PHYSICAL EFFECTS OF NANOPATICLES AND POLYMERS ON VESICLES

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Table of Contents

Acknowledgement I

Table of Contents II

Summary IV

List of Tables V

List of Figures V

List of Abbreviations VII

Chapter 1 Introduction 1

Chapter 2 Literature Review 4

2.1 Nanotechnology 4

2.2 Vesicles 6

2.3 Nanoparticles and Vesicles 11

2.4 Rheology 20

Chapter 3 Materials and Methods 24

3.1 Characteristics of EggPC 24

3.1.1 Materials 24

3.1.2 Preparation of EggPC 24

3.2 EggPC and Latex Nanoparticles 26

3.2.1 Materials 26

3.2.2 Preparation of Latex Nanoparticles 26

3.2.3 Experimental Method 26

3.3 EggPC and Gold Nanoparticles 28

3.3.1 Materials 28

3.3.2 Preparation of Gold Nanoparticles 28

3.3.3 Experimental Method 29

3.4 Rheology of EggPC, Gold nanoparticles and polyelectrolytes 30

3.4.1 Materials 30

3.4.2 Preparation of Mixture of EggPC, Gold Nanoparticles and Polyelectrolytes .30
3.4.3 Experimental Method....31
3.5 Characterization Methods 32
3.5.1 Dynamic Light Scattering (DLS) 32
3.5.2 SEM & FESEM....34
3.5.3 TEM 37
3.5.4 AFM 38
3.5.5 Zeta-potential Analyzer 39
3.5.6 Rheometer 41
Chapter 4 Results and Discussion 42
4.1 Characteristic of EggPC 42
4.1.1 Particle size, Morphology, Zeta potential, PH and Conductivity 42
4.1.2 Effect of preparation parameters on EggPC 45
4.1.3 Effect of pH 50
4.1.4 Effect of charged ions....52
4.2 EggPC and Nanoparticles....54
4.2.1 Characteristics of nanoparticles....54
4.2.2 Critical concentration 56
4.2.3 Effect of Microspheres on EggPC....59
4.2.4 Effect of gold nanoparticles on EggPC 64
4.3 Rheology of EggPC, Nanoparticles and Polyelectrolytes 80
Chapter 5 Conclusion 84
References....86

Summary

Vesicles are considered as model systems in biochemistry and they are found useful in cosmetics, pharmaceutical, genetic engineering and medical technology. Nanoparticles are generally regarded as a type of drug and they typically require drug carriers to transport them. Vesicles can be considered as drug carriers, this research project is to investigate the physical effects of nanoparticles on the properties of vesicles by experimental approaches from microscopic view.

Nanoparticles such as Latex and gold are chosen due to their physical and chemical properties. Laser light scatting along with imaging techniques such as Atomic Force Mocroscopy, Scanning Electron Microscopy, Field Emission Scanning Electron Microscopy, Transmission Electron Microscopy are used for investigation. Interactions between vesicles and nanoparticles were found mainly by adsorption at particle surface. The vesicles were observed to be stayed as particles not as bilayer membranes when interact with nanoparticles. The amount of vesicles adsorbed on nanoparticles increases with vesicles concentration. The effects of ion charges of aqueous solution and time factor on the interactions are also studied. The nature of the interactions was further understood by the means of rheology.

List of Tables

Table 1: Sample composition of EggPC with microsphere 27
Table 2: Sample composition of EggPC with gold nanoparticles 29
Table 3: Sample composition of rheology study. 31
Table 4: Zeta potential analysis of vesicles with gold nanoparticles.Sample No. 67
Table 5: Constants of power law equation determined by rheology experiments.80

List of Figures

Figure 1: Schematic illustration of a single bilayer vesicle 6
Figure 2: A schematic image of the complexes formed between vesicles and gold nanoparticles. (A) A vesicle with gold nanoparticles at the surface; (B) A vesicle with gold nanoparticles in the membrane; (C) A vesicle with encapsulating gold nanoparticles. 17
Figure 3: Schematic pictures of absorption of EggPC on latex or silica particles: (a) single vesicle layer model; (b) lipid molecular bilayer model. 19
Figure 4: Chemical structure of EggPC 24
Figure 5: Chemical Structure of Sodium Citrate 28
Figure 6: Chemical Structure of Poly(sodium 4-styrenesulfonate) 30
Figure 7: A typical Dynamic Laser Scattering result for unilamellar vesicles 42
Figure 8: AFM image and profile on vesicles obtained by tapping mode. 43
Figure 9: Effect of sonication on vesicle size distribution 46
Figure 10: Effect of centrifuge speed on vesicle size distribution 48
Figure 11: Effect of extrusion on vesicle size distribution 49

Figure 12: Effect of pH on vesicles size distribution 51
Figure 13: Results for effect of charged ions 53
Figure 14: A FESEM image on microsphere (D=300nm) 54
Figure 15: A TEM image on gold nanoparticles 55
Figure 16: Critical concentration of EggPC in DLS 57
Figure 17: Critical concentration of microspheres in DLS 57
Figure 18: Critical concentration of gold nanoparticles in DLS 58
Figure 19: Illustrations of effect of microspheres on EggPC vesicles on following compositions: (a) EggPC: MS (v:v) = 10:0; (b) EggPC: MS (v:v) = 8:2; (c) EggPC: MS (v:v) = 6:4; (d) EggPC: MS (v:v) = 4:6; (e) EggPC: MS (v:v) = 2:8; (f) EggPC: MS (v:v) = 0:10..... 62
Figure 20: Particle size distribution of EggPC with gold nanoparticle 64
Figure 21: SEM image of complex EggPC vesicles and gold nanoparticles..... 65
Figure 22: TEM image of complex of EggPC vesicles and gold nanoparticles. . 66
Figure 23(a-e): Vesicles with gold nanoparticles with presence of NaCl in 5 days. 72
Figure 24 (a-e): Vesicles with gold nanoparticles with presence of $MgCl_2$ in 5 days. 75
Figure 25 (a-e): Vesicles with gold nanoparticles with presence of $CaCl_2$ in 5 days 77
Figure 26 (a-e): Vesicles with gold nanoparticles with presence of $LaCl_3$ in 5 days 79
Figure 27: Rhelogy of NAPSS with EggPC and Gold NP at various concentration: (a) Concentration of NaPSS between 1% - 10%; (b) Concentration of NaPSS between 15% - 25%; (c) Concentration of NaPSS between 30% - 40%;.... 83

List of Abbreviations

AFM	Atomic Force Microscopy
$CaCl_2$	Calcium Chloride
DLS	Dynamic Light Scattering
EggPC	L-α-Phosphatidylcholine from egg yolk
FESEM	Field Emission Scanning Electron Microscopy
Gold NPs	Gold Nanoparticles
$LaCl_3$	Lanthanum Chloride
$MgCl_2$	Magnesium Chloride
NaCl	Sodium Chloride
NaPSS	Poly Sodium Styrene Sulfonate
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy

Chapter 1 Introduction

Vesicles are considered as model systems to better understand biochemical processes in biochemistry. They have potentials in acting as carriers for diagnostic agents and pharmaceuticals in pharmaceutical sciences. Nanoparticles are generally regarded as a type of drug and they typically require drug carriers to transport them. The interactions between vesicles and nanoparticles have become very important in order to minimize drugs toxicity and improve their effectiveness by delivering them efficiently and specifically to the affected areas of the target cell.

This research project is to investigate the physical effects of nanoparticles on the properties of vesicles by experimental approaches from microscopic point of view. Latex nanoparticles and gold nanoparticles exhibit interesting behaviors when they interact with liposomes due to their special chemical and physical properties. They are therefore chosen as the model nanoparticles in this research. The nature and strength of the interaction was further investigated by rheology with introducing polyelectrolytes into the mixture.

Vesicles made of natural phospholipids L-α-Phosphatidylcholine from egg yolk were employed. When composed of natural phospholipids, vesicles are often called

liposomes. Vesicle or liposome technology is a rapidly evolving field of inquiry in both the basic and applied sciences and engineering. Liposomes have been used extensively as models for the study of biological membrane structure and function. There are plenty reports on both natural and synthetic surfactant vesicles investigated in drug delivery and targeting, medical imaging, catalysis, energy conversion, and separations.

In general, suspensions of self-assembled surfactant aggregates, such as micelles, vesicles, microemulsions can be investigated using techniques such as electron microscopy, force microscopy, analytical untracentrifugation, sedimentation flow field fractionation, viscometry, NMR spectroscopy, gel chromatography and various scattering techniques. Microscopes offer the advantage of visualization in real space, therefore are of greatest value when it is suspected that the suspension consists of aggregates of unusual shape and widely varying size. These techniques, however, require the aggregates to be analyzed outside of their true aqueous environment and sample preparation protocols may lead to artifacts. Other characterization techniques, including those based on scattering methods, are best applied when the particles are somewhat homogeneous in size and shape or when the dynamics of the system are under investigation.

The scattering of light, x-rays and neutrons are very noninvasive methods for determining the structural properties, both static and dynamic, in situ. As a result, complex fluids of colloids, polymers and surfactant aggregates are commonly characterized by various scattering techniques. Among the vesicle dispersion properties that one may investigate by scattering techniques are geometric structure such as size, shape, lamellae (or bilayer) thickness and the number of lamellae; molecular weight; degree of polydispersity; vesicle-vesicle, vesicle-solvent and vesicle-other species (i.e., proteins, polymers, colloidal particles and others) interactions; membrane fluctuations and fluidities; inter-particle dispersion structural dynamics; lamellae permeabilities; lamellae inter-digitations; vesicle aggregation and fusion; the structure of any associated water or ions; and others. (Rosoff, 1996)

The thesis consists of five chapters. Chapter 1 gives a brief introduction to the project. Chapter 2 is a literature review on nanoparticles and polymers with vesicles. In Chapter 3, the materials and methods used in the experiments are described. The experimental results and discussions are presented in Chapter 4, followed by conclusions drawn from this project and some recommendations in Chapter 5.

Chapter 2 Literature Review

2.1 Nanotechnology

Nanotechnology is very useful in various industries like engineering, manufacturing, information technology, and especially in the field of biomedical engineering and this technology has advanced rapidly in recent years (Keller, 2007). In general, nanotechnology describes any activities at a magnitude of less than 100 nm. It is at this size that the properties of solid materials change, for example gold changes its color (Leydecker, 2008). At 100 nm and below things start to become particularly interesting. As the size of the material used decreases, certain phenomena become more significant, often due to the huge increase in available surface area, even allowing for new properties to be exhibited in substances which were previously thought to be inert.

Nanotechnology is a rapidly expanding field, encompassing the development of man-made materials in the 5–100 nanometer size range. This dimension vastly exceeds that of standard organic molecules, but its lower range approaches that of many proteins and biological macromolecules. The first practical applications of nanotechnology can be traced to advances in communications, engineering, physics,

chemistry, biology, robotics, and medicine. Nanotechnology has been utilized in medicine for therapeutic drug delivery and the development of treatments for a variety of diseases and disorders. The rise of nanomaterials correlates with further advances in these disciplines (Faraji and Wipf, 2009).

2.2 Vesicles

Vesicles are one kind of colloids made of lipid bilayers. A lipid molecule consists of a polar, hydrophilic head that is attached to hydrophobic tail. At appropriate concentrations, the lipid molecules in water "self-assemble" to form bilayers because hydrophobic tails try to avoid contact with the water. When such bilayers are broken up into small pieces, the fragments wrap themselves into closed structures known as vesicles and encapsulate some of the liquid inside (Hiemenz and Rajagopalan, 1997).

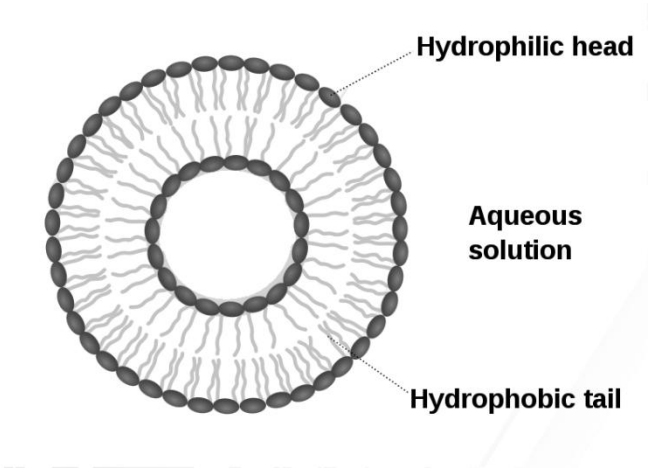


Figure 1: Schematic illustration of a single bilayer vesicle

A.D. Bangham discovered such vesicles during his research in 1961 (Hunter, 1992). He found out the appearance and the permeability of the phospholipids membranes of vesicles was similar to the properties of biological membranes. Since then, research on vesicles was conducted as the model for biological membranes.

As illustrated in Figure 1, vesicles are microscopic, fluid-filled pouches whose walls are made of layers of phospholipids identical to the phospholipids that make up cell membrane (Segota and Tezak, 2006). Just like a biological system, vesicles are naturally compartmentalized in three phases: the external aqueous phase, the hydrophobic interior of the bilayer and the internal aqueous phase (Myers, 2006).

This special "carrying capacity" structural property of vesicles leads them to be regarded as natural drug delivery systems. They are extremely useful in cosmetics, pharmaceutical, genetic engineering and medical technology. As known, drugs may cause side effects if they are administered in free form; the toxicity of drugs also delivers to other areas of the body which are not affected by the disease. Therefore the existence of vesicles makes it possible to improve the effectiveness of drugs and minimize their toxicity by encapsulating the drugs in vesicles and delivering them efficiently and specifically to the affected areas (Hiemenz and Rajagopalan, 1997).

Vesicles are characterized by their size, number of layers and surface charge. According to surface charge, vesicles are classified as anionic, cationic and neutral. If the vesicles are made of single bilayer, it is unilamellar vesicles; if they have more than one bilayer and consist of many concentric shells, they are called multilamellar vesicles. It has been observed that unilamellar vesicles are often found in diluted

solutions while multilamellar vesicles are usually found in more concentrated system (Regev and Guillemet, 1999). Unilamellar is the main focus in this research. Unilamellar vesicles can be classified by their sizes as small unilamellar vesicles, large unilamellar vesicles and giant unilamellar vesicles. They have a radius of 4-20nm, 50nm-10μm, > 10μm respectively.

Lipids are prone to decomposition by oxygen; they must be stored at low temperature in the dark and should be protected from air oxygen. Lipids decomposition is catalyzed by the glass walls of the container, so lipids are better stored as solutions. The choice of the solvent depends on the nature of the lipids. Phosphatidylcholines are kept in (9:1) mixtures of water saturated choloroform and methanol. Methanol, as well as other alcohols, can cause lipids esterification, though on the other hand, alcohols as free radical acceptors are capable of inhibiting the oxidation of lipids. The oxidation processes can be minimized by the addition of antioxidants and use of proper manufacturing conditions for dispersions, for example, the reduction of oxygen pressure by flushing with nitrogen or argon. In phospholipids, such as phosphatidylcholine, four ester bonds can be discerned. The two fatty acid ester bonds are the most labile bonds and are hydrolyzed first. If one fatty acid is left, lyso-phosphatidylcholine is formed, which can dramatically change the physico-chemical characteristics of the lipid bilayer. At low levels of degradation,

lyso-phosphatidylcholine and the hydrolysed free fatty acid chain cause a reduction of the bilayer permeability. For partially hydrogenated phosphatidylcholine and egg phosphatidyglycerol bilayers, an increase in permeability was only observed when over 10% of the phosphatidylcholine was hydrolyzed.

Liposomal aggregation, bilayer fusion, and drug leakage affect the shelf-life of liposomes. Aggregation is the formation of larger units composed of individual liposomes, but do not fuse into a new particle. This process is reversible by for example, applying mild shear forces, changing the temperature, or binding metal ions that initially induces aggregation. With aggregation, the small particles retain their identity, only their kinetic independence is lost (Hiemenz and Rajagopalan, 1997). Fusion of bilayers, however, is irreversible and consequently new liposomal structures are formed. In contrast to aggregation, fusion of liposomes may induce drug leakage, in particular when the encapsulated drug is water soluble and does not interact with the bilayer. In general, properly made, large liposomes do not fuse with time. However, bilayer defects may enhance fusion. These irregularities may disappear by a process termed 'annealing': incubating the liposomes at a temperature above the phase transition to allow differences in packing density between opposite sides of the bilayer leaflets to equalize by transmembrane 'flip-flop'. Bilayer defects can also be induced during a phase transition, so it is recommended to handle and store aqueous liposome

dispersions at a temperature well above or below the phase transition temperature range. Size effects play a role in the tendency to aggregate as well. Very small (<<100nm) liposomes are more prone to fusion than larger liposomes due to stress coming from the high curvature of their membrane. Phospholipids often come with aggregation problems, due to the tendency for the system to shift towards a lower free energy system, resulting in aggregation of vesicles into larger phospholipids in a bid to lower interfacial surface area. The reproducibility of the vesicle size and quantity may thus be problematic.

2.3 Nanoparticles and Vesicles

Nanoparticles currently are under intense scientific research because of their wide variety of potential applications in biomedical, optical, and electronic fields.

Nanoparticles are in solid state and either amorphous or crystalline. They are generally regarded as a type of drug and they typically require drug carriers to transport them. Due to unique property of vesicles, they have potential in acting as carriers for diagnostic agents and pharmaceuticals in pharmaceutical sciences.

Many kinds of molecules can be encapsulated in vesicles. For example, hydrophilic molecules can be encapsulated in the inner phase of vesicles, hydrophobic molecules can be encapsulated in the bilayer of the lipid membrane and also vesicles can be modified with many molecules at the surface.

The interactions between vesicles and nanoparticles have become very important in order to minimize drugs toxicity and improve their effectiveness by delivering them efficiently and specifically to the affected areas of the target cell.

Latex nanoparticles and gold nanoparticles exhibit interesting behavior when they

interact with vesicles due to their special chemical and physical properties. They are therefore chosen as the model nanoparticles in this research.

Monodispersed polystyrene latex particles formed aqueous phase-dispersed materials which were found in many practical applications, such as calibration standards and supports for biomolecules (Graillat et al., 1991). A model colloid system would preferably consist of monodisperse spherical polymer particles with known properties. The latex particles are sphereical and monodisperse and are considered to have well defined functional groups. As a result, they have been used extensively as models for fundamental phenomena research in colloid science (Elimelech and O'Melia, 1990).

