Physicochemical characteristics of liposomes for quercetin delivery

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Abstract

Quercetin is a highly potential antioxidant for treatment of chronic diseases including neurological disorders. This study aims to physicochemically characterize quercetin-encapsulated liposomes including composition, surface charge, vesicle size, viscosity, pH and efficiency of entrapment. Preparation of quercetin liposomes (QL) by thin film hydration and extrusion using various molar ratios of liposomes, consisted of egg phosphatidyl choline (EPC) cholesterol (Chol) and span 60. A molar ratio of EPC:Chol:span 60 of 1:1:1 with an addition of 0.12 M quercetin gave a mean vesicle diameter of about 200 nm. An increase in the ratio of EPC and Chol resulted in an increase the vesicle size of the liposomes. Sodium alginate (0.05%) and chitosan (0.05%) were used to comparatively coat the QL. Entrapment efficiency (EE) of QL in polymer systems was comparable with original water system at 80-90% of EE. Surface charges of QL were altered in the presence of polyethylene glycol 400 (PEG), chitosan and alginate. EE of QL could be reduced when the particle size decreases. In case of polymer coated QL, QL in PEG presented the lowest EE but it was increased by an increase of PEG concentration. In chitosan system, EE of QL was very slightly changed when the chitosan $\geq$ 12.5%, while in alginate system there was no effect on EE at over 12.5% of alginate. These EE results of each system were significantly difference depended on polymers concentration manner ($p<0.05$). QL coated polymers were found in the pH range of 3.9 to 6.8 that are suitable for widely applications especially for transdermal route.

Keywords: Quercetin, Liposomes, Permeation, Delivery

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1. Introduction

Recently, polyphenolic compounds have become interested for health use and so product development has been extensively invested. Quercetin, 3,5,7,3',4'-pentahydroxyflavone, one of highly potential polyphenolic compounds, is found in a wide variety of vegetables, fruits and some flowers such as onion, grape, berry, apple, tea, etc. Its structure, as shown in Figure 1, contains two potential sites to scavenge free radicals. It can protect skin from oxidative damage (Formica and Regelson, 1995), act as anti-inflammatory, antihistamine, antimicrobial, and especially antioxidant. Its activities also included to protect chronic diseases which caused by free radicals, such as cardiovascular diseases, cancer, cataracts, inflammation, allergies, artherosclerosis, hypertension and some infectious diseases. (Verma and Kinoshita, 1976; Stavric, 1994; Conquer *et al*., 1998; Huang et al., 2006) Moreover, it could exert benefits to protect neurological diseases such as Parkinson or Alzheimer's disease (Yankner, 1996; Multhaup et al., 1997).

At the present, the product of quercetin has been found only in nutritional supplement because it has some unclear information for pharmaceutical use. Quercetin presents almost insoluble in water and instability in some certain conditions (Makris and Rossiter, 2000; Pinelo et al., 2004). These solubility and stability properties are important to explain drug permeation into the body, which is needed to show its action at the active sites. To overcome its poor solubility and instability in aqueous system, an encapsulation or entrapment of quercetin into vesicles was proposed to be the strategy.

Liposomal vesicles were used as carrier device for drug in a form of circular lipid bilayer ranging form nano- to micrometer size. Entrapment of labile compound such as quercetin might protect and minimize its degradation, and also improve solubility and thus enhance permeation.

The design of liposomes preparation for encapsulate requires appropriate ratio of liposomes composition; phospholipids, cholesterol, sorbitan monostearate 60, and order of mixing and coating to modify surface charge. Egg phosphatidylcholine (EPC) was used as a main liposomes structure. Cholesterol (Chol) and sorbitan monostearate 60 (span 60) were also selected to make liposomes integrity. Three kinds of polymer; polyethylene glycol, chitosan and alginate, represented different charge were used to coat liposomes. The results this study presented by comparison of liposomes characteristic of with respect to size, surface charge, and entrapment efficiency.

