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Evaluation of Possible Natural Latex Substitutes from Artificially prepared latex using Transmission Electron Microscope

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Abstract
Rubber particles are the most abundance organelle in the laticifers of rubber tree. The bulk of rubber particles in NR latex exist in the form of particles dispersed in latex emulsion (cell cytosol). These naturally occurring particles are made-up of rubber core surrounded by a monolayer of non-rubber materials such as protein and lipids. In this report, transmission electron microscopy techniques were used to observe the differences between natural rubber (NR) particles and artificially prepared rubber particles from dried latex compound. The morphology of artificial rubber particles appeared to be similar to the particles of natural rubber but with different particle size distribution. More importantly, membrane-bound proteins (14 & 24 kD) that were associated with latex allergen were not found on the membrane of the artificially prepared rubber particles. This study is part of an effort to develop new NR-based rubber substitutes in addressing future energy crisis which is caused by dwindling global petroleum supply (Petroleum is the main raw material for many synthetic rubbers)
Key words: Rubber microscopy, artificial latex, protein allergy, Rubber particle size, double immunogold labelling.

1. Introduction

Natural rubber is a isoprenoid polymer, chemically termed as cis-1,4-polysioprene and it is composed of 320 to 35000 isoprene molecules (Oh et al., 1999; Kang et al., 2000; Derouet et al., 2003). Hevea brasiliensis has been the only commercial source at present, for natural rubber due to its abundance in the plant, ease of tapping, high rubber content and quality (Kang et al., 2000). Rubber particles are spherical bodies in young trees but in mature trees the particles are large, often present in pear-like shape (Gomez and Hamzah, 1989). The average size of rubber particles varies among latex-producing plant species as well as within the species itself (Shamsul Bahri et. al., 2013). Rubber particles of Hevea brasiliensis for instance range from between 50 μm to 1500 μm in diameter (Light and Dennis, 1989; Dennis and Light, 1989; Dennis et. al., 1989) and they can be classified into two distinct subsets of particles with mean diameters of 1.0 μm and 0.2 μm . These particles are likely to have originated from the rough endoplasmic reticulum (Cornish, 2001).

Rubber particles consist of a homogenous rubber core surrounded by a monolayer membrane of lipids, proteins and other components unique to each species of latex-bearing plants (Cornish, 2001; Singh et al., 2003). The surface of rubber particles contains enzymes and factors that are necessary for the generation of latex, which it is also the site of latex biosynthesis (Oh et al., 1999; Kang et al., 2000; Singh et al., 2003). The most complicated rubber particles are those of H. brasiliensis where they contain up to 80 different proteins with sizes ranging from 5 kDa to over 200 kDa (Cornish, 2001).

Hev b1 and Hev b3 are the two major proteins found on the surface of the rubber particles (Shamsul Bahri et al. 1993, Yeang et al. 1994; Yeang et al., 2002a). These two proteins are unique in the sense that they are insoluble in water compared to the rest and due to this property they remained strongly attached to the surface of the rubber particles even after repeated washing (Bahri and Hamzah, 1996). Both of them showed high similarity between each other in terms of amino acid sequence where Hev b3 is 47 % identical to Hev b1 and if only the similar regions of the paired proteins are considered, their amino acid similarity increases to 72 % (Yeang et al., 2002b). Hev b1 and Hev b3 have been recognised to be involved in rubber biosynthesis.

This study was being initiated to shed some light on the morphology and characteristic of both natural rubber and artificially prepared rubber. This was also part of an effort to look for Natural rubber substitutes for downstream applications.

2. Materials and Methods

2.1 Preparation of artificial latex from dried latex

Dried latex materials collected from the rubber trees were thoroughly washed and then passed through a creeper and a shredder to form biscuit-like material. Then, rubber cement was produced by immersing cut rubber biscuits in a solvent bath containing analar-grade organic solvent and left to swell. The rubber cement is subsequently

emulsified to give a stable rubber/solvent emulsion. The rubber/solvent composition that obtained is subjected to solvent removal process, giving dilute rubber latex. Thereafter, the dilute latex is concentrated to a total solids content of around 30%.

2.2 Determination of rubber particle property and size distribution.

Each rubber latex samples was treated with 1% osmium tetroxide, where 1 ml of 1% osmium tetroxide was used to fix 1 drop of each samples in Eppendorf's tubes. The fixed samples were left for 1 hour to ensure adequate fixation. After an hour of fixation, the samples were centrifuged at 10,000 rpm for 5 minutes and the supernatant was discarded. Subsequently, 1 ml of distilled water was added to the samples in order to remove excess osmium tetroxide and they were again subjected to centrifugation. This step was repeated twice. Next, a wire loop was dipped into each of the samples and a thin layer of rubber suspension was carefully transferred from the Eppendorf's tubes and deposited onto collodion coated-copper grids. The grids were left to dry at room temperature. Finally, the grids were visualised under transmission electron microscope (TEM) at 80 kV. The presence, size distribution and morphological properties of the rubber particles for each of the samples were then determined using an image analyser that attached to the transmission electron microscope.