Polystyrene latexes have traditionally been produced by emulsion polymerization using a water-soluble initiator, usually potassium persulfate which gives sulfate end groups which contribute to the particle stabilization (Graillat et al., 1991). The various factors affect the stability and monodispersity of the particle solution are surface charge, density, initiators, ionic strength, and temperature as well as monomer concentration. While there may be certain undesired properties under special preparation conditions, commercially available polystyrene particles are largely inert under normal conditions, and have a very narrow size distribution range, allowing for monodispersity. Small and similar charges are usually present on the particle surfaces

and this is effective in the prevention of aggregation behavior.

Gold nanoparticles have gained much attention in recent year due to their unique physical and chemical properties. Significantly different from those of bulk gold and gold atoms, these properties of gold nanoparticles are very much depending on their shapes and sizes (Daniel and Astruc, 2004; Burda et al., 2005). Gold nanoparticles have the ability to absorb light in the visible region of the spectrum and convert the absorbed light to heat (Link and El-Sayed, 1999; Link and El-Sayed, 2000). These properties have led gold nanoparticles to become a extremely useful materials for imaging and photothermal therapy in biomedical field (Govorov and Richardson, 2007; Jain et al., 2007). In this application, gold nanoparticles are generally regarded as a type of drug and they typically require drug carriers to preserve and transport them to the affected tissue and into the cells. Liposomes have been studied and then approved to be used as drug carriers. Therefore, liposomes are considered ideal partners with gold nanoparticles to deliver them to the target site in vivo (Kojima et al., 2008).

Any particle that has some linear dimension between 10^{-9} m and 10^{-6} m is defined as a colloid (Hiemenz and Rajagopalan, 1997). In this case, both nanoparticles and vesicles are considered colloids and the dispersion with them are called colloidal

systems.

Among numbers of aspects of colloidal system, the stability is an essential part of colloid system, as many functional applications of colloid systems depend heavily on the stability of the achieved dispersion. The stability of colloids may be either kinetic or thermodynamic. In colloid science, stability means small particles remaining uniformly distributed throughout a sample. Thus, the classical use of the term “colloid stability” is referred to kinetic stability not thermodynamically stability. Kinetic stability is a consequence of a force barrier against collision between the particles and possible coagulation subsequently. Many colloidal dispersions have kinetic stability, even though they are not thermodynamically stable. The coarsening process of a thermodynamically unstable dispersion is called aggregation. Aggregation is the process by which small particles clump together to form aggregates, but do not fuse into a new particle. The individual particles from which the aggregates are assembled are called primary particles. In aggregation, there is no reduction of surface, although certain surface sites may be blocked at the points at which the smaller particles touch. A colloid that is stable against aggregation is called kinetically stable (Hiemenz and Rajagopalan, 1997).

As primary particles of a dispersed system tend to associate into larger structure

known as aggregates, the nature of the inter-particle forces are seen to be responsible for this aggregation.

The tendency for aggregation can be attributed to van der Waals forces between atoms, molecules or particles. These forces originate from the dipole or induced-dipole interactions at the atomic level. Among three major types of van der Waals forces, dispersion force is always present. This force is a very general one which occurs between all particles in any suspension medium. When two colloidal particles approach each other, the atoms in one particle are able to interact with all of atoms of the other particles and these effects are to some extend additive (Hunter, 1992). The important outcome of this partial additive is that the force tends to exert its effect over a much longer range. The force is named Hamaker force. The potential energy due to van der Waals force between two colloidal particles of the same material immersed in a fluid is always negative and attractive. The force is strong at short inter-particle separations and its magnitude decreases with about the second power of the separation (Hunter, 2001).

If there are no repulsive interactions between particles, the dispersion will be unstable and the particles will aggregate. The repulsion force mainly comes from electrostatic interactions generally due to the net electrical charges on the particle surface. The electrostatic force that results from dissociation of certain ions from the particle

surface when in contact with the aqueous medium or from the adsorption of ions from the solution serves to counteract the attraction due to van der Waals force to prevent aggregation. The stability in this case is known as electrostatic stability (Hiemenz and Rajagopalan, 1997). The repulsive force between colloidal particles leads to a positive potential energy, which decreases roughly exponentially with distance (Hunter, 2001). The properties of this electrical double layer are best described by the Zeta-potential ζ and the Debye-Huckel length κ^{-1}, which is a measure of the screening length of the screening effect due to ions in an electrolyte.

A quantitative measure of kinetic stability is known as the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory. DLVO theory suggests the stability of particles in solution depends on its total potential energy function V_T, which can be expressed as:

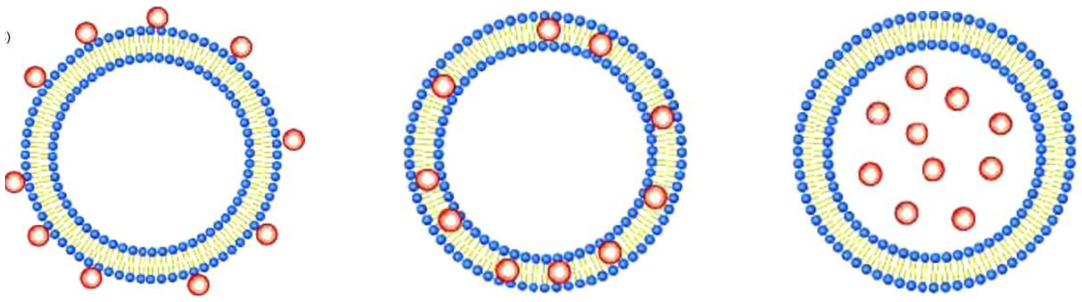
$$V_T = V_A + V_R + V_S$$

According to DLVO theory, the stability of colloidal system in a polar solution mainly depends on two conflicting forces, an attractive van der Waals force (V_A) and a repulsive electrostatic force (V_R); the solvent potential (V_S) often makes only a very marginal contribution to the colloid stability.

DLVO theory suggests that the energy barrier resulting from repulsive force prevents

particles from aggregation in solution, but if collisions take place with sufficient energy to overcome this barrier, the attractive force will allow them to attach to one another and subsequently aggregation occurs. According to the equation for DLVO theory, a negative total potential energy means attraction and a positive one represents repulsion.

Kojima et. al. (2008) suggest that there are three types of complexes of gold nanoparticles with vesicles as shown in Figure 2. The first is a vesicle with gold nanoparticles at its surface by physical adsorption; the second is a vesicle loaded with gold nanoparticles in its membrane by mixing lipid and gold nanoparticles possessing hydrophobic surfaces; the last one is a vesicle with gold nanoparticles encapsulated in its inner aqueous phase by reducing gold ions in the vesicles (Shioi and Hatton, 2002).



(A) (B) (C)

Figure 2: A schematic image of the complexes formed between vesicles and gold nanoparticles. (A) A vesicle with gold nanoparticles at the surface; (B) A vesicle with gold nanoparticles in the membrane; (C) A vesicle with encapsulating gold nanoparticles.

The advantage of the vesicles with gold nanoparticles at surface is that the complex comprising certain bioactive molecules such as drug, can be easily prepared by using liposomes containing them through simple mixing (Kojima et al., 2008).

Yang et al. (2003) stressed that the interactions between colloid particles and small vesicles are important. This is because the process involves the immobilization of vesicles on a colloid particle surface and it also provides useful information about the interaction between vesicles and solid particles in the colloid system. They studied the adsorption of EggPC vesicles on latex or silica particles and their aggregation behavior using DLS method and optical microscopy. They found that the adsorption of EggPC vesicles on solid particles was caused by electrostatic attraction in $LaCl_3$ aqueous solution. They also observed that the amount of EggPC adsorbed on both latex and silica particles surfaces increases with EggPC concentration and reaches a saturated value at a certain EggPC concentration. The depletion of EggPC in the bulk solution determines the amount of EggPC being adsorbed. They proposed 2 possible states of EggPC adsorption on latex and silica particles: vesicle-particle layer (a) and lipid molecular bilayer (b). The conclusion drawn was that EggPC vesicles existed on the solid particles surfaces as a particle state, not a bilayer membrane, and aggregation due to “particle bridges” was observed at certain concentration.

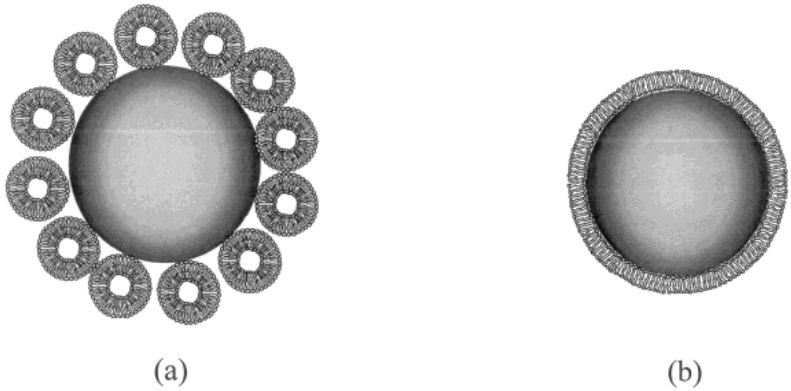


Figure 3: Schematic pictures of absorption of EggPC on latex or silica particles: (a) single vesicle layer model; (b) lipid molecular bilayer model.

There are various ways to induce vesicle aggregation, such as addition of divalent metal ions, decrease of pH and increase of temperature. Yao et al. (2007) reported the observation of such trend using hydrolyzed styrene-maleic anhydride copolymer (HSMA) with dodecyltrithylammonium bromide ($C_{12}Et_3$) mixture. They concluded the aggregation caused by decrease of hydration repulsion and hydrophobicity of vesicle surface had great influence on hydration force between vesicles.

Composite systems of lipid bilayer and polymer have drawn special attention due to their similarity to living systems such as plasma membranes and various organelle membranes, which mainly consist of complex polymers and lipids.

2.4 Rheology

Rheology is an important area of colloid and polymer science. Materials in colloidal state are often favored in industrial processing operations because of their large surface areas per unit volume which enhance chemical reactivity, adsorptive capacity, heat transfer rates, etc. The flow behavior and properties of colloids exert a significant influence on the performance, efficiency as well as the economy of the process. It also plays apart in quality control as it is a link of the products' microstructure and appeal and also marketability (Hiemenz and Rajagopalan, 1997).

When a stress is applied to a material, the material will be to some extent deformed. The study of the relation between the applied stress and the resulting deformation is called rheology. Even very dilute suspensions may show some unusual behavior patterns under the influence of a shearing stress. Very often, these unusual deformation patterns are sought after in the application of a colloidal system (Hunter, 1992).

Most of colloidal systems will show elastic behavior if it is examined on a short time scale; while exhibiting flow behavior over sufficiently long time. However, when a system does not show appreciable elastic behavior and has time-independent flow

behavior, it is a Newtonian fluid. For a Newtonian fluid, the relation between the shear stress and the strain rate is linear, the constant of proportionality being the coefficient of viscosity. Newtonian behavior is often observed in dilute stable dispersions of spherical particles. Lots of colloidal systems are reckoned as non-newtonians fluids. For a non-Newtonian fluid, the relation between the shear stress and the strain rate is nonlinear, and can even be time-dependent; these properties are the important and valuable characteristic of the colloidal system.

Colloids display a wide range of rheological behavior. Charged dispersions and sterically stabilized colloids may show elastic behavior like solids even at very low volume fractions. When the inter-particle interactions are not important, they behave as ordinary liquids under small shear forces. For behavior falls between these two extremems, it is known as viscoelastic. Therefore, it is important to understand how the interaction forces and fluid mechanics of the dispersions affect the flow behavior of dispersions (Hiemenz and Rajagopalan, 1997). For many colloidal dispersions, the elastic effects are not the primary role in the behavior, especially if the system is being sheared very strongly. In such system, the viscous aspects of the flow behavior are the primary concerns, even though the elastic properties have also to be recognized (Hunter, 2001).

Polyelectrolytes, sometimes called polysalts, are polymers whose repeating units each bear an electrolyte group. These groups will dissociate in aqueous solutions, making the polymers charged. Polyelectrolyte properties are similar to both electrolytes (salts) and polymers (high molecular weight compounds); their solutions are both electrically conductive and often viscous. With their unique properties, both natural and synthetic polyelectrolytes are being used in a wide range of technological and industrial fields. Many biological molecules are polyelectrolytes, such as polypeptides (all proteins) and DNA. Therefore, one of their important roles is in biology and biochemistry.

According to their dissociation in aqueous media, polyelectrolytes can be classified into two groups: strong polyelectrolytes and weak polyelectrolytes. Strong polyelectrolytes are fully dissociated upon dissolution, while weak polyelectrolytes are only partially dissociated except neutralization.

There are considerable interest in the interactions between the polyelectrolytes and colloidal particles. The nature and strength of the interactions depend on the properties of both polyelectrolytes and colloidal particles, composition of systems, solvent medium, etc. Some of the polyelectrolytes properties affecting these interactions are polyelectrolytes charge density, chain flexibility, conformation, hydrophobic properties, degree of polymerization and polyelectrolytes counterion

specificity (Vincekovic et al., 2005).

Theoretically, the presence of colloidal particles in the liquid increases the viscosity because of their effect it has on the flow behavior. In the case of nonrotating particle cutting across several velocity layers in the flowing liquid, it slows down the fluid so that the layers on opposite sides of the particle have the same velocity. The overall velocity gradient is thus reduced. Therefore, it results in an increase in viscosity. Alternatively, if a rotating particle exists in the flowing liquid, some of the energy that would otherwise keep the liquid flowing is taken by the particle, causing it to rotate. This will also increase the viscosity of the fluid. The increase in viscosity due to dispersed particles is expected to increase with the concentration of the particles. It can be described in terms of a power series in concentration, c:

$$\eta = A + Bc + Cc^2 + \ldots$$

The constants A, B, C ... are dependent on the size, shape orientation, etc of the dispersed particles (Hiemenz and Rajagopalan, 1997).

Chapter 3 Materials and Methods

3.1 Characteristics of EggPC

3.1.1 Materials

L-α-Phosphatidylcholine from egg yolk was purchased from Sigma-Aldrich. Sodium Chloride and Magnesium Chloride was purchased from BDH. Calcium Chloride and Lanthanum (lll) Chloride Heptahydrate were purchased from Nacalai Tesque Inc. Methanol and Chloroform were obtained from ChBE lab.

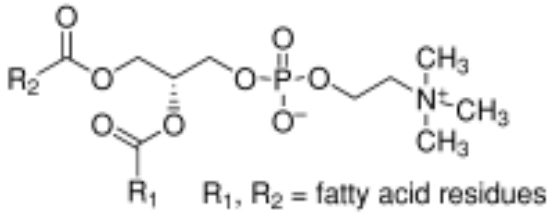


Figure 4: Chemical structure of EggPC

3.1.2 Preparation of EggPC

We followed the preparation procedure provided by our collaborator (Liang et al., 2004). L-α-Phosphatidylcholine(PC) mixed liposomes solution was prepared as follows. L-α-PC from egg yolk powder was dissolved in chloroform/methanol (2:1 v/v) mixture to get multilamellar vesicle solution. The solvent was evaporated under

pure nitrogen for 5 hours. A thin film of dried lipids was formed by evaporation of amphiphile in chloroform/methanol. 20mM NaCl was then added to dissolve the dried lipids layer to make a 1mM lipids solution. The mixture was sonicated with presence of ice in a bath type sonicator (Branson 2510) at $2^{o}C$ for 1 hour before centrifuged (Heraeus Instruments Biofuge Primo Centrifuge) for another 1 hour. Sample was obtained by extruding the solution through 0.2um membrane filter (Liang et al., 2004). The vesicles prepared were found to be uniformly dispersed and were used throughout the research.

3.2 EggPC and Latex Nanoparticles

3.2.1 Materials

Polystyrene Microsphere suspensions were purchased from Duke Scientific Corporation.

3.2.2 Preparation of Latex Nanoparticles

Microsphere was dissolved in ultra pure water in the w/w ratio of 1:10. The solution was mixed thoroughly to obtain a homogeneous solution.

3.2.3 Experimental Method

To study the effect of microsphere on EggPC vesicles, mixture of 0.2mg/ml EggPC unilamellar vesicles and microsphere solution were prepared in composition listed in Table 1.

Table 1: Sample composition of EggPC with microsphere

Sample No.	EggPC (ml)	Microsphere (ml)
S1	0	10
S2	2	8
S3	4	6
S4	6	4
S5	8	2
S6	10	0

3.3 EggPC and Gold Nanoparticles

3.3.1 Materials

Gold (lll) Chloride Trihydrate ($HAuCl_4 \cdot 3H_2O$) in ACS reagent Grade and Sodium Citrate were purchased from Sigma Aldrich. Silicon oil was obtained ChBE lab. Ultra pure water with resistivity of 18.0MΩ•cm was prepared by ELGA Purelab Ultra System.

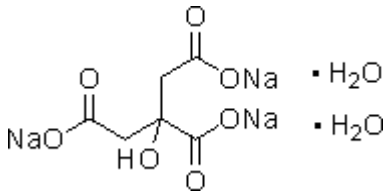


Figure 5: Chemical Structure of Sodium Citrate

3.3.2 Preparation of Gold Nanoparticles

Gold (lll) Chloride Trihydrate powder was dissolved in water to get 1mM gold solution. The gold solution was then transferred to a round-bottom flask attached to a condenser. The flask was immersed in pre-heated silicon oil (110^{o}C) for 10 minutes. In order to reduce the gold solution into gold nanoparticles, 38.8mM Sodium Citrate solution was subsequently added into the gold solution in v:v ratio of 10:1. This

mixture continued to be immersed in 110^{o}C silicon oil for another 10 minutes. The dark purple color mixture was then loaded with gold nanoparticles with a uniform particles size of 20nm.

3.3.3 Experimental Method

To study the effect of gold nanoparticles on EggPC vesicles, mixture of 0.2mg/ml EggPC unilamellar vesicles and microsphere solution were prepared in composition listed in **Error! Reference source not found.**. Sample S4 was chosen to be the composition for time study.

Table 2: Sample composition of EggPC with gold nanoparticles

Sample No.	EggPC (ml)	Gold Nanoparticles (ml)
S1	0	10
S2	2	8
S3	4	6
S4	6	4
S5	8	2
S6	10	0

3.4 Rheology of EggPC, Gold nanoparticles and polyelectrolytes

3.4.1 Materials

Poly(sodium 4-styrenesulfonate), one typical polyelectrolytes, with average molecular weight of 200,000 and 1,000,000 was purchased from Sigma Aldrich.