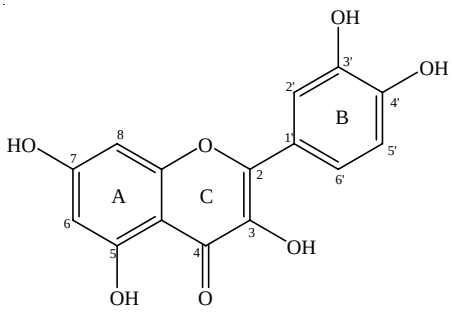

Figure 1. Structure of quercetin

This study was aimed to encapsulate quercetin in liposomes and study its physicochemical characteristic. The results were characterized by physicochemical properties of liposomes with respect to vesicle size, surface charge and entrapment efficiency.

2. Materials and methods

2.1. Materials

High-purity of egg L-α-phosphatidylcholine (EPC), cholesterol (Chol), quercetin dihydrate (98% HPLC purity), sorbitan monostearate 60 (span 60), chitosan, sodium alginate and polyethylene glycol 400 (PEG) were purchased from Sigma (St. Louis, Mo, U.S.A.). Other reagents used in this study include chloroform, ethanol, methanol and acetic acid were from BDH Laboratory Supplies (Poole, England). Other chemicals were at least reagent grade and all were used as received.

2.2. Preparation of liposomes and QL

Liposomal vesicles were prepared by thin film hydration with ultrasonic and extrusion technique (Priprem et al., 1999; Nii and Ishii, 2005). EPC, Chol, span 60, including quercetin as designed, were mixed and dissolved in choloform which was evaporated in a round bottom flask at 45 °C for 30 min until lipid film was formed. Then, the lipid film was hydrated to form vesicles which were then reduced and homogenized by sonication and extrusion via 0.2 and 0.1 μm PC membranes, respectively.

2.3. Study of liposomal compositions

An appropriate ratio of three compositions of liposomes including EPC, Chol and span 60 was optimized. The ratios of EPC, Chol and span 60 were varied by 2:1:1, 1:2:1, 1:1:1, 1:1:0, 1:1:0.5, 1:1:0.25 and 1:1:0.5 molar ratios. Each system was used for liposomes preparation following the process as above mentioned. Quercetin liposomes (QL) from each system were characterized by vesicle size and surface charge using laser diffraction techniques as well as an entrapment efficiency (EE).

2.4. Addition of polymers

Three polymers; chitosan, alginate and polyethylene glycol 400(PEG), were selected as each representing positive, negative and neutral charges, respectively. Quercetin liposome (EPC:Chol:span 60 at 1:1:1 molar ratio with additional of 0.12 M quercetin) vesicles were freshly prepared. In the final step of liposomes preparation, each polymeric system of PEG, chitosan and alginate was added to form liposome-coated polymer. Concentration of PEG solution in the suspension system was 25%, 12.5% and 5% (v/v), while the concentration of chitosan and alginate were 0.05%, 0.025% and 0.01% (v/v). Properties of each polymeric system with respect to viscosity and pH were determined before used. The vesicle-coated polymer were characterized their physicochemical properties.

2.5. Vesicle characterizations

2.5.1. Size distribution

The vesicles size distribution was determined by laser diffraction on a Mastersizer (hydro 2000 G/S/M model, Malvern Instruments, Worcestershire, UK). The measurements were performed three times at room temperature (25 1°C), using a 45 mm focus objective, and a beam length of 2.4 mm.

2.5.2. Zeta potential determination

Surface charges of vesicles were quantitatively analyzed by laser diffraction technique using Zetasizer (nano series model, Malvern instruments, Worcestershire, UK) at 25 °C, and illustrated as zeta potential.

2.5.3. Entrapment efficiency

An efficiency of quercetin loading was measured by ultracentrifugation method. All samples solution was centrifuged by using centrifugator at 10000 rpm for 1 hour. Free quercetin in supernatant was determined by UV-visible spectrophotometer (1240, Shimadzu, Japan) at 373 nm. The quercetin entrapment percentage was calculated from the relationship:

$$\%EE = \left(\frac{C_{tol} - C_{free}}{C_{tol}} \right) \; x \; 100$$

where EE is entrapment efficiency, C_{tol} is total quantity of added quercetin and $C_{f\backslash ree}$ is quantity of free quercetin detected from supernatant.

