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양친매성 고분자전해질 도입을 통한 리포좀의 안정도 증진에 관한 연구

조 은 철† · 임 형 준 · 김 준 오 · 장 이 섭

아모레퍼시픽 기술연구원 피부과학연구소
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Improved Stability of Liposome by Association of Amphiphilic Polyelectrolytes

Eun Chul Cho†, Hyung Jun Lim, Junoh Kim, and Ih Seop Chang

Amorepacific R&D Center, 314-1, Bora-dong, Giheung-gu, Yongin-si, Gyeonggi-do 446-729, Korea
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요 약: 일반적으로 cyclodextrin (CD)은 liposome의 구조를 불안정하게 만드는 것으로 알려져 있다. 본 연구에서는 양친매성 고분자전해질을 리포좀에 도입하여 CD에 대한 리포좀의 안정도를 증진시키는 연구를 수행하였다. Transmission electron microscopy와 photocorrelation spectroscopy 결과들로부터 β-CD (βCD)와 hydroxypropyl-βCD (HPβCD)를 함유하는 리포좀에 고분자가 도입되었을 때 CD를 함유하는 phosphatidylcholine (PC)-cholesterol (Chol) 리포좀보다 우수한 구조적 안정성을 나타내었다. 또한, rhaponticin (Rh)을 HPβCD에 포접시키고 이를 함유하는 PC-Chol 리포좀과 고분자가 도입된 리포좀의 안정도를 비교해 보았을 경우도 마찬가지로 고분자가 도입된 리포좀이 월등히 향상된 구조적인 안정성을 나타내는 것을 확인하였다. 이와 더불어 guinea pig의 피부조직을 사용하고 franz-cell을 통한 *in vitro* 피부흡수실험을 수행한 결과, HPβCD에 의해 가용화된 Rh의 피부흡수가 고분자가 도입된 리포좀에 의해 증진됨을 확인할 수 있었다. 상기 결과들은 양친매성 고분자 전해질의 도입에 따라 CD에 의해 가용화된 특정한 활성성분을 함유하는 리포좀의 구조적인 안정성을 효과적으로 증진시킬 수 있음을 확인시켜 주었으며, 이렇게 향상된 리포좀의 구조적인 안정성을 통해 약물전달시스템 측면에서 많은 응용이 가능할 것으로 생각된다.

Abstract: It has been generally known that liposomes become unstable when they contain cyclodextrins (CDs). Our present studies demonstrate that these liposomes can be stable by association of amphiphilic polyelectrolytes. Transmission electron microscopy and photocorrelation spectroscopy results showed that polymer-associated liposomes containing CDs (β-CD (βCD) and hydroxypropyl-βCD (HPβCD)) were more stable than phosphatidylcholine (PC)-cholesterol (Chol) liposomes containing these CDs. We also compared the stability of PC-Chol liposomes with polymer-associated liposomes containing HPβCD complexed with water-insoluble drug, rhaponticin (Rh). Two liposomes were relatively stable when HPβCD did not contain Rh, but Rh-HPβCD complexes triggered the disruption of PC-Chol liposomes. In contrast, polymer-associated liposomes containing Rh-HPβCD complexes maintained its stability over 6 months. The skin permeation test demonstrated that drugs solubilized by CDs were delivered better into the skin of guinea pig by using polymer-associated liposomes than by using PC-Chol liposomes. Above results showed that polymer-associated liposomes gave an effective way to stabilize the liposomes containing drug-loaded CDs, which gives an application of liposomes in drug delivery systems.

Keywords: *cyclodextrins, liposomes, vesicles, polyelectrolytes, rhaponticin*

† 주 저자 (e-mail: enjo@amorepacific.com)

1. Introduction

Drug-in-cyclodextrins-in-liposomes system as a new concept in biomedical applications has been proposed because cyclodextrins (CDs) feature a relatively non-polar cylindrical cavity, which can bind and thereby solubilize a wide variety of hydrophobic molecules[1-3]. This approach can be useful to increase drug solubility and stability[4,5] and better control the *in vivo* fate of poorly water-soluble drugs, avoiding the rapid release observed after conventional incorporation into the liposome lipid bilayer[6-8].