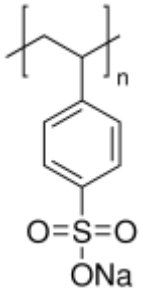


Figure 6: Chemical Structure of Poly(sodium 4-styrenesulfonate)

3.4.2 Preparation of Mixture of EggPC, Gold Nanoparticles and Polyelectrolytes

Poly(sodium 4-styrenesulfonate) was dissolved in Milli-Q water in a beaker. The solution was then left on a magnet stirrer to be stirred thoroughly until Poly(sodium 4-styrenesulfonate) was fully dissolved to achieve 20wt% NaPSS solution. The homogeneous solution was transferred into a refrigerator for relaxation. To prepare the mixture of polyelectrolytes with EggPC and gold nanoparticles, all individual

components are prepared separately, then mixed and stirred to achieve the desired compositions.

3.4.3 Experimental Method

To study the rheology of mixture with gold nanoparticles, EggPC vesicles and polyelectrolytes, 0.2mg/ml EggPC unilamellar vesicles solution, 1mg/ml gold nanoparticles solution and polyelectrolytes were prepared in composition listed in

Table 3. The measurement was done for similar combinations with final concentrations of 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35% and 40% NaPSS.

Table 3: Sample composition of rheology study.

Sample No.	NaPSS Concentration (wt%)	Contents
S1a	1%	NaPSS + H2O
S1b	1%	NaPSS + NaCl
S1c	1%	NaPSS + EggPC (in NaCl)
S1d	1%	NaPSS + EggPC(in NaCl) + Gold nanoparticles

3.5 Characterization Methods

3.5.1 Dynamic Light Scattering (DLS)

The presence of the huge numbers of particles per unit volume/weight and the polydisperse nature of particulate systems make light scattering become one of the most popular and important physical means for particle sizing. Laser light scattering can be applied for particles > 1 nm. Among many properties of a particulate material, the mean size and the size distribution are often the most important parameters that determine other macroscopic properties of the material and thus characterize the material (Muller et al., 1997). For instance, the physical stability of vesicle dispersions depends on particle size and particle size distribution, the particle size analysis is important in process control and particularly in quality assurance (Muller-Goymann, 2004).

DLS is a good method for investigating the dynamics of vesicles formation and their growth. Shioi et al. investigated the mixing of CTAB and SOS surfactant solutions using DLS. Sizes of the initial vesicles correlated well with the equilibrium sizes determined after 2 months (Shioi and Hatton, 2002).

The diffusion process in such system corresponds to the varying velocity of particles

in different directions, and only the component in the direction of scattering vector contributes to the frequency spectrum.

Dynamic light scattering (DLS) technique was employed to determine the size and size distribution of the nanoparticles, with a particle size analyzer (90 Plus, Brookhaven Instruments Corporation, US) at a fixed angle of 90°. The temperature was controlled at 25°C using a circulating water bath. The nanoparticles were suspended in prepared solution and sonicated to form uniform suspension. CONTIN correlation was used for the data analysis.

3.5.2 SEM & FESEM

The scanning electron microscope (SEM) and Field emission scanning electron microscope (FESEM) are able to provide some remarkable three-dimensional images. Field emission scanning electron microscope (FESEM, JSM-6700F, JEOL, Japan) at an accelerating voltage of 10kV was used to determine the surface morphology of the nanoparticles. Before the observation, samples were immobilized on metallic studs with double-sided conductive tape and then coated by a sputter coater (JFC-1300, JEOL, Japan) for 30s in a vacuum at a current intensity of 30mA.

Phospholipids have not been much investigated by means of electron microscopy at molecular resolution. This is because of phospholipid molecules is small with low electron density. Lack of highly developed sample preparation techniques also may have played some role. In spite of this, electron microscopy has been a valuable tool for studies of phospholipid aggregate structure. While preparing phospholipid samples for any sort of EM investigation, care must be taken to avoid osmotic stress, as this is prone to alter significantly the morphology of lipid vesicles. Temperature gradients caused by insufficiently rapid cryofixation are another potential source of artifacts.

It is advisable to use saturated and long acyl chains for biolayer core formation if a

compound requires a highly condensed bilayer with a low permeability. This can interfere with the preferred bilayer constitution for the in vivo application. For example, if in vivo a rather fast release is required, the optimum choice for bilayer constituents for prolonged shelf life and in vivo release characteristics may be mutually exclusive.

Freeze-drying technique has been implemented in some of the sample preparation for imaging purpose. It is a logical alternative to achieve the required long term stability on the shelf and the fast release in vivo. The major factors affecting the stability in a freeze-drying and rehydration cycle are bilayer structure, interaction compound/bilayer, liposome size, presence and type of cryoprotectant, process variables (freezing/drying time/temperature/rate, rehydration conditions). Of critical importance for the success of any freeze-drying of phospholipid suspensions is the interaction of the encapsulated substance with the bilayer. For molecules that strongly interact with the bilayer, freeze-drying generally does not present a problem, if proper cryoprotectants are present in the system. Retention and particle size remain then essentially unchanged upon rehydration. However, for hydrophilic compounds those do not interact intimately with a phosphlipid bilayer, the retention after a freezing-drying cycle tends to vary and to depend on the exact experimental conditions. Cyroprotectants play an important role in the physical stabilization of

liposomes during freeze-drying. However, there is no cyroprotectants introduced to the samples in this study. Therefore, in the absence of cryoprotectants, aggregation and fusion may occur during a freezing cycle.

Another disadvantage of freeze drying is the difficulty in establishing size classification of vesicles. Due to the fluid property of the vesicle dispersion prior to freezing, the sample film thickness increases from the center, where the film is thin, while the larger ones linger at the outside margin in the thicker part of the film. In this outer part the vesicles are out of the field of view. Therefore, the resulting distribution does not represent the true size distribution (Muller-Goymann, 2004).

3.5.3 TEM

The transmission electron microscope has a high-magnification power ranging from 100 to 500,000 under which the microstructure of colloidal systems can be easily visualized. The image formed is a two-dimensional representation of the actual structure. This is very helpful to have an idea of the surface topography of the sample. However, aqueous samples do not survive the high vacuum of a transmission electron microscope and water loss leads to microstructure changes (Muller-Goymann, 2004). Thus, special techniques of sample preparation are necessary prior to using TEM. Although Cryo-TEM has been proven to successfully overcome the problem (Swarbrick and Boylan, 2001), Cryo-TEM was not implemented in this research due to equipment restriction.

TEM (JEM 2010F, JEOL, Japan) examinations of the vesicles and nanoparticles were carried out with an electron kinetic energy of 200kV. A drop of sample was placed on a Formvar/carbon 200 mesh copper grid and dried with the presence of Nitrogen at 25^{o}C ambient condition. The grid was then attached to the sample holder of TEM for viewing.

3.5.4 AFM

AFM permits direct visualization of the lipid samples in situ. It is particularly suitable to monitor periodic lipid structures, potentially with a resolution greater than 1 nm. AFM imaging measurements were conducted using a Nanoscope IIIa atomic force microscope from Digital Instruments (Santa Barbara, CA) equipped with an E scanner. The scanner was calibrated following the standard procedures provided by Digital Instruments. Images were recorded in tapping mode using standard silicon nitride (Si_3N_4) integral tips (NP type) (Digital Instruments). The tapping mode was proven to show better-resolution images than contact mode and it is less likely to damage the sample than the contact mode because it eliminates friction force or drag between the sample and the tip (Liang et al., 2004). Room temperature was maintained at $22 \pm 1^{o}C$. Scanning rate between 1 to 5 Hz was used. Integral gain and proportional gain were chosen to be 0.5 and 0.75 respectively. The tip resonance frequency is in the range of 7.5 – 10 kHz. Its main disadvantage is difficulty in structural identifications and undesired conformational or phase changes that may result from the proximity of a scanning tip during the AFM measurements.

Due to equipment restriction, the fluid cell used for imaging under aqueous condition was not permitted. Therefore, only dry samples were imaged by AFM in this research.

3.5.5 Zeta-potential Analyzer

Techniques for probing the electric properties of phospholipid bilayers may be direct or indirect, depending on whether they rely on the used probes. In principle, direct methods are superior, as they are not perturbing. Zeta potentital measurement is one of the direct techniques chiefly based on the determination of the mobility of whole lipid vesicles in an external field. The zeta potential is defined as the electrostatic potential at the plane of shear between the membrane-associated and the stationary part of the double layer, the latter not being dragged through the measuring cell by the moving vesicles.

Experiments are difficult to perform in electrolytes with a salt concentration higher than 0.1 molar owing to heating problems caused by the ohmic currents. In dynamic experiments all results that show a multipeak distribution for a homogeneous suspension should be discarded.

The zero zeta potential point is called isoelectric point. It is the point where colloidal system is least stable.

Zeta potential is the difference in electrostatic potential between the layer of ions on

nanoparticle surface and the bulk liquid in which the nanoparticles are suspended. It indicates the amount of charge on the surface of the nanoparticles, and is used to predict their stability. The bigger the zeta potential, the more stable the nanoparticle suspension, because the repulsion between charged nanoparticles prevents the natural tendency of aggregation. Furthermore, surface charge also determines whether the nanoparticles will agglomerate in blood circulation and how they will interact with cells. The equipment used was ZetaSizer Nano-ZS (Malvern Instruments, UK).

3.5.6 Rheometer

Rheology is the study of the deformation and flow of materials under the influence of an applied stress. Different types of colloidal systems exhibit different rheological properties. (Kohler and Strnad, 1990; Roux et al., 1993)

The rheology measurements were carried out using Haake Rheostress 600 with a DC50 temperature controller (thermo Electron Corporation). A computer was linked to the rheometer to manipulate the controller, instruct the test routine and also evaluate the test results. A cone and plate sensor was chosen to do the measurement for all samples. It is ideal for measurement done on high viscosity at low shear rate.

The rheometer can be operated under test modes of controlled rate (CR), controlled stress (CS) and oscillation (OSC). The drive shaft of the rheometer is centered by an air bearing to ensure an almost frictionless transmission of the applied shear rate to the sample.

Chapter 4 Results and Discussion

4.1 Characteristic of EggPC

4.1.1 Particle size, Morphology, Zeta potential, PH and Conductivity

Strictly following the preparation procedure in Section 3.1.2, some relatively uniformly dispersed unilamellar vesicles were obtained as examined by Dynamic Light Scattering (Figure 7) and AFM (Figure 8).

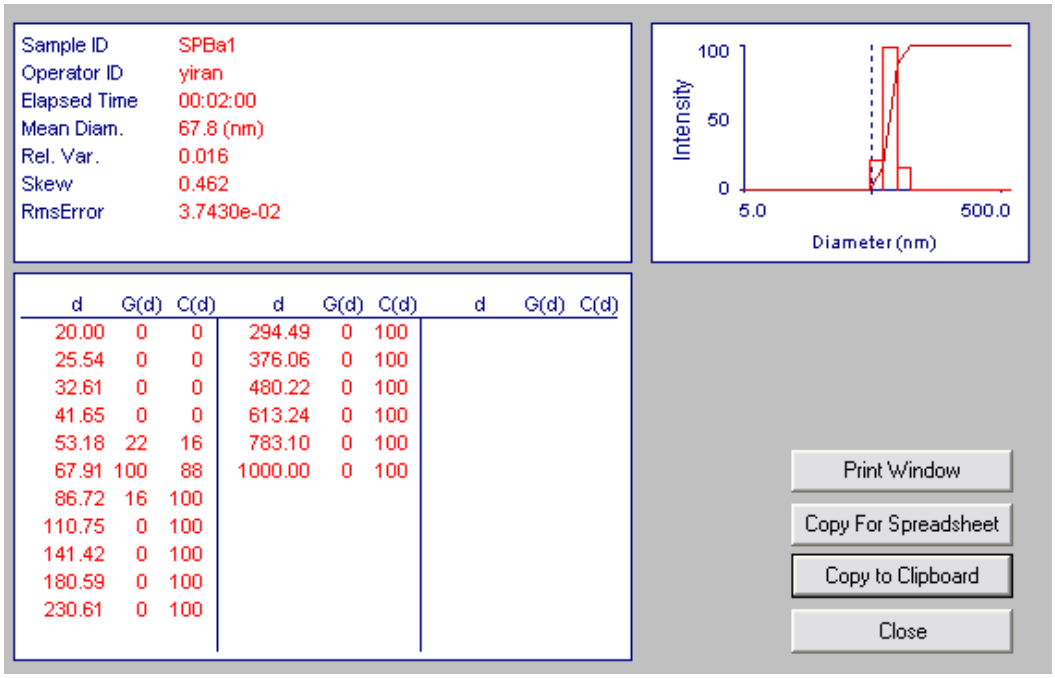


Figure 7: A typical Dynamic Laser Scattering result for unilamellar vesicles

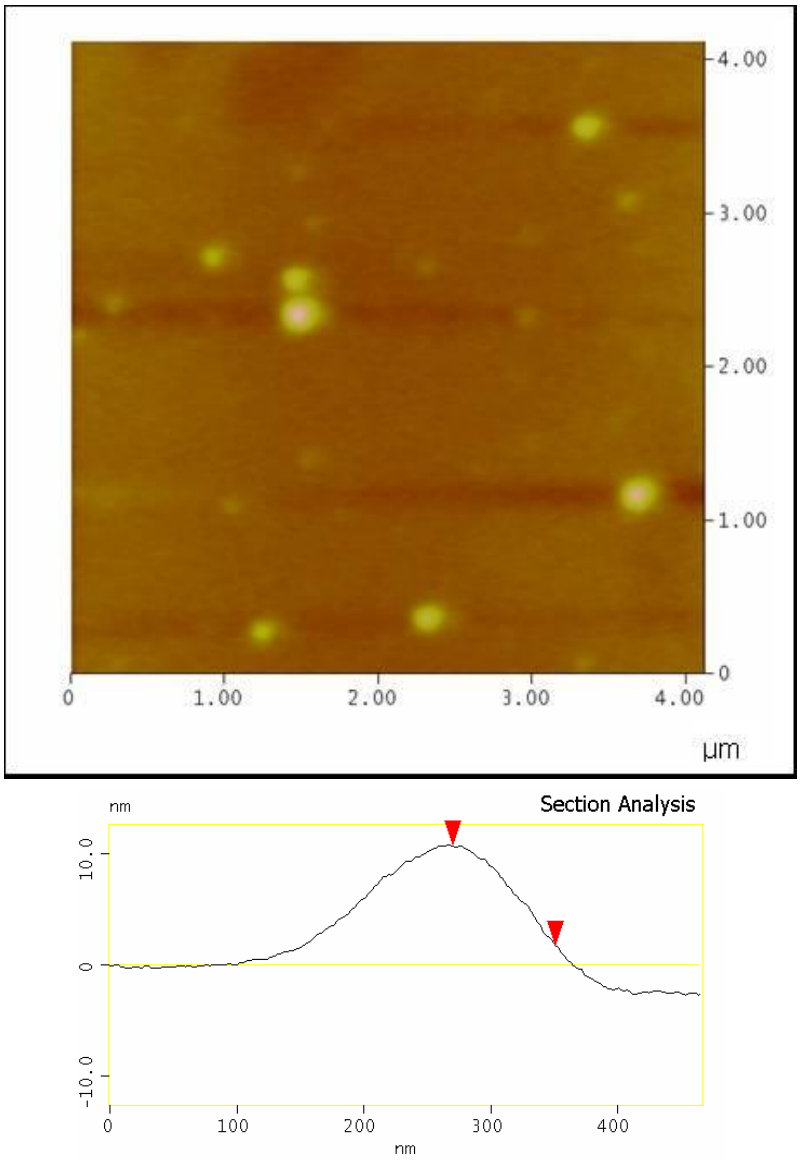


Figure 8: AFM image and profile on vesicles obtained by tapping mode.

The vesicle sizes measured by DLS were within the range of 50nm to 150nm. The diameters of vesicles in same sample measured from AFM were between 100nm to 200nm based on tapping mode. The AFM measurement results in a larger diameter than the DLS method. This observation agrees with finding of Liang et al. (2004). In

our study, only dry samples were allowed in AFM measurement due to equipment restriction. The vesicles had to be adsorbed and dried for 12 hours on the mica before the measurement. During the sample preparation, the soft vesicles flattened on the substrate, resulting in larger vesicle sizes (Reviakine and Brisson, 2000).

Zeta potential of EggPC vesicles are found to be -13mv, indicating that the stability is not very high. The pH of vesicles is about neutral and the conductivity is -12.8mS/m.

The above mentioned results are based on NaCl acting as solvent. In addition to NaCl, $MgCl_2$, $CaCl_2$ and $LaCl_3$ were also chosen to be examined. The mean size of vesicles in $MgCl_2$, $CaCl_2$ and $LaCl_3$ were found to be 191.3nm, 184.2nm and 171.4nm respectively.

4.1.2 Effect of preparation parameters on EggPC

The preparation methods can affect the vesicle size, its polydispersity, physical stability and other properties (Angelova et al., 1999; Feitosa et al., 2000; Lasic et al., 2001; Segota and Tezak, 2006). A narrow size distribution with high stability for a long time period is most favorable in many applications (Fendler, 1987). Therefore, the preparation method of vesicles becomes very important.

EggPC used in this study is a type of synthetic long-tailed phospholipids. When dispersed in aqueous solution, they form large multilamellar structures. In order to obtain unilamellar vesicles from multilamellar vesicles, some external energy input is required during preparation, as it is described in Section 3.1.2.

Some process parameters have been reported to have a major influence on vesicles size, such as homogeneity of the starting material; homogenizer type and valve, homogenization pressure and number of cycles, temperature, lipid composition and content (Torchilin and Weissig, 2003). In this section, some parameters involved in preparation of unilamellar vesicles other than above mentioned parameters were put into study to find out their influence on vesicle size and its size distribution. The parameters being varied were sonication process, centrifuge speed and extrusion

process.

Sonication, through vigorous agitation, may disrupt vesicles in multilamellar forms to produce unilamellar vesicles (Myers, 2006). However, some researchers suggested that sonication does not produce liposomes with a uniform distribution (Maguire et al., 2003). We compared the size distribution of vesicles prepared with and without sonication process from the analysis of dynamic light scattering. The finding is shown in Figure 9.

