Structural Transformations in Diluted Micellar and Lamellar Systems

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STRUCTURAL TRANSFORMATIONS IN DILUTED
MICELLAR AND LAMELLAR SYSTEMS

BY

BLANCA ZELAYA-RINCÓN

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ABSTRACT

The role of dilution by artificial hard water on nanostructures present in body wash samples provided by Procter and Gamble were investigated using time-resolved cryogenic transmission electron microscopy (cryo-TEM). Samples with and without perfume were examined at 10X, 20X, and 50X dilution. Micellar samples transformed to mostly unilamellar vesicles at 50X dilution, in contrast to the micelle to monomer transition seen in typical samples. At lower dilutions, a change in morphology from spherical to wormlike micelles was observed. For lamellar samples, lower dilution ratios show tightly packed multilamellar vesicles, while higher dilution ratios show more dispersed vesicles with less bilayers. Nanostructural transformations upon dilution were attributed to changes in curvature/packing parameters, which occurred due to dilution with hard water and addition of perfume. The systems experience changes in curvature in order to maintain equilibrium. Also, the addition of perfume in the lamellar samples caused an increase in the number of bilayers present in multilamellar vesicles, because of its role in increasing the packing parameter in the system.
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Figure 7. Graphs showing the areas of the vesicles present for each dilution in nm$^2$. The black bars indicate La samples and the red bars indicate LaP samples.
CHAPTER 1

INTRODUCTION

Body wash is an important consumer product. It consists of a complex mixture of surfactants and polymers, designed to produce rapid detergency action upon mixing with water and rubbing on skin, while providing the right feel for the user and being gentle on the skin [1, 2]. Its properties and performance are intimately related to the nanostructures present in the wash and changes to these nanostructures taking place because of dilution and mixing with water [1].

Micellar and lamellar systems can be found in many consumer products such as body wash, laundry detergent and shampoo [3]. These consumer product formulations often contain salts and perfume/raw materials, as well as different types of surfactants. There have been many studies focusing on the effects which the addition, removal, and change in concentration of these components have on these systems, as well as how shear affects the structures present in these systems [3-13]. Even so, there is still limited understanding as to how dilution affects the nanostructures present.

The skin barrier is a powerful film, made up of three major components: free fatty acids, ceramides, and cholesterol [14, 15]. A properly functioning skin barrier keeps out allergens, foreign materials, and reduces transepidermal water loss, therefore reducing skin dryness and irritation by keeping the balance between moisture and hydration, ultimately preventing skin diseases such as atopic dermatitis [15-17]. It is well known that certain surfactants such as SLS can be very harsh on the skin and actually strip the skin, meaning that although effective for cleansing all of the dirt and unwanted particles from skin, they also remove some of these major components of many of the skin barrier
[18]. This stripping of the skin barrier can cause slower skin regeneration after irritation occurs, and also makes penetration of foreign material and allergens easier, which can lead to conditions such as atopic dermatitis [16, 19]. Although there are now many gentler surfactants which are being studied and used, structural transformations in nanostructures present in these cleansing formulations can also have a drying effect, since it has been suggested that smaller nanostructures present in cleansing products tend to be more irritating to skin [19, 20].

This study focuses on the effects of dilution on the nanostructures present in micellar and lamellar systems. Specifically, the micellar and lamellar systems in this study are diluted with a salt solution (hard water), meaning that it may cause unexpected transformations to take place upon dilution [21]. However, there are many other factors which also need to be taken into consideration when diluting a system, such as mixing time, mixing method, whether or not perfume is present in the sample, and the sample preparation technique for cryogenic transmission electron microscopy (cryo-TEM).

Investigating what kinds of nanostructural transformations occur in different surfactant systems upon dilution with hard water, and using the results from this study in conjunction with previous knowledge regarding the maintenance of the skin barrier integrity, may be useful in the future optimization of body wash formulations, as well as cleansing formulations in general, to minimize skin irritation, dryness, and diseases such as atopic dermatitis.
CHAPTER 2
EXPERIMENTAL METHODS

Sample Preparation

Artificial hard water was made by adding 4.1 mg of calcium chloride dihydrate and 6.2 mg of magnesium chloride hexahydrate to 50 mL of DI water [22]. Total permanent water hardness was calculated by first calculating the concentration of Ca\(^{2+}\) and Mg\(^{2+}\) present in the DI water (in mg/L), since these are the prime cation contributors to water hardness [23]. These values were then expressed as equivalents of CaCO\(_3\) and were added together to obtain a total hardness value [23]. The hard water used throughout this study was calculated to have a total hardness of 117 mg/L which is classified as moderately hard [24]. This method for making hard water in a laboratory is considered to be standard and was used because it most closely imitates the water people have access to in their homes [25].