Meanwhile, CDs are known to remove lipid components from cell membranes by forming inclusion complexes[9]. Several groups have used differential scanning calorimetry to clarify the extraction of phospholipids from vesicles by CDs[10,11]. In addition, CDs-induced disruption or reorganization of phospholipid vesicles was monitored through carboxyfluorescein leakage from vesicles[12,13]. These studies demonstrated that the degree of destabilization of vesicles by CDs was highly sensitive to the concentration of CDs. Such destabilization effect of CDs on the vesicle formation might reduce the benefits of liposome carrier. Therefore, the improvement of the vesicle stability of liposomes in the presence of CDs is required.

In this study, we investigated the influence of β-CD (βCD), hydroxypropyl-βCD (HPβCD), and rhaponticin (Rh)-HPβCD complexes (Rh-HPβCD) on the vesicle stability of phosphatidylcholine (PC)-Cholesterol (Chol) liposomes and polymer-associated PC-Chol liposomes in aqueous media by using transmission electron microscopy (TEM), photocorrelation spectroscopy (PCS), and *in vitro* skin permeation test. Our present studies demonstrate that the stability of vesicles can be improved by association of amphiphilic polyelectrolytes.

2. Experimental Section

2.1. Preparation of Polymer-associated Liposome

The synthesis of poly (methylmethacrylic acid-co-stearyl methacrylate) (poly (MMAc-co-SM)) was described in our previous research[14,15]. The PC-Chol liposomes and polymer-associated liposomes containing CDs or Rh-HPβCD were prepared by using high pressure homogenization techniques[16].

Table 1. Composition of Liposomes and Polymer-associated Liposomes Containing CDs or Rh-HPβCD

Code	Composition (g)				
	Lipid components		Polymer	CD	Rh
	PC	Chol			
L-only	4.73	0.264	-	-	-
L-β	4.73	0.264	-	1.14 (βCD)	-
L-HPβ	4.73	0.264	-	5.84 (HPβCD)	-
PL-only	3.32	0.185	1.5	-	-
PL-β	3.32	0.185	1.5	1.14 (βCD)	-
PL-HPβ	3.32	0.185	1.5	5.84 (HPβCD)	-
L-HPβ-Rh1	4.73	0.264	-	5.84 (HPβCD)	1
L-HPβ-Rh2	4.73	0.264	-	11.7 (HPβCD)	2
PL-HPβ-Rh1	3.32	0.185	1.5	5.84 (HPβCD)	1
PL-HPβ-Rh2	3.32	0.185	1.5	11.7 (HPβCD)	2

Table 1 lists the compositions of lipid components, polymer, CDs, Rh for the preparation of liposomes and polymer-associated liposomes. The mole ratio of PC (S100-3; a mixture of oleoyl-palmitoyl and oleoyl-stearoyl, Lipoid) and Chol (Aldrich, USA) was 9 : 1 and the ratio of the lipid components (PC + Chol) and polymer was 7 : 3 on a weight basis. These components were solubilized in 40 mL ethanol by heating up to 60℃ and the solution was transferred into 300 mL aqueous solution containing CDs and the mixture was homogenized (5000 rpm) at 60℃. After 5 min, this mixture was undergone by the high pressure homogenizer (Microfluidics Corp., USA) with 1000 bar and 3 cycles. In case of Rh-HPβCD, the aqueous solution of HPβCD and Rh was homogenized at 60℃ and 9,000 rpm for 15 min before being homogenized with the ethanolic solution of lipid components and polymer. Ethanol and water were eliminated by using rotary evaporater and the resulting concentration of vesicles was 5 wt%.

2.2. Characterization of Polymer-associated Liposome

The vesicle sizes of PC-Chol liposomes and polymer-associated liposomes were measured at 25℃ by using PCS (Malvern Instruments 4500HS, USA) based on the principles outlined previously in the literature[17]. The samples were diluted to 0.1 mg/mL, and the intensity

of the He-Ne laser light (633 nm) scattered by the samples was detected at an angle of 90°. For each specimen, 10 autocorrelation functions were analyzed using the scattered intensity and the mean diameter of the nanoparticles calculated using the Stokes-Einstein equation. The size distribution was calculated using the CONTIN routine.

The vesicle structures were visualized by using TEM (Hithachi H-7600, Japan). Vesicles were stained with a 20 wt% phosphotungstic acid aqueous solution, and the stained solutions were floated on a gold-coated EM grid. This was immediately freeze-dried with liquid nitrogen and then lyophilized with a freeze dryer. Such prepared samples were observed by TEM.