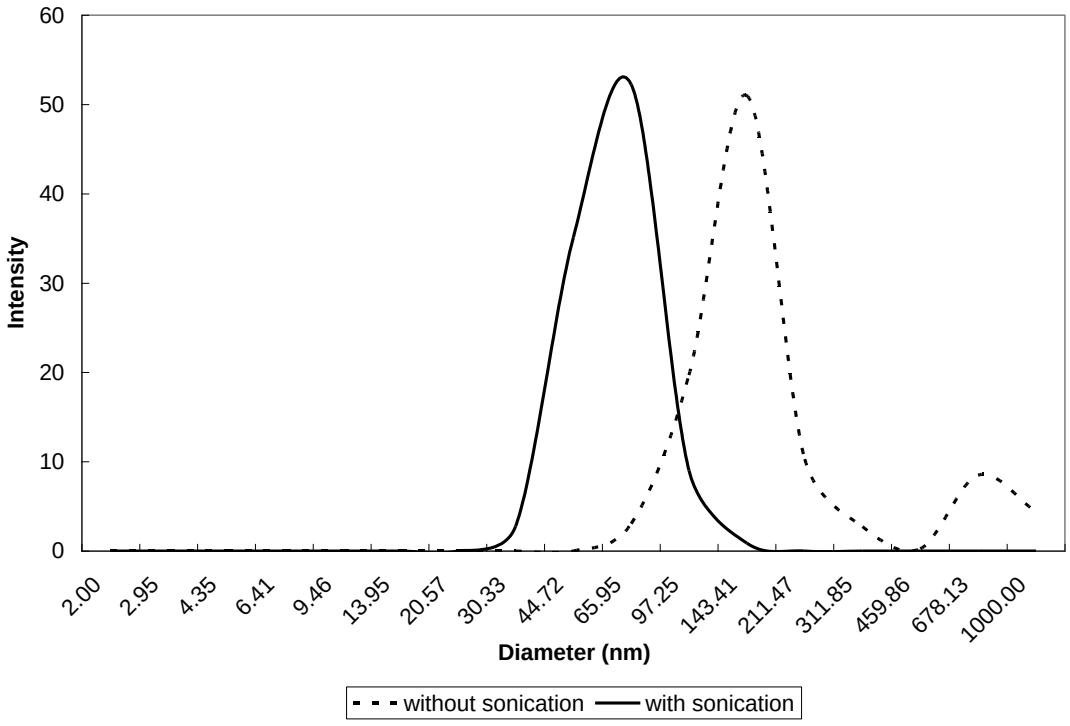


Figure 9: Effect of sonication on vesicle size distribution

If the solution did not go through the process of sonication, majority of the vesicles remained in multilamellar forms. Although centrifuge and extrusion process may remove partially large vesicles, the size distribution of unsonicated vesicles exhibits a stronger polydispersity with bigger average particle size than that of sonicated vesicles. On the other hand, the vesicles that underwent sonication show the uniformity and a narrow size distribution around 65nm. Therefore, in order to obtain uniform unilamellar vesicles, sonication process can not be omitted from the preparation procedure.

The purpose of centrifuge is to remove large lipid fragments after sonication process. As there are different centrifuge speeds reported by several researchers (Huang, 1969; Liang et al., 2004), it is interesting to find out if the centrifuge speed has any impact on the vesicle size distribution. The speeds ranging from 15443 – 18443 x g were tested and the result is shown in Figure 10.

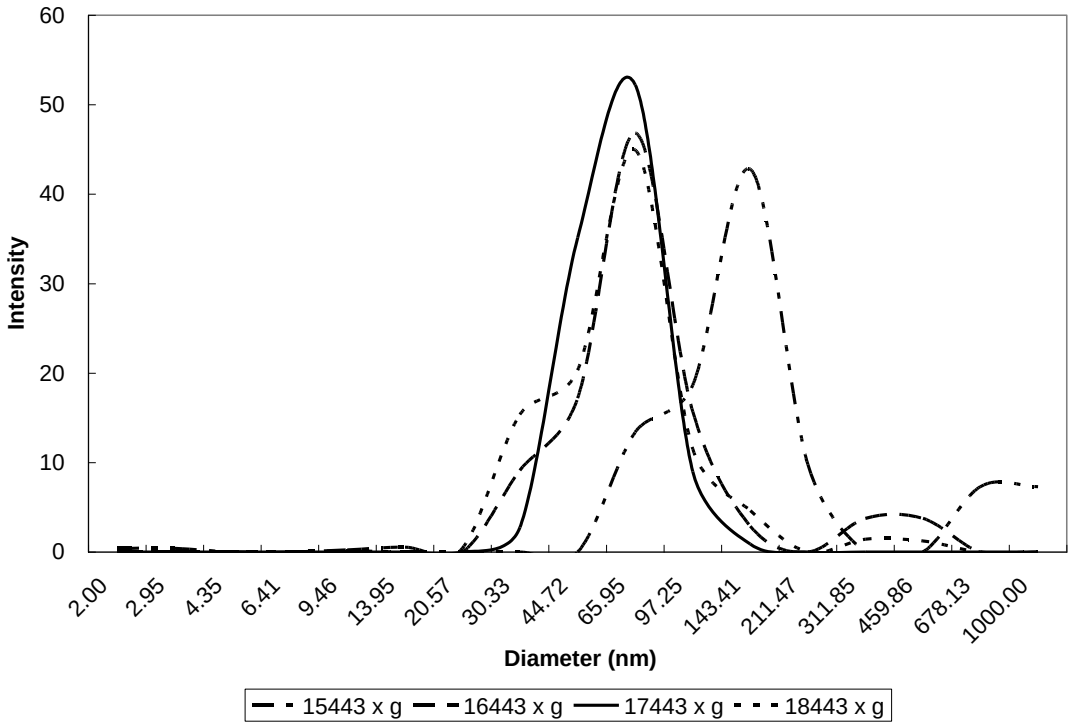


Figure 10: Effect of centrifuge speed on vesicle size distribution

Vesicles prepared at speed of 17443 x g have the narrowest and most uniform size distribution among all. As 15443 x *g* has the least centrifugal force among all, a high percentage of large lipid fragments possibly were not removed and remained in the solution. Speeds of 16443 and 18443 x g can produce vesicles with relative similar sizes to those at 17443 x g, however, with a wider size distribution. Therefore, 17443 x g provides the ideal centrifugal force to produce vesicles with a narrow size distribution..

Extrusion process uses shear forces to destroy the lamellar surface to form new fragment vesicles (Segota and Tezak, 2006). In essence, it ruptures polydisperse vesicles into monodisperse vesicles with a membrane filters of chosen pore size (Reviakine and Brisson, 2000). We used the extruder with port size of 200nm in preparation; ideally, all vesicles that went through extruder have particle size less than 200nm. However, it is evident from Figure 11 that there is obvious existence of giant vesicles in solutions without extrusion, reflected by a wider size distribution than that with extrusion.

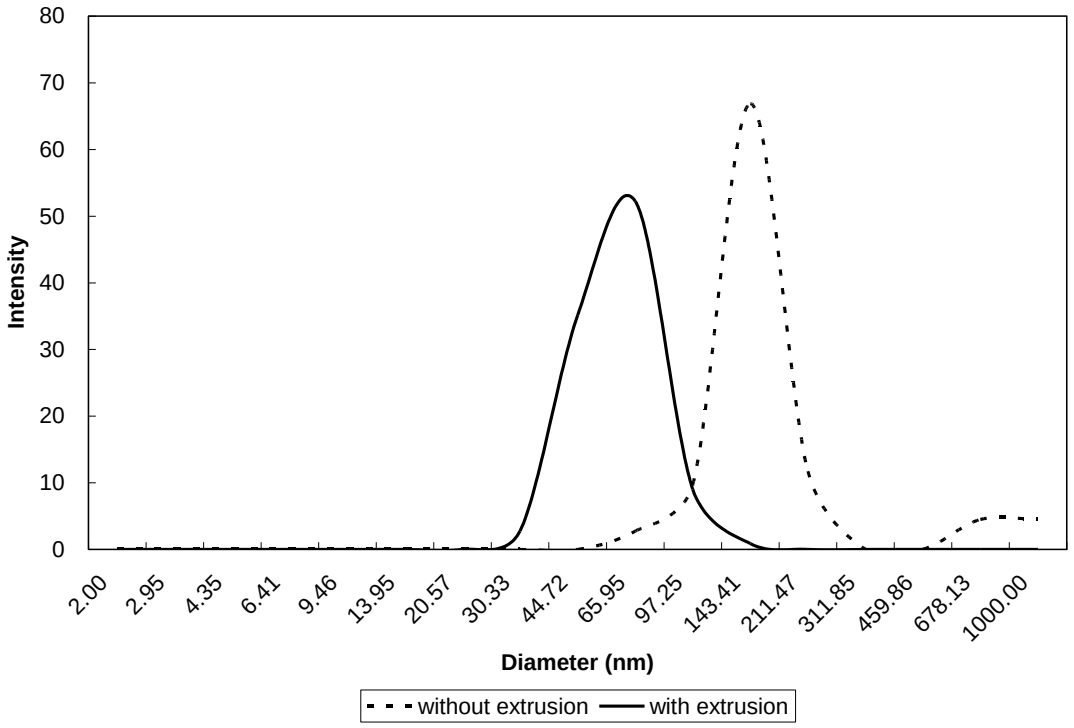


Figure 11: Effect of extrusion on vesicle size distribution

4.1.3 Effect of pH

The pH of the solution was adjusted to pH6.0, pH7.0 and pH8.0 and the size distribution of vesicles was measured accordingly. Some reports that decrease of solution pH will lower the vesicle charge density and then induce vesicles aggregation, because the binding of H+ to the vesicle surface will make the vesicle surface more hydrophobic. This will decrease the hydration repulsion force between vesicles (Yao et al., 2007). However, according to our experiment results presented in Figure 12, the size distribution of vesicles was not significantly affected by pH value within the specified range. As pH values of all samples used in experiments fall in the range of pH6.0 – pH8.0, the effects of pH value was not crucial in the study of size distribution.

However, pH becomes very important in the study related to zeta potential. That is because zeta potential values without pH readings are virtually meaningless. If the solution is more alkaline, particles inside the solution tend to acquire a more negative charge; while the solution becomes more acidic, the negative charges of the particles will be neutralized and eventually the particles become positively charged.

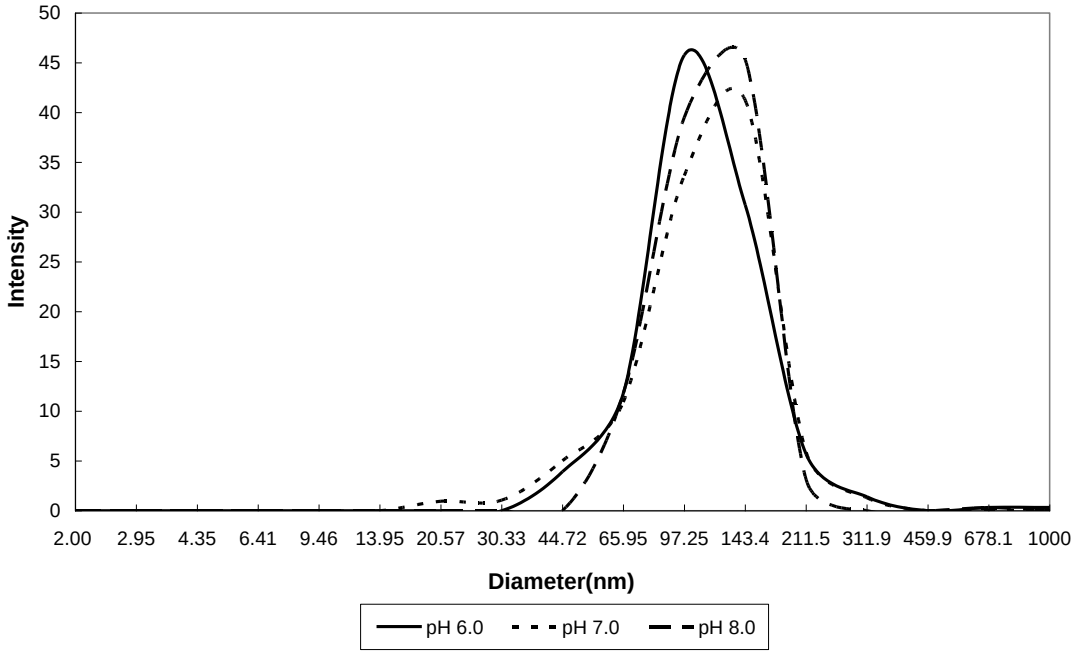


Figure 12: Effect of pH on vesicles size distribution

4.1.4 Effect of charged ions

The effects of Na^{+}, Mg^{2+}, Ca^{2+} and La^{3+} on vesicle size distribution were examined with the results shown in Figure 13. It can be seen that with addition of Na^{+}, the vesicles are mostly at the size of 97nm, which is the smallest among all solutions. The Mg^{2+} leads to the biggest vesicles size, while Ca^{2+} and La^{3+} give rise to mean sizes in between. Note that these ions were introduced to the samples during preparation of vesicles and the preparation procedures were all consistent. A possible reason for the size difference is that the vesicles aggregated to a different degree with each ion species present. Since the hydrated radii of the ions follow Na+ > La3+ > Ca2+ > Mg2+, it appears that the vesicles aggregation increases with the decrease of the hydrated radius. The least effective one has a large hydrated and small crystal radius, while the most effective cation has a small hydrated and large crystal radius.

The size of the ion affects their concentration in vesicle diffusion layer. The greater the hydration volume of the ion, the lower the ion concentration in diffusion layer is (Yao et al., 2007). The ions binding to the vesicle surface are expected to compete with water for the vesicle surface and destroy the water structure around them. As a result, the hydration repulsion was reduced and vesicle aggregation was promoted.

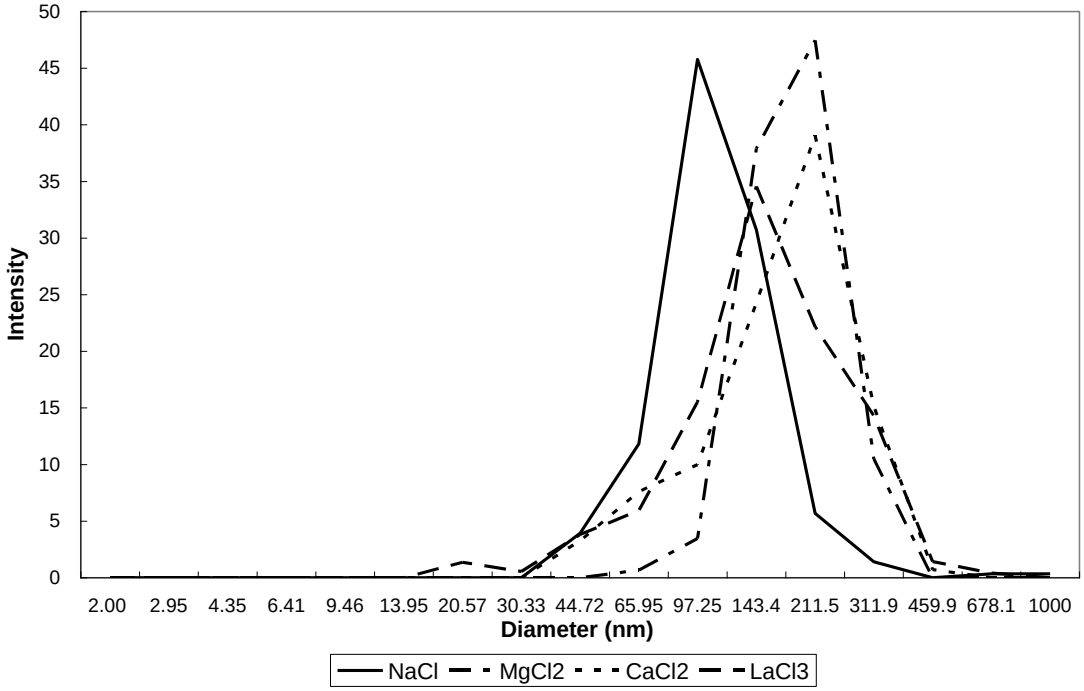


Figure 13: Results for effect of charged ions

4.2 EggPC and Nanoparticles

4.2.1 Characteristics of nanoparticles

Two types of nanoparticles were used in this section to study their effect on EggPC vesicles. They are latex microsphere and gold nanoparticles.

Polystyrene microspheres with size of 500nm and 300nm were prepared by dilution technique mentioned in section 3.3.1. The image of microspheres (300 nm) was represented in Figure 14. The results from DLS also showed an average particles size of 300nm.

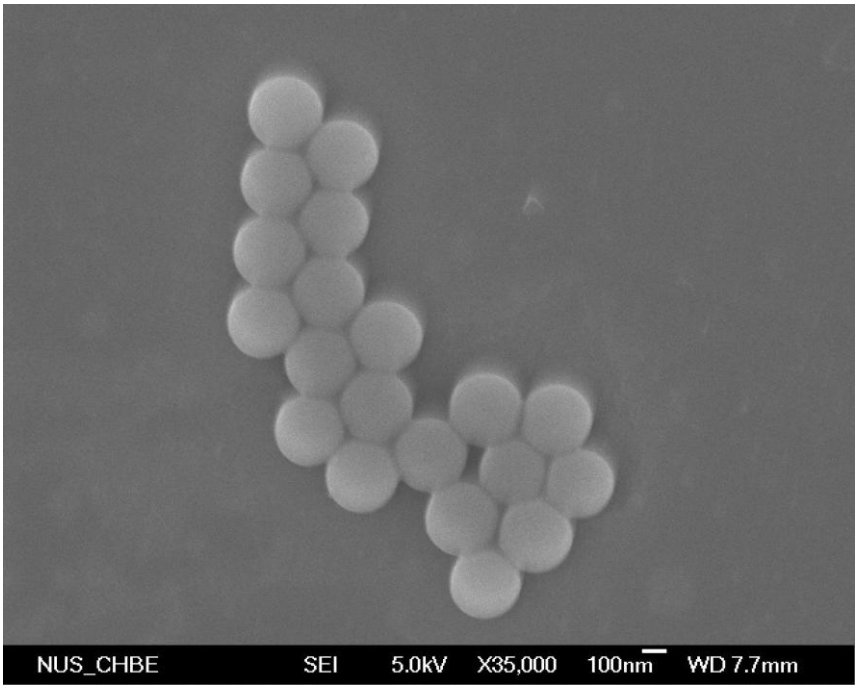


Figure 14: A FESEM image on microsphere (D=300nm)

Gold nanoparticles prepared by the method mentioned in section 3.3.2 were examined under TEM. A drop of gold nanoparticles solution was left on copper grid and dried in desiccator for 24 hours before imaging. The result is shown in Figure 15. The particle sizes were found to be relatively uniform around 20nm, which is consistent with results from DLS.