The body wash samples were provided by Procter and Gamble. Samples of 10X dilution were made by mixing 300 microliters of original sample with 2700 microliters of hard water. Samples at 20X dilution were made by mixing 150 microliters of original sample with 2850 microliters of hard water, and samples at 50X dilution were made by mixing 60 microliters of original sample with 2940 microliters of hard water.

Sample Mixing

When body wash is used in the shower, a substantial amount of foam is produced with ease via dilution and scrubbing action. In order to mimic this production of foam samples were vortex mixed for 15 seconds, and then vitrified within 20 seconds after mixing. This mixing time of 15 seconds was chosen through personal experience and
inquiry about how long (on average) acquaintances spent using body wash while showering. Only the liquid layer was imaged.

The four original samples received from Procter and Gamble were: micellar no perfume (Mi), micellar with perfume (MiP), lamellar no perfume (La) and lamellar with perfume (LaP). Dilution will be indicated after these labels in order to indicate samples being referred to throughout this study (e.g. Mi10x would refer to the micellar sample with no perfume at 10x dilution).

Table 1. Chemical names and structures of surfactants and salt present in original samples received from Procter and Gamble.

| Chemical Name          | Formula                      | Structure                                      | Molecular Weight | Sample Presence |
|------------------------|------------------------------|-----------------------------------------------|------------------|-----------------|
| Sodium Trideceth-2 Sulfate | C_{19}H_{38}NaO_{7}S [26] | ![Structure](image) | 434.564          | Mi, MiP, La, LaP |
| Cocamidopropyl Betaine | C_{19}H_{38}N_{2}O_{3}[27]  | ![Structure](image) | 342.524          | Mi, MiP, La, LaP |
| Trideceth-3            | C_{13}H_{27}(CH_{2}CH_{2}O)_{3}OH | C_{13}H_{27} \rightarrow \text{branched hydrocarbon; approximately 2-3 methyl branches at random positions} | 332.525          | La, LaP         |
| Sodium Chloride        | NaCl                         | Na^+ —Cl^-                                     | 58.44            | La, LaP         |

Note: Information for Trideceth-3 (formula and structure) given by Procter and Gamble

The table above shows the main/important components of the samples, which are mainly surfactants which were present. The chemical names were given by Procter and Gamble.

**Cryogenic Transmission Electron Microscopy (cryo-TEM)**

A blotless method was chosen for cryo-TEM sample preparation to avoid artifacts created by shear [28]. After pipetting the sample onto a holey carbon grid, excess liquid
was removed via syringe or capillary tube. The syringe (or capillary tube) was placed parallel to the plane of the grid as seen in the figure below.

![Blotless method schematic for cryo-TEM sample preparation](image)

**Figure 1.** Blotless method schematic for cryo-TEM sample preparation

This geometry allowed sample to be thinned out without introducing any flow within the grid holes, therefore removing any shear-induced artifacts from the sample and images [28]. The sample was then vitrified in ethane and stored in a liquid nitrogen dewar until it was imaged. The grid was placed on a Gatan 626 DH cryo holder, inserted into a JEOL 2100 TEM. The sample’s temperature was maintained at -165°C during imaging.

**Image Analysis**

In order to estimate vesicle size (area in nm²) ImageJ was used. The diameter of the vesicles was measured directly when round vesicles were present. However, for irregularly shaped vesicles the diameter had to be estimated in order to calculate the area as accurately as possible. The particle analysis functions were tested, but were not used
due to the complex nature of the systems imaged and low image contrast. Manual analysis proved to be the most effective choice for this study.

Results obtained through ImageJ analysis were averaged and the mean areas were plotted. Standard deviations are reported as error bars. They were also graphed as histograms. The outliers in the data were not included in the graphs, due to the fact their large values distorted the axis, making the smaller vesicle areas more difficult to visualize. However, they are included and highlighted in yellow in the Appendix.
CHAPTER 3
RESULTS AND DISCUSSION

Images of the micellar samples with and without perfume at the different dilutions are shown in Figure 2.

Figure 2. Summary of Micellar sample, vortex mixed for 15 seconds, cryo-frozen within 20 seconds using blotless method: A,B,C,D) no perfume; E,F,G,H) with perfume; A,E) 10x dilution; B,F) 20x dilution; C,D, G, H) 50x dilution. Red arrows indicate micelles. Blue arrows indicate initial transition to small vesicles. Purple arrows indicate vesicles.

