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Effect of lipid composition on the physical properties of liposomes: a light scattering study

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EFFECT OF LIPID COMPOSITION ON THE PHYSICAL PROPERTIES OF LIPOSOMES: A LIGHT SCATTERING STUDY

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ABSTRACT

The aim of this study was to investigate the effect of lipid composition on the physical properties of liposomes. Eight different types of liposomes were prepared for this study. Liposomes consisting of phosphatidylcholine (PC) and cholesterol were prepared in three different compositions 80/20, 50/50 and 20/80 respectively. Similarly liposomes consisting of PC and negatively charged phosphatidylserine (PS) were also prepared in three different compositions 80/20, 50/50 and 20/80 respectively. Two liposomes with the compositions 2:2:1:1 (cholesterol:PC:phosphatidylglycerol (PG): phosphatidylethanolamine (PE)) which resembles the myelin membrane and 10:5:7.5:16 (PC:PE:PS:cholesterol) which mimics rat's neural membranes were also prepared.

The liposomes were prepared by freeze-drying method. The experiments were carried out at three pHs 4, 7 and 9 and at three different temperatures 15^{o}C, 25^{o}C and 45^{o}C. The ionic strength of the buffer used was 40mM. The size and polydispersity indices were measured by dynamic light scattering. The size of the liposomes obtained was small and reasonably monodispersed. Static light scattering experiments were performed using a Brookhaven temperature controlled goniometer equipped with a 2W argon ion laser. The data was analyzed to obtain second virial coefficients. The second virial coefficients obtained were positive for all liposomes at all pHs and all temperatures which indicates that the interactions are repulsive under these conditions. Zeta potentials of liposomes were determined using zetasizer nano ZS90. The zeta potentials obtained were all highly negative at above pHs and temperatures indicating that the liposomes were stable. The structure of the liposomes was analyzed using static light scattering by using Guinier and Kratky plots.

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1. INTRODUCTION

Liposomes are spherical vesicles in which an aqueous volume is enclosed by a lipid bilayer membrane. Although the main components of liposomes are phospholipids some contain cholesterol. Phospholipids are the major structural components of biological membranes such as the cell membrane; therefore, liposomes have been used as biological membrane mimics. Liposomes can be negatively or positively charged and they may have proteins inserted into the membrane. The fluidity of the membrane may be reduced by replacing the phospholipid by cholesterol. They range in size from about 100nm to several microns in diameter. Liposomes can be classified based on structural parameters as:

1. Multilamellar Vesicles or MLV (> 500 nm)
2. Small Unilamellar Vesicles or SUV (20-100 nm)
3. Large Unilamellar Vesicles or LUV (> 100 nm)
4. Oligolamellar Vesicles (100-1000 nm)

1.1. STABILITY OF LIPOSOMES

Liposomes may undergo chemical degradation or physical changes during preparation or upon storage. The lipids used in the preparation of liposomes are unsaturated and hence susceptible to oxidation. The higher the level of unsaturation, the more susceptible they are. Thus, liposomes should be prepared in the absence of oxygen and procedures which involve high temperatures should be avoided. They should be stored at low temperatures and in the dark.

1.2. USES OF LIPOSOMES

Liposomes have been used in separations (van Zanten *et al.*, 1992), as carriers in the cosmetic industry (Junginger *et al.*, 1991) and in drug delivery, and as model biomembranes. Liposomes have been also used as a model for human skin in permeation enhancement studies (El Maghraby *et al.*, 2008). One of their major applications is in drug delivery (Drabu *et al.*, 2010) in part because of their versatility since they can encapsulate both hydrophobic and hydrophilic drugs. For example, liposomes have been

used as carriers of proteins and peptide antigens (Busquets *et al.*, 2003). Liposome formulated drugs are used to treat cancer and local fungal infections because they are biologically inert, biocompatible and do not seem to cause any toxic side effects (Schwendener, 2007). Liposomes are widely used as biomembrane models (Bangham *et al.*, 1965, Chan and Boxer, 2007, Chaaterjee and Agarwal, 1988). Papahadjopoulos *et al.* (1973) used liposomes as models for biological membranes and studied their properties and interactions with cholesterol and proteins. Sessa *et al.* (1968) worked on phospholipid spherules (liposomes) as a model for biological membranes. Liposomes are used in cell physiology to study the functions of proteins in natural membranes (Malathi *et al.*, 1983). They are commonly used to test antimicrobial properties of peptides. Epand *et al.* (2003) evaluated the relationship between antimicrobial peptides and membrane bilayers, as well as the biological activities of the peptides. Busquets *et al.* (2003) used liposomes to study the interactions between peptides derived from viral hepatitis proteins with cell membranes. Liposomes have also been used to study the effect of biomembranes on the formation of proteinaceous fibrils. For example, Zhao *et al.* (2004) showed that membranes containing phosphatidylserine (PS) induce a rapid formation of fibrils by a variety of proteins such as lysozyme, insulin, glyceraldehyde-3-phosphate dehydrogenase, myoglobin etc. They suggested that phosphatidylserine as well as other acidic phospholipids could provide the physiological low-pH environment natural in cellular membranes, enhancing protein fibrillation in vivo. Recently, liposomes have been used to study the fibrillation of tailored peptides near solid surfaces. Naik *et al.* (2011) found that the presence of interfaces affects the kinetics of fibril formation. They found that although surfaces seem to act as nucleation sites, some inhibit fibril formation.

1.3. PREPARATION OF LIPOSOMES

Liposomes may be prepared by a variety of methods including: 1) hand shaken, 2) sonication, 3) freeze drying, 4) reverse phase evaporation, etc. All these methods of preparing liposomes have the following common steps: a) drying down lipids from an organic solvent, b) dispersion of the lipid in an aqueous media and c) purification of the resultant liposomes.

The preparation of hand-shaken vesicles starts with the coating of the bottom of a round bottomed flask with a lipid dissolved in an organic solvent. The flask is then attached to a rotary evaporator and the organic solvent is evaporated. The dried lipids are then hydrated and hand-shaken. The lipids form bubbles on the surface of the flask. The shaking makes the bubbles pop off and close to form spherical vesicles. The vesicles formed are MLV because not much energy has been added to help them rearrange to a more stable form.

Hand-shaken vesicles may be sonicated to form more stables structures. For example, if hand-shaken vesicles are sonicated for around 30 minutes the extra energy allows the lipids to rearrange into a more stable SUV type of structure.

Drugs may be entrapped into vesicles prepared by freeze drying. In this method, liposomes are made by adding the solution with the material to be entrapped. The resulting solution is freeze-dried and rehydrated. The material to be entrapped is in close contact with the lipids, so upon reforming more material is captured. This method is only important for entrapment.

Formation of liposomes by reverse-phase evaporation consists of the addition of an aqueous phase to a lipid dissolved in an organic solvent. The dispersion is sonicated and the organic phase is slowly evaporated. The organic solvent and aqueous phase will not actually mix, but the aqueous phase will form small pockets within the organic solvent. Reverse micelles will form around the aqueous phase. When evaporation of organic phase reaches a critical point, some of the micelles will dissolve and that lipid will reassemble around nearby micelles making liposomes. Trace amounts of organic phase may remain in the preparation when this method is used.

Vesicles can also be made by freeze drying. In this method, a lipid is dissolved in tertbutyl alcohol and mixed with a sucrose solution in a single phase. The dispersion is freeze-dried and rehydrated This method was used because it requires fewer steps and the liposomes produced are fairly mono-disperse and small in size, which are desired for these studies. Also it is known that freeze-drying increases the stability of liposomes (Crowe *et al.,* 1986).

The size, molecular weight and osmotic second virial coefficients of liposomes can be measured by light scattering methods. Dynamic light scattering is widely used to

determine the size of liposome dispersions. The second virial coefficient, which gives information about the interactions between liposomes, can be determined by both dynamic and static light scattering but the latter is the preferred method. There are a few second virial coefficient studies of liposomes. Bordi *et al.* (2007) used both dynamic and static light scattering to study interactions in polyelectrolyte-liposome clusters. Lin *et al.* (2007) measured the second virial coefficient of liposomes by isothermal titration calorimetry and qualitatively described the repulsive forces of different liposomes suspensions. Riske *et al.* (1997) studied the temperature dependence of the intensity of the scattered light by aqueous dispersions of the anionic lipid DMPG (dimyristol phosphatidylglycerol) at different ionic strengths. They found that the second virial coefficient was negative at 13 ^{o}C but it was positive at 22 and 40 ^{o}C. The temperature of the main transition (T_m ~ 22.5 ^{o}C) was found to increase with increasing ionic strength. For low ionic strength, a DMPG second transition temperature (~ 35 ^{o}C) was found. They found that below T_m the liposomes tend to aggregate and show a negative second virial coefficient. At the main phase transition, the second virial coefficient becomes positive. They claim that the increased ionization at T_m could be related to the melting of the hydrocarbon chain. Stigter *et al.* (1992) studied the interactions among the phoshpholipid head groups in monolayers and bilayers. They presented experimental pressure-area isotherms for different phospholipids at the oil/water interface covering a broad range of surface densities. They showed that at low surface densities, phosphatidylcholine (PC) head-groups have strong repulsions (which increase with temperature) whereas phosphatidylethanolamine (PE) interactions are much weaker and have no significant temperature dependence.

Measurements of zeta potential of liposomes have been made to determine their stability. Carrion *et al.* (1994) investigated the effect of incorporating phosphatic acid on the zeta potential of PC liposomes in the presence of neutral electrolytes. Experiments were done at pH 7.2 (PC liposomes are negatively charged at this pH) and 25 ^{o}C. They showed that increasing the concentration of phosphatic acid in lipid bilayers resulted in higher zeta potentials and physical stability of the liposomes. McLaughlin *et al.* (1983) examined the effect of dimethonium on the zeta potential of multilamellar vesicles formed from the negatively charged lipid phosphatidylserine and from 5:1

phosphatidylcholine/phosphatidylserine mixtures in solutions containing 0.1, 0.01 and 0.001 M sodium, cesium, or tetramethylammonium chloride.

There are a few studies aimed at understanding the effect of cholesterol on the stability of vesicles. Zeta potential measurements of PC vesicles with different amounts of cholesterol incorporated were done by Liu *et al.* (2000) to study inter-liposome forces. They found that the PC vesicles made of egg-PC are negatively charged at pH 7.4 indicating that there is a weak electrostatic repulsive force between the PC vesicles at that pH. Their experiment also demonstrated that the incorporation of cholesterol into the system can elevate the negative zeta potential. They speculated that the cholesterol incorporated into the bilayer reduces the surface binding affinity among the cations in the buffer solution and the bilayer. They also observed that the zeta potential of PC vesicles with or without cholesterol is higher at 310 than at 298 K. It is possible that some hydrophilic headgroups become more hydrophobic at higher temperatures increasing their ability to dehydrate and to bind with the cation in the buffer. Wiedmer *et al.* (2002) studied the effects of pH and various buffers on the electrophoretic mobilities and sizes of the zwitterionic liposomes with and without cholesterol. They observed that the effect of pH on the sizes of liposomes (POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) or POPC/cholesterol) in the same buffer was negligible. When results obtained using different buffers were compared, they observed differences in the sizes of the liposomes. The smallest sizes were observed in phosphate buffer at pH 7.4 while the larger particles were observed in 3-((1,1-Dimethyl-2-hydroxyethyl)amino)-2-hydroxypropanesulfonic acid (AMPSO) (pH 8.25), 3-[N,N-bis(2-Hydroxyethyl)amino]-2-hydroxypropanesulfonic Acid (DIPSO) (pH 7.4), and N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pHs 7.4 and 8.25) buffers. In those buffers, the cholesterol containing liposomes were larger than the ones without cholesterol. They also observed a change in electrophoretic mobilities of liposomes with the change in buffer. The values reported were slightly higher in AMPSO buffer at pH 8.25. Arriaga *et al.* (2009) studied the stiffening effects of cholesterol on large unilamellar 1-palmitoyl-2-oleyl-sn-glycero-phosphatidylcholine vesicles by means of neutron spin echo in combination with dynamic light scattering. The combined analysis of neutron spin echo and dynamic light scattering data allowed them to calculate the bending elastic constant (kappa) of the

vesicle bilayer. A stiffening effect, a manifestation of an increase in kappa with increasing cholesterol molar ratio, was found from these measurements. Sulkowski *et al.* (2005) used electron paramagnetic resonance **(**EPR) and nuclear magnetic resonance **(**NMR) spectroscopy to determine the influence of temperature (297-340 K), cholesterol and pH on L- α -dipalmitoyl-phosphatidylcholine (DPPC) liposome membranes. They found that liposome membranes prepared in acid environment were stiffer below the transition temperature (~314 K), and exhibited greater fluidity above this temperature. They also observed that the use of more than 0.5 mol of cholesterol per 4 mol of DPPC for the preparation of liposomes did not significantly affect the fluidity of the obtained liposome membranes. Liu *et al.* (2003) prepared liposomes from mixtures of egg-PC, cholesterols and distearoyl-phosphatidyl-ethanolamine covalently attached poly(ethylene glycol) (PEG, mol. wt. 2000) (DSPE-PEG2000). They studied the effect of PEG2000-grafted lipids on the surface properties of egg-PC liposomal bilayer membranes through zeta potential and interaction potential measurements using microcalorimetry. They observed that the absolute value of the zeta potential of PEG2000-grafted PC liposomes decreased from -19 to -8 mV when increasing DSPE-PEG2000 from 0 to 7 mol fraction, and the repulsive interaction potential of PEG2000-grafted PC liposomes decreased compared with that of liposomes not grafted to PEG.

In this study, the surface chemistry of the liposomes was varied by using different phospholipids and by replacing some of the phospholipids with various amounts of cholesterol. The hypothesis was that the osmotic second virial coefficients will capture these differences in surface chemistry. It is known that cholesterol affects the fluidity of membranes but, of course, it would highly speculative to expect that second virial coefficients would capture that. It has been suggested by others that the presence of cholesterol changes the surface charge and therefore that should be captured by second virial coefficient measurements. Experiments were performed at different pHs and temperatures to produce a wide enough data base to reach meaningful conclusions. The second virial coefficients were measured using static light scattering and the liposomes were further characterized by measuring their sizes by dynamic light scattering and their surface charges by zeta potential measurements (Edwards, *et al.*, 2006, van Zanten, 1994, van Zanten and Monbouquette, 1991, van Zanten and Monbouquette, 1994).

Experiments were also done using polystyrene latexes having different surface chemistries. This was done to gain some general understanding of the effect of surface chemistry on the second virial coefficient without the additional complexities that are added by using liposomes. The results of these experiments are included in Appendix A with a brief explanation.