2.3 Double-immunogold localisation of membrane-bound proteins

Each latex samples was treated with 1% osmium tetroxide, where 1 ml of 1% osmium tetroxide was used to fix 1 drop of each of the samples in Eppendorf's tubes. After an hour of fixation, the samples were centrifuged at 10,000 rpm for 5 minutes and the supernatant was discarded. Subsequently, 1 ml of distilled water was added to the samples in order to remove excess osmium tetroxide and they were again subjected to centrifugation. This step was repeated twice. Next, wire loops were then dipped into each of the samples and a thin layer of rubber suspension was carefully transferred from the Eppendorf's tubes and deposited onto a 'formvar' coated-nickel. The grids were left to dry at room temperature prior to labelling. The grids were treated with blocking solution for half an hour and then they were washed with washing solution for 3 times of 5 minutes wash. Incubation with the first primary antibody, rabbit IgG anti-Hev b1 (1:200 in Phosphate Buffer Saline (PBS)) was carried out for 30 minutes. Next, the grids were washed with the washing solution for 5 times of 5 minutes wash. Incubation with the first secondary antibody, goat anti-rabbit IgG conjugated to 10 nm gold particles (1:20 in PBS), for Hev b1 protein detection, was done for half an hour. Again, the grids were washed with the washing solution for 3 times of 10 minutes wash. The next incubation with the second primary antibody, rabbit IgG anti-Hev b3 (1:200 with PBS) was done for 30 minutes. Later, the grids were washed with the washing solution for 5 times of 5 minutes wash. Incubation with the second secondary antibody was later being carried out by using goat anti-rabbit IgG (1:50 with PBS) conjugated to 30 nm gold particles, for Hev b3 protein labelling for 30 mins. The grids were then washed with the washing solution for 3 times of 10 minutes wash and it was followed by another 3 times of 5 minutes wash with distilled water. After the washings, the grids were left to dry at room temperature. Finally, visualisation of the gold-labelled rubber particles was done using a transmission electron microscope (TEM) at 80 kV.

3. Results and Discussion.

3.1 Determination of rubber particle property and size distribution.

Rubber particles were observed as spherically shaped bodies and some were pear-like shaped for fresh field latex, highly ammoniated (HA) latex concentrate, creamed latex and distilled latex respectively as shown in Figure 1.