Table 1 Polymeric properties in viscosity and pH

Polymer	**Concentration (%, v/v)**	**Viscosity (cP) at 26±1°C**	**pH**
Polyethylene glycol	5	10.65 ± 0.07	4.13 ± 0.005
	12.5	9.14 ± 0.02	4.56 ± 0.002
	25	10.70 ± 0.85	4.51 ± 0.004
Chitosan	0.01	0.48 ± 0.00	3.76 ± 0.001
	0.025	2.56 ± 0.11	4.04 ± 0.001
	0.05	3.15 ± 0.04	3.94 ± 0.001
Sodium alginate	0.01	0.40 ± 0.09	6.85 ± 0.002
	0.025	2.75 ± 0.19	6.64 ± 0.001
	0.05	6.75 ± 0.04	6.76 ± 0.001

3. Results

3.1. Composition of QL

A series of lipid composition to form liposomes were investigated by varying each lipid and surfactant and displaying the results as molar ratio. For comparison, mean vesicle diameter and entrapment efficiency (EE) of each composition (or molar ratio) which gave vesicle size of less than 250 nm, were plotted to display possibilities of correlation as shown in Figure 2. The results which were not shown in Figure 2 were quercetin liposome (QL) with EPC:Chol:span 60 of 2:2:1 due to the vesicle size were rather large. Figure 2 suggests that a molar ratio of EPC:Chol:span 60 of 1:1:1 was optimized as it gave high EE and small vesicle size. This ratio could give an appropriate molecular orientation of EPC, Chol and span 60 to accommodate molecules of quercetin. Alterations of these well-oriented molecules of the mixtures lead to enlarge vesicle size and reduce EE.

3.2. Surface charges of QL

Zeta potential was used to determine the charge of the liposomes to suggest the physicochemical properties of the dispersion system. Figure 3 was plotted between zeta potential and vesicle size of each ratio system. They showed the same charge between -35 to -20 mV. It indicated that these liposomes formulation exhibit negative surface.

3.3. Effect of polymer on QL

Three polymers including polyethylene glycol 400, chitosan and alginate were used in order to coat liposomal vesicles. Varying the concentration of polymers, the measurement of viscosity and pH value for each was shown in Table. It was design such that all polymeric systems gave a similar range of viscosity. The pH of the systems was observed to be acidic. Decrease in pH of the system was found not more than 4 when chitosan was added. PEG and alginate increased the pH to 4 and 6, respectively.

Addition of PEG, chitosan and alginate affects the characteristic of QL is resulted by the factors of average mean size, surface charge and %EE. Figure 4 presents the comparison of an affect of polymers on vesicle size. PEG and alginate revealed there is no effect on size of QL, while the result was contrast in chitosan system. A positive charge of chitosan, the largest molecule, resulted to strong reaction with negative charge of EPC, leading to coated and increased vesicle size. Increasing of zeta potential, positive charge of chitosan is able to inhibit fewer negative charge of EPC. In contrast, reducing of zeta potential, negative charge of alginate was distribution in the surrounding interfacial region to increase negative charge. A large zeta potential in positive and negative charge, found at 0.05%chitosan and 0.05% alginate, tend to decrease tendency of vesicle fusion, resulting to enhance liposome stability. Figure 5 shows an efficiency of liposome in polymeric system to encapsulate quercetin. An efficiency of quercetin loading in liposomal vesicles was gently affected by these polymers. The efficiency was increased by the progress of polymeric concentration. In PEG system, the vesicles presented the least EE about 70% in the system of 5% PEG and then up to over 80% in the higher concentration system. Percentage of EE in chitosan and alginate systems was over than 80%. At these conditions, they were able to compare with original one in water system. That can be suggested that quercetin was encapsulated in liposome with shield by polymer at interfacial region.