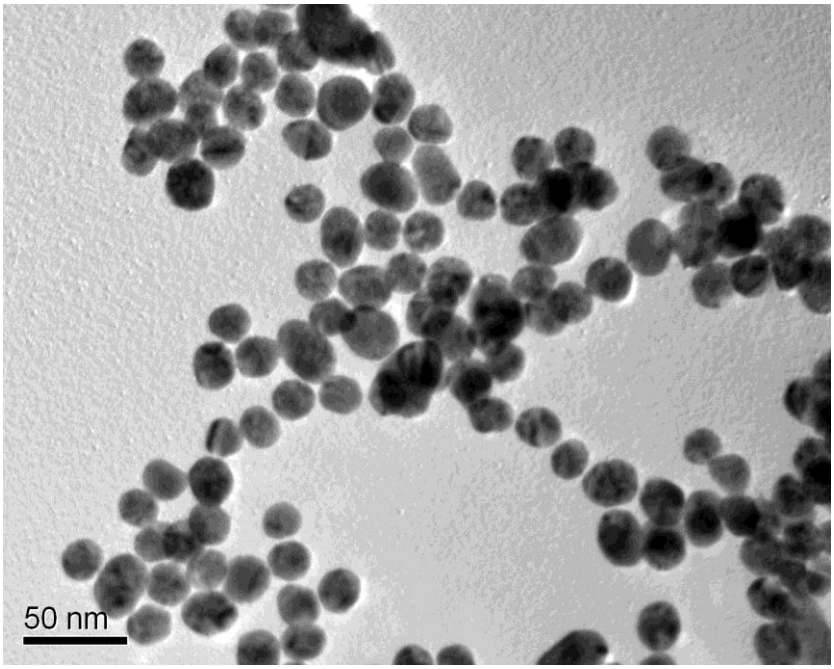


Figure 15: A TEM image on gold nanoparticles

4.2.2 Critical concentration

In the case of concentrated suspensions under the examination of DLS, the detected scattering may result from multiple scattering. Therefore, the size distribution varies considerably for each run of tests. This makes the results inconsistent throughout the experiments. In order to minimize the effect of multiple scattering, the critical concentration of each category of sample must be found prior to investigation of size distribution.

The samples of EggPC, microspheres and gold nanoparticles were prepared in different concentration specified in Figure 16; Figure 17; Figure 18. There are 10 samples for each type of solution. Each sample was tested for 5 runs. The minimum average size and maximum average size were complied. The critical concentration was reached when the gap between minimum and maximum average size was getting consistent; in other words, the average particle size did not vary with the change of concentration.

The critical concentration was found to be 0.1mg/ml, 0.2mg/ml and 0.1mM for EggPC, microspheres and gold nanoparticles respectively. These concentrations will be used for study of interaction in mixtures.

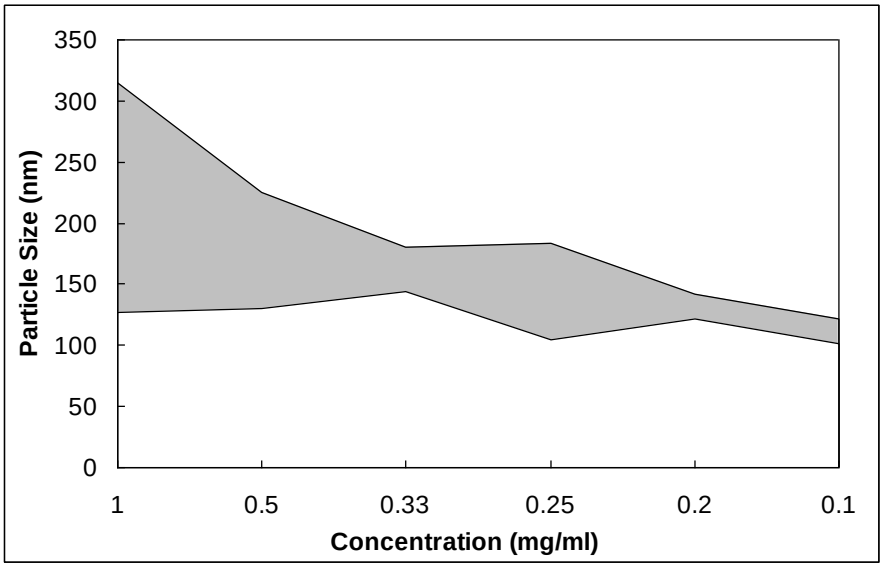


Figure 16: Critical concentration of EggPC in DLS

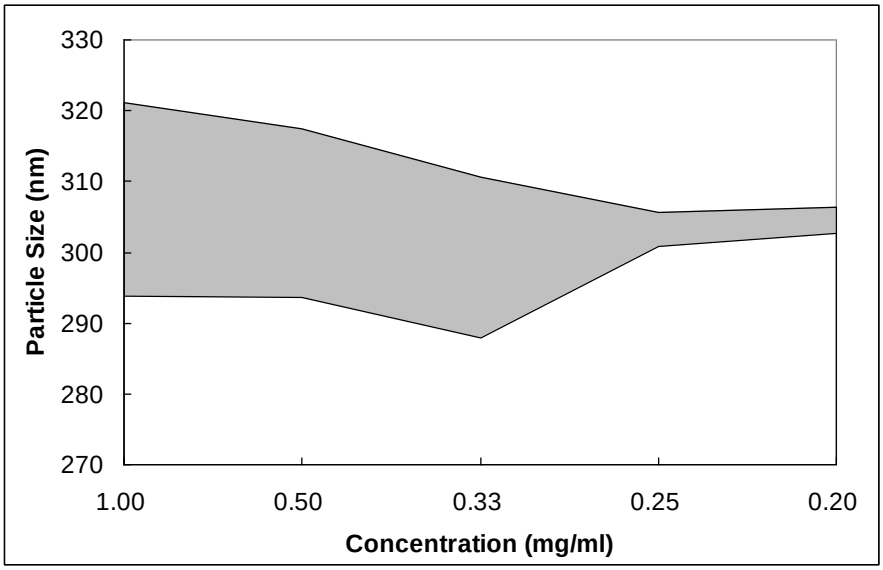


Figure 17: Critical concentration of microspheres in DLS

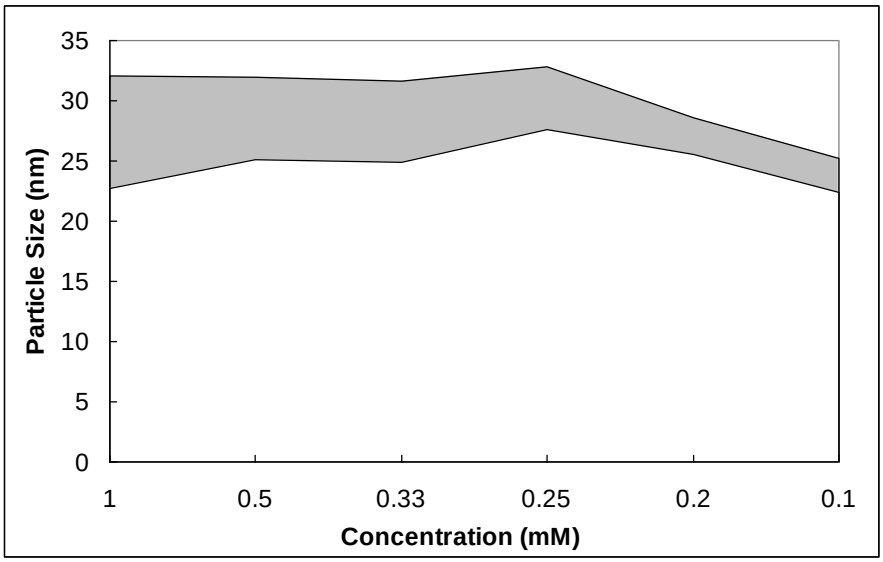
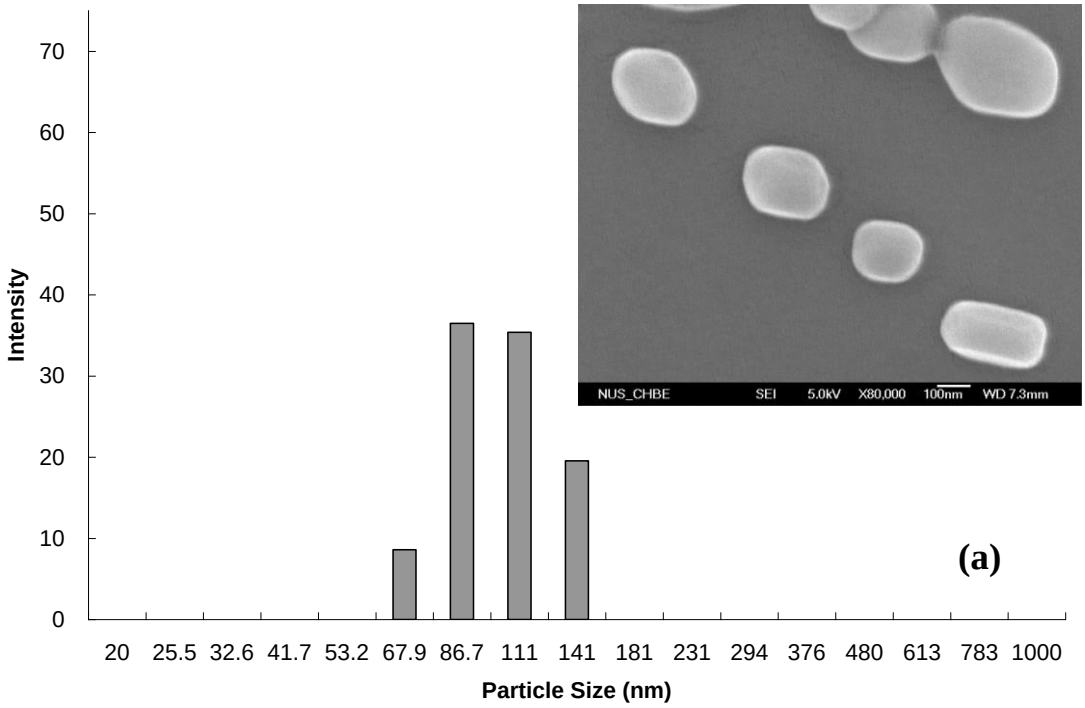
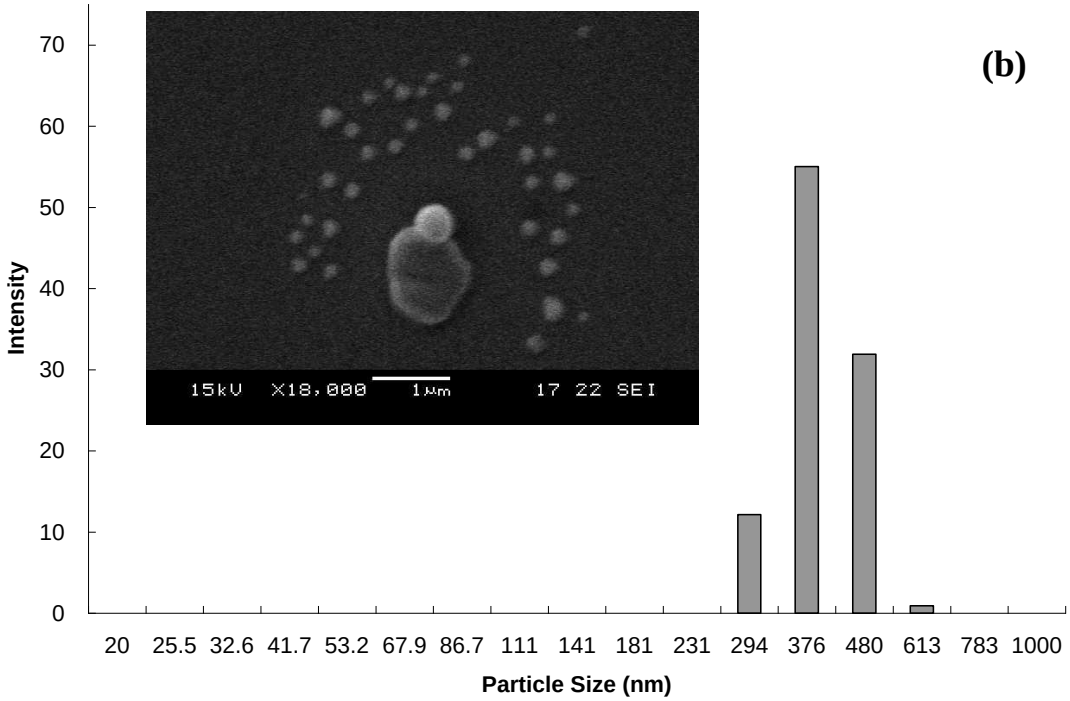


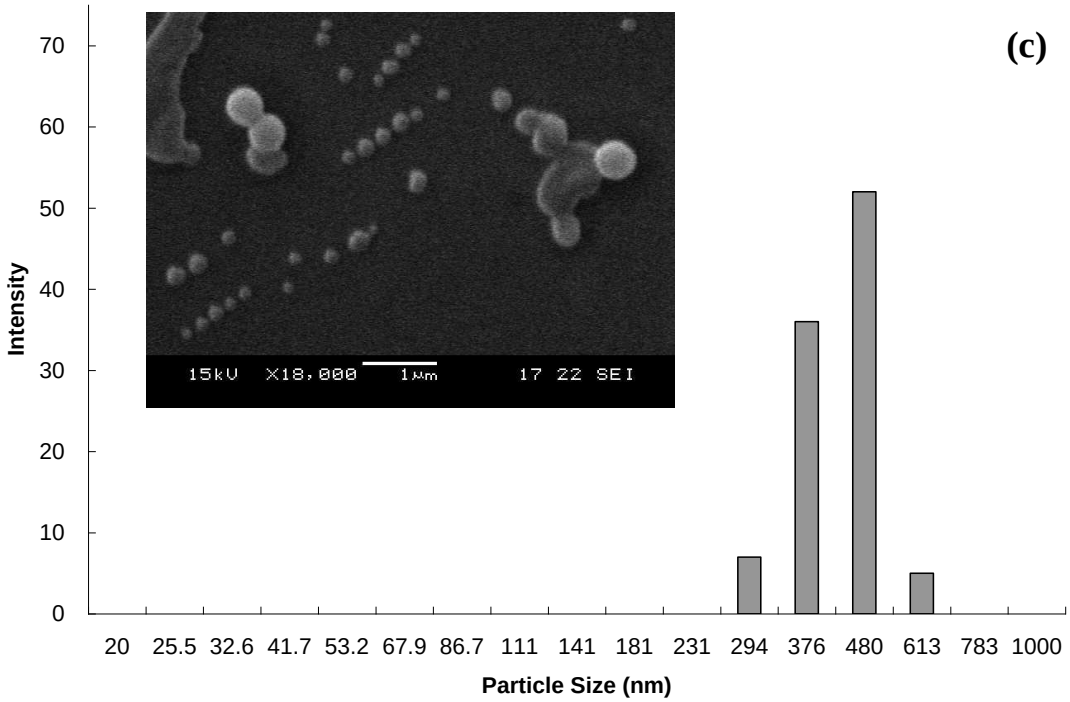
Figure 18: Critical concentration of gold nanoparticles in DLS

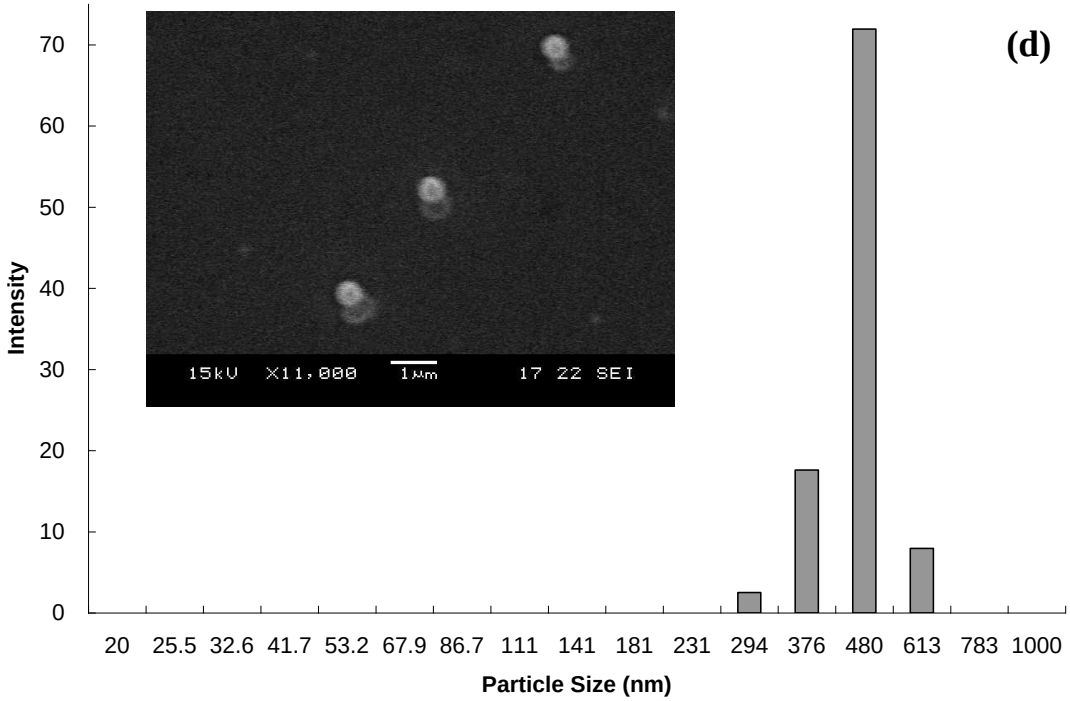
4.2.3 Effect of Microspheres on EggPC

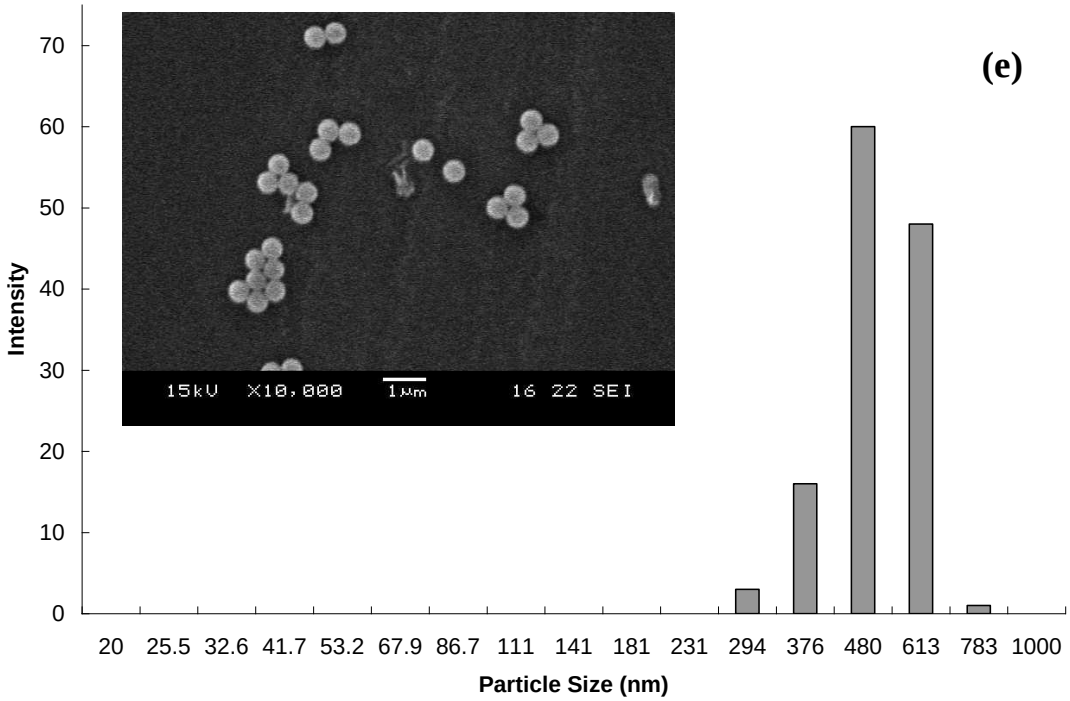
When vesicles are mixed with latex particles at different concentrations, the results are shown as in Figure 19 (a-f).