From these images, it can be seen that in general, as the dilution increases the size of the structures increases in the micellar system with no perfume. Also, when less dilute, there are no vesicles present in the system. Only micelles and wormlike micelles can be seen.

Figure 3 shows a graph indicating how the area of the vesicles present in the samples changes due to dilution.
Figure 3. Graph showing how the area of the vesicles present changes due to dilution for the micellar samples with and without perfume at 10x, 20x, and 50x dilution. The black squares and error bars indicate area values (in nm$^2$) for Mi samples at the different dilutions, and the red circles and error bars indicate area values (in nm$^2$) for MiP samples at the different dilutions.

From this graph, the size increase due to dilution as seen in the Mi and MiP images from figure 2 can be confirmed. Figure 4 shows graphs of the micellar samples at 20 and 50 times dilution. When compared to figure 3 above, the overall size increase upon dilution can be confirmed, as well as the fact that there is more variability in the micellar samples at 50x dilution. It should be noted that for both the Mi and MiP samples at 50 times dilution, there are some larger values which were included in these graphs, which are highlighted in the appendix. These larger values also contribute to the large standard deviation seen figure 3.
Figure 4. Graphs showing the areas of the vesicles present for each dilution in nm². The black bars indicate Mi samples and the red bars indicate MiP samples.

This is different from what is usually expected, as micelles would usually transform into monomers upon dilution, since the surfactant concentration in the system would be below the CMC [29]. However, in this case the systems were diluted with hard water, which is a salt solution. The addition of salts to micellar systems have been shown to increase the packing parameter by reducing headgroup repulsion, even at low surfactant concentrations, therefore inducing micelle/wormlike micelle formation [3, 30, 31]. As more salt solution is added to the system, the packing parameter continues to increase, and eventually vesicle formation becomes more favorable, as seen in the vesicle images in figure 2 [21, 32]. Initially, salt is absent from the original micellar samples, as shown in table 1, which further suggests that the reason for vesicle formation is the addition of salt via hard water. However, as the dilution increases and the surfactant ratio decreases, the addition of salt would have less of an impact and the system would follow the logical transition from vesicles to micelles and eventually to monomers.

It is known that the addition of perfume may alter the curvature and packing constraints of a system, depending on whether it acts as a co-surfactant and/or co-solvent,
therefore causing changes in the structures present [33-35]. It is more commonly assumed that perfume acts as a co-surfactant, allowing the formation of vesicles with more bilayers [33-35]. However, in the micellar samples, the perfume does not seem to have much of an effect. The only noticeable effect is that the standard deviation for the samples with perfume is larger than the standard deviation for the no perfume samples, meaning that there is a larger size distribution in the perfume samples. Given the large standard deviation overall, there is not a noticeable difference in the sizes of the structures found in the Mi and MiP samples.

The remaining figures show images of the lamellar systems with and without perfume at 10x, 20x, and 50x dilution.

**Figure 5.** Summary of Lamellar sample, vortex mixed for 15 seconds, cryo-frozen within 20 seconds using blotless method: A,B,C) no perfume; D,E,F) with perfume; A,D) 10x dilution; B,E) 20x dilution; C,F) 50x dilution. Purple arrows indicate unilamellar vesicles. Green arrows indicate multilamellar vesicles. Pink arrows indicate bilamellar vesicles. As dilution increases, curvature increases, causing transformations from larger vesicles to smaller vesicles with less layers.
As a general trend, both lamellar systems (with and without perfume) show a decrease in vesicle size with increase in dilution ratio. This is quite different from the micellar samples which showed changes from wormlike micelles and micelles to vesicles. In lamellar systems, the addition of salt has less of an effect on the structural transformations, while the effects of perfume are more obvious. This can also be attributed to the fact that there was already some salt present in the original lamellar samples before the addition of hard water, as shown in Table 1. The only thing that salt may have an effect on is an increase in the lamellar repeat distance [36].

**Figure 6.** Graph showing how the area of the vesicles present changes due to dilution for the lamellar samples with and without perfume at 10x, 20x, and 50x dilution. The black squares and error bars indicate area values (in nm$^2$) for La samples at the different dilutions, and the red circles and error bars indicate area values (in nm$^2$) for LaP samples at the different dilutions.
Figure 6 shows a graph indicating how the area of the vesicles present in the samples changes as a function of dilution. However, like the micellar samples, the standard deviation is larger for the LaP samples, and smaller for the La samples, meaning that the size distribution is larger for the LaP samples. Given the large standard deviation, one might say that there is no change, however this is due to a smaller number of outliers present throughout the samples. Overall, it can be seen through the images that the area does decrease with an increase in dilution.