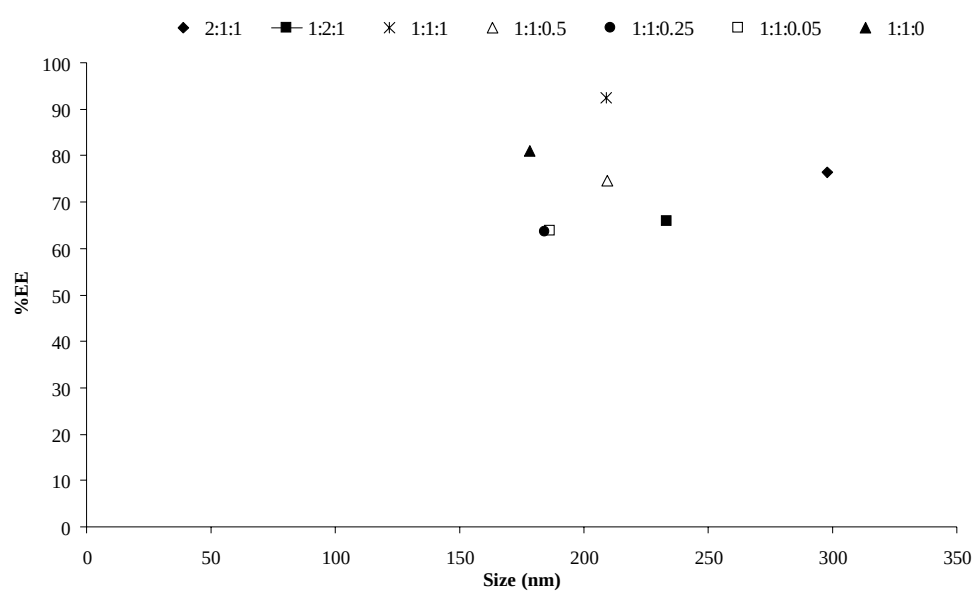

Figure 2. Relation between vesicle mean size and %EE of liposomal vesicle from each composition ratio (EPC:Chol:span 60)

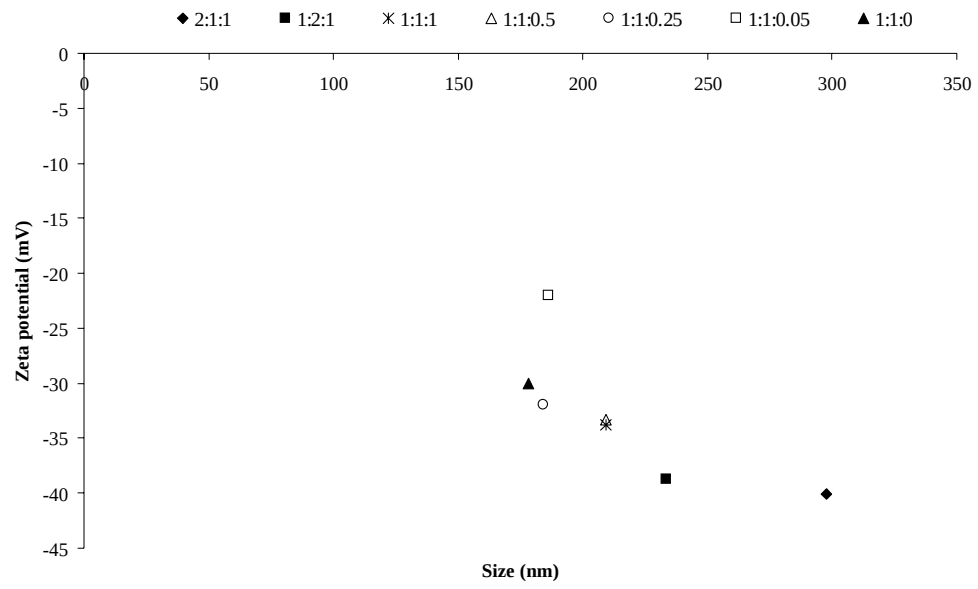

Figure 3. Relation between zeta potential and size of liposomal vesicle from each composition ratio (EPC:Chol:span 60)