2.3. *In vitro* Skin Permeation Test

To compare the Rh delivery efficiency of the PC-Chol liposomes, polymer-associated liposomes, and vesicle-free solution, we measured the amount of Rh transported into the skin for three carriers: Rh-HPβCD, PC-Chol liposomes containing Rh-HPβCD, polymer-associated liposomes containing Rh-HPβCD. The concentration of Rh was 2 wt% in all cases. The skin permeation test was carried out using Franz-type diffusion cells (Hansan Research, Korea) for 18 h at 32℃, and the skin tested was the abdominal part of albino guinea pigs (Charles Liver, China). After 18 h, 1 mL of receptor solution of the diffusion cells was withdrawn and the amount of Rh was determined using high performance liquid chromatography (Hewllet Packard 1100, USA).

3. Results and Discussion

3.1. Structure of PC-Chol Liposomes and Polymer-associated Liposomes

The structure and stability of polymer-associated liposomes were well described in previous results [14,15]. Briefly, when poly (MMAc-co-SM) content was approximately 30 wt% in all components (PC, Chol, and polymer) used for the preparation of polymer-associated liposome, most of PC-Chol liposomes transitioned into the polymer-associated liposome. The association of the stearyl group in poly (MMAc-co-SM) with the bilayer of PC-Chol liposome was confirmed by using microcalorimetry and TEM. As shown in Figure 1, both PC-Chol liposomes and polymer-associated liposomes have a similar vesicular structure although they have different vesicle sizes.

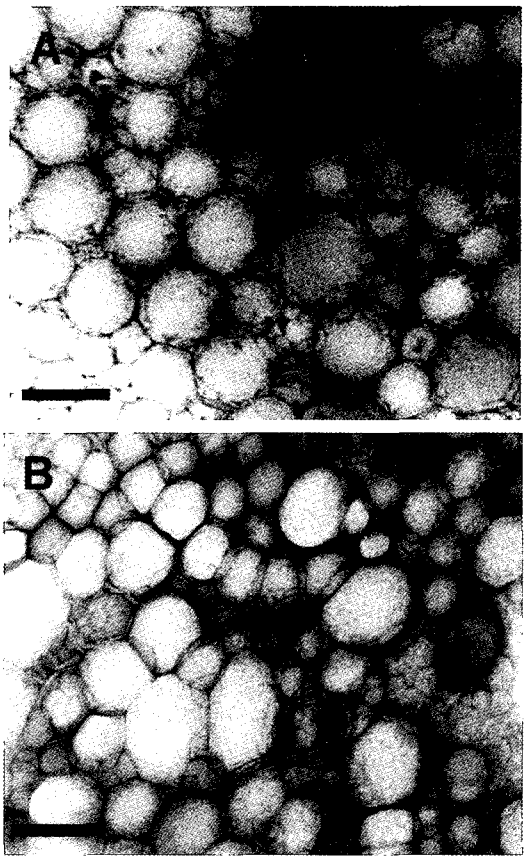

Figure 1. TEM images of PC-Chol liposomes (A) and polymer-associated liposomes (B). Scale bar is 200 nm.

3.2. Stability of PC-Chol Liposomes and Polymer-associated Liposomes Containing CDs or Rh-HPβCD

Table 2 summarizes the average vesicle sizes and polydispersity indexes (PDI) of PC-Chol liposomes and polymer-associated liposomes for various CDs monitored for 1 month by using PCS. As shown in Table 2, PC-Chol liposomes containing CDs showed the gradual increase of vesicle size or PDI as a function of time, indicating that CDs caused the disruption of vesicles.

In contrast to PC-Chol liposomes, polymer associated liposomes exhibited a similar vesicle size and PDI for one month regardless of the presence and type of CDs. These results indicate that polymer-associated lipo-

Table 2. Vesicle Size[a] and Polydispersity Index[b] (shown in parentheses) of PC-Chol Liposomes and Polymer-associated Liposomes Containing CDs or Rh-HPβCD