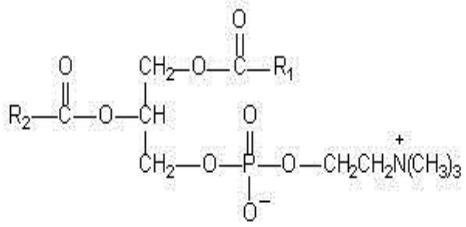
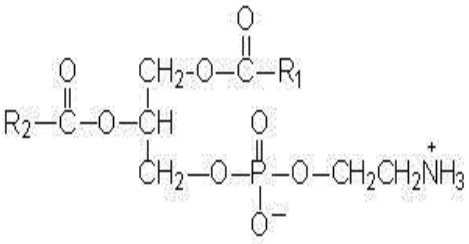
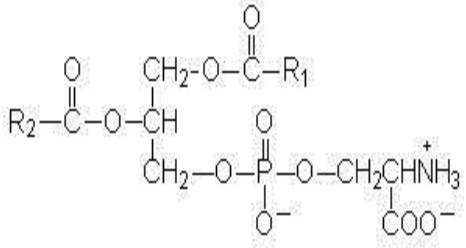
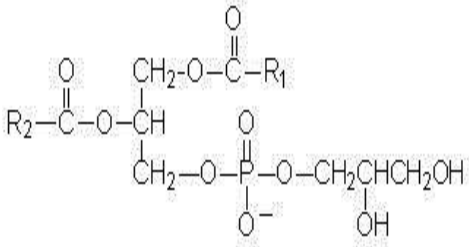
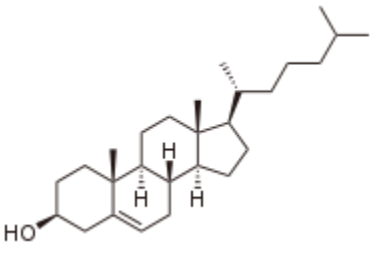
This project is a small portion of a larger project aimed at the understanding of the effect of solid liquid interfaces on the formation of amyloid fibrils by tailored peptides. The tailored peptides consist, for the most part, of diblock peptides having a positively charge block (lysine) and a neutral block (leucine and isoleucine). There was an interest in understanding how these peptides interact in solution. Therefore, a few experiments were done adsorbing these peptides on polystyrene latex and measuring the second virial coefficient of the resulting modified latex. The results of this work are included in Appendix B together with a brief explanation.

The following liposomes were prepared:

1) Phosphatidylcholine/Cholesterol (80%/20%; 50%/50%; 20%/80%). The replacement of a phospholipid by cholesterol decreases the fluidity of the membrane and decreases the surface charge.
2) PC / Phosphatidylserine (80%/20%; 50%/50%; 20%/80%). PS is an acidic phospholipid and has a negative charge at pH 7. As the PS/PC ratio increases, the electrostatic repulsive forces may increase with increase in pH due to a higher negative charge.
3) 2:2:1:1 Cholesterol/PC/Phosphatidylglycerol (PG)/Phosphatidylethanolamine. The composition of these liposomes is similar to that of the myelin membrane (the insulating material in the brain).
4) 10:5:7.5:16 PC/PE/Phosphatidylserine/Cholesterol. The composition of these liposomes is similar to that of a rat neural membrane.

The chemical structure and some of the characteristics of the lipids used in this work are presented in Table 1.1.

Table 1.1. Lipids: Structure and relevant properties

Name	Structure	Comments
Phosphatidylcholine		This class of phospholipids is also called the lecithins. At physiological pH, phosphatidylcholines are neutral zwitterions.
Phosphatidylethanolamine		These molecules are neutral zwitterions at physiological pH.
Phosphatidylserine		Phosphatidylserines will carry a net charge of –1 at physiological pH.
Phosphatidylglycerol		Phosphatidylglycerols exhibit a net charge of –1 at physiological pH.
Cholesterol		Cholesterol is used to modulate the fluidity of membranes by interacting with the lipid components.

Second virial coefficients were measured by static light scattering. In this method a beam of polarized light (laser) is focused on a solution and the scattered light from the particles in the solution is detected at various angles by a photodiode detector. The angular dependent intensity of the scattered light measured is proportional to the concentration and molar mass of the particles in solution. The data is analyzed using Eq. 1 first developed by Zimm (1948).

$$\frac{Kc}{R(\theta)} = \frac{1}{M_w}\left(1 + 16n_o\pi^2 R_g^2 sin^2\left(\frac{\theta}{2}\right)\left(\frac{1}{3\lambda_o^2}\right)\right) + 2cB_{22} \quad (1)$$

Where K is an optical constant, c is the concentration, $R(_\theta)$ is the Rayleigh ratio, θ is the scattering angle, λ_o is the wavelength of the incident radiation in the empty space, R_g is the radius of gyration, M_w is the weight-average molecular weight, n_o is the refractive index of the solvent, and B_{22} is the second virial coefficient. The weight average molecular weight and second virial coefficient can be determined if the intensity of scattered light from several dispersions of different concentrations is measured and the data is analyzed according to Zimm (1948).

2. MATERIALS AND METHODS

2.1. MATERIALS

L-α- PhosphatidylCholine from egg yolk (~99%), L-α- Phosphatidyl-L-serine from Glycine max (soybean), L-α-Phosphatidyl-DL-glycerol, L-α-Phosphatidylethanolamine from egg yolk (~98%) and cholesterol (~99%) were purchased from Sigma (St. Louis, MO, USA). All chemicals were used as received without further purification.

2.2. METHODS

2.2.1. Liposome Preparation. The liposomes were prepared by freeze drying (Li and Deng, 2004). 20 mg total of lipid (must be lower than 30 mg/mL total) was dissolved in 1 mL of tert-ButylAlcohol (tBA). 150 mg of sucrose (must be 7.5 times as large per weight as the amount of lipid. The addition of sucrose helps determining size) was dissolved in 1 mL of water. The two solutions were mixed at a volume ratio between 1:1 and 1:2. The mixture should be optically clear. To ensure proper mixing the solution was sonicated sometimes for 30 seconds. The solution was then filtered through a 0.22 µm filter into a 10 mL vial. The sample was then freeze-dried by freezing for 8 hours and drying for as long as it takes to dry. After drying the sample was stored in the freezer.

2.2.2. Dynamic Light Scattering. The size of the liposomes was monitored in a FoQels back scattering instrument operating at a wavelength of 830 nm. The size of the liposomes was monitored at pHs 4, 7, and 9 and at temperatures 15^{o}C, 25^{o}C and 45^{o}C. Measuring time was one minute. The liposomes were dissolved in buffer solutions at different pHs. All the buffer solutions were prepared with nanopure water. The buffer solutions were all filtered through a 0.45 µm and then through a 0.02 µm syringe filter. The liposomes stock solution was sonicated for few seconds and then the stock solution was filtered through 0.2 µm filter prior to the experiments. From the stock solution, around 2 mL of sample was taken into a four clear sides methacrylate cuvette.

2.2.3. Static Light Scattering. The second virial coefficients of the liposomes were measured in a research goniometer illuminated with a 2W Laser at 548 nm. Liposomes stock solutions of concentration 1 mg/mL were made. The stock solution was

sonicated for few seconds (~30 seconds) and then filtered through 0.02 micron filter. From the stock solution several samples were made in the concentration range of 0.02 mg/mL – 0.1 mg/mL for light scattering readings at 3 different pHs. The buffers typically used were 40mM acetate buffer (pH=4), phosphate buffer (pH=7) and carbonate buffer (pH=9). The second virial coefficients were measured at three different temperatures 15^{o}C, 25^{o}C and 45^{o}C respectively. The readings were taken at 8 different angles ranging from 30^{o} to 140^{o}. All the measurements were done in duplicate and /or repeat at a later time.

All the buffer solutions for this purpose were prepared with nano pure water. The buffer solutions were thoroughly filtered before use, first with a 0.45 µm filtration unit and then through a 0.02 µm filter. The liposomes solutions were placed in 10 mL glass vials. All glass vials were cleaned prior to use as follows. The glass vials were rinsed with a lukewarm soap solution and then sonicated for around 5 minutes in a soap solution. They were then rinsed with deionised water. These glass vials were kept in a sulfuric acid solution overnight. The vials from the sulfuric acid solution were cleaned the other day with tap water followed by nanopure water, ultrafiltered water and ultra-filtered ethanol. Gloves were used at this stage to avoid any stain marks or contamination. These cleaned vials were kept under the fume hood and were allowed to dry.

2.2.4. Zeta Potential Measurement. The zeta potentials and electrophoretic mobilities of liposomes were determined in a Zetasizer Nano ZS90 (Malvern Instruments, UK). The liposomes solution was placed in the sample holder and a laser beam at 633 nm was focused on the sample. Scattered light was detected by using a photomultiplier tube. The liposomes solutions were prepared at pHs 4, 7 and 9. All the buffer solutions were filtered through a 0.45 µm filter and then through a 0.02 µm syringe filter. The liposomes stock solutions were made by dissolution in buffer solutions. The stock solutions were sonicated for 30 seconds and then filtered through a 0.2 µm filter. The concentration of the liposomes prepared was 0.1 mg/mL. The solutions were filled in the folded capillary cells for measuring the zeta potentials. The capillary cells were thoroughly rinsed with ethanol and then with deionized water before filling with liposomes solution to avoid any contamination. The measurements were taken at three temperatures 15^{o}C, 25^{o}C and 40^{o}C respectively. All the measurements were done in duplicated.

3. RESULTS AND DISCUSSION

The sizes, osmotic second virial coefficients and zeta potentials of various liposomes were measured. The studies were carried out using liposomes with different PC/cholesterol and PC/PS ratios. In addition two liposomes with a chemical composition similar to actual biological membranes were used. All the experiments were carried out at pHs 4, 7 and 9 and at 15, 25 and 45 ^{o}C. The ionic strength of all the buffers was 40mM.

The sizes of the liposomes were determined by dynamic light scattering. The effect of incorporation of cholesterol in vesicles was also studied by measuring their sizes with different cholesterol composition. The polydispersity index (PDI) which measures the width of the size distributions was used to monitor the quality of the data. The dynamic light scattered data is shown in Tables 3.1 and 3.2. The data is representative of any given liposome batch. The extrusion method that was used makes the exact control of sizes from batch to batch nearly impossible. So, there was more interest in understanding general trends than in singularities, which are likely caused by the non-uniformity of the preparation method.

The polydispersity indices obtained from DLS readings are in general small which indicate that the liposomes were fairly monodispersed and that the sizes (obtained from cumulants analysis) are reliable. There are some studies on the effect of cholesterol on the size of liposomes (Arriaga, *et al.*, 2009; Wiedmer *et al.*, 2002). The data from Table 3.2 has been plotted in Figure 3.1, panels left and right. It is evident that for a given pH and for a given temperature the size increases as the amount of cholesterol increases. This is in agreement with the finding of Sulkowski *et al.* (2005) who found that the presence of cholesterol in liposome membrane causes an increase in the distance between the phospholipid chains and limits the possibility of interaction between electronic shells of polar head-groups of PC in the bilayer. The presence of cholesterol in liposome bilayer affects the conformation of the phospholipids. The figure also shows that the effect of the amount of cholesterol on the size of the liposomes is greater at pH 4, which is closer to the isoelectric point of PC containing liposomes (Petelska and Figaszewski, 1973).

Table 3.1. Sizes and polydispersity indices of PC/PS liposomes at three pHs and three temperatures.

Composition PC/PS (%)	pH	Temperature (15 °C)		Temperature (25 °C)		Temperature (45 °C)	
		Size (nm)	PDI	Size (nm)	PDI	Size (nm)	PDI
20/80	4	219	0.17	203	0.005	197	0.005
50/50	4	173	0.2	191	0.005	178	0.3
80/20	4	184	0.09	228	0.03	210	0.1
20/80	7	182	0.31	171	0.4	153	0.45
50/50	7	165	0.005	190	0.005	207	0.005
80/20	7	176	0.12	168	0.09	183	0.07
20/80	9	185	0.14	189	0.005	157	0.005
50/50	9	225	0.02	171	0.005	206	0.005
80/20	9	208	0.12	208	0.1	200	0.06

Table 3.2. Sizes and polydispersity indices of PC/C liposomes at three pHs and three temperatures.

Composition PC/C (%)	pH	Temperature (15 °C)		Temperature (25 °C)		Temperature (45 °C)	
		Size (nm)	PDI	Size (nm)	PDI	Size (nm)	PDI
20/80	4	212	0.14	208	0.17	233	0.3
50/50	4	189	0.005	191	0.005	208	0.005
80/20	4	173	0.15	174	0.09	180	0.005
20/80	7	368	0.1	359	0.23	381	0.17
50/50	7	241	0.19	271	0.12	307	0.1
80/20	7	223	0.08	239	0.2	248	0.12
20/80	9	316	0.1	306	0.1	338	0.18
50/50	9	233	0.12	250	0.32	291	0.4
80/20	9	206	0.1	227	0.23	274	0.2

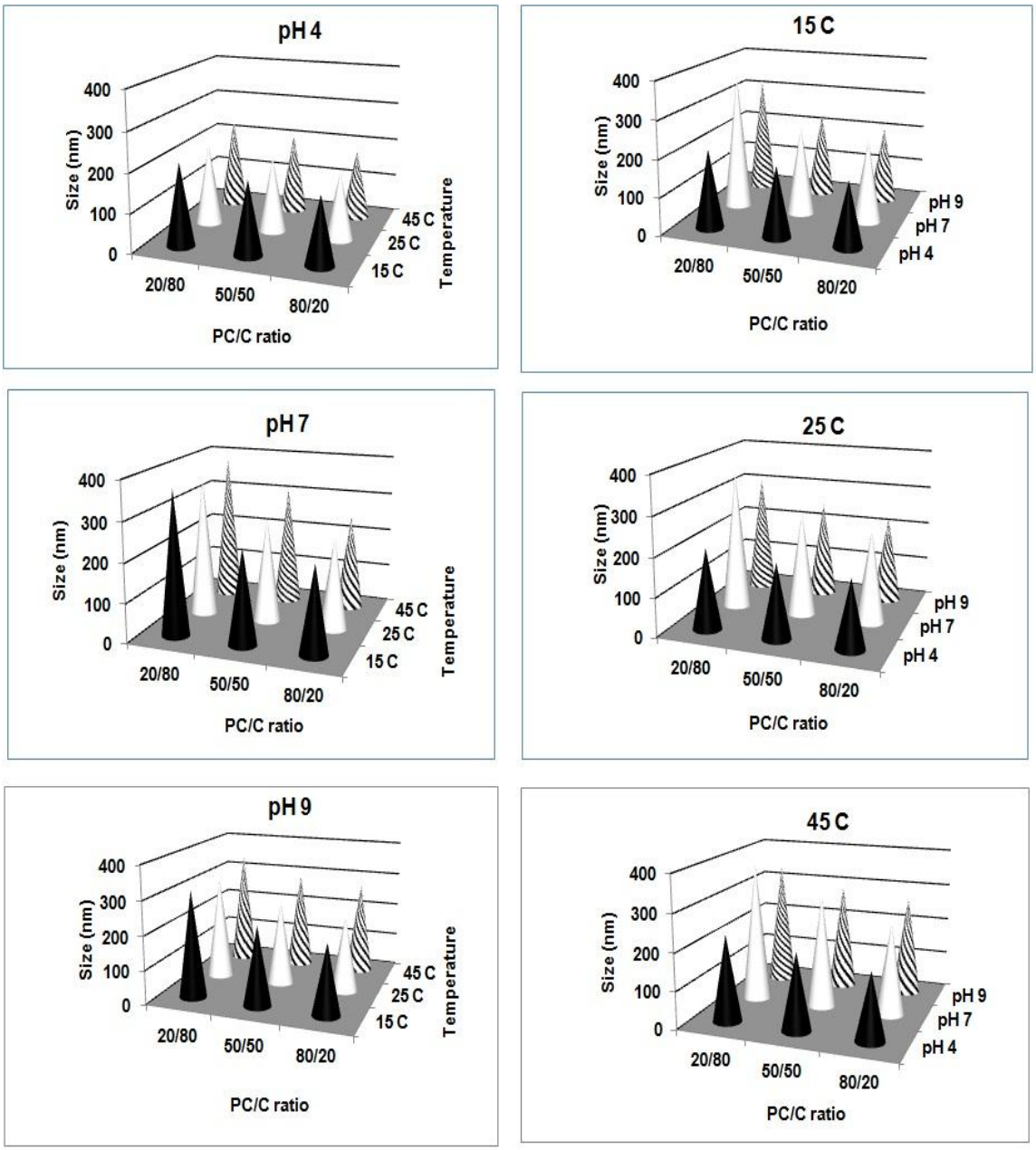


Figure 3.1. Size of PC/C liposomes from DLS measurements.The left panels are plots of size vs composition and pH as a parameter. The right panels are plots of size vs. composition and temperature as a parameter.