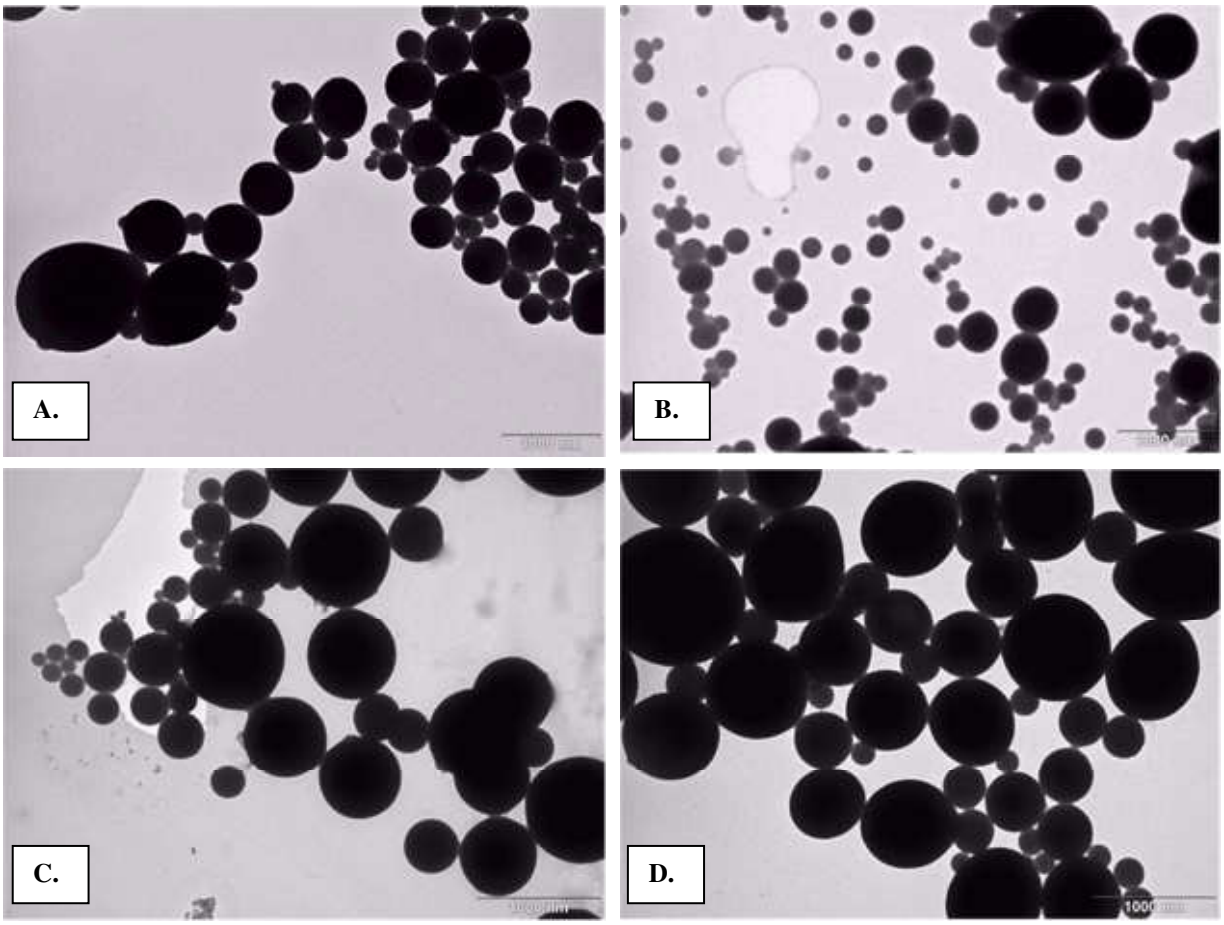

Figure 1: Whole mount of rubber particles viewed under TEM. **A.** Rubber particles of osmicated fresh field latex sample. Note the presence of spherical and pear-like shaped rubber particles. **B**. Rubber particles of osmicated HA latex concentrate sample. **C.** Rubber particles of osmicated creamed latex sample. **D.** Rubber particles of osmicated distilled latex sample.

For the fresh field latex sample, the largest rubber particle observed was 1320.00 nm while the smallest was 35.92 nm. In terms of their size distribution, rubber particles in the size range of between 100 nm to 200 nm were the most abundant in fresh field latex as shown in Table 1 and they made up 43.2 % of the total rubber particles observed. On the other hand, rubber particles that were more than 1100 nm in diameter were the least observed which comprised only a fraction of 0.2 % from the total rubber particles present.

Particle Size Class	Size Range (nm)	Relative Abundance (%)
1	0 – 100	21.6
2	100 – 200	43.2
3	200 – 300	20.5
4	300 – 400	3.8
5	400 – 500	2.3
6	500 – 600	1.8
7	600 – 700	2.3
8	700 – 800	1.8
9	800 – 900	0.9
10	900 – 1000	0.5
11	1000 – 1100	0.7
12	1100 – 1200	0.2
13	1200 – 1300	0.2
14	1300 – 1400	0.2
TOTAL		**100.00**

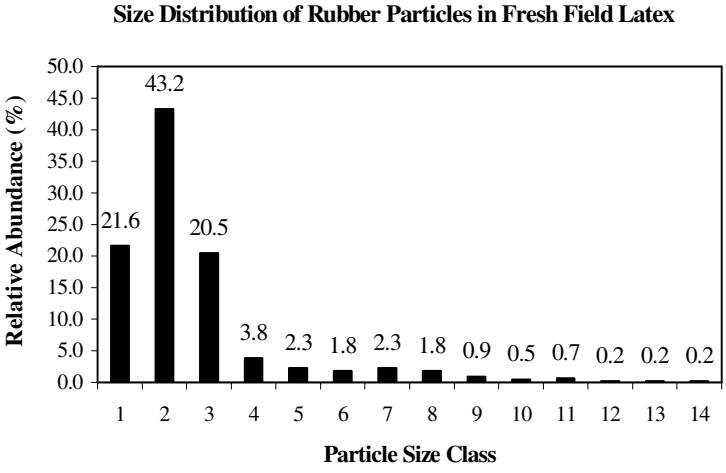

Table 1: Relative abundance of rubber particle size in fresh field latex sample and size distribution of rubber particles in fresh field latex sample

In HA latex concentrate sample, the largest rubber particle observed was 1328.98 nm and the smallest was 80.82 nm. Rubber particles with sizes of between 200 nm to 300 nm were the most abundant to be found where the made up 35.3 % of the total rubber particles, while those that were more than 1100 nm were the fewest observed as depicted in Table 2.

Particle Size Class	Size Range (nm)	Relative Abundance (%)
1	0 – 100	0.69
2	100 – 200	32.18
3	200 – 300	35.29
4	300 – 400	7.61
5	400 – 500	6.23
6	500 – 600	2.77
7	600 – 700	4.84
8	700 – 800	5.54
9	800 – 900	1.38
10	900 – 1000	1.38
11	1000 – 1100	1.38
12	1100 – 1200	0.35
13	1200 – 1300	0.00
14	1300 – 1400	0.35
TOTAL		100.00

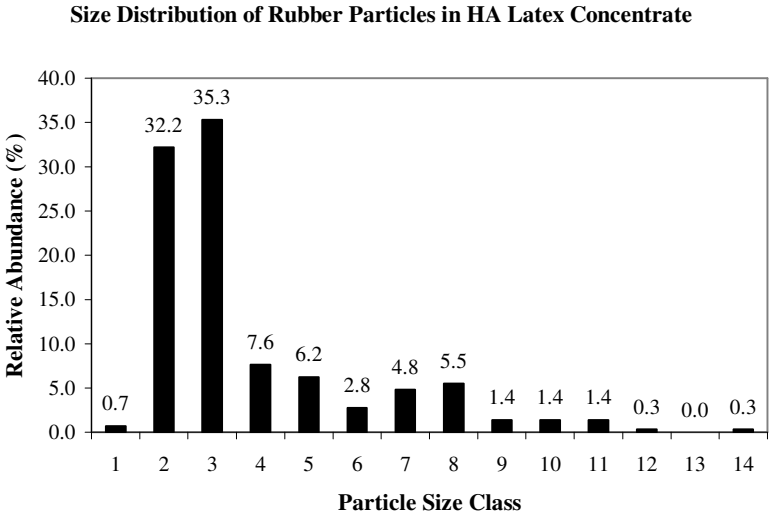

Table 2: Relative abundance of rubber particle size in HA latex concentrate sample and size distribution of rubber particles in HA latex concentrate sample

For the creamed latex sample, the smallest rubber particles observed was 85.71 nm and the largest diameter was 2295.49 nm. In fact it was the largest rubber particles observed in all of the latex samples. Rubber particles with sizes that ranged from 300 nm to 400 nm and those of 700 nm to 800 nm, were relatively the most abundant to be found, where both of them scored 12.81 % of the total rubber particles counted as displayed in Table 3. Meanwhile, rubber particles that were less that 100 nm in diameter and those that were more than 1300 nm were the least to be observed with their relative abundance of less that 0.8 % as presented in Table 3.