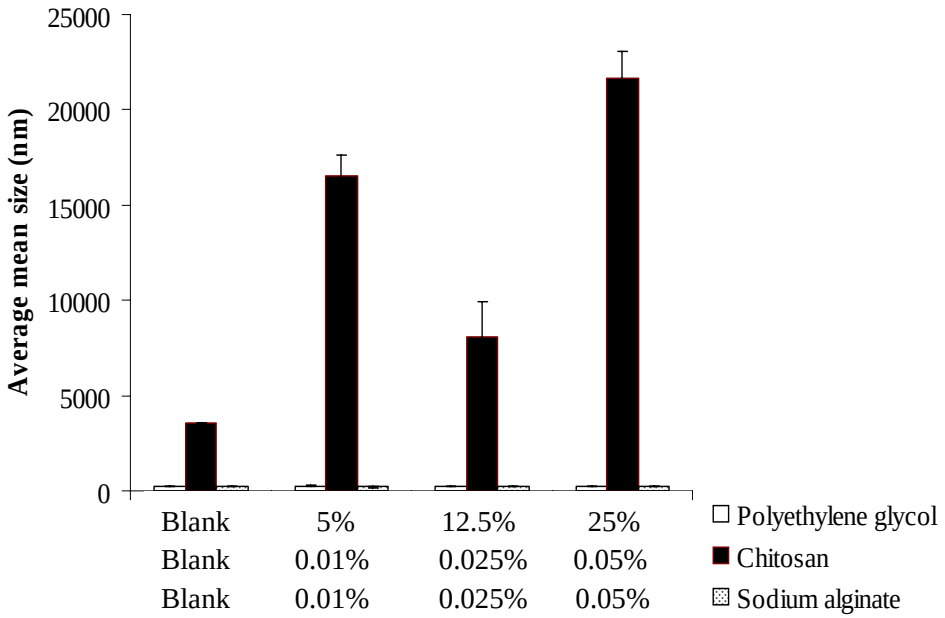

Figure 4. Average mean size of vesicle in three different polymeric systems of polyethylene glycol, chitosan and alginate

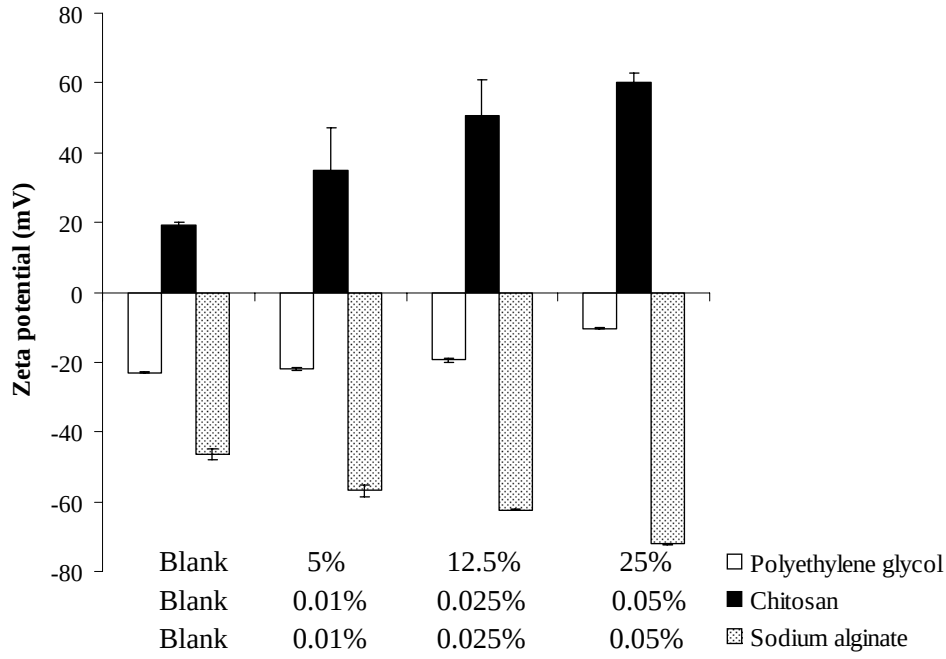

Figure 5. Zeta potential of vesicle in three different polymeric system of polyethylene glycol, chitosan and alginate