Figure 3.2, panels left and right, shows the change in size of the liposomes as some of the phosphatidylcholine is replaced by phosphatidylserine. Once again, pH and temperature are the parameters. In general, liposomes seem to be larger at pH 4. The effect of temperature on size at any pH seems to be negligible.

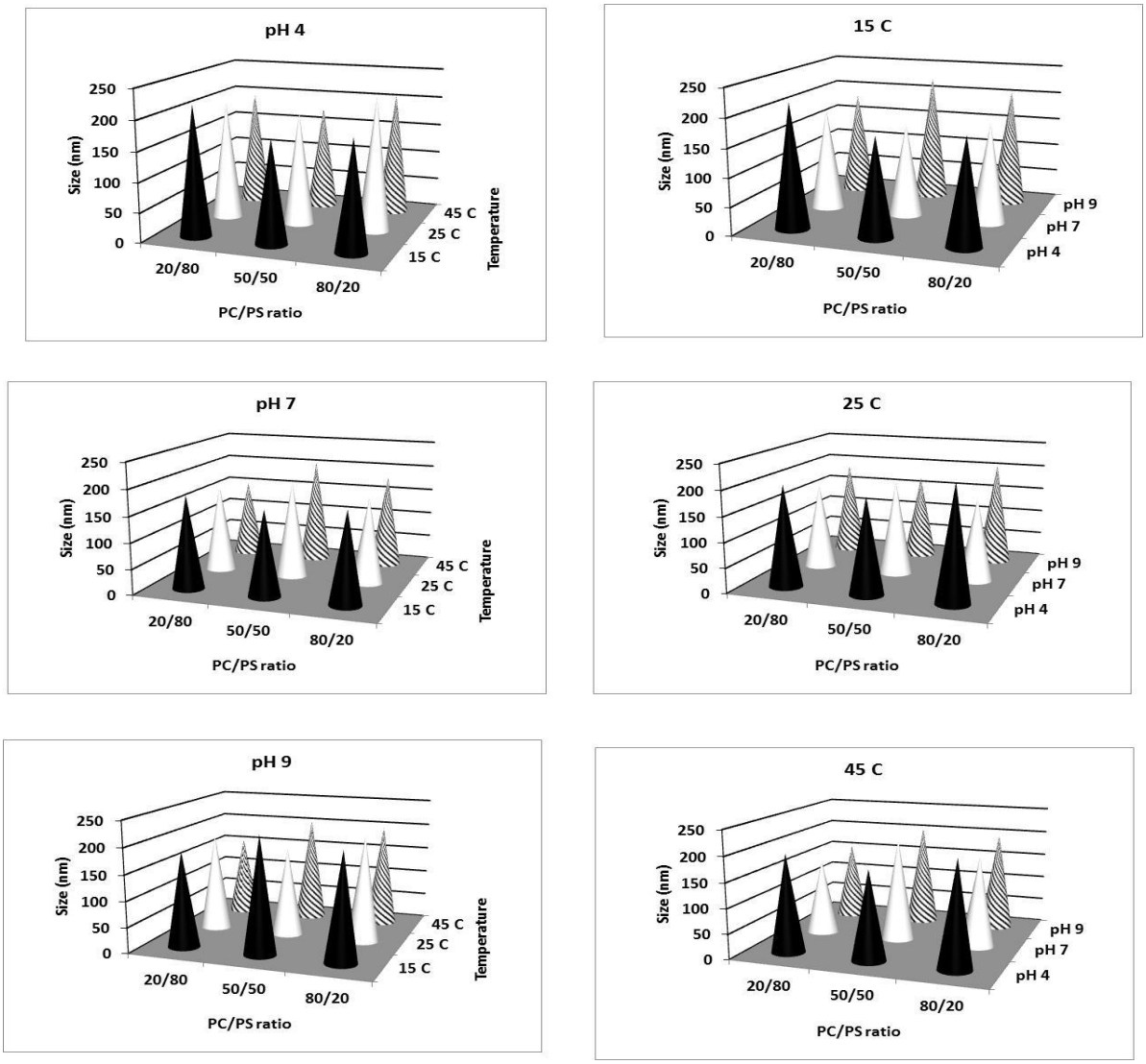


Figure 3.2. Size of PC/PS liposomes from DLS measurements. The left panels are plots of size vs. PC/PS ratio and temperature as a parameter. The right panels are plots of size vs. PC/PS ratio and pH as a parameter.

The zeta potentials of the liposomes used in this work are presented in Tables 3.3, 3.4 and 3.5. The zeta potential is always negative indicating than the liposomes are stable at the pHs and temperatures explored in this work. The isoelectric point of liposomes containing PC is around 4 (Petelska and Figaszewski, 2000). The net charge exhibited at physiological pH is caused by adsorption of ions present in the solution. A few observations are worthwhile. The zeta potential remains nearly constant from pH 4 to pH 7 and then decreases from pH 7 to pH 9 for all PC/C compositions and all temperatures.

No major temperature effects are observed. The same trend is observed when PC is replaced by PS but there is a more pronounced effect of temperature. At high temperatures the zeta potential is less sensitive to a change in pH. Replacing PC by cholesterol does not affect the zeta potential at pH 4. However, the zeta potential decreases from 20% to 50% cholesterol and then remains constant at pHs 7 and 9 at all temperatures; this is opposite to the trend observed by Liu et al. (2000). The zeta potential decreases at pHs 4 and 7 as the amount of PC replaced by PS increases for all temperatures. A minimum is observed when half of the PC has been replaced by PS at pH 9 and all temperatures. The product of the zeta potential times the hydrodynamic radius is proportional to the surface charge. It increases as the amount of PC increases for PC/C liposomes at all temperatures and pHs but the changes are more obvious at pHs 4 and 9. On the contrary the product of the zeta potential times the hydrodynamic radius decreases as the amount of PC increases for PC/PS liposomes at all temperatures and pHs 4 and 7. The product exhibits a minimum at pH 9 and all temperatures.

Table 3.3. Zeta potential (in mV) and electrophoretic mobilities (in μmcm/Vs) of various PC/C liposomes at three pHs and three temperatures.

Composition PC/C (%)	pH	15 ^{o}C		25 ^{o}C		40 ^{o}C	
		Zeta Potential	Mobility	Zeta Potential	Mobility	Zeta Potential	Mobility
20/80	4	-21.1	-1.34	-21.7	-1.71	-16.0	-1.61
50/50	4	-20.9	-1.33	-21.2	-1.66	-15.8	-1.59
80/20	4	-21.9	-1.39	-27.2	-2.13	-17.0	-1.70
20/80	7	-26.2	-1.667	-33.1	-2.59	-21	-2.10
50/50	7	-19.5	-1.24	-19.9	-1.56	-19.5	-1.95
80/20	7	-28.0	-1.79	-26.8	-2.10	-27.7	-2.78
20/80	9	-47.3	-3.01	-47.3	-3.71	-60.5	-6.06
50/50	9	-46	-2.92	-44.9	-3.52	-54.9	-5.50
80/20	9	-56.8	-3.614	-57.7	-4.52	-56.1	-5.62

Table 3.4. Zeta potential (in mV) and electrophoretic mobilities (in μm cm/Vs) of various PC/PS liposomes at three pHs and three temperatures.

Composition PC/PS (%)	pH	15 °C		25 °C		40 °C	
		Zeta Potential	Mobility	Zeta Potential	Mobility	Zeta Potential	Mobility
20/80	4	-24.9	-1.58	-20.9	-1.64	-18.7	-1.88
50/50	4	-42.1	-2.68	-44.4	-3.48	-34.3	-3.43
80/20	4	-38.6	-2.45	-49.1	-3.85	-40.2	-4.02
20/80	7	-57.2	-3.64	-46.8	-3.66	-36.7	-3.68
50/50	7	-69.5	-4.42	-53.7	-4.22	-36.3	-3.63
80/20	7	-103	-6.56	-103	-8.08	-34.2	-3.43
20/80	9	-106	-6.76	-106.5	-8.34	-119	-11.89
50/50	9	-64	-4.07	-64.9	-5.09	-61.8	-6.19
80/20	9	-95.6	-6.08	-102.2	-8.00	-89.9	-9.01

Table 3.5. Zeta potential (in mV) and electrophoretic mobilities (in μm cm/Vs) of two biological membrane mimics at three pHs and three temperatures.

Composition	pH	15 °C		25 °C		40 °C	
		Zeta Potential	Mobility	Zeta Potential	Mobility	Zeta Potential	Mobility
10:5:7.5:16 PC/PE/PS/C	4	-48.4	-3.08	-53.8	-4.22	-44.1	-4.42
	7	-120	-7.64	-117	-9.19	-67.7	-6.78
	9	-106	-6.76	-107	-8.39	-99.6	-9.98
2:2:1:1 C/PC/PG/PE	4	-62.5	-3.98	-64.5	-5.06	-54.6	-5.47
	7	-99.1	-6.30	-92.5	-7.26	-72.9	-7.30
	9	-108.5	-6.89	-104.7	-8.20	-109	-10.87

Structural and solution properties of the liposome solutions may be obtained by analyzing static light scattering data. Zimm plots can be used to obtain values of the second (osmotic) virial coefficients as explained earlier. Structural properties, like size and shape, are also accessible from scattering data. The angular dependence of the scattered light is a function (for dilute, non-interacting solutions) of the shape and size of the scatters. The form factor, P (θ) is related to the scattering intensity, I(θ) by,

$$P\ \theta\ = \frac{I\ \theta}{I\ \theta=0} \tag{2}$$

θ is the scattering angle. In a scattering experiment the Raleigh ratio, R (θ) was measured, which is related to the scattering intensity by,

$$R\ \theta\ = \frac{I\ \theta}{I_o} r^2 \tag{3}$$

Where I_o is the intensity of the incident light beam and r is the distance between the scattering volume and the detector. Therefore, measuring the Raleigh ratio allows the determination of the form factor. The form factor takes simple forms for simple shapes. For a hollow sphere it is,

$$P\ \theta\ = \frac{I\ \theta}{I\ \theta=0} = \left(\frac{\sin\ QR}{RQ}\right)^2 \tag{4}$$

where R is the hydrodynamic radius of the sphere, and the momentum transfer, Q, is related to the scattering angle and the wave length of the radiation, λ, by,

$$Q = \frac{4\pi}{\lambda} sin\frac{\theta}{2} \tag{5}$$

For a solid hard sphere the form factor is,

$$P\ \theta\ = \frac{I\ \theta}{I\ \theta=0} = \left(\frac{3}{R^3Q^3}\ \sin\ RQ\ - RQcos\ RQ\right)^2 \tag{6}$$

More complex equations have been derived for polydisperse systems (Stauch and Schubert, 2002). The scattering data collected at various angles may be presented in a variety of ways. One of the most common ones is the Guinier plot,

$$ln\ \frac{I\ \theta}{I\ \theta=0} \cong -\frac{{R_g}^2 Q^2}{3} \quad (7)$$

where R_g is the radius of gyration. For hollow spheres $R=R_g$ whereas for solid spheres,

$$R = \sqrt{\frac{5}{3}} R_g \quad (8)$$

Guinier plot is a good approximation when $QR_g < 1$. The range of Qs used in this work goes from 4.25910^{-3} to $3.186\ 10^{-2}$ nm^{-1}. Therefore, the entire Q range is usable if the particles are smaller than ~30 nm. These liposomes are 50 nm in diameter or larger. Therefore, the higher Q range is not usable (angles higher than 90^o) for Guinier analysis. Often the low angle range curves upwards because of the presence of aggregates so the usable angular range is from 30 to 90 degrees. Two Guinier plots are shown in Figure 3.3.

The data in Figure 3.3 (left panel) exhibits the typical upwards curvature of Guinier plots for polydisperse preparations. The data on the right panel of Figure 3.3 at pHs 9 and 7 are reasonable good. Fitting to these curves yield radius of gyration of 104±6 nm at pH 4, 86 ± 3nm at pH 7 and 71 ± 1 nm at pH 9.

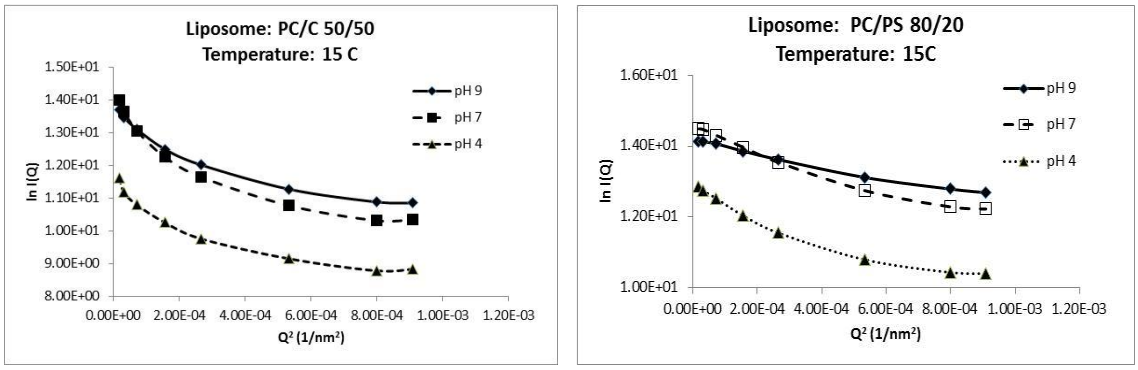


Figure 3.3. Guinier plots (ln I(Q) vs Q^2) for two liposome preparations at 15 ^oC and three pHs. An evident upward curvature is present for PC/C (50/50) liposomes. The data is of better quality for PC/PS liposomes.

An alternative to Guinier analysis is to plot $Q^2I(Q)$ vs. Q, which is known as the Kratky plot. For solid and hollow spheres, Kratky plots look like as shown in Figure 3.4.

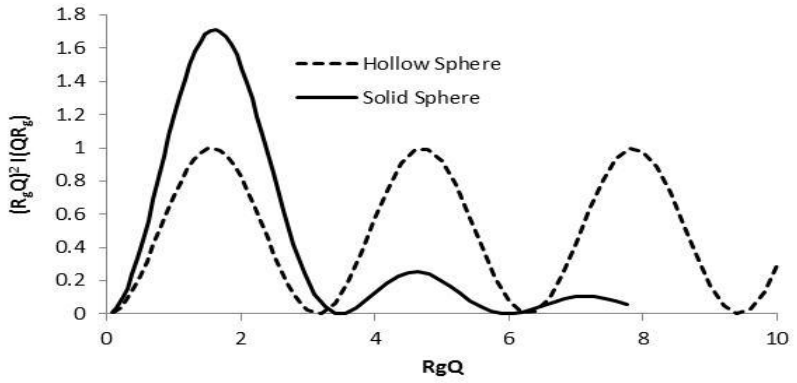


Figure 3.4. Kratkly plot for solid and hollow spheres. The maxima are at π/2, 3π/2, 2π, etc.