(b)
Intensity
70
60
50
40
30
20
10
0
15kV X18,000 1μm 17 22 SEI
20 25.5 32.6 41.7 53.2 67.9 86.7 111 141 181 231 294 376 480 613 783 1000
Particle Size (nm)


(c)
Intensity
70
60
50
40
30
20
10
0
15kV X18,000 1μm 17 22 SEI
20 25.5 32.6 41.7 53.2 67.9 86.7 111 141 181 231 294 376 480 613 783 1000
Particle Size (nm)


(d)
Intensity
70
60
50
40
30
20
10
0
15kV X11,000 1μm 17 22 SEI
20 25.5 32.6 41.7 53.2 67.9 86.7 111 141 181 231 294 376 480 613 783 1000
Particle Size (nm)


(e)
Intensity
70
60
50
40
30
20
10
0
15kV X10,000 1μm 16 22 SEI
20 25.5 32.6 41.7 53.2 67.9 86.7 111 141 181 231 294 376 480 613 783 1000
Particle Size (nm)

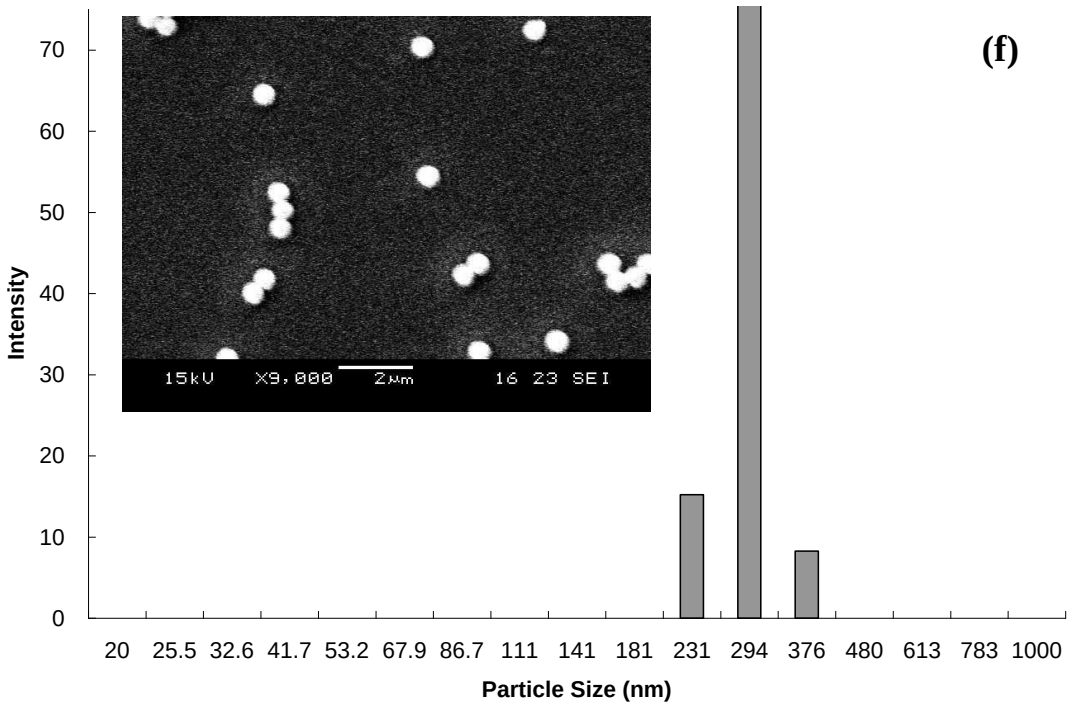


Figure 19: Illustrations of effect of microspheres on EggPC vesicles on following compositions: (a) EggPC: MS (v:v) = 10:0; (b) EggPC: MS (v:v) = 8:2; (c) EggPC: MS (v:v) = 6:4; (d) EggPC: MS (v:v) = 4:6; (e) EggPC: MS (v:v) = 2:8; (f) EggPC: MS (v:v) = 0:10.

In Figure 19(a), since no latex nanoparticles are presented in the mixture, only vesicles with average size of 100nm can be detected. When a small portion of latex nanoparticles were introduced to vesicles solutions in Figure 19(b), the size distribution shifted very obviously from 100nm range to 400nm range. The images showed that some latex particles were attached to large vesicles. As the amount of

latex particles increaseed in vesicle solution in Figure 19(c), the size distribution shifts more to the right, with more bigger aggregated particles present. We can see from the image that more latex particles become attached either with each other or with vesicles. A similar trend was observed in Figure 19(d) with slightly more latex particles than vesicles in the solution. When the majority of a solution was the latex particles (Figure 19(e)), we can only see very little vesicles along with latex particles aggregating together by their own kind. Figure 19(f) shows the result for the case where only latex particles are present in the solution without vesicles.

It can be concluded that the vesicles interact with latex nanoparticles and aggregation happens. They attract each other to form composite particles. No penetration of nanoparticles into vesicles was observed. The amount of vesicles complexed with latex particles increases with increasing vesicles concentration.

4.2.4 Effect of gold nanoparticles on EggPC

Samples of gold nanoparticles mixed with EggPC at compositions described in Table 2: Sample composition of EggPC with gold nanoparticles were examined by DLS.

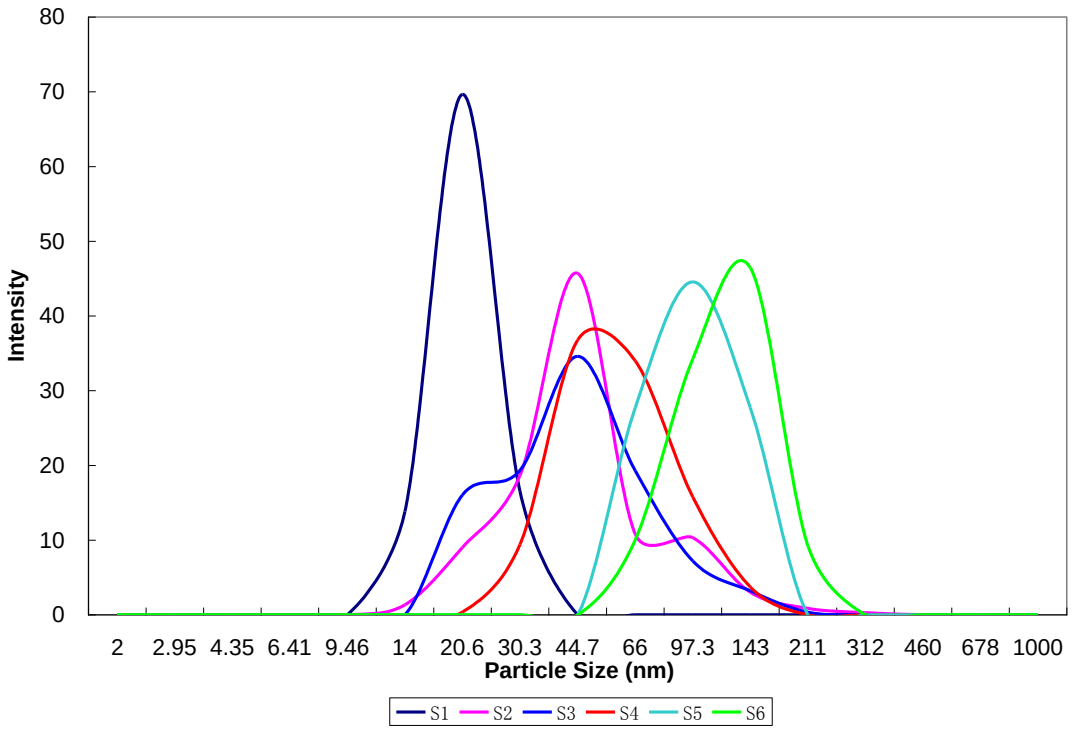


Figure 20: Particle size distribution of EggPC with gold nanoparticle

As the mixture contains less gold nanoparticles, the particle size distribution curve of the mixture will shift to the right with more particles matching the size of EggPC. Since the size of gold particle has been measured to be around 20nm, it is obvious that gold nanoparticles are hardly detectable in S5 by DLS when the EggPC vesicles

considerably outnumber the gold nanoparticles. Therefore, most gold nanoparticles can aggregate with the vesicles. For sample S4, DLS shows a smooth size distribution curve which covers from 20nm to 200nm. This reflects the existence of free nanoparticles and vesicle. This composition was a critical point for the disappearance of free nanoparticles in S5; therefore it was chosen to study the time evolution of size distribution.

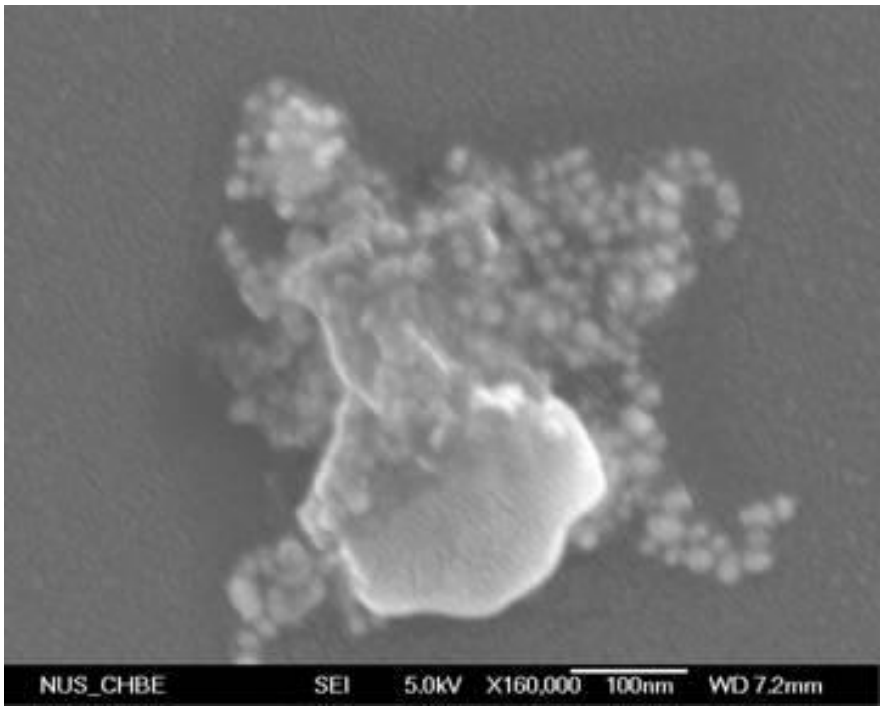


Figure 21: SEM image of complex EggPC vesicles and gold nanoparticles.

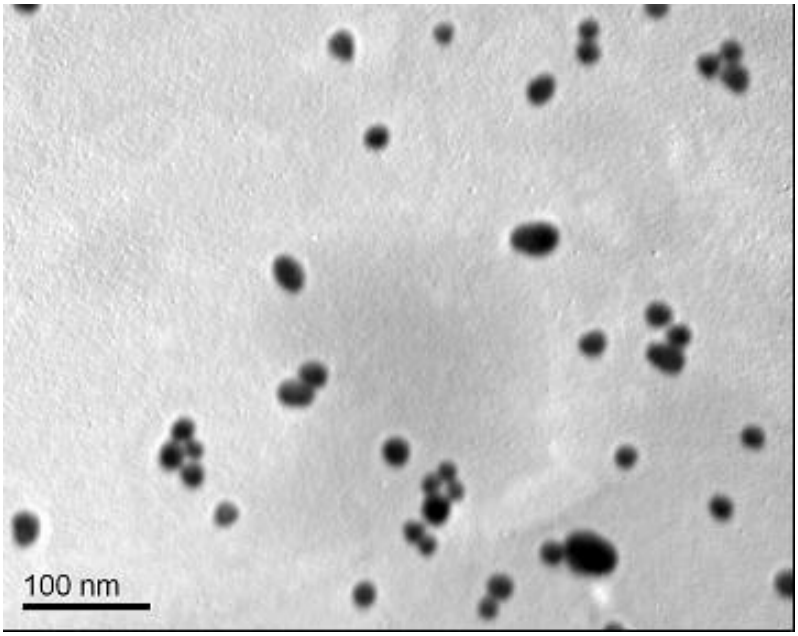


Figure 22: TEM image of complex of EggPC vesicles and gold nanoparticles.

As it can be seen from Figure 20 to Figure 22, the gold nanoparticles in the complex aggregate largely when there are more gold nanoparticles than vesicles in the solution. Vesicles remain intact when interacting with gold nanoparticles in such case. On the other hand, when more vesicles are present in solution than gold nanoparticles, there are no large aggregates of gold nanoparticles. It is observed that gold nanoparticles stay at the boundary surface of vesicles, indicating that gold NPs complex with vesicles. The vesicle aggregation may be an artificial product brought by TEM sample preparation; because the light scattering results suggest that the size distribution of complex only varies slightly with that of the intact vesicles. The small deviation could be due to attachment of gold nanoparticles to vesicles.

Many reports explained vesicle aggregation through classical DLVO theory, in which the electrostatic double-layer repulsion was screened by addition of salt. The distance between two particles is governed by several nonspecific long range and short range interactions. At long range, a repulsive electrostatic force dominates an attractive van der Waals force. The surface charge arising from ion binding was insignificant in monovalent salt solution (NaCl in this case) (Komatsu and Okada, 1995).

Zeta potential values were monitored (

Table 4) to examine the interactions between vesicles and gold nanoparticles.

Table 4: Zeta potential analysis of vesicles with gold nanoparticles.

Sample No.	Zeta Potential	pH
S1	-14.6	5.890
S2	-11.7	5.910
S3	-11.3	5.920
S4	-8.05	6.005
S5	-11.2	6.648
S6	-13.3	7.001

It is noted that the zeta potential of various composition of vesicles and gold nanoparticles solution only differed by at most 6 mV. The slight increase of zeta potential means the electrostatic repulsion only varied little. The small value of zeta potential implies low stability. By taking consideration of only attractive van der Waals and repulsive electrostatic forces, particles should become irreversible adhesive to each other with no water remaining between them in the absence of strong electrostatic repulsion force. This seems not be the case as we observed. Therefore, the interactions between vesicles and gold nanoparticles cannot be simply explained by DLVO theory. Other factors such as hydration repulsion force could also play a part in the process of vesicles or particles aggregation and shall be taken into consideration. The hydration repulsion force acting as strong repulsive force exists in short range below 1-3nm. It has been found to occur in many systems by using the osmotic pressure technique on phosphatidylcholines multilayers. The highly hydrated head group of EggPC ensures that their bilayers do not adhere or aggregate completely and will not fuse, even under the condition of no repulsive electrostatic force present (Komatsu and Okada, 1995).

The time evolution of size distribution for mixtures of vesicles and nanoparticles were investigated. The results are presented in Figure 23 to Figure 26. The graphs are arranged in time sequence from day 1 to day 5 in each case.

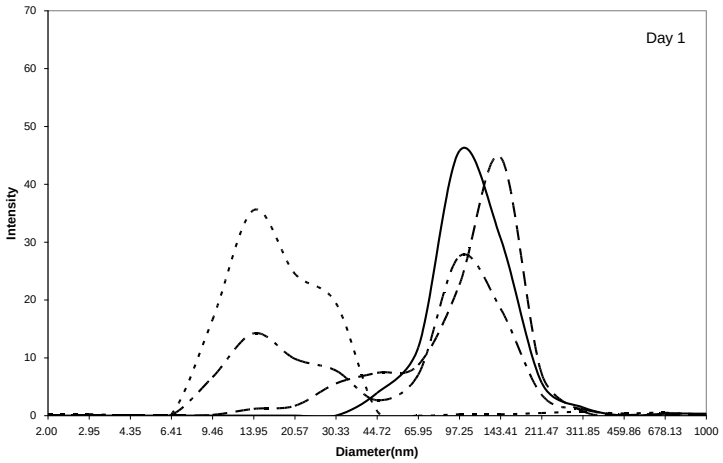
The size distribution of gold nanoparticles does not vary much according to image results. In this case, we make an assumption that the changes of size distribution of gold nanoparticles are negligible in the time factor study.

If EggPC does not interact with gold nanoparticles at all, the size distribution of their mixture is a superposition of individual results. Such superposition curves are also shown in Figure 23 to Figure 26 along actual measurement of size distribution for the mixture. From the comparsion, we can conclude that there are some interactions between EggPC and gold nanoparticles when they are mixed.

