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# Effects of operating parameters on the efficiency of liposomal encapsulation of enzymes

Sang Youn Hwang[a,1], Hak Kyung Kim[a,2], Jaebum Choo[b], Gi Hun Seong[b], Thai Bao Dieu Hien[c], E.K. Lee[c,*]

[a] Department of Chemical Engineering, Hanyang University, Ansan, Republic of Korea
[b] Department of Bionano Engineering, Hanyang University, Ansan, Republic of Korea
[c] College of Bionanotechnology, Gachon University, Seongnam, Republic of Korea

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ABSTRACT

Encapsulation of active proteins in the hydrophilic core of vesicular liposomes is important for developing a therapeutic protein carrier system. The efficiency of liposomal encapsulation of proteins is generally low. A better understanding of the fundamental mechanisms of encapsulation is needed to increase this efficiency. In this study we investigated the effects of operating parameters such as phospholipid concentration, buffer pH and ionic strength, protein size and surface charge, and liposome size on the enzyme encapsulation yield. Four model enzymes of different molecular weights and isoelectric points (trypsin, horseradish peroxidase, enterokinase and hyaluronidase) were encapsulated into three different sized liposomes (125 nm, 194 nm, and 314 nm in mean diameter). Relatively inert and neutral DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine) was used as the main phospholipid in the liposomes. Size exclusion chromatography was used to separate the enzyme-encapsulated liposomes from the free enzyme, and the encapsulation yield was determined from the peak area. The encapsulation yield was generally low ranging from ca. 5% to 20%, and did not depend much on the molecular weight of the enzyme encapsulated. Larger liposomes had higher encapsulation yields. The electrostatic interaction between the phospholipid and enzyme was the most significant parameter in determining the encapsulation yield. Thus adjusting buffer pH and ionic strength and adding charged phospholipids to the liposome preparation to impart electric charge to the lipid bilayer could significantly improve the yield. This approach can be used to optimize the liposomal encapsulation of clinically significant proteins.



## 1. Introduction

Liposomes are colloidal vesicles of a few nanometers to micrometers in diameter with one or more lipid bilayers surrounding a hydrophilic core [1]. Liposomes have been approved by the US FDA as in vivo carriers of drugs because of their biocompatibility [2]. They are favored as drug delivery vehicles for two main reasons. First, liposomes can encapsulate either hydrophilic substances in the hydrophilic core or hydrophobic substances in the lipid bilayer space [2,3]. Second, liposome surfaces can be readily modified with an appropriate moiety for targeted delivery and also with a biocompatible polymer, such as polyethylene glycol (PEG), to significantly improve in-serum stability by reducing blood opsonization and macrophage clearance [4,5]. Thus, liposomes are a good candidate for in vivo delivery of protein/peptide drugs.

Liposomal encapsulation of low molecular weight drugs, such as doxorubicin, has been extensively studied and some of them are commercialized [2]. However, the encapsulation of larger therapeutic proteins, nucleotides, and enzymes is still a growing area in the early stages of development. Several pharmaceutical proteins have been encapsulated in liposomes for various purposes. For instance, liposome-encapsulated asparaginase was used as an anti-P1534 tumor agent and significantly reduced antibody production [6]. Liposome-encapsulated superoxide peroxidase reduced oxidative stress in gerbil brain cells and resulted in decreased membrane peroxidation [7]. Encapsulating tissue plasminogen activator (tPA) in liposomes reduced the required dose by four-fold compared with the native protein because of improved thrombolytic activity [8]. Recombinant interleukin-2 was encapsulated, improving the in-serum stability by eight-fold [9]. In sum, liposomal encapsulating of therapeutic proteins is becoming an integral part of targeted delivery and in vivo uptake [1,10].

For protein encapsulation, small unilamellar vesicles (ULVs) around 100 nm in diameter as well as large unilamellar vesicles

* Corresponding author.
*E-mail address:* eklee@kyungwon.ac.kr (E.K. Lee).
[1] Hanmi Research Center, Hanmi Pharmaceuticals Co. Ltd., Hwaseong-si, Republic of Korea.
[2] QA Team, Celltrion Inc., Incheon, Republic of Korea.