Particle Size Class	Size Range (nm)	Relative Abundance (%)
1	0 – 100	0.36
2	100 – 200	6.05
3	200 – 300	10.32
4	300 – 400	12.81
5	400 – 500	9.96
6	500 – 600	7.83
7	600 – 700	11.74
8	700 – 800	12.81
9	800 – 900	10.68
10	900 – 1000	6.41
11	1000 – 1100	2.85
12	1100 – 1200	2.49
13	1200 – 1300	2.85
14	1300 – 1400	0.71
15	1400 – 1500	0.36
16	1500 – 1600	0.36
17	1600 – 1700	0.71
18	1700 – 1800	0.36
19	1800 – 1900	0.00
20	1900 – 2000	0.00
21	2000 – 2100	0.00
22	2100 – 2200	0.00
23	2200 – 2300	0.36
TOTAL		**100.00**

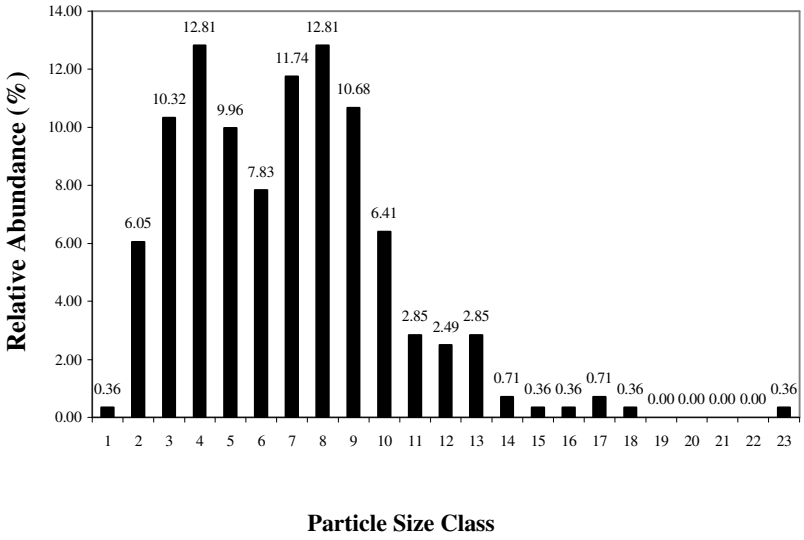

Table 3: Relative abundance of rubber particle size in creamed latex sample and size distribution of rubber particles in creamed latex sample

The largest rubber particle of distilled latex sample was 1574.59 nm while the smallest was 42.86 nm. Rubber particles with diameters of between 100 nm to 200 nm were the most plenty found in the sample where they comprised 23.03 % of the total rubber particles, while those that were more than 1300 nm in diameter were the least observed as exhibited in Table 4.

Particle Size Class	Size Range (nm)	Relative Abundance (%)
1	0 – 100	5.05
2	100 – 200	23.03
3	200 – 300	18.61
4	300 – 400	11.67
5	400 – 500	7.57
6	500 – 600	6.31
7	600 – 700	5.36
8	700 – 800	5.36
9	800 – 900	3.79
10	900 – 1000	3.79
11	1000 – 1100	3.79
12	1100 – 1200	2.21
13	1200 – 1300	1.26
14	1300 – 1400	0.63
15	1400 – 1500	0.95
16	1500 – 1600	0.63
TOTAL		100.00

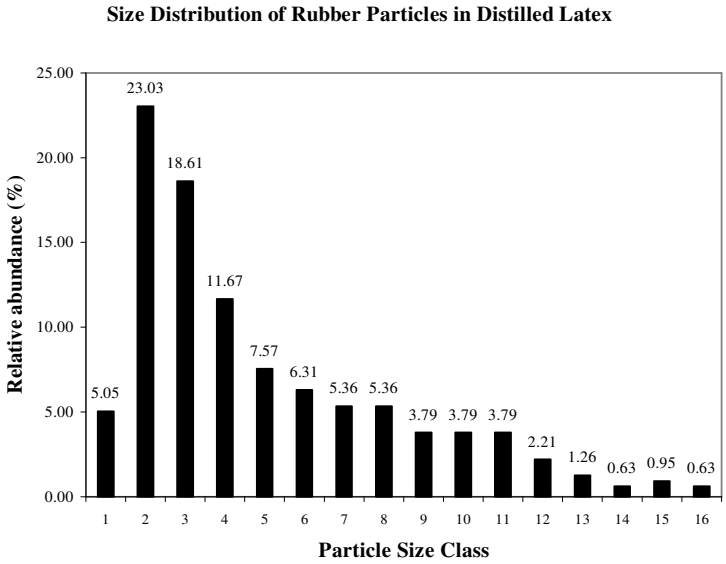

Table 4: Relative abundance of rubber particle size in distilled latex sample and size distribution of rubber particles in distilled latex sample

Overall, for these 4 samples namely the fresh field latex, HA latex concentrate, creamed latex and distilled latex, small rubber particles with diameters ranging from 100 nm to 400 nm, dominated the total rubber particles counted. This finding correlated well with the previously reported study by Singh et al., 2003. The shape of the rubber particles observed in this research matched to the one described earlier by Gomez and Hamzah, 1989.