Figure 7. Graphs showing the areas of the vesicles present for each dilution in nm$^2$. The black bars indicate La samples and the red bars indicate LaP samples.
The general trend showing a decrease in size with dilution increase shown in the graph is in agreement with the visual results shown in figures 6 and 7. Another outcome that is initially surprising is that the average vesicle size at 20x dilution is slightly larger than the average vesicle size at 10 times dilution. However, upon closer inspection this makes sense for a few reasons. First, the variability for the samples at 20 times dilution are larger, meaning that the ranges of vesicle sizes are larger. Also, although the majority of the vesicles are smaller and have less bilayers at 20 times dilution, there are a few which are larger and contain many smaller vesicles within.

In aqueous solutions, surfactants often aggregate into structures, due to enthalpic or entropic driving forces [37, 38]. The curvature of this aggregate can change depending on many variables such as temperature, surfactant concentration, pH, as well as addition of electrolytes/salt, head group size, surfactant tail length, and number/types of surfactants present [39-41]. Structures formed in these systems depend on the curvature of these films, and in some instances these films form micelles by closing up [39]. Similarly, in systems with multiple surfactants present, surfactant bilayers may close up and form vesicles [39]. More specifically, the flexibility/rigidity of the film, which depends on the packing parameter, dictates what kind of aggregates are formed in the system; tail length and flexibility also have an effect on structures formed and on transitions that take place in mixed surfactant systems [30, 42-44].

Perfume seems to take on a co-surfactant role in the lamellar samples, due to the fact that the systems with perfume contain vesicles with many more layers than the ones found in the no perfume systems. By acting as a co-surfactant, the perfume would increase the surfactant efficiency by increasing the hydrophobicity of the surfactant, and
therefore increasing the packing parameter, causing vesicles to form more readily [34]. Although effects of shearing have been known to produce multilamellar vesicles in mixed surfactant systems, and are a factor in the structures present, the same shear was applied to the samples over the same timescale, therefore the increase in layer number from the no perfume sample to the sample with perfume can be directly attributed to the co-surfactant qualities of the perfume [45-49]. Also, since the amount of hard water added at each dilution is the same, salt cannot account for the difference in layers seen in the La and LaP samples.

Dilution expands the water layer, lowering the surfactant concentration present in the system. In order to maintain equilibrium, curvature must increase, causing the transition from larger vesicles to smaller unilamellar and bilamellar as seen in figure 5 [50, 51]. Therefore, a logical progression of expected structures observed with increasing dilution would be: multilamellar vesicles→unilamellar/bilamellar vesicles→micelles.

Since the micellar samples have a different formulation than the lamellar samples, the progression would be slightly different: wormlike micelle/micelle→unilamellar/bilamellar→micelle, and eventually monomers. After dilution, the size of the structures would initially increase, and there would be a transition to vesicles, however upon further increase of the hydrophilic layer, the decrease in surfactant density would cause larger structures to break up and would transform into a more energetically stable micellar structure.
CHAPTER 4

CONCLUSION

As the dilution increases, initially micellar and lamellar systems seem to behave differently. Both micellar systems show an overall increase in the sizes of the nanostructures present, shown by the formation of larger, unilamellar vesicles from wormlike micelles/micelles. For lamellar samples, lower dilution ratios show tightly packed multilamellar vesicles, while higher dilution ratios show more dispersed vesicles with less bilayers. However, it has been predicted that both systems would eventually show transitions to from vesicles to micelles, and eventually monomers at even higher dilutions.

The effects of perfume on the nanostructures present in the samples were also considered, and it was found that the addition of perfume in lamellar samples caused more bilayers to form, though this did not always indicate a larger vesicles size. These effects indicated the role of perfume as a co-surfactant in the lamellar sample. In the micellar sample, the role of perfume was negligible. The mean area calculated for the samples with perfume was slightly larger than the no perfume sample, but due to the large standard deviation, it can’t be said that perfume made a meaningful difference in the formulation.

Suggestions for future work

In order to see nanostructural transformations at smaller dilution increments, future experiments with more dilution ratios in between those used in this study (such as 15x, 30x, and 40x) should be tested. Also, in order to achieve a better understanding of what
the final nanostructures present are at higher dilutions, dilutions such as 120x, 150x, and 200x should be tested.