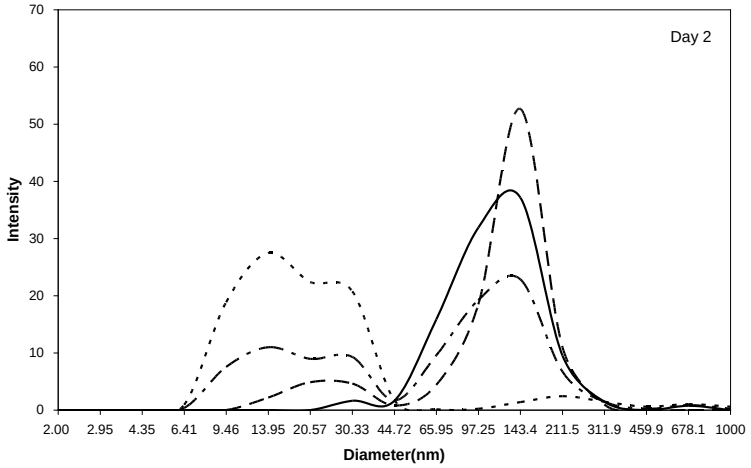
It is obvious that once EggPC mixed with gold nanoparticles in the presence of NaCl, the intensity of particles ranging from 6.41nm-44.72nm diminished dramatically comparing with only gold NPs in the solution. It is a hint that the amount of intact gold NPs decreases. The shifting of the mixture curve shifted more to the right in the size distribution graph means that more bigger particles have been detected in the solution. We regard this as a signal that the attractive force has dominated over other forces to allow gold NPs to attach to EggPC to form bigger complexes. This trend remains unchanged until day 3. From day 4 onwards, the EggPC starts to aggregate among themselves. The size distribution curve starts showing one more peak at larger

size. The liposomal curvature is found to be influential on the molecular packing of the phospholipid molecules in bilayer. In order to dissipate an excess of surface energy originating from the distortion of their molecular packing, lipid rearrange their molecular packing and aggregation occurs to achieve a more stable state. The aggregation of EggPC vesicles indicates that the excess energy from high curvature of vesicles is too weak to overcome the repulsive hydration force within first 3 days. Starting from day 4, significant aggregation takes place. As a result, this behavior of EggPC affects the size distribution of mixture more significantly in a later date as shown in Figure 23 (d) & (e).

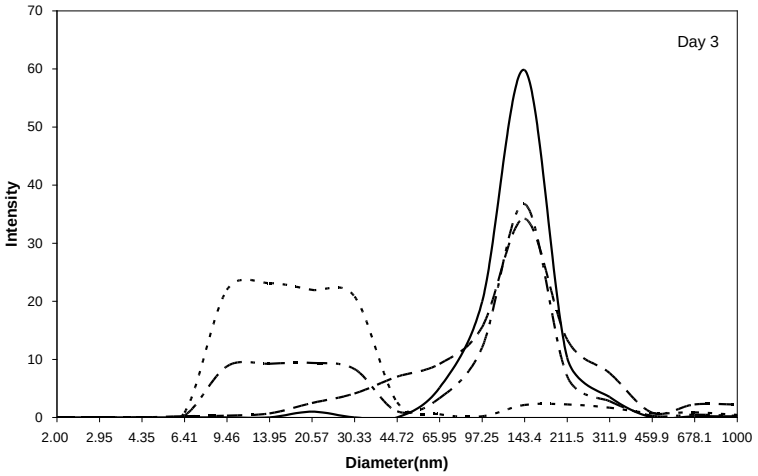
(a)



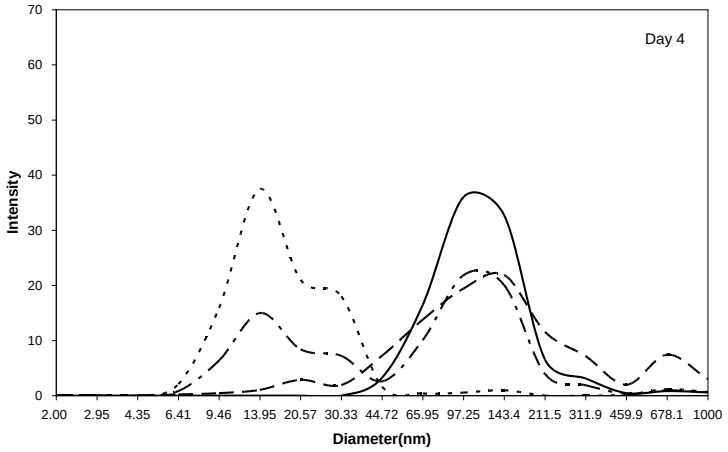
(b)


Day 2
Intensity
70
60
50
40
30
20
10
0
2.00 2.95 4.35 6.41 9.46 13.95 20.57 30.33 44.72 65.95 97.25 143.4 211.5 311.9 459.9 678.1 1000
Diameter(nm)

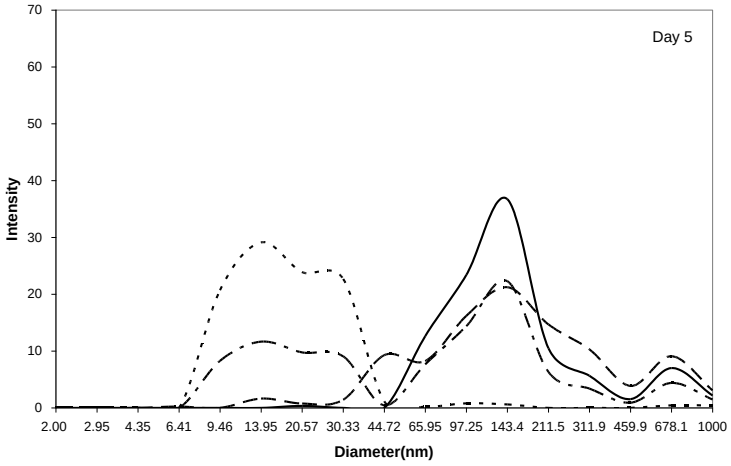
(c)


Day 3
Intensity
70
60
50
40
30
20
10
0
2.00 2.95 4.35 6.41 9.46 13.95 20.57 30.33 44.72 65.95 97.25 143.4 211.5 311.9 459.9 678.1 1000
Diameter(nm)

(d)


Day 4
Intensity
70
60
50
40
30
20
10
0
2.00 2.95 4.35 6.41 9.46 13.95 20.57 30.33 44.72 65.95 97.25 143.4 211.5 311.9 459.9 678.1 1000
Diameter(nm)

(e)



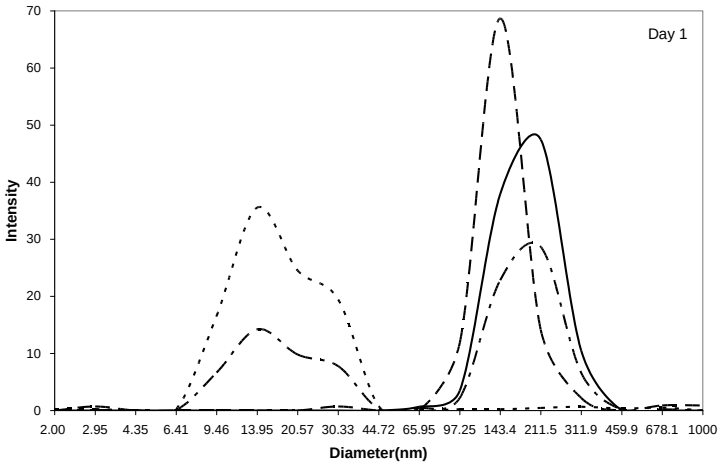
… Gold Nanoparticles ─ Vesicles

- - - Mixture (Measured) –•–Mixture (Superposition)

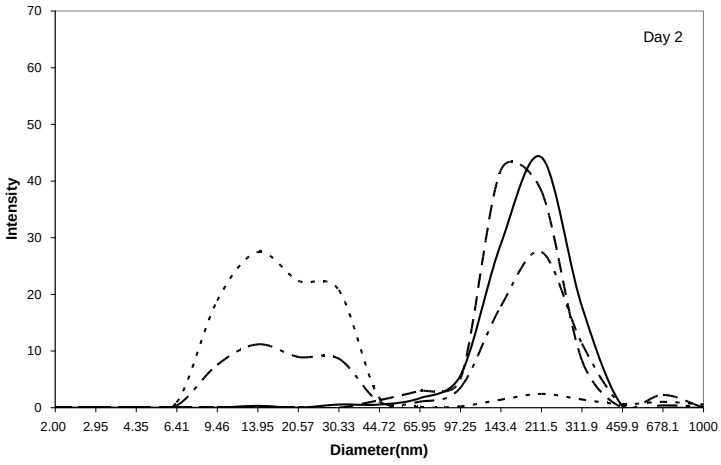
Figure 23(a-e): Vesicles with gold nanoparticles with presence of NaCl in 5 days.

The results for mixtures with presence of $MgCl_2$, $CaCl_2$ and $LaCl_3$ show some difference from NaCl. In day 1, the size distribution of mixture shifted to the left as compared to the case without added NPs. This interesting observation could be due to the attachment of NPs on vesicles, thereby hindering the aggregation of vesicles at early stage. However, such behavior cannot persist in a later time.

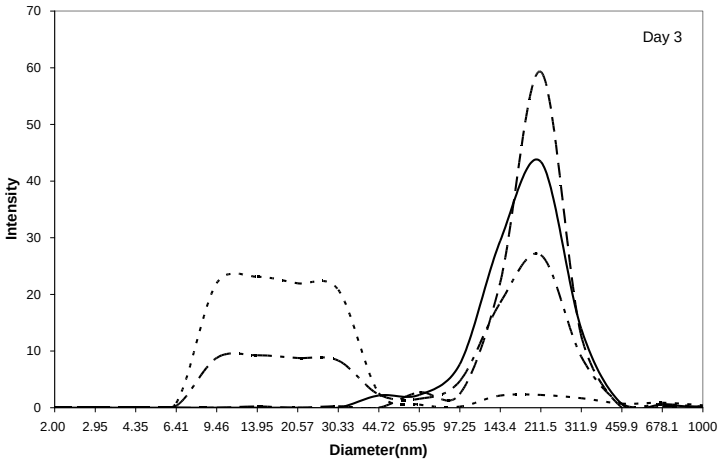
(a)


Day 1
Intensity
70
60
50
40
30
20
10
0
2.00 2.95 4.35 6.41 9.46 13.95 20.57 30.33 44.72 65.95 97.25 143.4 211.5 311.9 459.9 678.1 1000
Diameter(nm)

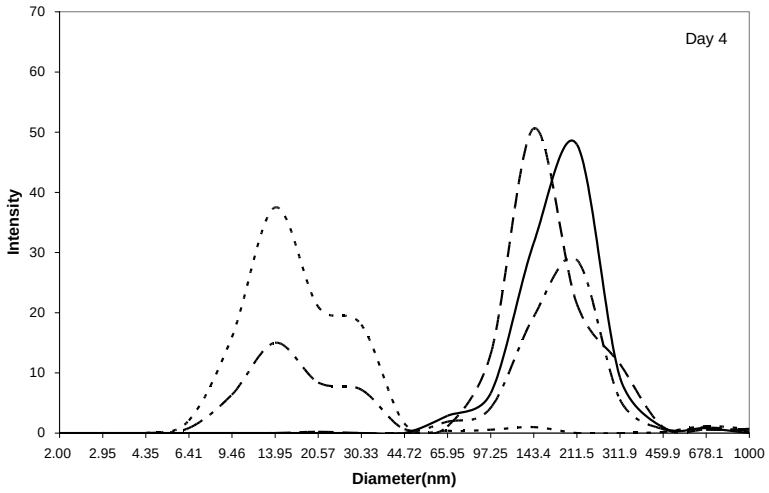
(b)


Day 2
Intensity
70
60
50
40
30
20
10
0
2.00 2.95 4.35 6.41 9.46 13.95 20.57 30.33 44.72 65.95 97.25 143.4 211.5 311.9 459.9 678.1 1000
Diameter(nm)

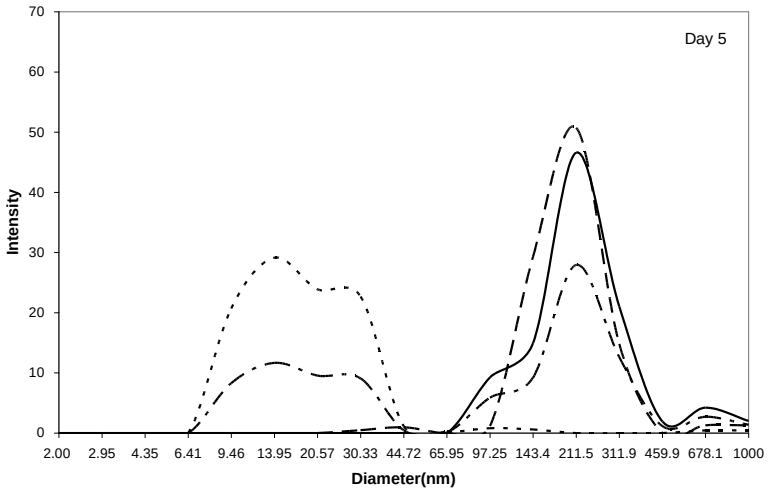
(c)


Day 3
Intensity
70
60
50
40
30
20
10
0
2.00 2.95 4.35 6.41 9.46 13.95 20.57 30.33 44.72 65.95 97.25 143.4 211.5 311.9 459.9 678.1 1000
Diameter(nm)

(d)



(e)

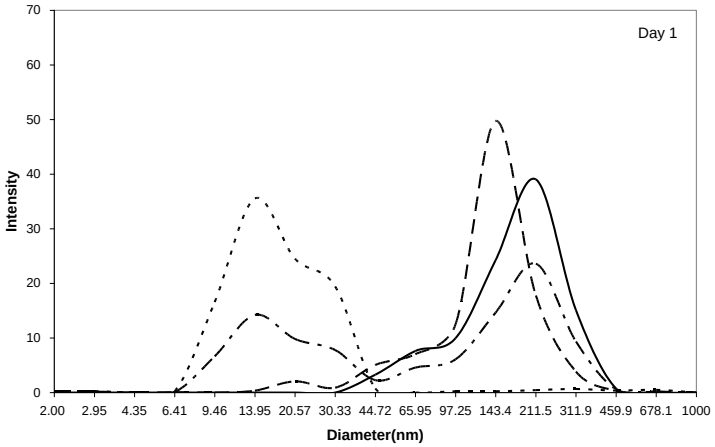


… Gold Nanoparticles ─ Vesicles

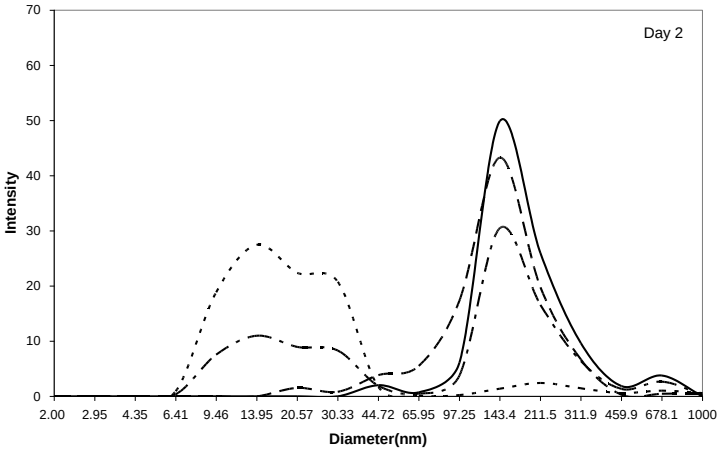
- - - Mixture (Measured) –•–Mixture (Superposition)

Figure 24 (a-e): Vesicles with gold nanoparticles with presence of $MgCl_2$ in 5 days.

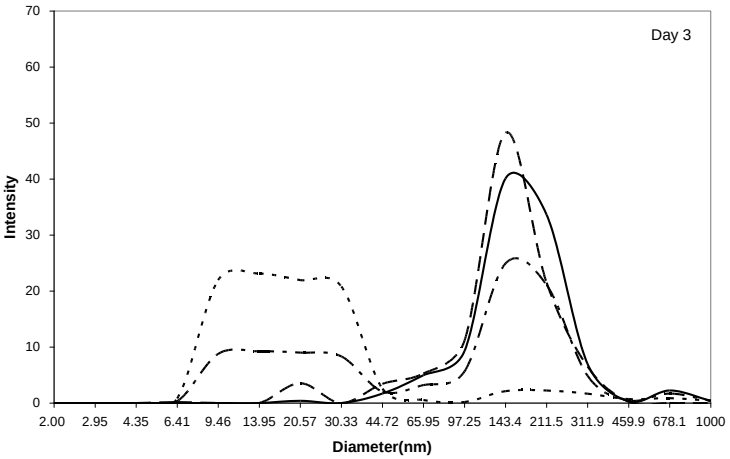
(a)


Day 1
Intensity
70
60
50
40
30
20
10
0
2.00 2.95 4.35 6.41 9.46 13.95 20.57 30.33 44.72 65.95 97.25 143.4 211.5 311.9 459.9 678.1 1000
Diameter(nm)

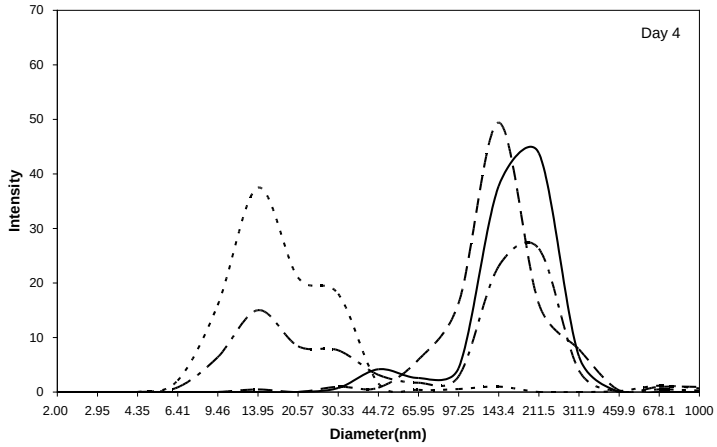
(b)


Day 2
Intensity
70
60
50
40
30
20
10
0
2.00 2.95 4.35 6.41 9.46 13.95 20.57 30.33 44.72 65.95 97.25 143.4 211.5 311.9 459.9 678.1 1000
Diameter(nm)