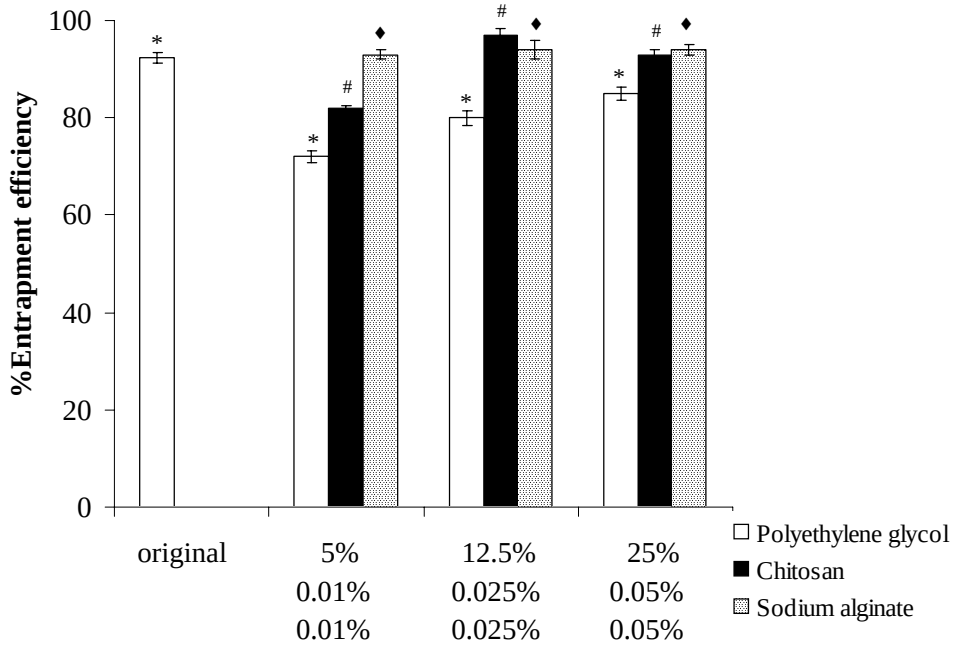

Figure 6. Entrapment efficiency of vesicles in three different polymeric systems of polyethylene glycol, chitosan and alginate The symbol sign (*, # and ◆) above the column indicates significant difference ($p < 0.05$) depended on polymers concentration manner.

4. Discussion

Quercetin liposomes were successfully prepared by using EPC, Chol, and span 60. EPC was used as a main structure of liposomes, while Chol and span 60 are additional composition used for integrity liposomes making. Appropriate ratio will give the suitable orientation of each composition to form liposome, resulting to integrity, stability and high efficiency of entrapment. The result showed that decreasing of span 60 reduced vesicle size, while an increase of EPC and Chol enhanced the size. An optimum ratio was found at equal ratio of 1:1:1, which revealed the highest efficiency of entrapment. As EPC structure has two carbon chains, which are one straight chain configuration and another tilt chain. This is able to bring other adjacent molecule like either cholesterol and/or span 60 into closer proximity. Cholesterol has been severally inserted into phospholipids membranes with its hydroxyl group oriented towards the aqueous surface, and the aliphatic chain aligned parallel to the acyl chain in the centre of bilayer. Additional of micelle, long chain of span 60 was inserted into bilayer of liposomes in order to give high elasticity and deformable vesicle (Honeywell-Nguyen et al., 2002). These appropriate molecular orientations of EPC, Chol and span 60 gave small vesicle size and high EE. The present of surface charge of this vesicle can be found in negative charge. Since the charge, represented by phosphate group, is not neutralized by the headgroup of