Some other suggestions for future work would be to use methods such as DLS in order to investigate the sizes of the nanostructures present more closely. The values obtained through DLS could then be compared to the values obtained through cryo-TEM in this study.
## APPENDIX

| Mi 10x | Vesicle Area (nm²) | MiP 10x | Vesicle Area (nm²) |
|--------|--------------------|---------|--------------------|
| 0      | 0                  | 0       | 0                  |
| Mi 20x | Vesicle Area (nm²) | MiP 20x | Vesicle Area (nm²) |
| 42.32532772 |                      | 183.87789 |
| 44.81710942 |                      | 211.189171 |
| 49.78909449 |                      | 263.3670509 |
| 62.23935184 |                      | 300.6419543 |
| 79.6748612  |                      | 318.0353066 |
| 89.61795482 |                      | 360.2862681 |
| 93.22756703 |                      | 362.7801743 |
| 99.57874818 |                      | 422.4047888 |
| 99.57874818 |                      | 422.4047888 |
| 102.0703453 |                      | 459.6565245 |
| 113.3802556 |                      | 459.6565245 |
| 123.7416044 |                      | 482.0389679 |
| 123.7416044 |                      | 496.9400445 |
| 143.5420081 |                      | 509.3441833 |
| 150.9617705 |                      | 516.8117082 |
| 160.8757322 |                      | 526.7715779 |
| 160.8757322 |                      | 549.1341884 |
| 161.8213296 |                      | 576.4637668 |
| 161.8213296 |                      | 598.8052658 |
| 161.8213296 |                      | 598.8052658 |
| 169.3013109 |                      | 636.0608005 |
| 171.3136871 |                      | 648.5034402 |
| 179.2447069 |                      | 680.8077613 |
| 179.2447069 |                      | 715.6031469 |
| 181.4106426 |                      | 715.6031469 |
| 181.745018  |                      | 720.5427485 |
| 181.745018  |                      | 720.5427485 |
| 184.2385881 |                      | 720.5427485 |
| 184.2385881 |                      | 725.5470817 |
| 186.4343988 |                      | 740.4228214 |
| 186.4343988 |                      | 777.7287692 |
| 199.1813921 |                      | 787.6461489 |
| 199.1813921 |                      | 807.518294 |
| 200.4591964 |                      | 966.5711107 |
| 201.5648988 |                      | 1046.060052 |
| 204.1398141 | 1195.14202 |
| 210.3657219 | 1195.14202 |
| 210.3657219 | 1217.488406 |
| 211.6272826 | 1292.0692 |
| 211.6272826 | 1316.907499 |
| 211.6272826 | 1364.083948 |
| 214.1617185 | 1552.909006 |
| 222.7540624 | 1813.857619 |
| 224.0787149 | 1900.779814 |
| 224.0787149 | 1928.155871 |
| 240.0611026 |                 |
| 242.5390652 |                 |
| 243.9765764 |                 |
| 247.4773913 |                 |
| 247.4773913 |                 |
| 248.9574074 |                 |
| 257.3895936 |                 |
| 258.9274346 |                 |
| 263.885065  |                 |
| 271.36586  |                 |
| 279.6621433 |                 |
| 279.6621433 |                 |
| 279.6621433 |                 |
| 279.6621433 |                 |
| 279.6621433 |                 |
| 279.6621433 |                 |
| 281.3244982 |                 |
| 281.3244982 |                 |
| 281.3244982 |                 |
| 281.3244982 |                 |
| 281.3244982 |                 |
| 281.3244982 |                 |
| 281.3244982 |                 |
| 288.8058084 |                 |
| 288.8058084 |                 |
| 289.559339  |                 |
| 289.559339  |                 |
| 301.256921  |                 |
| 301.256921  |                 |
| 301.9341102 |                 |
| 303.7230713 |                 |
| 309.371014  |                 |
| Mi 50x | Vesicle Area (nm²) | MiP 50x | Vesicle Area (nm²) |
|--------|------------------|--------|------------------|