For polydisperse samples the peaks broaden and make the differences between hollow and solid spheres difficult to distinguish. The information of I(Q) vs. Q was collected for all liposome preparations at three temperatures and three pHs. The complete data set as I(Q) vs Q and Kratky plots is in Appendix C. An inspection of the I(Q) vs Q curves shows that some have an upward curvature, which is a characteristic of aggregation. A representative Kratky plot is shown in Figure 3.5. The presence of polydisperse hollow spheres is obvious.

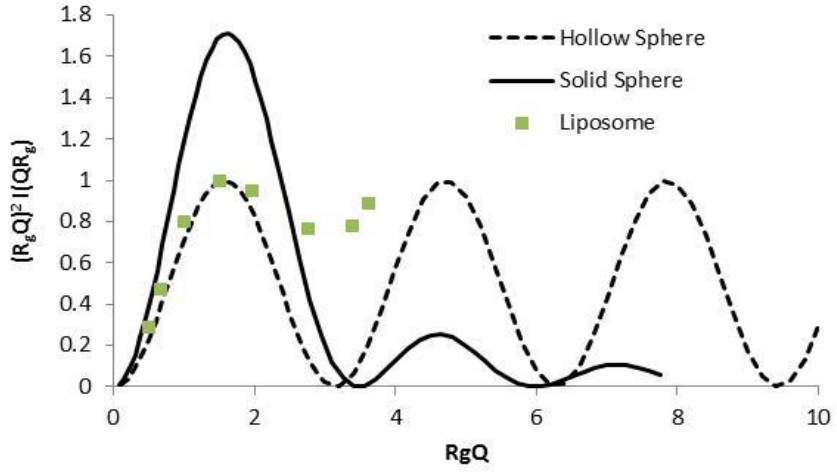


Figure 3.5. Kratky plot for Cholesterol/PC/PG/PE, 2:2:1:1 at 25^0C and pH 4. The curves corresponding to hollow and solid spheres are included as reference.

The scattered intensity at 90^{o} was interrogated to discard (or confirm) any drastic change in the size and shape of the liposomes caused by a lipid phase transition like the one observed by Riske *et al.* (1997). No such a transition has been identified in this work at any angle.

The second virial coefficients of liposomes were measured at different pHs and different temperatures. The values of second virial coefficient (B_{22}), radius of gyration (R_g) and molecular weight (Mw) of all liposomes at different pHs and different temperatures are summarized in Tables 3.6, 3.7 and 3.8.

The second virial coefficients are all positive, which indicates that the net interactions between liposomes are repulsive. Although the samples are reasonable monodisperse, the slight aggregation suggests that the second virial coefficients obtained from Zimm plots must be taken very carefully.

The second virial coefficients of PC/C liposomes with different cholesterol content are positive, indicating overall net repulsive forces between the PC/C vesicles. The second virial coefficients of liposomes with high cholesterol content shown in Figures 3.6, 3.7 and 3.8 are of the order of 10^{-4} while those with less cholesterol content are of the order of 10^{-5} , which clearly indicates that repulsive forces increase as cholesterol is incorporated. Liu *et al.* (2000) postulated that incorporating of cholesterol into the bilayer causes the lipid vesicles to change their packaging geometrical structures which include lipid vesicle size, the curvatures of surface bilayer and surface bilayer rigidity. Mcintosh *et al.* (1989) postulated that the incorporation of cholesterol in the lipid vesicles affects the short distance repulsive force among the lipid vesicles. It was observed that the increase in cholesterol content increases the net repulsive forces among PC vesicles.

Table 3.6. Results from Zimm analysis of PC/C liposomes

PC/C (%)	pH	15 ^{0}C			25 ^{0}C			45 ^{0}C		
		B_{22} (cm^3mol/g^2)	R_g (nm)	Mw (g/mol)	B_{22} (cm^3mol/g^2)	R_g (nm)	Mw (g/mol)	B_{22} (cm^3mol/g^2)	R_g (nm)	Mw (g/mol)
20/80	4	$3.75\pm0.32\times10^{-4}$	185±0.4	$1.1\pm0.1\times10^{7}$	$5.0\pm0.2\times10^{-4}$	258±0.8	$2.2\pm0.3\times10^{7}$	$4.43\pm0.33\times10^{-4}$	359±1.8	$6.1\pm1.8\times10^{7}$
50/50	4	$3.2\pm0.4\times10^{-4}$	258±0.3	$9.0\pm1.0\times10^{6}$	$3.8\pm0.6\times10^{-4}$	246±0.3	$8.3\pm1.0\times10^{6}$	$3.9\pm0.7\times10^{-4}$	255±0.4	$8.1\pm1.0\times10^{6}$
80/20	4	$3.0\pm0.68\times10^{-5}$	179±0.3	$5.1\pm0.3\times10^{7}$	$4.5\pm0.8\times10^{-5}$	185±0.4	$4.4\pm0.3\times10^{7}$	$6.47\pm0.87\times10^{-5}$	184±0.4	$4.6\pm0.3\times10^{7}$
20/80	7	$6.95\pm0.97\times10^{-4}$	143±0.8	$4.9\pm0.3\times10^{6}$	$6.5\pm0.7\times10^{-4}$	157±0.7	$5.6\pm0.3\times10^{6}$	$6.46\pm0.80\times10^{-4}$	165±0.8	$6.1\pm0.5\times10^{6}$
50/50	7	$3.83\pm0.82\times10^{-4}$	187±0.6	$7.1\pm0.5\times10^{6}$	$5.5\pm1.1\times10^{-4}$	214±0.9	$7.9\pm1.4\times10^{6}$	$5.0\pm0.9\times10^{-4}$	195±0.8	$5.8\pm0.8\times10^{6}$
80/20	7	$1.09\pm0.18\times10^{-5}$	196±0.3	$1.6\pm0.1\times10^{8}$	$1.5\pm0.2\times10^{-5}$	207±0.3	$1.6\pm0.1\times10^{8}$	$1.69\pm0.27\times10^{-5}$	212±0.5	$1.6\pm0.1\times10^{8}$
20/80	9	$2.82\pm0.17\times10^{-4}$	130±0.2	$8.8\pm0.2\times10^{6}$	$2.9\pm0.2\times10^{-4}$	128±0.2	$8.2\pm0.1\times10^{6}$	$3.64\pm0.39\times10^{-4}$	125±0.4	$6.9\pm0.2\times10^{6}$
50/50	9	$1.26\pm0.24\times10^{-4}$	142±0.2	$9.2\pm0.2\times10^{6}$	$2.1\pm0.4\times10^{-4}$	150±0.3	$9.4\pm0.5\times10^{6}$	$2.56\pm0.59\times10^{-4}$	135±0.5	$6.7\pm0.4\times10^{6}$
80/20	9	$8.9\pm2.1\times10^{-6}$	225±0.4	$1.8\pm0.1\times10^{8}$	$1.4\pm0.3\times10^{-5}$	240±0.6	$2.1\pm0.2\times10^{8}$	$1.25\pm0.31\times10^{-5}$	235±0.5	$2.1\pm0.2\times10^{8}$

Table 3.7. Results from Zimm analysis of PC/PS liposomes

PC/PS(%)	pH	15 ^{0}C			25 ^{0}C			45 ^{0}C		
		B_{22} (cm^3mol/g^2)	R_g (nm)	Mw (g/mol)	B_{22} (cm^3mol/g^2)	R_g (nm)	Mw (g/mol)	B_{22} (cm^3mol/g^2)	R_g (nm)	Mw (g/mol)
20/80	4	$1.35\pm0.87\times10^{-5}$	176±0.3	$3.12\pm0.03\times10^{7}$	$2.8\pm1.3\times10^{-5}$	182±0.4	$3.23\pm0.19\times10^{7}$	$5.6\pm1.3\times10^{-5}$	190±0.4	$3.54\pm0.24\times10^{7}$
50/50	4	$1.12\pm0.73\times10^{-5}$	162±0.2	$3.42\pm0.09\times10^{7}$	$1.8\pm1.0\times10^{-5}$	166±0.3	$3.31\pm0.10\times10^{7}$	$1.6\pm1.3\times10^{-5}$	172±0.4	$3.52\pm0.22\times10^{7}$
80/20	4	$5.0\pm1.6\times10^{-5}$	144±0.4	$1.82\pm0.06\times10^{7}$	$4.74\pm0.82\times10^{-5}$	210±0.4	$4.18\pm0.16\times10^{7}$	$3.36\pm0.82\times10^{-5}$	201±0.3	$3.82\pm0.13\times10^{7}$
20/80	7	$4.90\pm0.30\times10^{-4}$	111±0.6	$1.31\pm0.08\times10^{7}$	$5.24\pm0.29\times10^{-4}$	110±0.5	$1.19\pm0.06\times10^{7}$	$5.67\pm0.41\times10^{-4}$	117 ±0.6	$1.03\pm0.06\times10^{7}$
50/50	7	$6.86\pm0.12\times10^{-4}$	98.4±0.4	$4.58\pm0.13\times10^{7}$	$8.09\pm0.20\times10^{-4}$	111±0.5	$2.72\pm0.18\times10^{7}$	$8.48\pm0.44\times10^{-4}$	110±0.6	$1.78\pm0.14\times10^{7}$
80/20	7	$3.39\pm0.78\times10^{-5}$	227±0.8	$6.97\pm0.38\times10^{7}$	$3.09\pm0.77\times10^{-5}$	241±0.8	$6.73\pm0.36\times10^{7}$	$2.08\pm0.89\times10^{-5}$	233±0.8	$5.82\pm0.31\times10^{7}$
20/80	9	$1.88\pm0.95\times10^{-3}$	115±1.3	$1.08\pm0.17\times10^{6}$	$3.0\pm1.4\times10^{-3}$	113±1.5	$8.4\pm1.6\times10^{5}$	$6.0\pm1.3\times10^{-3}$	114±1.8	$8.8\pm2.0\times10^{5}$
50/50	9	$1.04\pm0.47\times10^{-3}$	112±1.1	$1.30\pm0.11\times10^{6}$	$1.54\pm0.47\times10^{-3}$	127±0.9	$1.48\pm0.13\times10^{6}$	$1.8\pm1.1\times10^{-3}$	110±1.6	$1.30\pm0.25\times10^{6}$
80/20	9	$6.43\pm0.8\times10^{-5}$	140±0.4	$3.22\pm0.08\times10^{7}$	$7.92\pm0.85\times10^{-5}$	144±0.4	$3.17\pm0.08\times10^{7}$	$9.14\pm0.90\times10^{-5}$	141±0.4	$3.1\pm0.088\times10^{7}$

Table 3.8. Results from Zimm analysis of biological membrane mimics liposomes

	pH	15 ^{0}C			25 ^{0}C			45 ^{0}C		
		B_{22} (cm^3mol/g^2)	R_g (nm)	Mw (g/mol)	B_{22} (cm^3mol/g^2)	R_g (nm)	Mw (g/mol)	B_{22} (cm^3mol/g^2)	R_g (nm)	Mw (g/mol)
10:5:7.5:16 PC/PE/PS/C	4	$2.62\pm0.74\times10^{-5}$	245±0.5	$6.34\pm0.32\times10^{7}$	$2.70\pm0.84\times10^{-5}$	242±0.5	$5.58\pm0.28\times10^{7}$	$3.0\pm0.65\times10^{-5}$	232±0.6	$5.25\pm0.60\times10^{7}$
	7	$1.27\pm0.30\times10^{-5}$	231±0.5	$1.79\pm0.09\times10^{8}$	$1.01\pm0.32\times10^{-5}$	232±0.5	$1.7\pm0.086\times10^{8}$	$9.9\pm3.4\times10^{-6}$	235±0.5	$1.67\pm0.08\times10^{8}$
	9	$7.9\pm1.3\times10^{-6}$	277±0.6	$4.10\pm0.25\times10^{8}$	$6.6\pm1.3\times10^{-6}$	287±0.6	$4.32\pm0.28\times10^{8}$	$7.6\pm1.9\times10^{-6}$	288±0.8	$4.74\pm0.68\times10^{8}$
2:2:1:1 C/PC/PG/PE	4	$1.58\pm0.55\times10^{-4}$	188±0.5	$8.74\pm0.66\times10^{6}$	$2.07\pm0.48\times10^{-4}$	193±0.5	$9.25\pm0.68\times10^{6}$	$2.12\pm0.49\times10^{-4}$	197±0.5	$9.18\pm0.70\times10^{6}$
	7	$1.89\pm0.19\times10^{-4}$	192±0.5	$2.34\pm0.16\times10^{7}$	$1.81\pm0.35\times10^{-4}$	176±0.6	$1.89\pm0.21\times10^{7}$	$2.05\pm0.27\times10^{-4}$	198±0.6	$2.09\pm0.18\times10^{7}$
	9	$4.31\pm0.96\times10^{-5}$	177±0.4	$3.58\pm0.11\times10^{7}$	$4.38\pm0.88\times10^{-5}$	172±0.4	$3.27\pm0.09\times10^{7}$	$4.09\pm0.94\times10^{-5}$	181±0.5	$3.19\pm0.09\times10^{7}$

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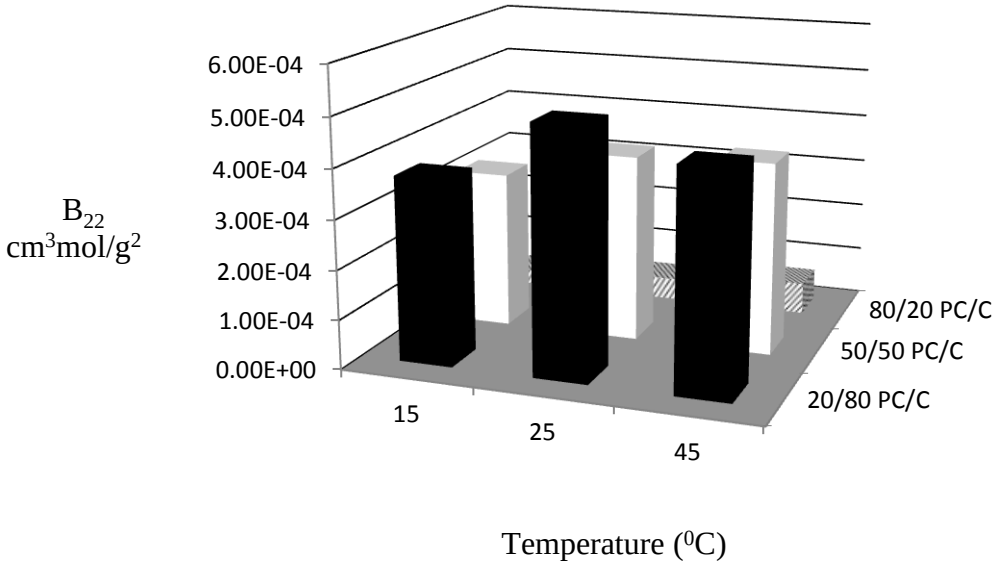


Figure 3.6. Second virial coefficients of PC/C liposomes suspensions with different ratios measured at pH 4 at three different temperatures.