On the contrary, rubber particles of the other 3 samples namely 10 % rubber solvent emulsion (RSE) with 1 part soap, 10 % RSE with 2 parts soap and 15 % RSE with 2 parts soap, were not morphologically as same as those observed in the previous 4 samples. In the 10 % RSE with 1 part soap sample, its rubber particles were clustered together forming a massive structure while still maintaining their spherical shape property as depicted in Figure 2 (A). Meanwhile, for the 10 % RSE with 2 parts soap and 15 % RSE with 2 parts soap samples, the rubber particles were utterly distorted. They were attached to each other, forming lumps and they were no longer globularly shaped as shown in Figure 2 (B & C) respectively. The collodion films of the 2 latter samples were covered with artefacts of what was believed to have originated from the deformed rubber particles.

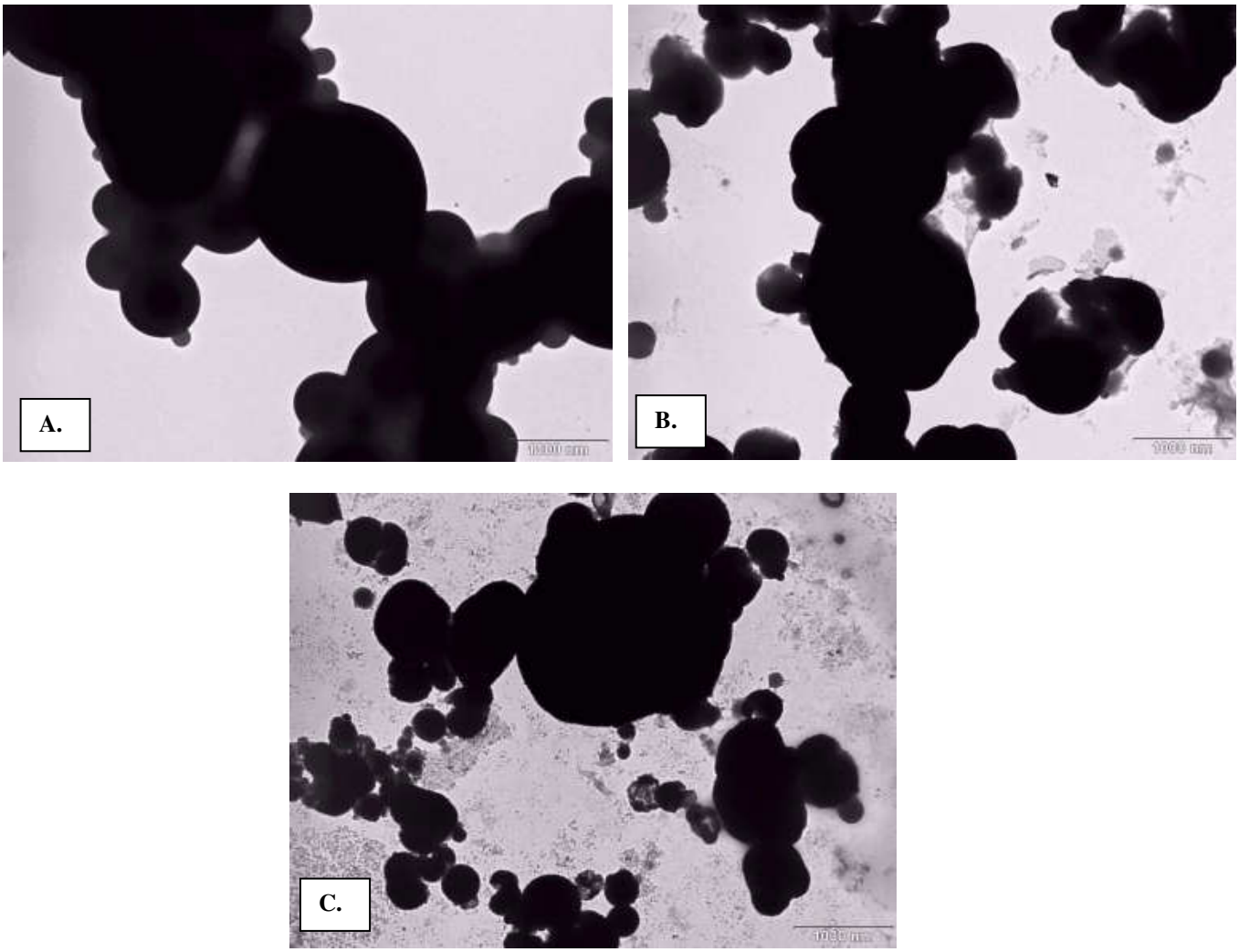

Figure 2 : Rubber particles. **A.** Rubber particles of 10 % RSE with 1 part soap. Note the spherical shape of the rubber particles even though they were clustered together. **B.** Rubber particles of 10 % RSE with 2 parts soap. They were ruptured and attached to each other forming lumps. **C.** Rubber particles of 15 % RSE with 2 parts soap. Note the broken rubber particles and its debris, present on the collodion film.