|        | 222.595368       |        | 226.4196413      |
|        | 281.5921218      |        | 226.4196413      |
| 289.3784021 | 254.4124594 |
| 289.3784021 | 280.7599328 |
| 354.2892796 | 323.0173 |
| 365.0455901 | 325.6347525 |
| 378.4067986 | 337.9022948 |
| 378.4067986 | 348.541135 |
| 391.5871472 | 370.2111853 |
| 396.230641 | 370.2111853 |
| 426.7874991 | 379.0621828 |
| 429.5749148 | 390.080404 |
| 429.5749148 | 397.5371375 |
| 431.8187463 | 407.49385 |
| 431.8187463 | 419.8949728 |
| 436.2869213 | 429.9423555 |
| 436.2869213 | 432.4819774 |
| 439.9969851 | 442.2678374 |
| 439.9969851 | 442.2678374 |
| 442.8644385 | 447.2393049 |
| 445.1800656 | 457.910154 |
| 445.1800656 | 459.6565245 |
| 445.1800656 | 460.4548857 |
| 457.5688616 | 470.6274192 |
| 457.5688616 | 470.6274192 |
| 469.4361768 | 479.5516307 |
| 471.8971748 | 486.9938289 |
| 500.4628168 | 490.9916689 |
| 510.3847636 | 501.8912927 |
| 514.7989683 | 501.8912927 |
| 518.1826144 | 508.7843106 |
| 518.1826144 | 508.7843106 |
| 520.8489689 | 509.3441833 |
| 535.8823827 | 513.8744266 |
| 540.3228669 | 521.5368653 |
| 543.1281269 | 549.1341884 |
| 549.9652025 | 553.587425 |
| 553.587425 | 559.0640056 |
| 556.4686762 | 559.0640056 |
| 563.1786155 | 562.2115869 |
| 571.961654 | 562.2115869 |
| 575.7405415 | 568.9982368 |
| 578.7214417 | 578.7214417 |
| 598.371616 | 598.371616 |
| 637.9844267 | 637.9844267 |
| 642.1547614 | 642.1547614 |
| 642.3793577 | 642.3793577 |
| 655.5638995 | 655.5638995 |
| 659.882266 | 659.882266 |
| 663.3471663 | 663.3471663 |
| 673.1523377 | 673.1523377 |
| 677.6204309 | 677.6204309 |
| 681.1315314 | 681.1315314 |
| 690.7872542 | 690.7872542 |
| 695.3133993 | 695.3133993 |
| 703.9867122 | 703.9867122 |
| 708.6030062 | 708.6030062 |
| 712.2879813 | 712.2879813 |
| 717.4535065 | 717.4535065 |
| 721.2089923 | 721.2089923 |
| 726.3111673 | 726.3111673 |
| 726.3111673 | 726.3111673 |
| 748.4505991 | 748.4505991 |
| 752.8697369 | 752.8697369 |
| 752.8697369 | 752.8697369 |
| 756.8141973 | 756.8141973 |
| 788.3427185 | 788.3427185 |
| 796.3753979 | 796.3753979 |
| 797.1759008 | 797.1759008 |
| 801.5858549 | 801.5858549 |
| 819.347314 | 819.347314 |
| 823.6146084 | 823.6146084 |
| 849.1928549 | 849.1928549 |
| 849.1928549 | 849.1928549 |
| 859.1907387 | 859.1907387 |
| La 10x          | Vesicle Area (nm²) | LaP 10x          | Vesicle Area (nm²) |
|----------------|--------------------|------------------|--------------------|
| 859.1907387 | 1124.465738        | 859.1907387      | 1144.764699        |
| 859.1907387 | 1383.991325        | 863.6645991      | 1562.843121        |
| 868.0456318 | 15509.47401        | 876.9984098      | 27562.78479        |
| 888.7426766 | 45181.64971        | 894.6175955      | 48501.27477        |
| 894.6175955 | 57058.48246        | 901.9485365      |                    |
| 915.1445459 |                    | 943.7833045      |                    |
| 978.7876988 |                    | 978.7876988      |                    |
| 978.7876988 |                    | 978.7876988      |                    |
| 978.7876988 |                    | 978.7876988      |                    |
| 989.9653323 |                    | 996.5009375      |                    |
| 1007.553916 |                    | 1036.337294      |                    |
| 1073.583356 |                    | 1085.054707      |                    |
| 1085.054707 |                    | 1107.177453      |                    |
| 1151.491175 |                    | 1161.919904      |                    |
| 1210.879941 |                    | 1219.777761      |                    |
| 1244.477241 |                    | 1291.050017      |                    |
| 1439.328906 |                    | 1598.783145      |                    |
| 7783.29847  |                    | 29938.63035      |                    |
| 30750.33057 |                    | 64908.13204      |                    |

