs.

Both *H. brasiliensis* and *P. argentatum* rubber particles were well preserved and maintained their integrity following aldehyde and OsO_4 fixation, as shown in both SEM and TEM preparations. Both types of particles contain primarily high-

molecular-weight rubber, which is more viscous and therefore less mobile than the low-molecular-weight rubber of *F. elastica* and *E. lactiflua*. The high-molecular-weight rubber also seemed to be better stabilized after fixation with aldehydes and OsO_4.

Cryo-SEM and Freeze-Fractured Particles

Despite the care taken in preparation for cryo-SEM, rubber particles of *H. brasiliensis* and *P. argentatum* were larger than expected, which indicated coalescence of two or more particles, in agreement with the findings on the fusion of latex particles of *C. silvatica* (Condon and Fineran 1989). Similarly, oil bodies, another hydrophobic body surrounded by a monolayer membrane (Yatsu and Jacks 1972; Tzen and Huang 1992), readily coalesce, yielding larger spherical droplets (D. F. Wood, unpublished results).

Rubber particles from *F. elastica* did not fuse, and they maintained their integrity and spherical shapes throughout specimen preparation. The greater stability of the rubber particles may be attributed to the rigidity of the outer membrane of the particle (because of their unusually long fatty acids; Siler et al. 1997) and to the large amount of integral membrane protein surface protein (Cornish et al. 1999).

The SEM freeze fractures showed that the isolated rubber particles of *F. elastica*, *H. brasiliensis*, and *P. argentatum* contained a homogeneous rubber core surrounded by a discrete membrane. However, as described earlier, *P. argentatum* particles fractured in three distinct manners, unlike *F. elastica* and *H. brasiliensis* particles. Two different rubber particle suspensions of *P. argentatum* were studied in this report. Sample 1 was stored in 30% glycerol before study, and sample 2 was stored in 10% glycerol before study. The particles in sample 1 tended to be larger and tended to have fracturing characteristics similar to those of *F. elastica* and *H. brasiliensis*, exhibiting several fracture planes in the core material, as was also observed for *F. elastica* and *H. brasiliensis* particles. However, sample 2 of *P. argentatum* had slightly smaller particles that either fractured with extremely smooth fracture planes or that exhibited plastic deformation during fracturing under liquid nitrogen. There appeared to be resistance as the central portion of the particles pulled away and then fractured (fig. 3*F*). The fracturing anomaly indicates that *P. argentatum* rubber retains some flexibility at low temperatures and can undergo plastic deformation. We have not determined why the two samples behaved differently, but they were collected at different times of the year, from different parts of the plant, and would likely contain different levels of resin dissolved in the rubber core. Nonetheless, these observations indicate that *P. argentatum* rubber should be investigated for its possible effectiveness in low-temperature applications.

Conclusions

Our electron micrographs indicate that the surface of the rubber particle is distinct from the homogeneous interior and that this surface takes the form of a contiguous membrane-like structure. That this surface structure is a monolayer biomembrane is concluded from the single-track TEM surface layers and the single layer seen by SEM freeze fractures as well as from electron paramagnetic resonance data (Cornish et al. 1999) and biochemical analysis of the rubber particles' lipid and protein components (Siler et al. 1997). An intact monolayer biomembrane provides a compatible interface between the hydrophobic rubber particle interior and the aqueous surrounding cytosol and is consistent with evidence for the presence of monolayer membranes surrounding other biological particles with hydrophobic interiors, such as oil bodies (Yatsu and Jacks 1972; Tzen and Huang 1992), and various prokaryotic inclusion bodies, including sulfur globules (Schmidt et al. 1971) and poly (β-hydroxyalkanoate) granules (Preusting et al. 1991; Mayer et al. 1996; Mayer and Hoppert 1997). The four rubber-producing species examined in this article are members of three different superorders of the Dicotyledonae. Their similar rubber particle structures leads us to the general conclusion that rubber particles consist of a homogeneous rubber core surrounded by an intact monolayer biomembrane.

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Literature Cited

Archer BL, BG Audley 1987 New aspects of rubber biosynthesis. Bot J Linn Soc 94:181–196.

Archer BL, BG Audley, EG Cockbain, GP McSweeney 1963 The biosynthesis of rubber: incorporation of mevalonate and isopentenyl pyrophosphate into rubber by *Hevea brasiliensis* latex fractions. Biochem J 89:565–574.

Backhaus RA, S Walsh 1983 The ontogeny of rubber formation in guayule, *Parthenium argentatum* Gray. Bot Gaz 144:391–400.

Condon JM, BA Fineran 1989 The effect of chemical fixation and dehydration on the preservation of latex in *Calystegia silvatica* (Convolvulaceae): examination of exudate and latex *in situ* by light and scanning electron microscopy. J Exp Bot 40:925–939.