The addition of ammonia into the rubber latex had no effect over the physical properties of the rubber particles as shown for the HA latex concentrate sample. The addition of ammonia was done in order to prevent self-coagulation of the latex and also retarding the growth of bacteria (Ownby, 2002). Thus, the rubber particles remained unattached to each other as in the fresh field latex sample and as the result no lumps were observed in the HA latex concentrate sample.

Distilled latex resulted from the removal of cyclohexane used in the latex extraction from cuplumps. Creamed latex on the other hand, originated from distilled latex treated with ammonia and creaming agent as shown in Figure 3. Thus, from the result obtained for both of them, neither the distillation step nor the addition of ammonia and the creaming agent had any effect over the morphology features of the rubber particles. They remained globular resembling the rubber particles of the fresh field latex sample.

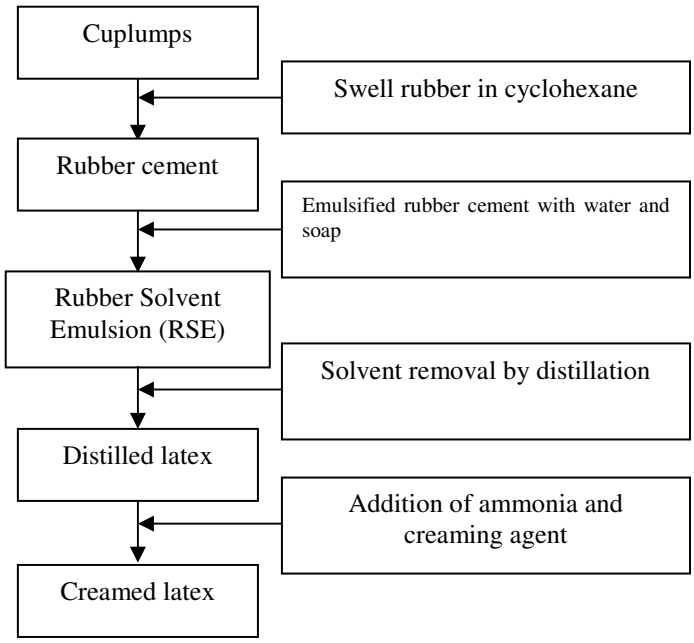

Figure 3: Flow diagram of creamed latex production

The addition of soap as in the 10 % RSE with 1 part soap, 10 % RSE with 2 parts soap and 15 % RSE with 2 parts soap samples, had resulted in the clustering of the rubber particles. By increasing the presence of soap from 1 part to 2 parts, the rubber particles were totally ruptured and destroyed. Thus, suggesting that soap had a lysis effect over the rubber particles.

3. 2 Double-immunogold localisation of Hev b 1 and Hev b 3 proteins.

The utilisation of different sizes of electron opaque markers such as colloidal gold had enabled the detection of more than one type of molecule simultaneously in the same sample (Doerr-Schott and Lichte, 1986; Robinson and Vandré, 1997; Takizawa et al., 1998; Yi et al., 2001). The detection of the gold particles was only possible in the fresh field latex and in the HA latex concentrate, while the rest of the samples showed no presence of the gold particles.

Fresh field latex sample showed immunogold labelling for both Hev b1 and Hev b3 proteins as shown in Figure 4 (A). The small gold particles, which acted as the marker for Hev b1, were found on both the small or the large rubber particles. Thus showing that Hev b1 was present on the membrane of all of the rubber particles regarding of their sizes. On the other hand, the 30 nm gold particle was mainly detected on the small rubber particles with diameters of below 400 nm.

Meanwhile, for the HA latex concentrate sample, the 10 nm gold particles were found to be present on both the small and large rubber particles as in the fresh field latex as displayed in Figure 4 (B). On the other hand, the 30 nm gold particles could be detected on the membrane of the small rubber particles. The amount of the 30 nm gold particles was less observed than those found in the fresh field latex sample.