choline, thus surface charge of all ratios presented almost in the same region. However, different change of liposome can take place with the passage of time. The phospholipids can undergo chemical degradation, oxidation and hydrolysis, leading to unstable form. Especially liposome in aqueous system, the suspension may aggregate, fuse, or leak their content. This study tries to overcome the problem of liposome instability by using polymers. Polymers, represented different charge, were used to design surface charge of liposome. PEG, chitosan and alginate were selected for this study for neutral, positive and negative charge, respectively. PEG, in the last decade, has been increased interest to stabilized lipid vesicles (sterically stabilized liposomes or stealth liposomes) for drug delivery (Kaul and Amiji, 2002; Angelini et al., 2007). PEG can shield the surface of liposome by covalently linked to the headgroup of lipids, thus providing long-lived liposomes. PEG is not only able to stabilize liposome, it can also decrease the leakage of entrapped-substance (Hashizaki et al., 2005) and present long circulation *in vivo* (Kaul and Amiji, 2002). Chitosan, a polysaccharide, has a positive charge and is mucoadhesive. Therefore, it has been used extensively in drug delivery applications. Chitosan has been used to minimize the disruptive influences of liposome by the formation of a polymeric membrane around the liposome (Guo et al., 2003; Agnihotri et al.,2004). Alginate, an anionic biopolymer consisting of linear chains of α-L-glucuronic acid and β-D-mannuronic acid residues, is biocompatible and non-toxic. Before each polymer was used, their properties including viscosity and pH were proved. Viscosity of each polymeric system were adjusted, followed our previous study which quercetin liposome in 25%PEG was injected to rat brain via intranasal administration. The study showed results of quercetin liposomes to reduce anxiety and enhance cognitive function [Priprem et al., 2008]. As well as pH value of these three polymeric systems, found in the range of 3.9 to 6.8, are suitable for widely application especially for transdermal route (pH of normal skin ~ 4-6.5) and mucosal route (pH between 6.3 -7.3). Nature of each polymer effected to vesicles properties which characterized by size, surface charge and EE. Chitosan only presented significantly effect to increase vesicle size. Because the positive charge of chitosan might directly interact with negative charge of phosphate group of phosphatidyl choline. Moreover, chitosan represents long-chain polymers having molecular mass up to several million Daltons, that gave the size of liposome is bigger than the others polymer as well as display zeta potential in positive charge. Whereas, in PEG and alginate systems, size of liposome were not change from original one. Since PEG and alginate represent neutral and negative charge which there is no strongly react with liposomes like chitosan. Thus, both polymers will shield at interfacial region of liposome, resulting to display zeta potential of liposome close to neutral in PEG and negative charge in alginate system. The further concentration effect of each polymer on zeta potential of liposomes was proved. The effect was enhanced by an increasing concentration of polymer which found an increase zeta potential in positive and negative charge with the progress of chitosan and alginate concentration, respectively. Then the zeta potential of liposome approached to neutral with an increase of PEG. A large of zeta potential in

positive and negative charge at the surface (> 60mV) is able to improve the stability of vesicles due to there is a force to prevent the vesicles coming together and flocculating. A long chain of polymer is also play steric hindrance, leading to stabilize liposome, as well. Consequently, the products from this study, display different surface charge showed beneficial to decrease possibility tendency of fusion, enhance liposome stability and increase entrapment efficiency. It can be suggested that they are potential products for widely applications, especially for drug delivery system.

5. Conclusion

Quercetin liposomes (QL) can be prepared by the ratio of EPC, Chol and span 60 at 1:1:1 including quercetin at 100ug/mL. Three polymers were applied for QL, resulting to vesicles size about 200 nm, except in chitosan system which higher due to its strong reaction and larger structure. High concentration of chitosan and alginate resulted a large zeta potential of > +60 and −60 mV, respectively, as well as maintain high efficiency of quercetin entrapment. These polymers were not only used for surface charge design, they also useful for reveal steric effect of vesicle to decrease possibility of vesicle fusion. This characteristic may gave the product stay in the circulation in longer time. The results gave the required different charge products with stabilized and still maintain high entrapment efficiency. These QL products can support for various applications with various administration routes.