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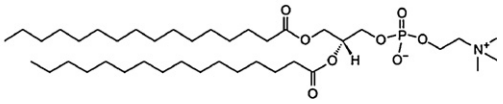
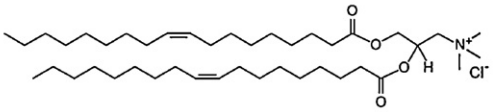
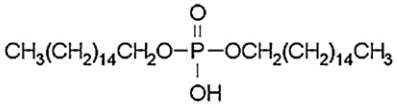
1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)
Neutral charge
MW = 734.04



1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)
Positive charge
MW = 698.55



Dicetyl phosphate (DCP)
Negative charge
MW = 546.86




Cholesterol
MW = 386.66

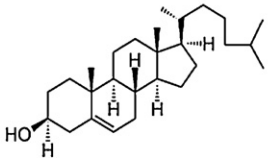



**Fig. 1.** Molecular structures of phospholipids used in this study.

(LUVs), 200–800 nm, are frequently used [2]. Various forms and sizes of liposomes can be manufactured using different preparation methods such as lipid film hydration or hand shaking, sonication, freeze-dried rehydration, reverse-phase evaporation, and detergent depletion [11,12]. Among these, the reverse-phase evaporation and detergent depletion methods are not suitable to encapsulate proteins for in vivo use since the organic solvents and/or detergents used can denature proteins and are difficult to remove completely [11]. To encapsulate large molecules such as proteins, polypeptides, nucleic acids, and antibodies, ULVs or LUVs prepared by the film hydration method followed by extrusion seemed to be the liposome of choice [12]. Common methods to isolate liposomes containing protein include ultracentrifugation, ultrafiltration, and size exclusion chromatography (SEC). The high pressure applied in ultracentrifugation (>100,000 × $g$) may damage the liposomes to release their contents [13]. Membrane fouling in ultrafiltration may result in incomplete separation between liposomes and unencapsulated materials [11]. SEC could successfully separate liposomes and unencapsulated material, thus minimizing the loss of the encapsulated material [14,15].

Several operating parameters influence the liposomal encapsulation yield of proteins; they include phospholipid type and concentration, buffer salt and its concentration, pH and ionic strength, liposome size (diameter), and protein concentration [13]. Using egg phosphatidylcholine and tissue-type plasminogen activator (t-PA) as a model phospholipid and protein, Heeremans et al. investigated the effects of the several operating parameters on liposomal encapsulation yield [13]. They found that: (1) the encapsulation yield was proportional to the phospholipid concentration up to 100 mM, where it reached a plateau; (2) a buffer pH near the protein's pI value and a lower ionic strength (10 mM HEPES buffer) provided a higher yield; (3) a lower protein concentration (0.3 mg/ml) provided a better yield than higher concentrations (0.75 and 1.5 mg/ml); and (4) larger liposomes (ca. 0.5 μm mean diameter) encapsulated about 1.4-fold more protein than smaller liposomes (ca. 0.23 μm). In order to optimize protein encapsulation, however, mechanistic and comprehensive understanding of how protein size (i.e., molecular weight), liposome diameter, and electric charge differences between the phospholipid and protein affect encapsulation is needed.

In this study, we used DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine), which is neutral and relatively inert, as the main phospholipid for liposome preparation. Phosphatidylcholine with carbon chains of 16 or 18 carbons is common in mammalian cell membranes [16] and it has a relatively high phase transition temperature ($T_m$ = 41.5 °C) because it is saturated [17]. Since it is uncharged, electrostatic interactions between liposomes are minimal, making it less prone to aggregation. We focused on the effects of liposome size (nominal diameter of 100 nm, 200 nm, and 400 nm) and enzyme size (molecular weight) on the encapsulation yield. The following four enzymes with different molecular weights and isoelectric points were used: trypsin (TRP; 24 kDa, pI 10.5), horseradish peroxidase (HRP; 40 kDa, pI 7.2), enterokinase (ENT; 55 kDa, pI 4.2), and hyaluronidase (HYA; 150 kDa, pI 4.9). Furthermore, the effect of electrostatic interactions between the enzyme surface and the liposomal bilayer was studied by determining how adding a charged phospholipid to the liposomes affected the encapsulation yield. We found that the protein encapsulation yield depended primarily on the electrostatic associations between the enzyme and phospholipid, which suggests that adding an appropriate charged phospholipid to the liposome composition as well as adjusting the buffer pH and ionic strength could improve the yield.