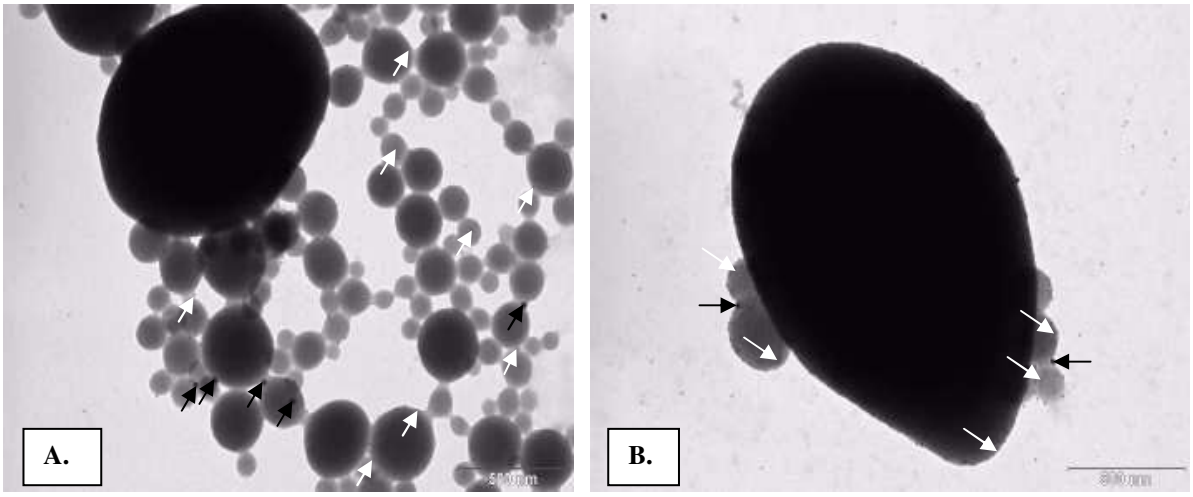

Figure 4: Immunogold–labelled rubber particles. **A.** Immunogold–labelled rubber particles of osmicated fresh field latex sample. The white and black arrows indicated the 10 nm and 30 nm gold particles respectively. **B.** Immunogold–labelled rubber particles of osmicated HA latex concentrate sample. The white and black arrows indicated the 10 nm and 30 nm gold particles respectively.

According to Yeang et al., 2002b, the treatment of latex with ammonia could result in minor reduction of the membrane bound-Hev b3 where a small amount of it would be solubilised. Ownby, 2002 noted that the introduction of ammonia would cause accelerated hydrolysis of proteins present in the latex. Thus, this could explain the decrease in the amount of large gold particles visualised in the ammoniated latex sample.

For the other two samples, namely the distilled and creamed latex samples, no labelling was observed as demonstrated in Figure 5 (A & B) respectively. The absence of these gold particles could be due to the denaturation of the rubber particle membrane proteins during the distillation step. The destruction of the proteins resulted in the loss of antigenic sites for the gold-conjugated antibodies. As the result, no labelling took place.

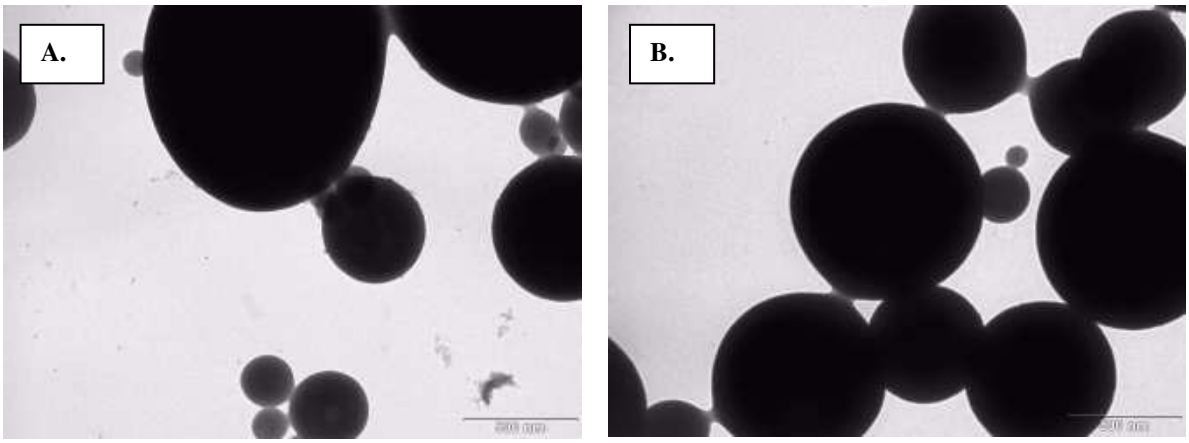

Figure 5: **A.** Immunogold–labelled rubber particles of the osmicated distilled latex sample. No gold particles observed. **B.** Immunogold–labelled rubber particles of the osmicaed creamed latex sample. No gold particles observed.

For the 10 % RSE with 1 part soap, 10 % RSE with 2 parts soap and 15 % RSE with 2 parts soap, none of them were immunogold-labeled as shown in Figure 6 (A, B & C) respectively. This was expected from the earlier

observation of osmicated samples, where the membranes of the rubber particles were destroyed and as the result no membrane-bound proteins were present. Thus no labelling was observed.