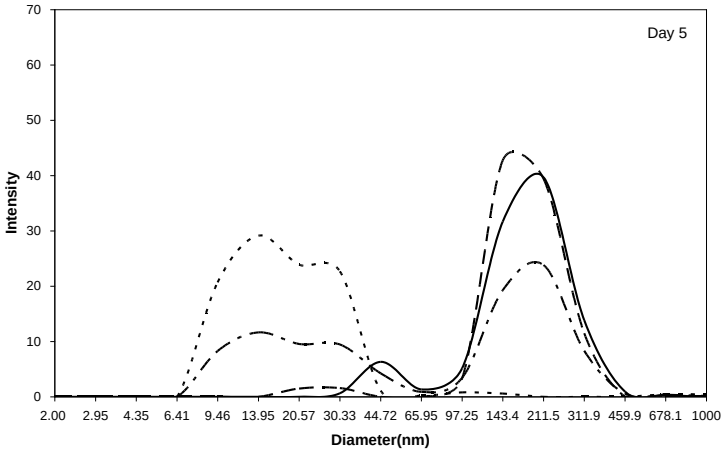
(c)


Day 3
Intensity
70
60
50
40
30
20
10
0
2.00 2.95 4.35 6.41 9.46 13.95 20.57 30.33 44.72 65.95 97.25 143.4 211.5 311.9 459.9 678.1 1000
Diameter(nm)

(d)



(e)

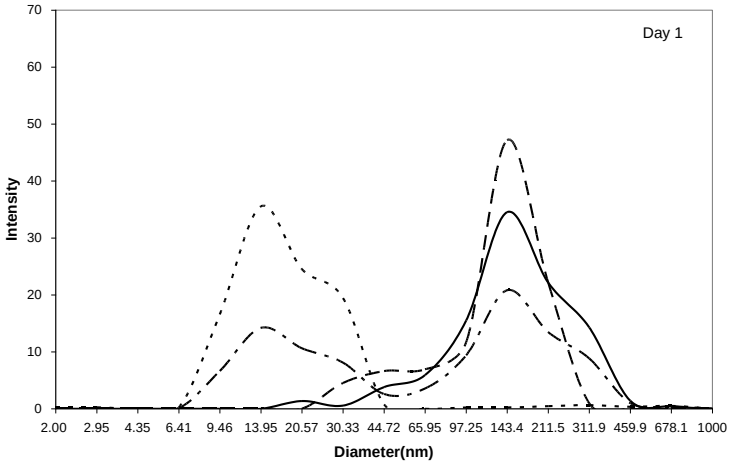


… Gold Nanoparticles ─ Vesicles

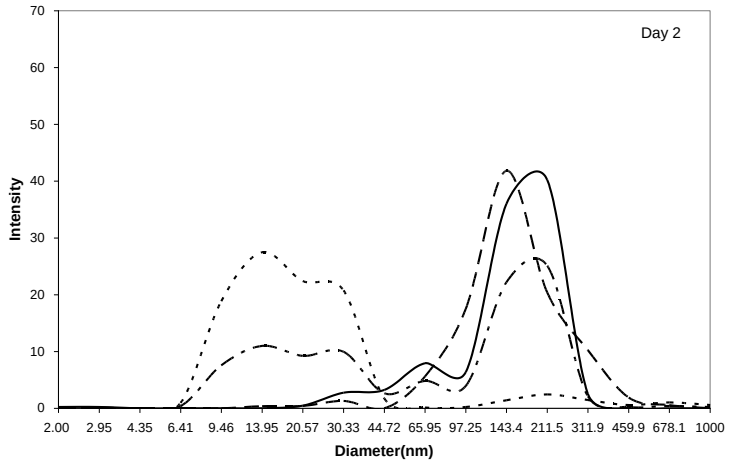
- - - Mixture (Measured) −•−Mixture (Predicted)

Figure 25 (a-e): Vesicles with gold nanoparticles with presence of $CaCl_2$ in 5 days

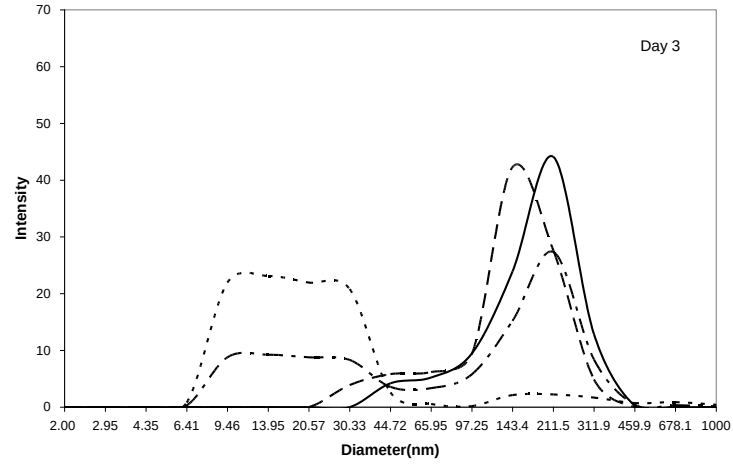
(a)


Day 1
Intensity
70
60
50
40
30
20
10
0
2.00 2.95 4.35 6.41 9.46 13.95 20.57 30.33 44.72 65.95 97.25 143.4 211.5 311.9 459.9 678.1 1000
Diameter(nm)

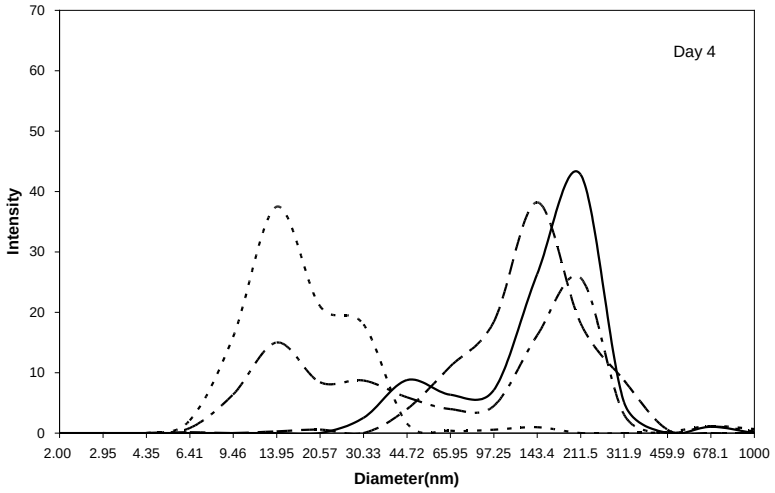
(b)


Day 2
Intensity
70
60
50
40
30
20
10
0
2.00 2.95 4.35 6.41 9.46 13.95 20.57 30.33 44.72 65.95 97.25 143.4 211.5 311.9 459.9 678.1 1000
Diameter(nm)

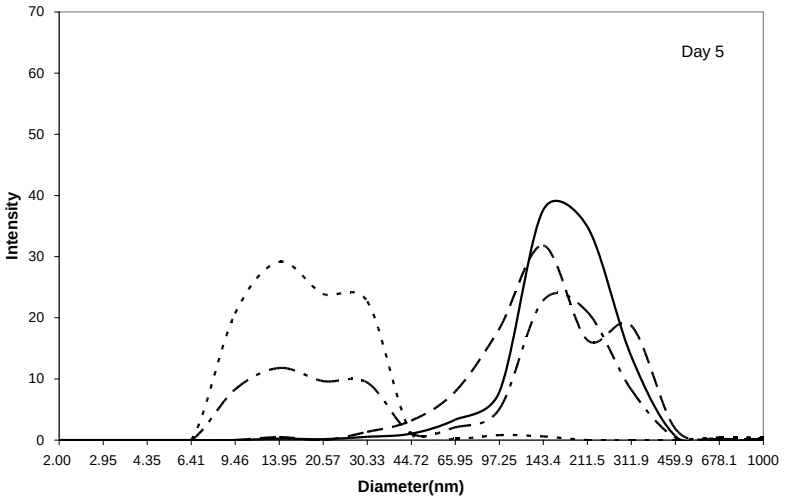
(c)


Day 3
Intensity
70
60
50
40
30
20
10
0
2.00 2.95 4.35 6.41 9.46 13.95 20.57 30.33 44.72 65.95 97.25 143.4 211.5 311.9 459.9 678.1 1000
Diameter(nm)

(d)



(e)



… Gold Nanoparticles ─ Vesicles

- - - Mixture (Measured) −•−Mixture (Superposition)

Figure 26 (a-e): Vesicles with gold nanoparticles with presence of $LaCl_3$ in 5 days

4.3 Rheology of EggPC, Nanoparticles and Polyelectrolytes

The flow properties of NaPSS in H_2O, NaPSS in NaCl, NaPSS in NaCl and EggPC, and also NaPSS in NaCl with EggPC and gold nanoparticles are examined and presented in Figure 27 as a function of NaPSS concentration in solution.

In overview, the viscosity increases with increasing of NaPSS concentration in all cases. In dilute regime, the viscosity increases almost linearly; while it increases in the exponential manner in non-dilute regime. According to a power law, the constants are determined to be:

Table 5: Constants of power law equation determined by rheology experiments.

Samples	Polynomial			Exponential
	A	B	C	
NaPSS + H2O	940.20	-728.86	108.37	$\eta = 6.8527e^{0.1841c}$
NaPSS + NaCl	837.43	-652.61	97.538	$\eta = 6.4502e^{0.1831c}$
NaPSS + EggPC (in NaCl)	742.56	-574.79	85.309	$\eta = 5.998e^{0.1849c}$
NaPSS + EggPC(in NaCl) + Gold nanoparticles	668.25	-518.37	77.148	$\eta = 5.1811e^{0.1851c}$

For solutions with only NaPSS, the viscosity shows strong dependence on its concentration. It is obvious that the viscosity increases with the increasing of its concentration. This is due to the increasing resistance to the flow via increased polyelectrolyte chain entanglement at higher NaPSS concentration.

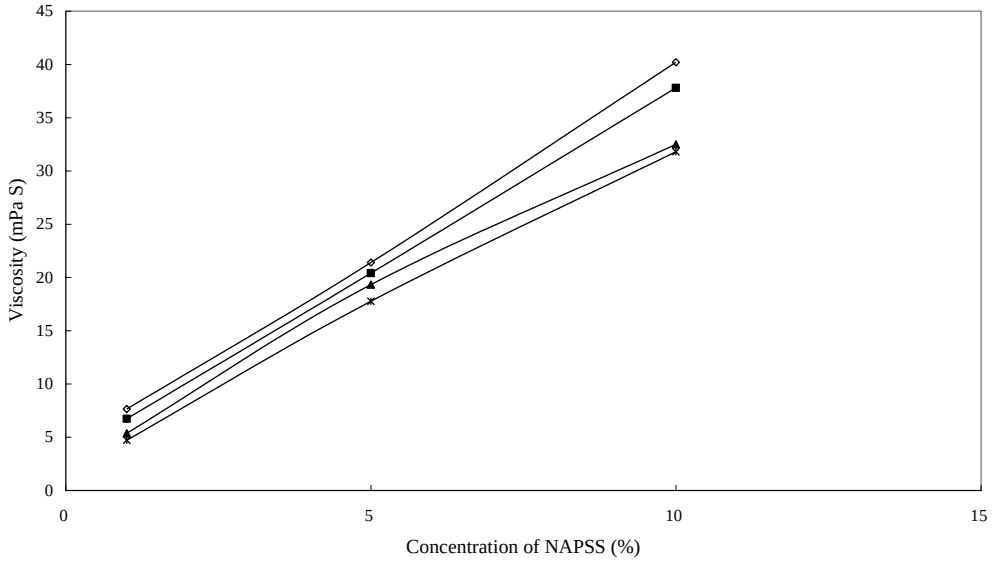
With added salt, the viscosity of NaPSS solution decreases. It is due to conformational changes of polyelectrolyte chains, a typical behavior of polyelectrolyte. With added salt, the electrostatic repulsions between negatively charged chains are guarded. This allows a more compact structure to form. The contraction of polyelectrolyte structure reduces the possibility of entanglement, leading to reduction in effective volume fraction. As a result, there is smaller resistance to the shear flow and the viscosity decreases.

With additional EggPC into the solution, the viscosity decreases further. This observation may be explained by tertiary electroviscous effect. Vesicles are adsorbed to the backbone of polyelectrolytes which causes the conformational changes of the polyelectrolyte.

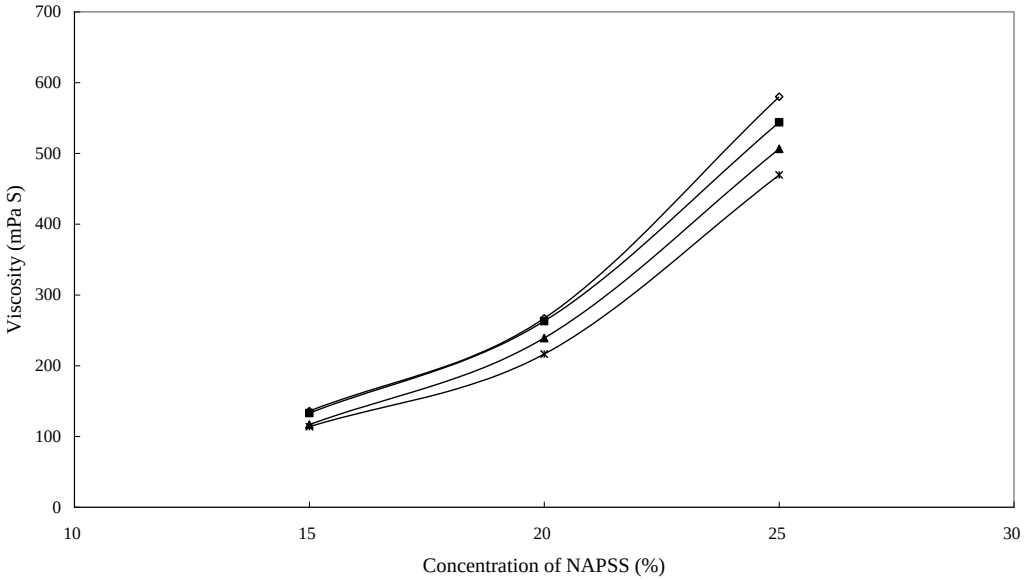
With gold NPs together with EggPC in the NaPSS solution, the viscosity reaches the

lowest among all combinations. Gold NPs are highly asymmetrical particles which can form volume-filling networks even at low concentration. From the results of Section 4.2.4, we know gold NPs are adsorbed to EggPC vesicles efficiently once they are mixed. With vesicles adsorbed to polyelectrolytes, and gold NPs attached to vesicles, the polyelectrolyte conformation is further changed and the mixture appears to flow more freely. This leads to the decrease of viscosity of the system.

(a)



(b)



(c)

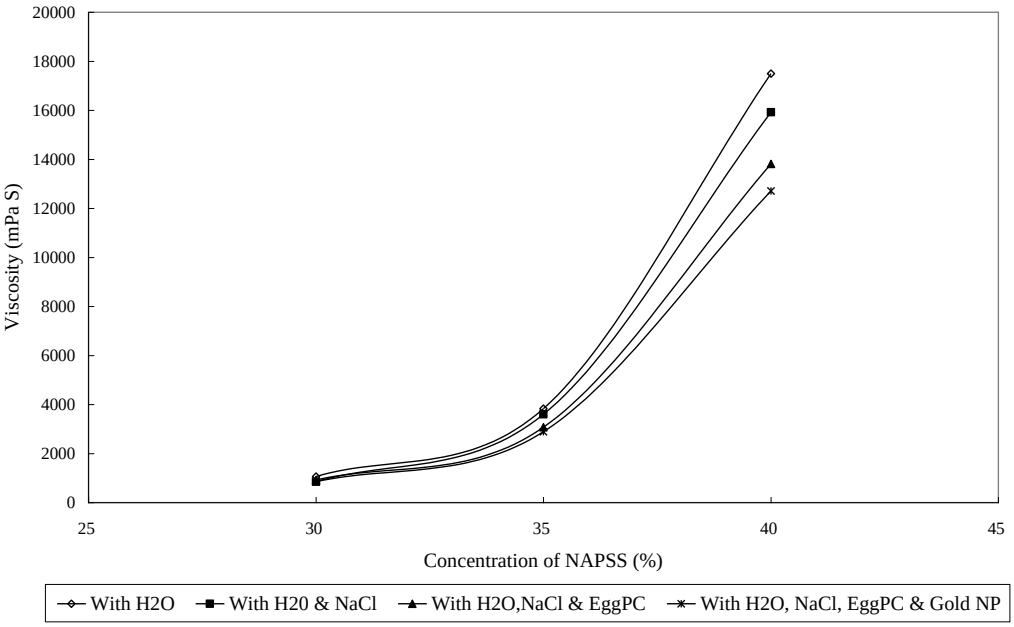


Figure 27: Rhelogy of NAPSS with EggPC and Gold NP at various concentration: (a) Concentration of NaPSS between 1% - 10%; (b) Concentration of NaPSS between 15% - 25%; (c) Concentration of NaPSS between 30% - 40%;

Chapter 5 Conclusion

The unilamellar EggPC vesicles were successfully formed and a series of studies on external factors influencing vesicles size distribution were also conducted and discussed. We found that preparation parameters (sonication, centrifuge speed and extrusion) as well as solution environment (pH and charged ions) have effects on vesicles size.

Vesicles and latex nanoparticles can attract each other to form composite particles. The amount of vesicles adsorbed on latex particles increases with increasing of vesicles concentration. Gold NPs can be associated with vesicles at the surface without disturbing the membrane.

Vesicles are more complex than rigid particles. The interactions between vesicles and nanoparticles cannot be simply explained by the DLVO theory. Other mechanisms such as hydration force should also be taken into account. The ion bindings to the vesicles surface are expected to compete with water for the vesicles surface and destroy the water structure near the vesicle surface. As a result, the hydration repulsion is reduced and the vesicles aggregation is promoted.

Viscosity was particularly chosen to understand the flow properties and particles interactions. Both vesicles and nanoparticles have effects on viscosity. With additional vesicles and nanoparticles to the polyelectrolyte solution, the viscosity of mixture decreases due to conformational changes of polyelectrolyte chains caused by interaction of vesicles and nanoparticles.

Dynamic light scattering (DLS) technique was heavily employed in this project to determine the size and size distribution of particles and vesicles. It is a good method for investigating the dynamics of vesicles formation and their growth quantitatively. However, it is not easy to determine the types of interactions and the exact time of occurrence.

In sample preparation, drying samples were inevitable in some of the EM imaging. The fluid property of the vesicle dispersion changes during drying, and the sample film thickness increases from the center to the periphery. Therefore, more larger particles stay in the peripheral area when the film is thin; whereas smaller particles stay near the center area. This reflects a disadvantage of conventional dying technique. The true size distribution cannot be accurately determined by randomly choosing a small region for analysis.

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