Cornish K 1993 The separate roles of plant *cis* and *trans* prenyl transferases in *cis*-1,4-polyisoprene biosysthesis. Eur J Biochem 218: 267–271.

Cornish K, RA Backhaus 1990 Rubber transferase activity in rubber particles of guayule. Phytochemistry 29:3808–3813.

Cornish K, DL Bartlett 1997 Stabilisation of particle integrity and particle bound *cis*-prenyl transferase activity in stored, purified rubber particles. Phytochem Anal 8:130–134.

Cornish K, DJ Siler 1996 Characterization of *cis*-prenyl transferase activity localised in a buoyant fraction of rubber particles from *Ficus elastica* latex. Plant Physiol Biochem 34:377–384.

Cornish K, DJ Siler, O-K Grosjean, N Goodman 1993 Fundamental similarities in rubber particle architecture and function in three evolutionarily divergent plant species. J Nat Rubber Res 8:275–285.

Cornish K, DF Wood, JJ Windle 1999 Rubber particles are shown to consist of a homogeneous rubber core enclosed by a contiguous, monolayer biomembrane using a combination of transmission electron microscopy and electron paramagnetic resonance spectroscopy. Planta 210:85–96.

de Faÿ E, J-L Jacob 1989 The anatomical organization of the laticiferous system in the bark. Pages 3–14, 51–55 *in* J d'Auzac, J-L Jacob, H Chrestin, eds. Physiology of rubber tree latex: the laticiferous cell and latex—a model of cytoplasm. CRC, Boca Raton, Fla.

Dennis MS, DR Light 1989 Rubber elongation factor from *Hevea brasiliensis*: identification, characterization, and role in rubber biosynthesis. J Biol Chem 264:18606–18617.

Gilliland MG, J van Staden 1983 Detection of rubber in guayule (*Parthenium argentatum* Gray) at the ultrastructural level. Z Pflanzenphysiol Bodenkd 110:285–291.

Goss R 1991 The morphology, anatomy, and ultrastructure of guayule. Pages 33–45 *in* JW Whitworth, EE Whitehead, eds. Guayule: natural rubber. USDA, Washington, D.C.

Madhavan S, GA Greenblatt, MA Foster, CR Benedict 1989 Stimulation of isopentenyl pyrophosphate incorporation into polyisoprene in extracts from guayule plants (*Parthenium argentatum* Gray) by low temperature and 2-(3,4-dichlorophenoxy) triethylamine. Plant Physiol 89:506–511.

Mayer F, M Hoppert 1997 Determination of the thickness of the boundary layer surrounding bacterial PHA inclusion bodies, and implications for models describing the molecular architecture of this layer. J Basic Microbiol 37:45–52.

Mayer M, MH Madkour, U Pieper-Furst, R Wieczorek, M Liebergesell, A Steinbuchel 1996 Electron microscopic observations on the macromolecular organization of the boundary layer of bacterial PHA inclusion bodies. J Gen Appl Microbiol 42:445–455.

Preusting H, J Kingma, B Witholt 1991 Physiology and polyester formation of *Pseudomonas oleovorans* in continuous two-liquid-phase cultures. Enzyme Microbiol Technol 13:770–779.

Reynolds ES 1963 The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J Cell Biol 17:208–212.

Schmidt GL, GL Nicolson, MD Kamen 1971 Composition of the sulfur particle of *Chromatium vinosum*. J Bacteriol 105:1137–1141.

Siler DJ, K Cornish 1994 Hypoallergenicity of guayule rubber particle proteins compared to *Hevea* latex proteins. Ind Crops Prod 2: 307–313.

Siler DJ, K Cornish, RG Hamilton 1996 Absence of cross-reactivity of IgE antibodies from subjects allergic to *Hevea brasiliensis* latex with a new source of natural rubber latex from guayule (*Parthenium argentatum*). J Allergy Clin Immunol 98:895–902.

Siler DJ, M Goodrich-Tanrikulu, K Cornish, AE Stafford, TA McKeon 1997 Composition of rubber particles of *Hevea brasiliensis*, *Parthenium argentatum*, *Ficus elastica*, and *Euphorbia lactiflua* indicates unconventional surface structure. Plant Physiol Biochem 35: 881–889.

Spurr AR 1969 A low-viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct Res 26:31–43.

Tzen JTC, AHC Huang 1992 Surface structure and properties of plant seed oil bodies. J Cell Biol 117:327–335.

Yatsu LY, TJ Jacks 1972 Spherosome membranes: half-unit membranes. Plant Physiol 49:937–943.