| La 10x          | Vesicle Area (nm²) | LaP 10x          | Vesicle Area (nm²) |
|----------------|--------------------|------------------|--------------------|
| 2089.630009    |                    | 1053.525733      |                    |
| 2594.013761    |                    | 2149.277687      |                    |
| 3349.316181    |                    | 2370.389752      |                    |
| 4021.668495 | 2516.95929 |
| 4235.868017 | 3702.197021 |
| 4462.508966 | 4119.598545 |
| 4676.240436 | 4315.848234 |
| 5153.251822 | 4633.914622 |
| 6147.12604 | 6452.809448 |
| 7238.983476 | 8075.113946 |
| 7255.279501 | 10460.36065 |
| 7575.788397 | 12674.27104 |
| 7754.708842 | 30067.26877 |
| 8301.254 | 33538.51951 |
| 8525.933824 | 62514.27271 |
| 9504.00894 | 68222.36667 |
| 10080.46815 | 72743.41471 |
| 11792.80942 | 90645.21763 |
| 12339.87727 | 93841.65773 |
| 12584.83458 | 119887.0455 |
| 12584.83458 | 194128.4685 |
| 12587.41961 | 246902.0286 |
| 12951.13605 | 314160.7237 |
| 13191.0625 | 510115.3117 |
| 13337.22161 | 646879.4385 |
| 13390.08524 | |
| 13680.63101 | |
| 13698.0509 | |
| 13743.10359 | |
| 13906.909 | |
| 14808.78808 | |
| 16888.64416 | |
| 17223.8255 | |
| 18014.04449 | |
| 18393.31741 | |
| 20675.14602 | |
| 21529.93342 | |
| 23281.44249 | |
| 23736.09621 | |
| 24966.54615 | |
| 25760.85984 | |
| 25780.77744 | |
| 33027.292 | |
| La 20x | Vesicle Area (nm²) | LaP 20x | Vesicle Area (nm²) |
|--------|-------------------|---------|-------------------|
| 33710.45205 | 248.4822046 |
| 38217.08737 | 310.5880568 |
| 40731.13944 | 318.0353066 |
| 43198.45064 | 340.4157236 |
| 46131.86552 | 360.2862681 |
| 50102.99954 | 370.2111853 |
| 51226.0484 | 422.4047888 |
| 53991.50597 | 449.7166644 |
| 54625.12998 | 606.2449852 |
| 57606.80638 | 606.2449852 |
| 74343.54147 | 658.426075 |
| 88585.46777 | 725.5470817 |
| 108629.282 | 735.4634914 |
| 155405.4709 | 757.8385175 |
| 440871.5595 | 787.6461489 |
| La 20x Vesicle Area (nm²) | 906.905826 | LaP 20x Vesicle Area (nm²) |
| 839.8184021 | 805.0521681 |
| 906.905826 | 807.518294 |
| 940.4646761 | 827.3830744 |
| 970.3771023 | 837.3546898 |
| 974.0246139 | 974.0246139 |
| 1046.060052 | 974.0246139 |
| 1080.155915 | 978.9540626 |
| 1205.089213 | 1053.525733 |
| 1229.826742 | 1056.000993 |
| 1277.140371 | 1098.231077 |
| 1322.12265 | 1137.998133 |
| 1396.415595 | 1224.983198 |
| 1403.843677 | 1224.983198 |
| 1559.061627 | 1354.151727 |
| 1589.017878 | 1441.145072 |
| 1629.9754 | 1503.232451 |
| 1629.9754 | 2367.887862 |
| 1672.178792 | 2516.95929 |
| 1674.716543 | 4258.749103 |
| 1681.323701 | 9036.787149 |
| 1711.986839 | 12920.49417 |
| 2089.630009 | 27217.52461 |
| 2099.60775 | 64048.05115 |
| 2397.737348 | 94001.35767 |
| 2516.95929 | **161455.5257** |
| 2633.795263 | **216320.3589** |
| 2753.002795 | **216665.909** |
| 2818.863344 | **1043219.071** |
| 2886.168919 | |
| 2946.855155 | |
| 3508.41357 | |
| 3888.578953 | |
| 3968.00764 | |
| 4047.562743 | |
| 4238.877317 | |
| 4405.385663 | |
| 4810.3942 | |
| 4942.083091 | |
| 5014.115237 | |
| 5260.042854 | |
| 5399.271172 | |
| 5588.047064 | |
| 5588.047064 | |
| 5749.517937 | |
| 5916.014886 | |
| 6005.51025 | |
| 6087.515545 | |
| La 50x | Vesicle Area (nm²) | LaP 50x | Vesicle Area (nm²) |
|--------|--------------------|---------|--------------------|
| 6124.772747 | 89.09855295 | 211.189171 |
| 6298.68917 | 178.2021065 | 323.0173 |