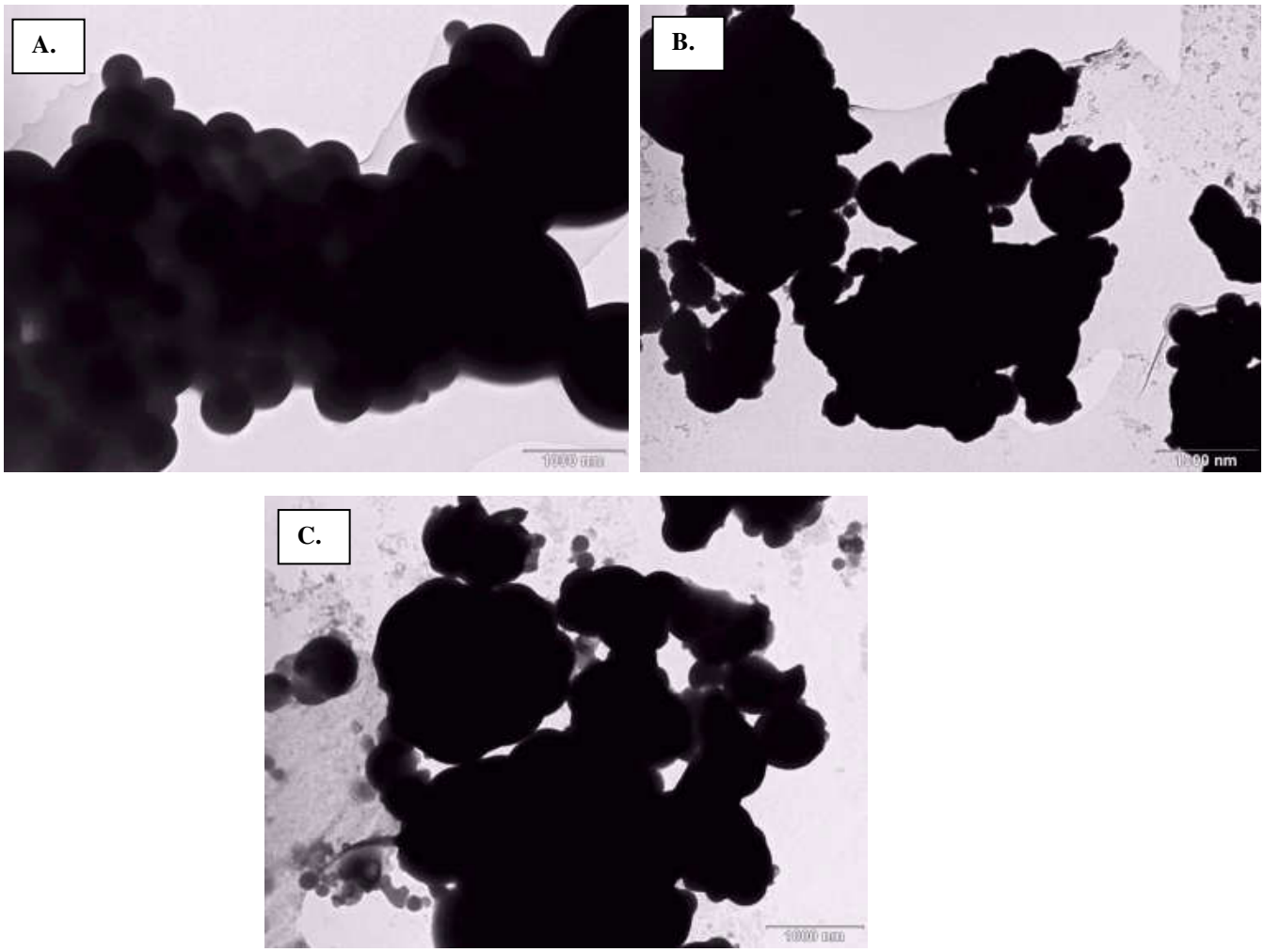

Figure 6: Immunogold–labelled rubber particles. **A.** Immunogold–labelled rubber particles of the osmicated 10 % RSE with 1 part soap sample. No gold particles observed. **B.** Immunogold–labelled rubber particles of osmicated 10 % RSE with 2 parts soap sample. No gold particles observed. **C.** Immunogold–labelled rubber particles of the osmicated 10 % RSE with 2 parts soap sample. No gold particles observed.

The fixation in osmium tetroxide, had provided a better view of the rubber particles under a transmission electron microscope and it had also rendered them sufficiently hard for further observation under the hot environment of the sample chamber of the electron microscope (Gomez and Hamzah, 1989). The utilisation of osmium tetroxide at high concentrations could cause distortion to the antigenic properties of the proteins of interest (Bahri and Hamzah, 1996), thus preventing them from being recognised by the antibodies. Therefore, in order to overcome this problem, compromise must be made (Ramandeep et al., 2001), where the fixation step must not be too long and been carried out as mildly as possible.
On the other hand, the usage of osmium tetroxide in immunolabelling electron microscopy is restricted due to the reason that only a few of antigens are known to resist exposure to osmium treatment. According to Ramandeep et al., 2001, the use of 1 % osmium tetroxide in the fixation of E. coli cells, caused 90 % to 95 % loss in antibody binding.

Immunogold labelling can only be carried out on samples that are deposited onto coated grids of nickel or gold. Copper grids are not suitable because the components of the solutions used in the preparation of the samples may corrode the metal and the released salts will compromise the samples via the formation of precipitates and clusters of irregular sizes (Sierralta, 2001). On the other hand, gold grids are more preferred than nickels ones, where they lack of paramagnetic properties that will result in disturbance of electron beam stability during observation under electron microscope (Sierralta, 2001).

4. Conclusion.

The rubber particles observed in the fresh field latex, HA latex concentrate, creamed latex and distilled latex samples were globular bodies and some were pear-like in shape. Small rubber particles with diameters of less than 400 nm were the most abundantly found in these samples. For the 10 % RSE with 1 part soap, 10 % RSE with 2 parts soap and 15 % RSE with 2 parts soap, the rubber particles were totally distorted and clustered to each other.

Both the Hev b 1 and Hev b 3 proteins were present on the membrane of rubber particles for fresh field latex and HA latex concentrate samples. However, they were not detected on the rubber particles of the other samples. Hev b 1 was found on both the large and small rubber particles while Hev b 3 was localised mostly on the small rubber particles.

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