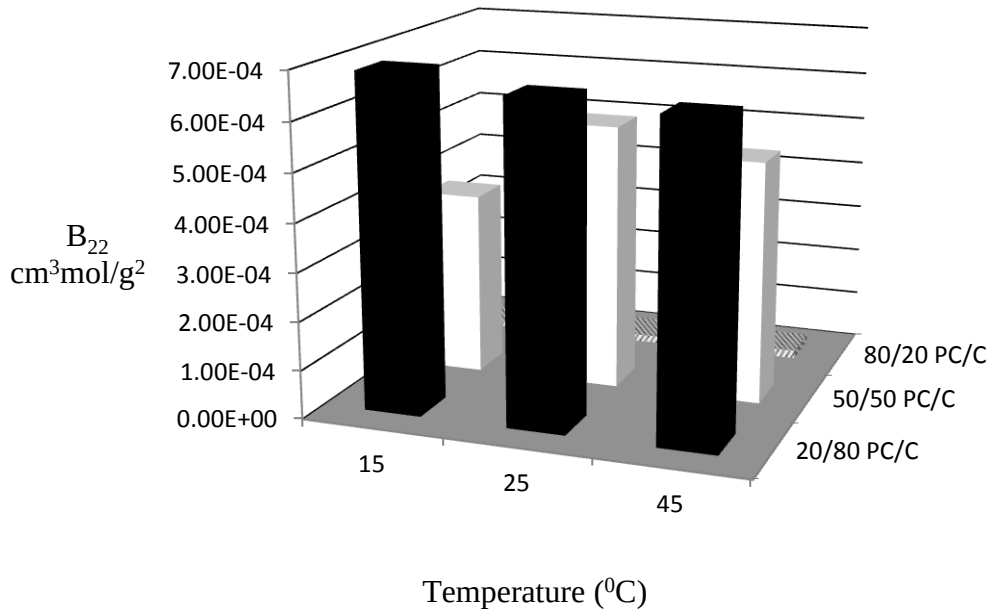


Figure 3.7. Second virial coefficients of PC/C liposomes suspensions with different ratios measured at pH 7 at three different temperatures.

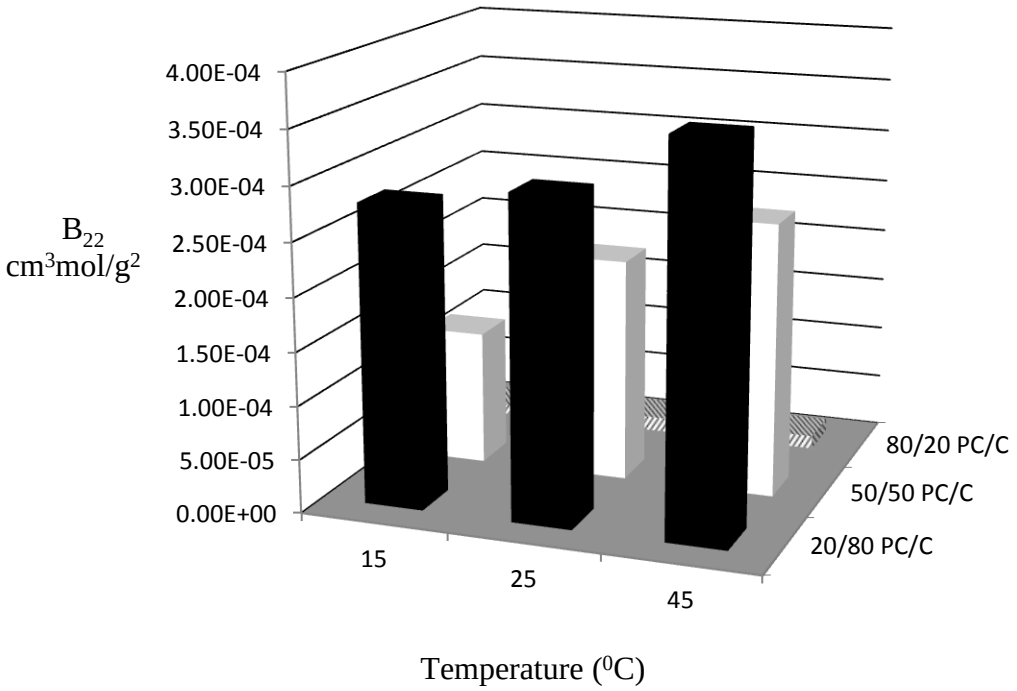


Figure 3.8. Second virial coefficients of PC/C liposomes suspensions with different ratios measured at pH 9 at three different temperatures.

Liposomes were prepared with the acidic phospholipid PS and the amphoteric phospholipid PC at different ratios to see how that ratio affects the second virial coefficients. In Figures 3.9, 3.10 and 3.11, the second virial coefficients of liposomes with different PS composition are plotted against temperature at 3 pHs. PS is negatively charged at pH 7 and it is believed that the electrostatic repulsive forces are the major contributing factor to the force field between lipid vesicular surfaces. It was observed that as the pH was increased from 4 to 9, the second virial coefficients increased, which implies that the net repulsive forces increases with the increase in pH. An increase in pH causes an increase in the negative charge of PS which results in an increase of net electrostatic repulsive forces between the liposomes. This increase in repulsive forces is manifested by an increase in the second virial coefficient as observed in Figures 3.9, 3.10 and 3.11.

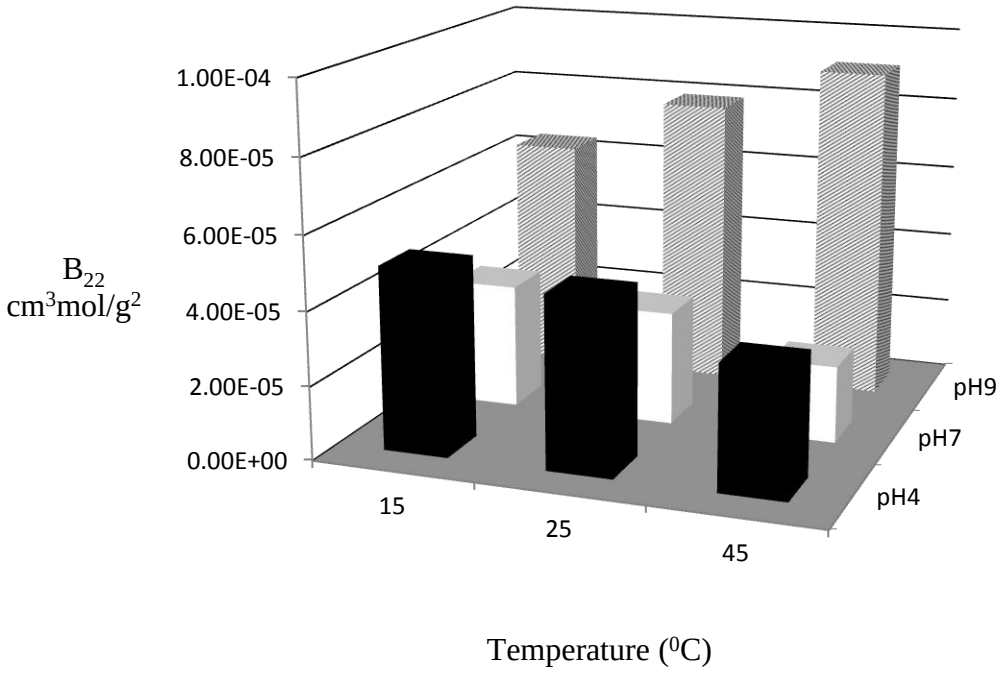


Figure 3.9. Second virial coefficients of 80/20 PC/PS liposomes suspensions at 3 different pHs measured at three different temperatures.

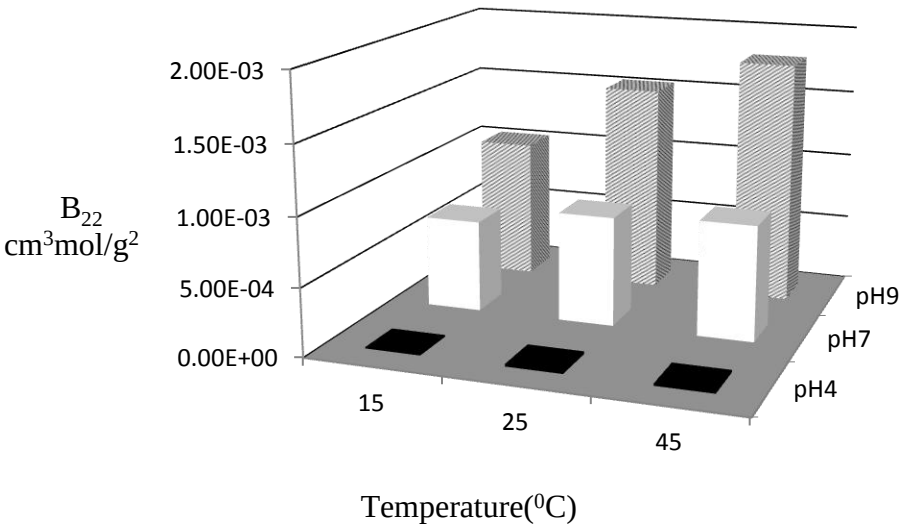


Figure 3.10. Second virial coefficients of 50/50 PC/PS liposomes suspensions at 3 different pHs measured at three different temperatures.

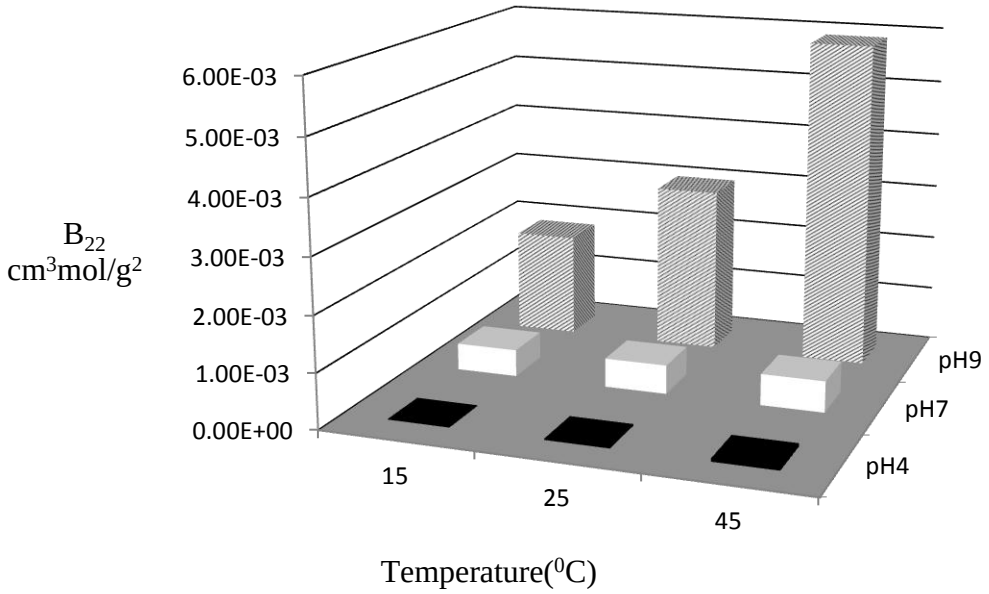
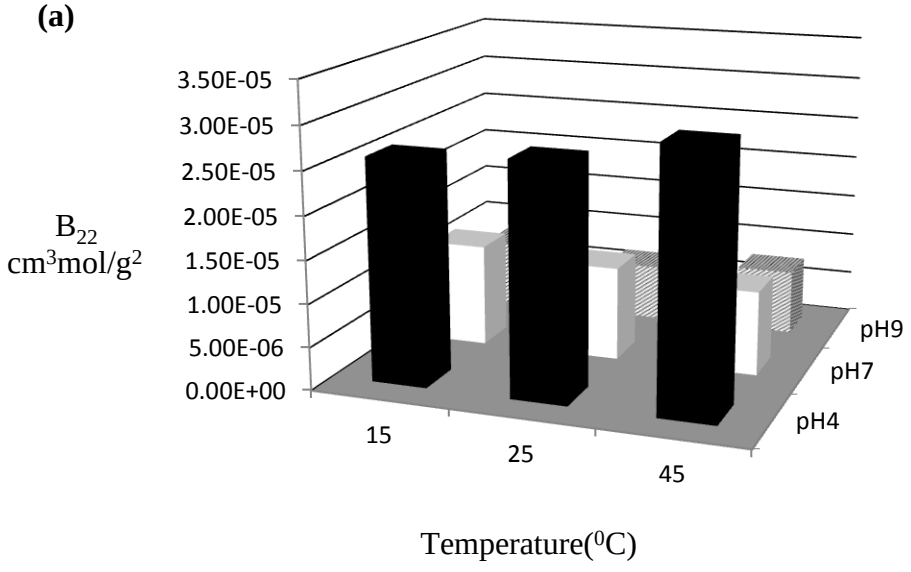


Figure 3.11. Second virial coefficients of 20/80 PC/PS liposomes suspensions at 3 different pHs measured at three different temperatures.

Liposomes with different phospholipids were prepared with cholesterol incorporated and the second virial coefficients of these liposomes were measured at three different pHs and three different temperatures as shown in Figure 3.12. Liposomes with the composition 10:5:7.5:16 PC/PE/PS/Cholesterol showed a decrease in the values of second virial coefficients with increase in pH. The phospholipids PC and PE are amphoteric and have different properties compared to phospholipids PS or PG. Even though PS is negatively charged and the electrostatic repulsive forces are dominant at neutral pH, the incorporation of lipids PC and PE seems to reduce the repulsive forces.

(a)



(b)

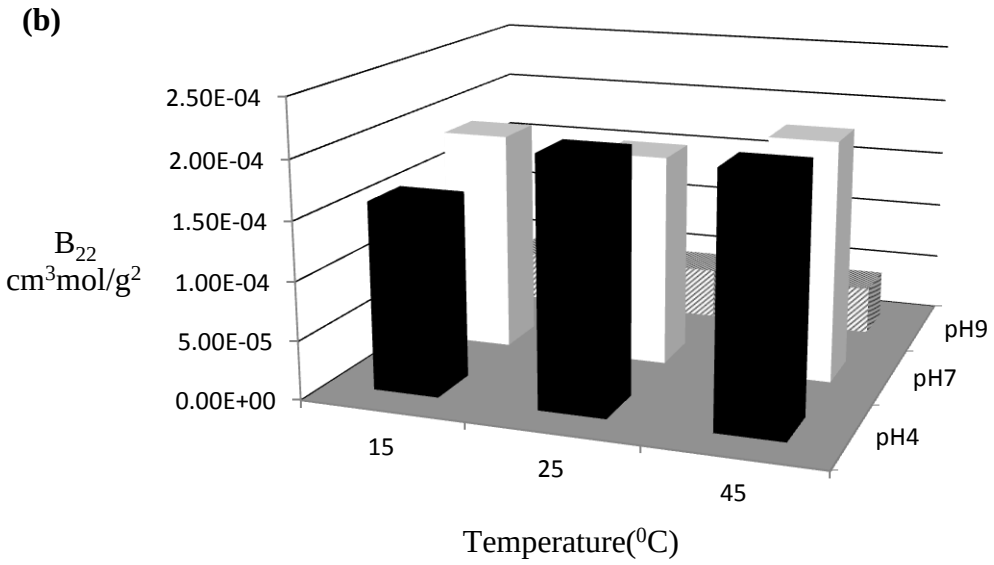


Figure 3.12. Second virial coefficients of liposomes (a) 10:5:7.5:16 PC/PE/PS/Cholesterol; (b) 2:2:1:1 Cholesterol/PC/PG/PE measured at 3 pHs and 3 different temperatures.

4. CONCLUSIONS

A detailed light scattering study of the solution properties of four different types of liposomes was conducted. The data suggests that replacing phosphatidylcholine by cholesterol increases liposomes' sizes. Replacing PC by PS does not have a clear effect on liposome's size. Replacing PC by cholesterol does not affect the zeta potential at pH 4. However, the zeta potential decreases from 20% to 50% cholesterol and then remains constant at pHs 7 and 9 at all temperatures. The zeta potential decreases at pHs 4 and 7 as the amount of PC replaced by PS increases for all temperatures. A minimum is observed when half of the PC has been replaced by PS at pH 9 and all temperatures. The second virial coefficients of all liposomes are positive, indicating overall net repulsive forces between the vesicles. The second virial coefficients of liposomes with high cholesterol content are of the order of 10^{-4} cm^3 mol/ g^2 while those with less cholesterol content are of the order of 10^{-5} cm^3 mol/ g^2.