| 6348.584351 | 184.2385881 | 449.7166644 |
| 6899.968617 | 210.3657219 | 541.6420844 |
| 7195.616981 | 210.3657219 | 549.0926542 |
| 11292.96367 | 220.2743468 | 613.2038289 |
| 14111.6618 | 247.4773913 | 658.8354662 |
| 18371.68924 | 263.885065 | 732.2944287 |
| 52627.13268 | 309.371014 | 793.1274898 |
| 62758.69456 | 321.7443267 | 793.1274898 |
| 121678.9654 | 321.7443267 | 1013.582857 |
| 89.09855295 | 323.6547292 | 1054.101116 |
| 178.2021065 | 338.5868518 | 1097.174041 |
| 184.2385881 | 360.9931241 | 1127.618041 |
| 210.3657219 | 361.3299657 | 1228.956684 |
| 210.3657219 | 363.4894713 | 1279.675322 |
| 220.2743468 | 368.7461844 | 1279.675322 |
| 247.4773913 | 400.9369942 | 1388.610895 |
| 263.885065 | 420.7307472 | 1391.121885 |
| 309.371014 | 420.7307472 | 1411.424638 |
| 321.7443267 | 445.479296 | 1455.986145 |
| 323.6547292 | 445.479296 | 1464.655935 |
| 338.5868518 | 447.9517828 | 1552.909006 |
| 360.9931241 | 447.9517828 | 1583.723215 |
| 361.3299657 | 450.6192188 | 1621.756479 |
| 363.4894713 | 460.5689938 | 1654.684705 |
| 368.7461844 | 460.5689938 | 1707.882411 |
| 460.5689938 | 1745.888206 |
| 485.0791091 | 1890.361959 |
| 487.5415844 | 2108.225519 |
| 507.3460518 | 2141.150407 |
| 510.3847636 | 2153.881754 |
| 514.7587535 | 2283.101454 |
| 546.9350397 | 2308.23882 |
| 546.9350397 | 2354.022086 |
| 550.2146292 | 2354.022086 |
| 550.2146292 | 2447.375827 |
| 556.845043 | 2452.903099 |
| 560.1541666 | 2579.590081 |
| 562.631932 | 2627.704251 |
| 603.8470822 | 2648.643139 |
| 607.4675611 | 2725.63644 |
| 609.9601817 | 2882.170994 |
| 618.7024997 | 2894.649869 |
| 618.7024997 | 3167.420492 |
| 637.3578109 | 3180.40171 |
| 639.8212869 | 3259.8584 |
| 643.457967 | 3296.696966 |
| 655.8362191 | 3309.532298 |
| 659.7456798 | 3746.871451 |
| 677.1591195 | 3960.530573 |
| 677.1591195 | 4534.549905 |
| 689.6231174 | 5131.263216 |
| 715.2238813 | 5194.559267 |
| 726.9800721 | 5478.615889 |
| 732.5822426 | 5667.560934 |
| 736.9062364 | 5749.517937 |
| 774.6178078 | 6580.724994 |
| 774.6178078 | 6631.290085 |
| 774.6178078 | 6842.814706 |
| 784.5153955 | 6943.026959 |
| 784.5153955 | 8777.675917 |
| 806.6119319 | 9471.55145 |
| 809.1308609 | 10375.8745 |
| 816.609906 | 16580.02732 |
| 863.7166891 | 20641.51837 |
| 975.0759236 | 22351.92326 |
|                   |                   |
|-------------------|-------------------|
| 989.9653323       | 31908.55956       |
| 1120.309081       | 37839.30072       |
| 1125.297993       | 43342.24259       |
| 1244.789896       | 47321.10526       |
| 1274.671214       | 60532.45006       |
| 1294.618917       | **110106.9972**   |
| 1356.827239       | **128041.7271**   |
| 1366.769249       | **212890.657**    |
| 1443.972496       |                   |
| 1458.895782       |                   |
| 1526.134315       |                   |
| 1645.61263        |                   |
| 1692.899296       |                   |
| 1843.797826       |                   |
| 1854.773696       |                   |
| 2519.449743       |                   |
| 2990.021333       |                   |
| 5599.182326       |                   |
| 6555.156254       |                   |
| 9923.591274       |                   |
| 26927.64037       |                   |
| 33181.76545       |                   |
| **241883.912**    |                   |
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