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References

Agnihotri, S.A., Mallikarjuna, N.N. and Aminabhavi, T.M. 2004. Review: Recent advances on chitosan-based micro- and nanoparticles in drug delivery. **J. Control. Rel.** 100: 5-28.

Angelini, G., Boncompagni, S., De Maria, P., De Nardi, M., Fontana, A., Gasbarri, C. and Menna, E. 2007. Layer-by-layer deposition of shortened nanotubes or polyethylene glycol-derivatized nanotubes on liposomes: A tool for increasing liposome stability. **Carbon.** 45: 2479-2485.

Conquer, J.A., Maiani, G., Azzini, E., Raguzzini, A. and Holub, B.J. 1998. Supplementations with quercetin markedly increase plasma quercetin concentration without effect on selected risk factors for heart disease in healthy subjects. **J Nutr.** 128: 593-597.

Formica, J.V. and Regelson, W. 1995. Review of the biology of quercetin and related bioflavonoids. **Food Chem Toxicol.** 33: 1061-1080.

Hashizaki, K., Taguchi, H., Itoh, C., Sakai, H., Abe, M., Saito, Y. and Ogawa, N. 2005. Effects of poly(ethylene glycol) (PEG) concentration on the permeability of PEG-grafted liposomes. Chem. **Pharm.** Bull. 53(1): 27-31.

Honeywell-Nguyen P., de Graaff A.M., Wouter Groenink H.W. and Bouwstra J.A. 2002. The *in vivo* and *in vitro* interactions of elastic and rigid vesicles with human skin. **Biochim Biophys Acta.** 1573: 130-140.

Huang, S.L., Hsu, C.L. and Yen, G.C. 2006. Growth inhibitory effect of quercetin on SW 872 human liposarcoma cells. **Life Sci.** 79: 203-209.

Guo, J., Ping, Q., Jiang, G. Huang, L. and Tong, Y. 2003. Chitosan-coated liposomes: characterization and interaction with leuprolide. **Inter J Pharm.** 260: 167-173.

Kaul, G. and Amiji, M. 2002. Long-circulating poly(ethylene glycol)-modified gelatin nanoparticles for intracellular delivery. **Pharm. Res.** 19(7): 1061-1067.

Makris, D.P. and Rossiter, J.T. 2000. Heat-induced, metal-catalyzed oxidative degradation of quercetin and rutin (quercetin 3-*O*-rhamnosylglucoside) in aqueous model systems. **J. Agric. Food Chem.** 48: 3830-3838.

Multhaup, G., Ruppert, T., Schlicksupp, A., Hesse, L., Beher, D., Masters, C.L. and Beyreuther, K. 1997. Reactive oxygen species and Alzheimer's disease. **Biochem. Pharmacol.** 54: 533-539.

Nii, T. and Ishii, F. 2005. Encapsulation efficiency of water-soluble and insoluble drugs in liposomes prepared by the microencapsulation vesicle method. **Int J Pharm.** 298: 198-205.

Pinelo, M., Manzocco, L., Nu ez, M.J. and Nicoli, M.C. 2004. Solvent effect on quercetin antioxidant capacity. **Food Chem.** 88: 201-207.

Priprem, A., Rahman, Y.E., Juhn, S.K., Lekkaraju, A. and Pituksuteepong, T. 1999. Liposome-encapsulated ampicilin. **Mahidol J.** 6: 55-57.

Priprem, A., Watanatorn, J., Sutthiparinyanont, S., Phachonpai, W. and Muchimapura, S. 2008. Anxiety and cognitive effects of quercetin liposomes in rats. **Nanomedicine: Nanotec. Biol. Med.** 4: 70-78.

Stavric, B. 1994. Quercetin in our diet from potent mutagen to probable anticarcinogen. **Clin Biochem.** 27: 245-248.

Verma, S.D. and Kinoshita, J.H. 1976. Inhibition of lens aldose reductase by flavonoids. Their possible role in the prevention of diabetic cataracts. **Biochem Pharmacol.** 25: 2502-2513.

Yankner, B.A. 1996. Mechanisms of neuronal degeneration in Alzheimer's disease. **Neuron** 16: 921-932.