APPENDIX A

SECOND VIRIAL COEFFICIENTS, ZETA POTENTIALS AND MOBILITIES OF POLYSTYRENE LATEX

Materials

Polystyrene latex was purchased from Polysciences, Inc. (Lot # 560167). The nominal diameter of the latex was 107 nm and the concentration was 2.64 % (solids-latex). The Latex was used as received without further purification.

Methods

Static Light Scattering. The second virial coefficients of the Polystyrene latex were measured in a research goniometer illuminated with a 2W Laser at 548 nm. 10 mL of Polystyrene stock solution of concentration 0.026 mg/mL was made. The stock solution was filtered through 0.02 micron filter. From the stock solution several samples were made in the concentration range of 0.0005 mg/mL – 0.0026 mg/mL for light scattering readings at 3 different pHs. The buffers were prepared at pHs 2, 4 and 6. All the buffer solutions for this purpose were prepared with nanopure water. The buffer solutions were thoroughly filtered before use, first with a 0.45 μm filtration unit and then through a 0.02 μm filter. The polystyrene solutions were placed in 10 mL glass vials for measuring the second virial coefficients. The second virial coefficients were measured at three different temperatures 15^{o}C, 25^{o}C and 45^{o}C respectively. The readings were taken at 8 different angles ranging from 30^{o} to 140^{o}. All the measurements were done in duplicate.

Zeta Potential Measurement. The zeta potentials and electrophoretic mobilities of Polystyrene were determined in the instrument zetasizer nano ZS90 (Malvern Instruments, UK). The Polystyrene solution was placed in the sample holder and laser light of wavelength 633 nm was focused on the sample. Scattered light was detected by using a photomultiplier tube. The Polystyrene solutions were prepared at pH's 4, 7 and 9. All the buffer solutions were filtered through a 0.45 μm filter and then through a 0.02 μm syringe filter. The Polystyrene stock solutions were made by dissolution in buffer solutions. The stock solutions were filtered through 0.2 μm filter. The concentration of the solutions prepared was 0.001 wt %. The solutions were filled in the folded capillary cells for measuring the zeta potentials. The capillary cells were thoroughly rinsed with ethanol and then with deionized water before filling with Polystyrene solution to avoid

any contamination. The measurements were taken at 25^{o}C. All the measurements were done in duplicate.

Table A.1. Properties of Polystyrene latex are tabulated below:

Temperature (^{o}C)	Second Virial Coefficients B_{22} (cm^3mol/g^2)x10^{-6}			Zeta Potentials (mV)			Mobilities (μm cm/Vs)		
	pH 2	pH 4	pH 6	pH 4	pH 7	pH 9	pH 4	pH 7	pH 9
15	3.93	4.96	2.97	-	-	-	-	-	-
25	4.39	5.68	2.28	-66	-128	-97	-5.1	-10.1	-7.6
45	4.49	5.53	1.00	-	-	-	-	-	-

APPENDIX B

SECOND VIRIAL COEFFICIENTS OF POLYSTYRENE LATEXES "DECORATED" WITH LEUCINE AND ISOLEUCINE

Materials

Dimethylformamide (DMF) and trifluoracetic acid (TFA) were purchased from Acros organics, HBTU (O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate), the FMOC protected amino acids and the Wang resin were purchased from CS Bio, Menlo California, diisopropylethylamine (DIEA) was purchased from Fisher Scientific, ethyl ether, dichloromethane (DCM) peptide synthesis grade was purchased from Fisher Scientific.

Methods

The two peptides (7 Isoleucine + 3 Lysine) and (7 Leucine + 3 Lysine) were prepared using CS Bio 336 peptide synthesizer. The peptides were synthesized according to the procedure described in Kunal *et al.* (2011).

Static Light Scattering. The 2 peptides were adsorbed on the surface of polystyrene latex. The peptides used for this purpose were (7 Leucine + 3 Lysine) and (7 Isoleucine + 3 Lysine). Peptide stock solution of 1mg/mL was made and 0.01% Polystyrene latex suspension was prepared in buffer at different pHs. 5mL of peptide solution was added to 5 mL of PS latex suspension. 2mL of this stock solution was taken for the DLS analysis. The DLS readings were obtained after the adsorption. There was an increase of about ~20nm in size after adsorption. From the stock solution, five different dilute samples were made in the concentration range of 0.01 - 0.05 mg/mL for determining the second virial coefficients of peptides. The second virial coefficients were measured at three different temperatures 15^{o}C, 25^{o}C and 45^{o}C respectively. The readings were taken at 8 different angles ranging from 30^{o} to 140^{o}. All the measurements were done in duplicate. The measurements were also taken in the presence of NaCl at two different concentrations of 50mM and 100mM by adding appropriate amounts of salt.

Table B.1. The second virial coefficients of the peptides adsorbed on latex surface.

Peptide	Second Virial Coefficients B_{22} (cm^3mol/g^2)		
7 Isoleucine + 3 Lysine	pH 2	pH 4	pH 6
15 ^oC	$4.08x10^{-4}$	$5.83x10^{-4}$	$7.59x10^{-5}$
25 ^oC	$1.88x10^{-4}$	$6.54x10^{-4}$	$4.69x10^{-5}$
45 ^oC	$6.17x10^{-4}$	$7.95x10^{-4}$	$4.90x10^{-5}$
7 Leucine + 3 Lysine	pH 2	pH 4	pH 6
15 ^oC	$-2.29x10^{-4}$	$-2.12x10^{-4}$	$-2.60x10^{-4}$
25 ^oC	$-2.74x10^{-4}$	$-2.30x10^{-4}$	$-4.02x10^{-4}$
45 ^oC	$-2.24x10^{-4}$	$-5.20x10^{-5}$	$-5.22x10^{-4}$

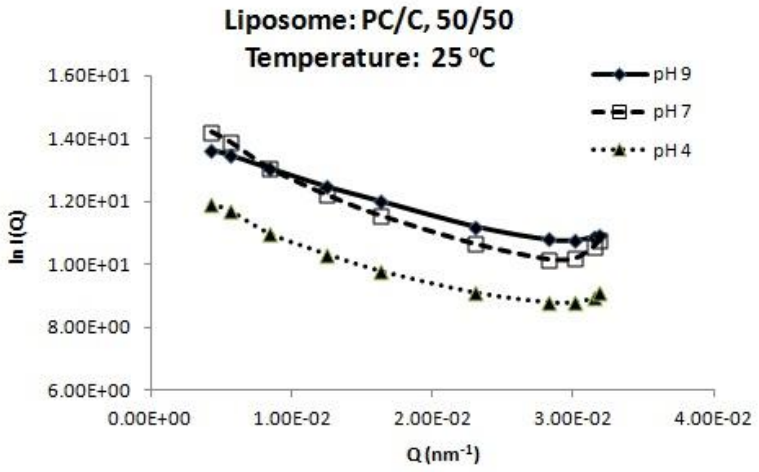
Table B.2. The second virial coefficients of these peptides adsorbed on latex surface were also measured in the presence of NaCl salt at pH 4.

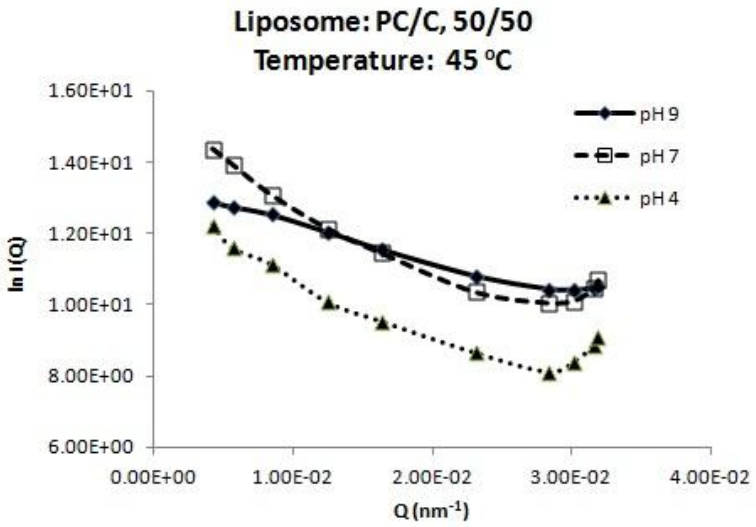
Peptide	Second Virial Coefficients B_{22} (cm^3mol/g^2)	
7 IsoLeucine + 3 Lysine	50 mM NaCl	100 mM NaCl
15 ^oC	$2.78x10^{-5}$	$-1.3x10^{-5}$
25 ^oC	$1.06x10^{-5}$	$-1.43x10^{-5}$
45 ^oC	$2.83x10^{-5}$	$-1.23x10^{-5}$
7 Leucine + 3 Lysine	50 mM NaCl	100 mM NaCl
15 ^oC	$-1.09x10^{-4}$	$-2.0x10^{-5}$
25 ^oC	$-1.01x10^{-4}$	$-3.30x10^{-5}$
45 ^oC	$-1.45x10^{-4}$	$-4.10x10^{-5}$

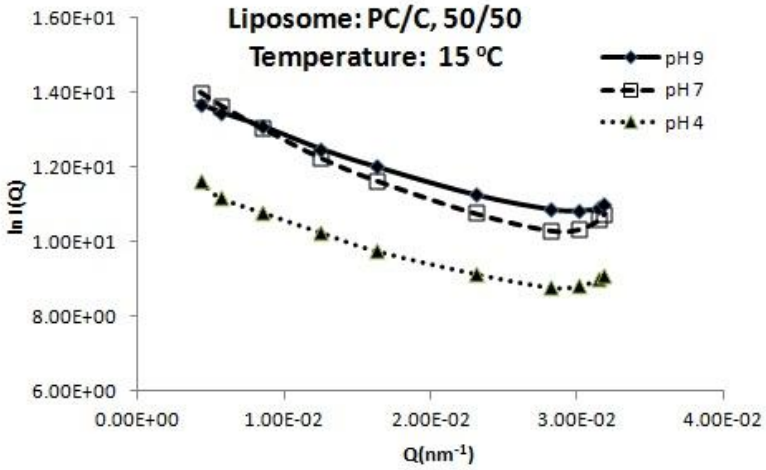
APPENDIX C

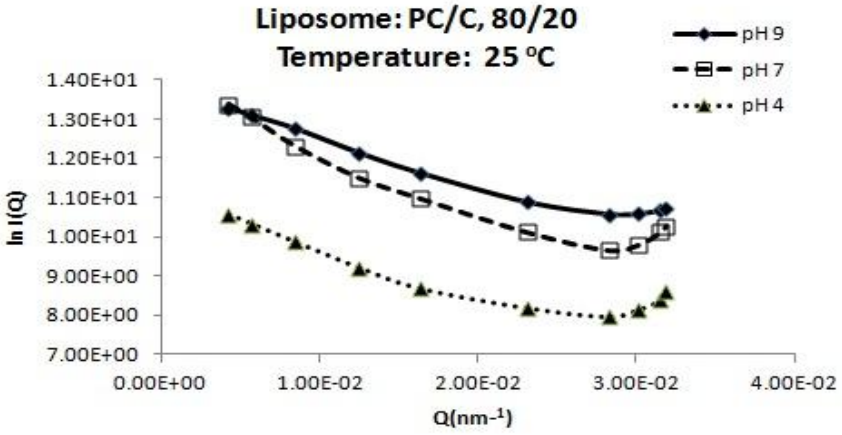
COLLECTION OF STATIC LIGHT SCATTERING DATA FOR VARIOUS LIPOSOMES.

Plots of Intensity vs. momentum transfer





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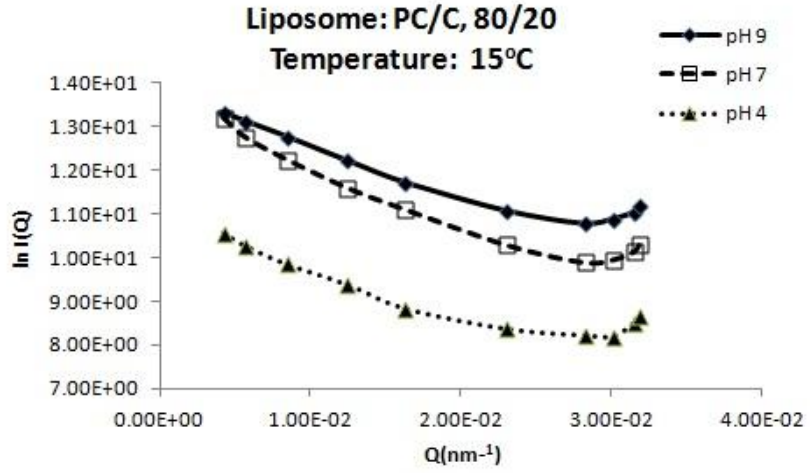
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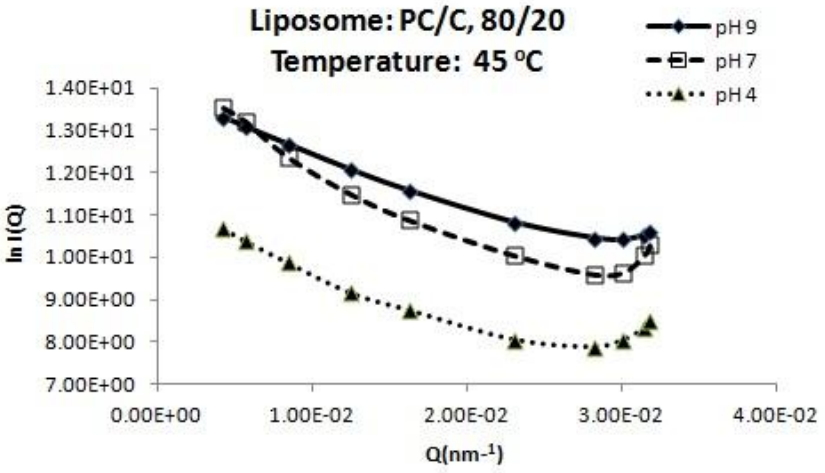
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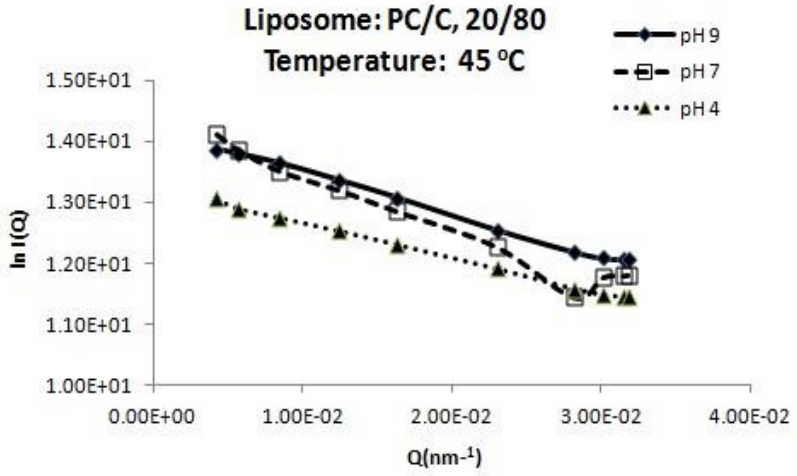
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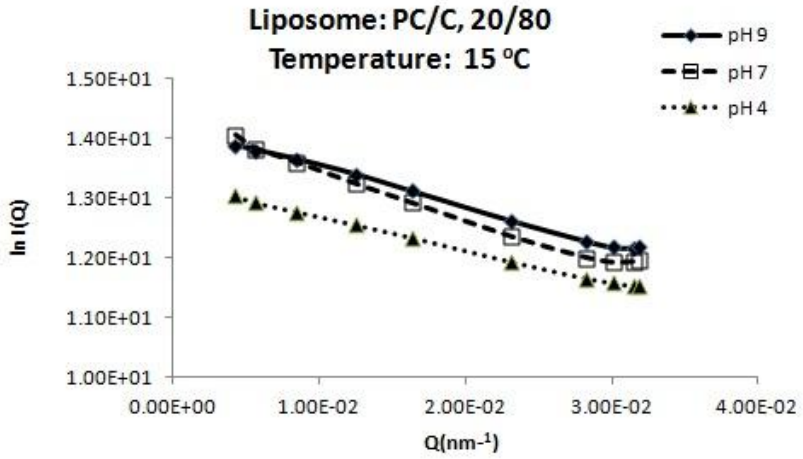
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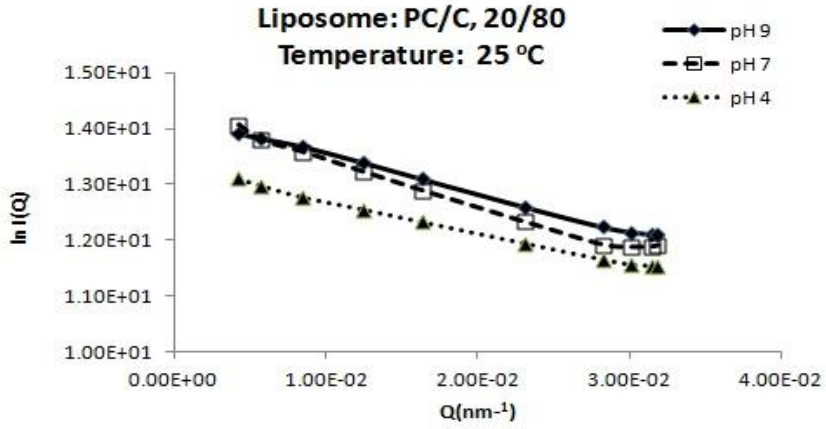
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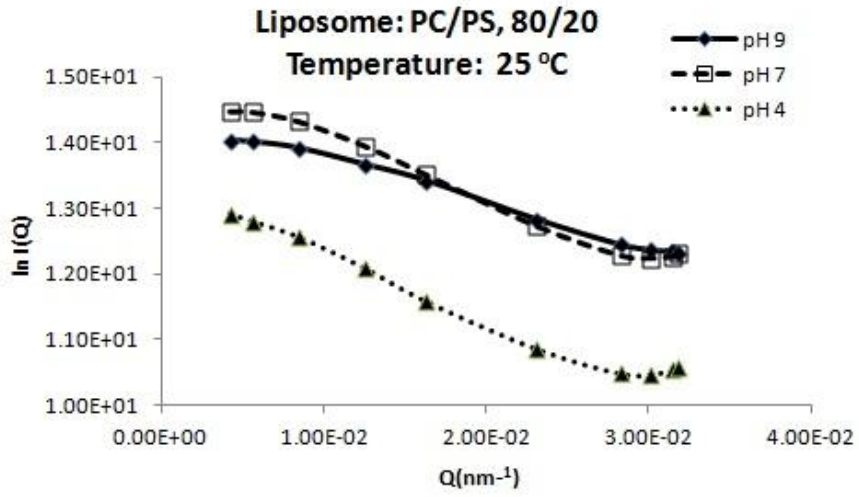
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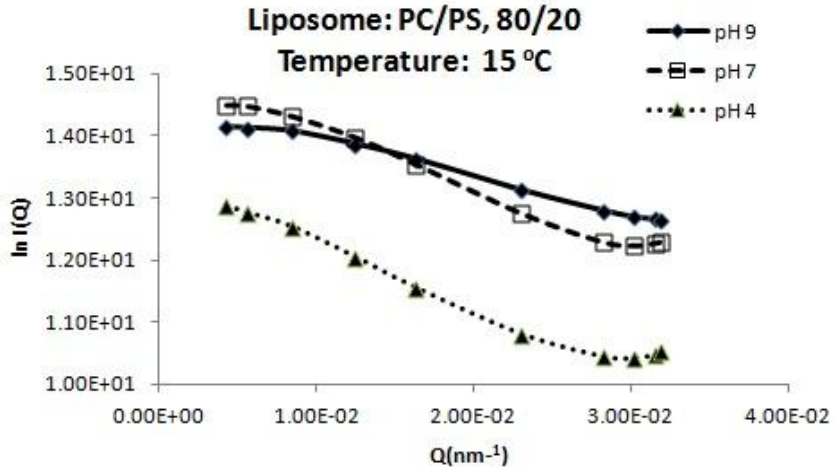
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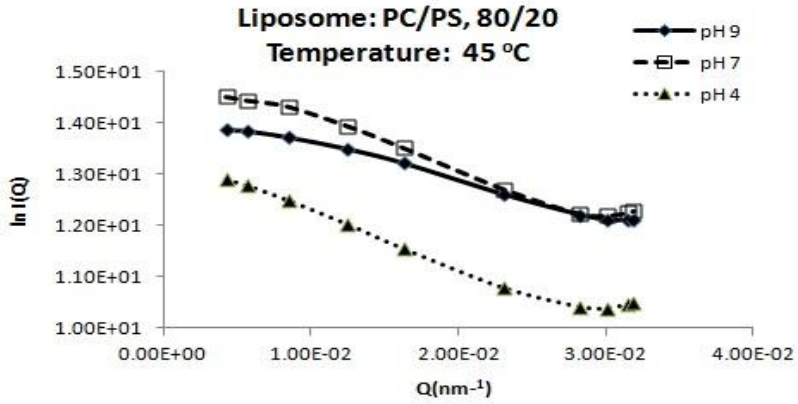
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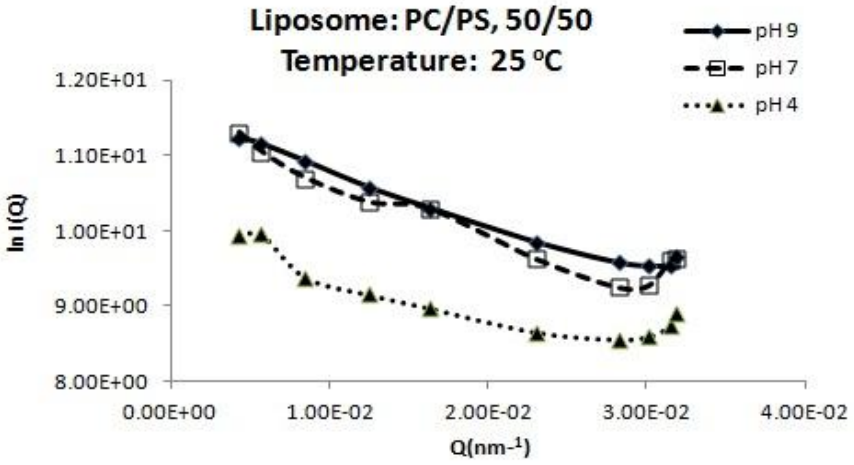
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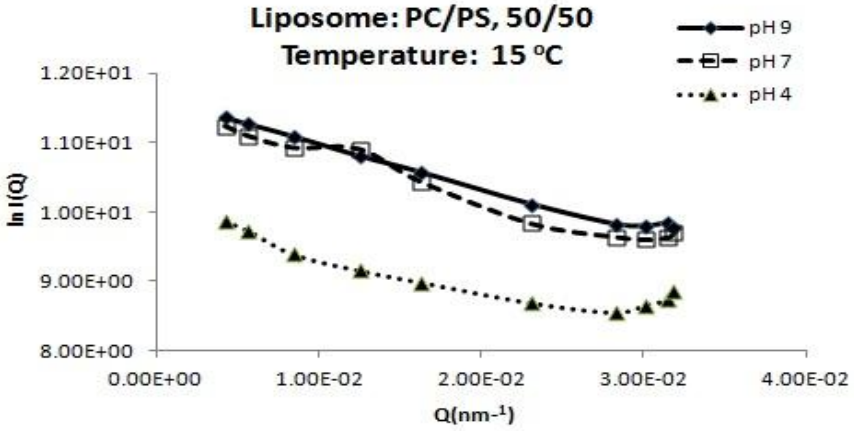
Q(nm-1)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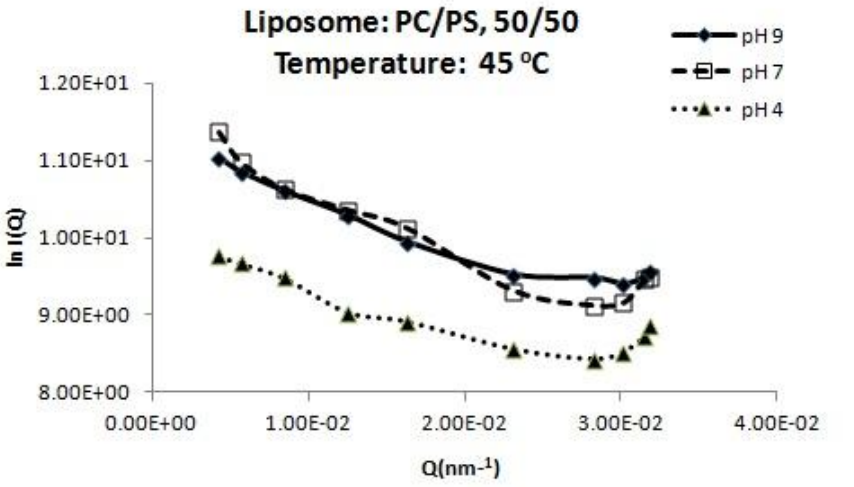
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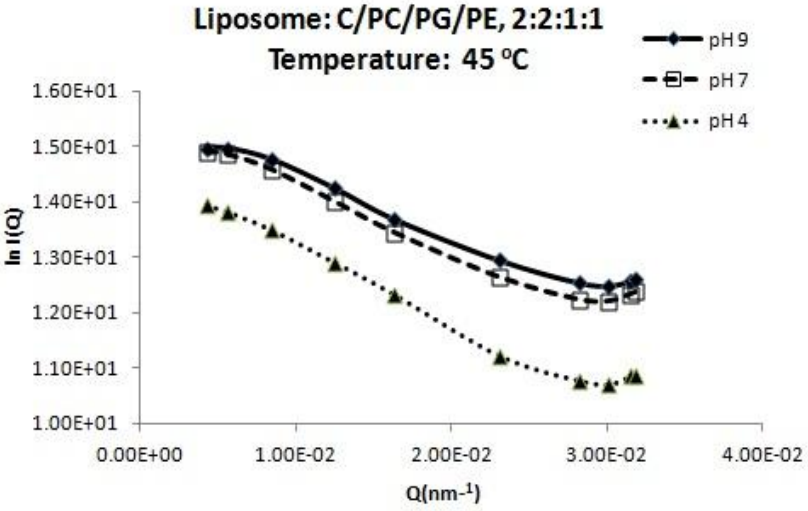
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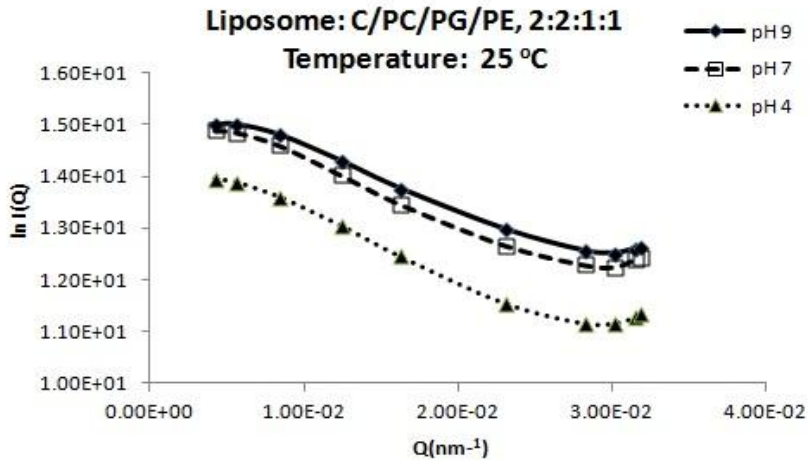
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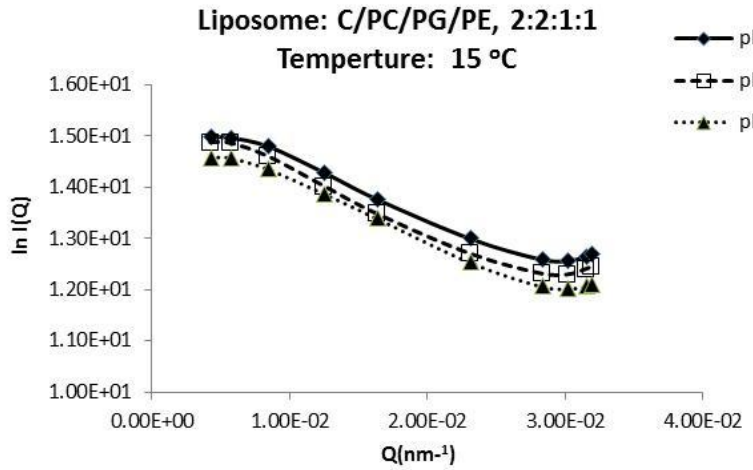
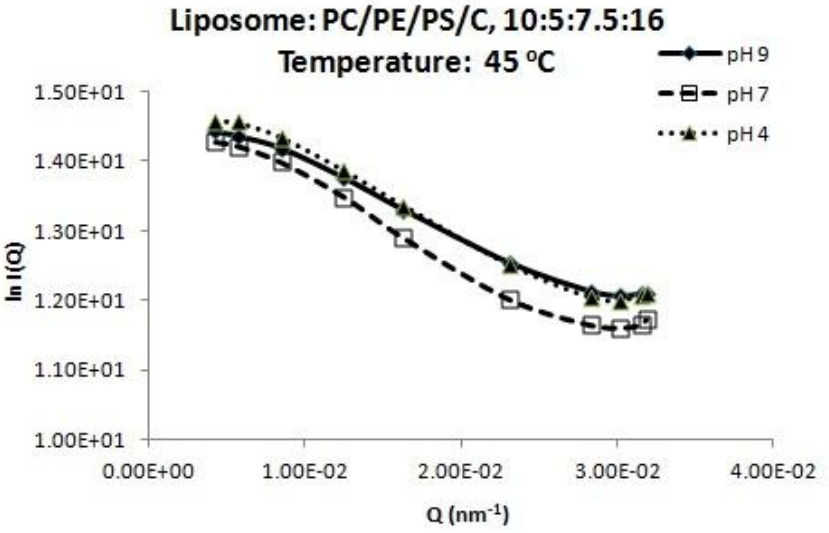
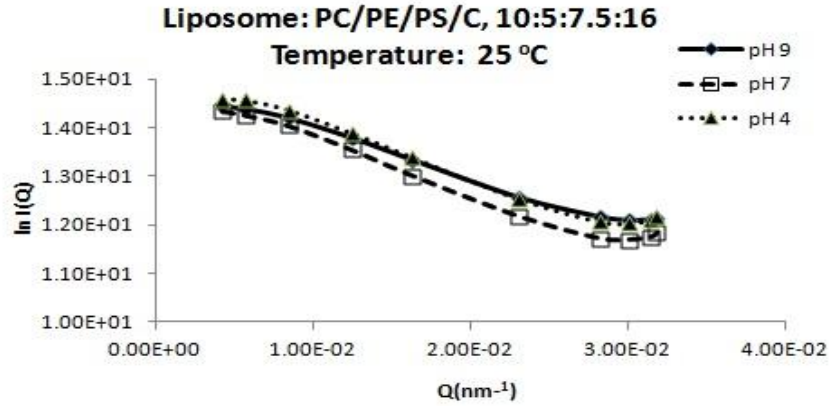
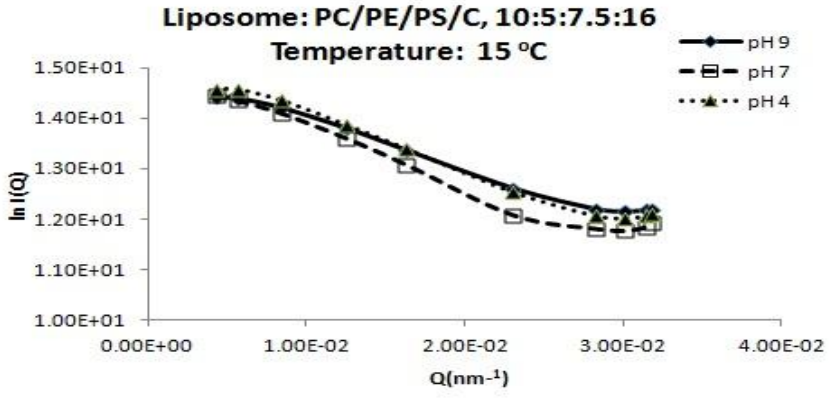
Q(nm-1)

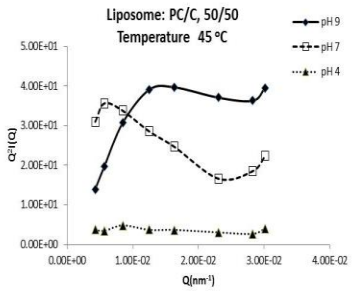

Liposome: C/PC/PG/PE, 2:2:1:1
Temperture: 15 °C
pH 9
pH 7
pH 4
1.60E+01
1.50E+01
1.40E+01
1.30E+01
1.20E+01
1.10E+01
1.00E+01
ln I(Q)
0.00E+00
1.00E-02
2.00E-02
3.00E-02
4.00E-02
Q(nm-1)

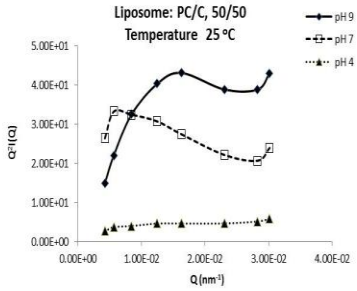

Liposome: PC/PE/PS/C, 10:5:7.5:16
Temperature: 45 °C
pH 9
pH 7
pH 4
1.50E+01
1.40E+01
1.30E+01
1.20E+01
1.10E+01
1.00E+01
ln I(Q)
0.00E+00
1.00E-02
2.00E-02
3.00E-02
4.00E-02
Q (nm-1)

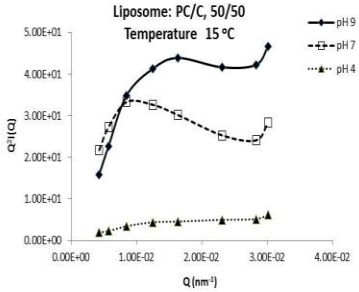

Liposome: PC/PE/PS/C, 10:5:7.5:16
Temperature: 25 °C
pH 9
pH 7
pH 4
1.50E+01
1.40E+01
1.30E+01
1.20E+01
1.10E+01
1.00E+01
ln I(Q)
0.00E+00
1.00E-02
2.00E-02
3.00E-02
4.00E-02
Q(nm-1)

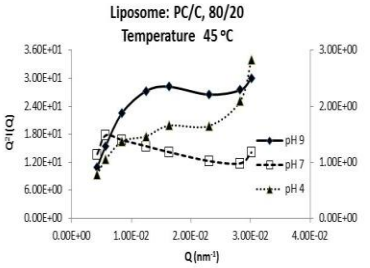

Liposome: PC/PE/PS/C, 10:5:7.5:16
Temperature: 15 °C
pH 9
pH 7
pH 4
1.50E+01
1.40E+01
1.30E+01
1.20E+01
1.10E+01
1.00E+01
ln I(Q)
0.00E+00
1.00E-02
2.00E-02
3.00E-02
4.00E-02
Q(nm-1)

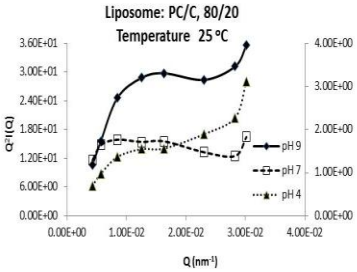
Kratky Plots

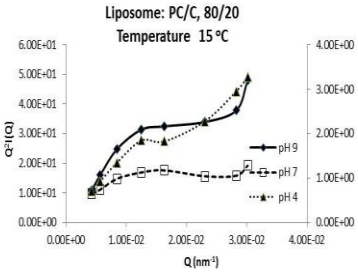


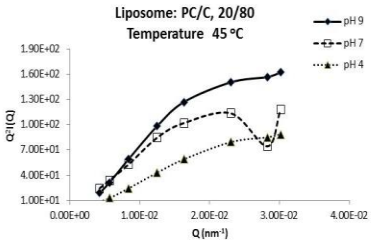


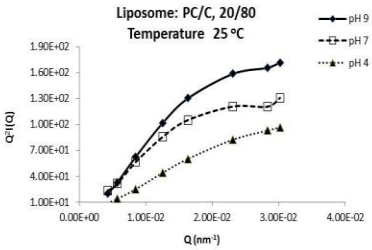


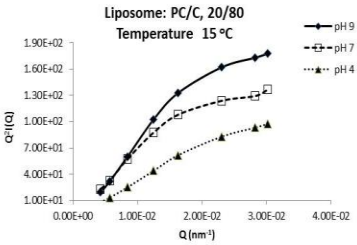


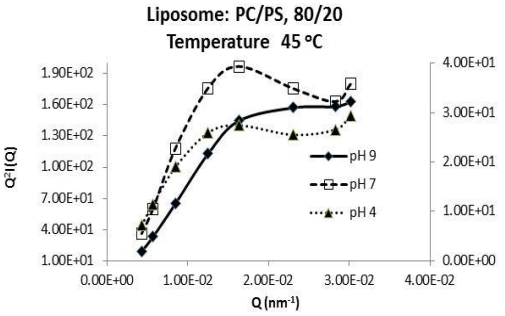


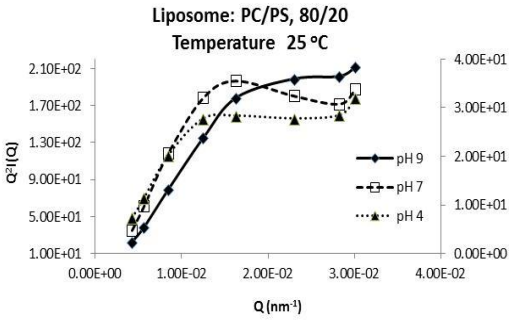


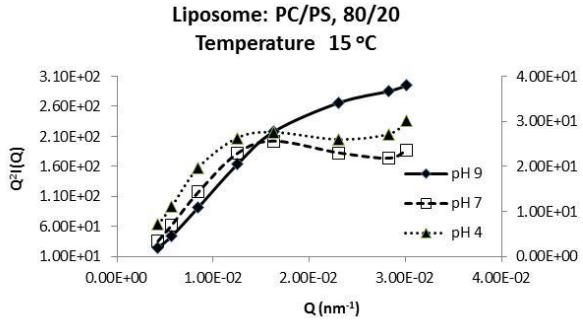


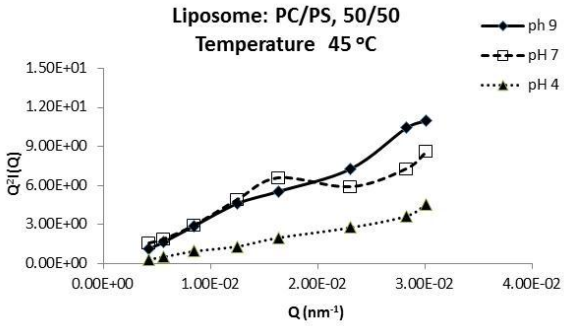


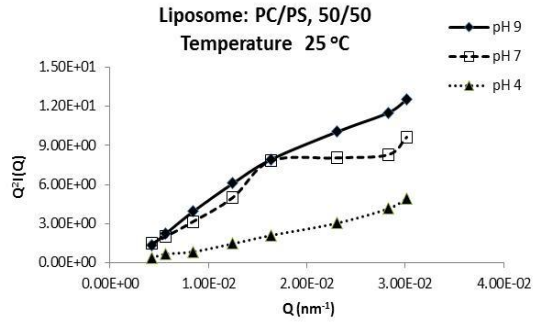


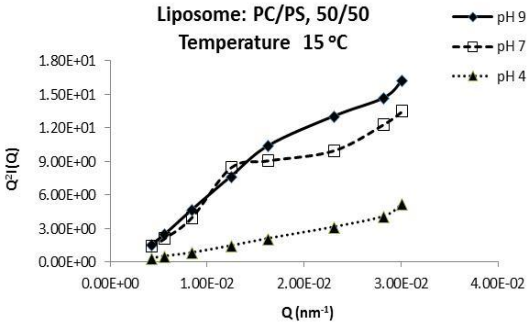

Liposome: PC/PS, 80/20
Temperature 45 °C
Q²I(Q)
Q (nm⁻¹)
pH 9
pH 7
pH 4

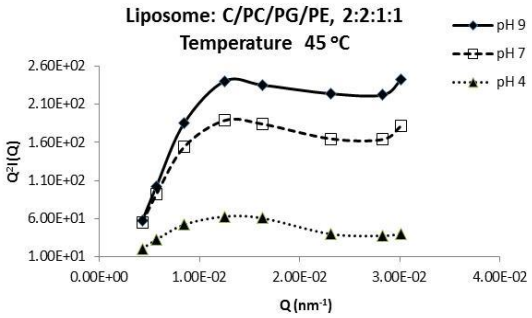

Liposome: PC/PS, 80/20
Temperature 25 °C
Q²I(Q)
Q (nm⁻¹)
pH 9
pH 7
pH 4

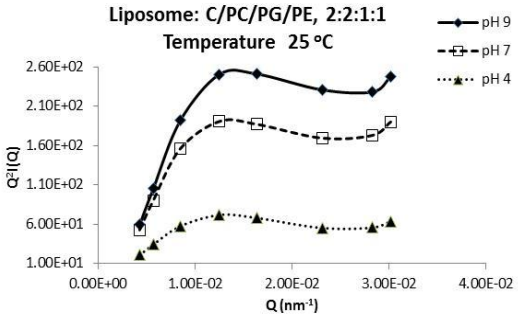

Liposome: PC/PS, 80/20
Temperature 15 °C
Q²I(Q)
Q (nm⁻¹)
pH 9
pH 7
pH 4

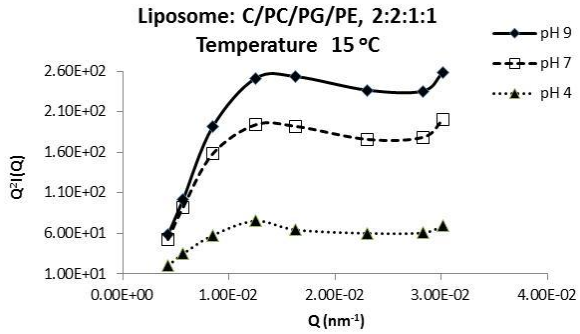

Liposome: PC/PS, 50/50
Temperature 45 °C
Q²I(Q)
Q (nm⁻¹)
ph 9
pH 7
pH 4

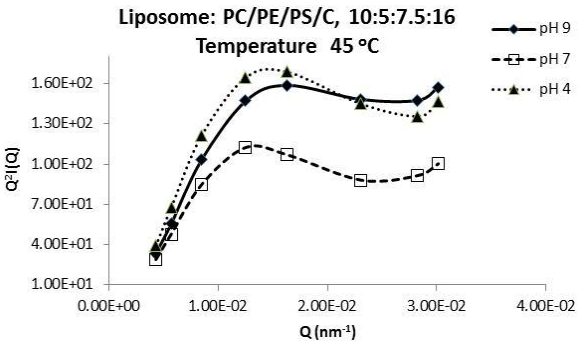

Liposome: PC/PS, 50/50
Temperature 25 °C
Q²I(Q)
Q (nm⁻¹)
pH 9
pH 7
pH 4

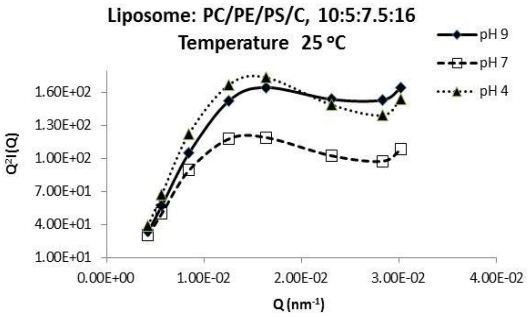

Liposome: PC/PS, 50/50
Temperature 15 °C
Q²I(Q)
Q (nm⁻¹)
pH 9
pH 7
pH 4

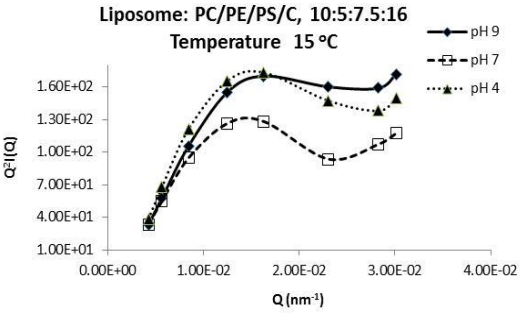

Liposome: C/PC/PG/PE, 2:2:1:1
Temperature 45 °C
pH 9
pH 7
pH 4
Q²I(Q)
Q (nm⁻¹)


Liposome: C/PC/PG/PE, 2:2:1:1
Temperature 25 °C
pH 9
pH 7
pH 4
Q²I(Q)
Q (nm⁻¹)


Liposome: C/PC/PG/PE, 2:2:1:1
Temperature 15 °C
pH 9
pH 7
pH 4
Q²I(Q)
Q (nm⁻¹)


Liposome: PC/PE/PS/C, 10:5:7.5:16
Temperature 45 °C
pH 9
pH 7
pH 4
Q²I(Q)
Q (nm⁻¹)


Liposome: PC/PE/PS/C, 10:5:7.5:16
Temperature 25 °C
pH 9
pH 7
pH 4
Q²I(Q)
Q (nm⁻¹)


Liposome: PC/PE/PS/C, 10:5:7.5:16
Temperature 15 °C
pH 9
pH 7
pH 4
Q²I(Q)
Q (nm⁻¹)

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VITA

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