Code	Vesicle size (nm)			
	0 week	1 week	2 weeks	4 weeks
L-only	123 ± 4 (0.22)	125 ± 5 (0.24)	121 ± 6 (0.26)	120 ± 5 (0.24)
L-β	122 ± 7 (0.24)	125 ± 6 (0.19)	121 ± 6 (0.20)	139 ± 14 (0.37)
L-HPβ	105 ± 4 (0.17)	112 ± 7 (0.28)	112 ± 6 (0.27)	109 ± 8 (0.31)
PL-only	197 ± 7 (0.24)	192 ± 5 (0.20)	193 ± 7 (0.23)	195 ± 7 (0.25)
PL-β	189 ± 6 (0.20)	189 ± 6 (0.18)	188 ± 5 (0.17)	187 ± 7 (0.23)
PL-HPβ	185 ± 5 (0.20)	183 ± 5 (0.18)	182 ± 5 (0.19)	172 ± 6 (0.20)
L-HPβ-Rh1	130 ± 4 (0.24)	129 ± 4 (0.25)	123 ± 11 (0.33)	125 ± 10 (0.32)
L-HPβ-Rh2	230 ± 15 (0.36)	234 ± 12 (0.34)	243 ± 21 (0.39)	247 ± 19 (0.38)
PL-HPβ-Rh1	183 ± 4 (0.20)	184 ± 3 (0.17)	181 ± 5 (0.25)	186 ± 4 (0.23)
PL-HPβ-Rh2	181 ± 2 (0.15)	184 ± 4 (0.16)	181 ± 4 (0.17)	180 ± 4 (0.18)

[a] Vesicle size were measured by photocorrelation spectroscopy in deionized water. Average values and standard deviations were obtained by measuring three different samples.

[b] Polydispersity index is defined as the broadness in the size distribution of the particles, the variance is divided by the mean square of the particle size[17].

somes have much more stable vesicle formation than PC-Chol liposomes. We obtained the similar results by observing TEM images (data not shown). As shown in Table 2, L-HPβ-Rh1 and PL-HPβ-Rh1 showed good vesicle stability for 1 month. However, when the concentration of Rh-HPβCD was increased by twice, L-HPβ-Rh2 showed an increase in the vesicle size and PDI, while PL-HPβ-Rh2 maintained the similar vesicle size and PDI. The disruption of L-HPβ-Rh2 was accelerated as a function of time, and the collapsed vesicle structure was confirmed by the photograph (Figure 2E) and TEM image (Figure 3B) of L-HPβ-Rh2. PL-HPβ-Rh1, L-HPβ-Rh1, and PL-HPβ-Rh2 showed homogeneous vesicle solutions with no precipitation (Figures 2A, 2B, and 2D), whereas L-HPβ-Rh2 was precipitated (Figure 2E). TEM image of L-HPβ-Rh2 (Figure 3B) also indicated that the vesicle structure was extremely disrupted. In addition, although L-HPβ-Rh1 showed no precipitation, TEM image of L-HPβ-Rh1 showed lots of lamellar phases and indistinct boundary of vesicles (Figure 3A).

The mechanism underlying the enhanced stability of polymer-associated liposomes observed in aqueous media containing various CDs is difficult to explain. However, it can be speculated that the structure of the polymer-associated liposomes act to stabilize the vesicles in

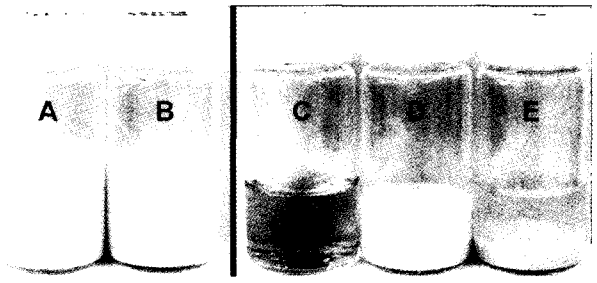

Figure 2. Photographs of samples after four weeks of storage at 4℃: (A) PL-HPβ-Rh1, (B) L-HPβ-Rh1, (C) Rh (2 wt%)-HPβCD (11.7 wt%) complex aqueous solution, (D) PL-HPβ-Rh2, (E) L-HPβ-Rh2.

aqueous media containing various CDs. Specifically, the stearyl groups in the polymer associate to form a bilayer structure with PC and Chol[14,15]; thus, the structure of the polymer-associated liposomes may be strengthened by the liposome constituents being chained to a polymer binder. Such physical binding of lipid components disturbs effectively CDs in extracting lipid components from vesicle structure. In addition, the carboxyl groups of polymer covering the liposomes serve to stabilize the vesicles because this charged surface, as a barrier function, prevents CDs from interacting directly with lipid components of vesicles.

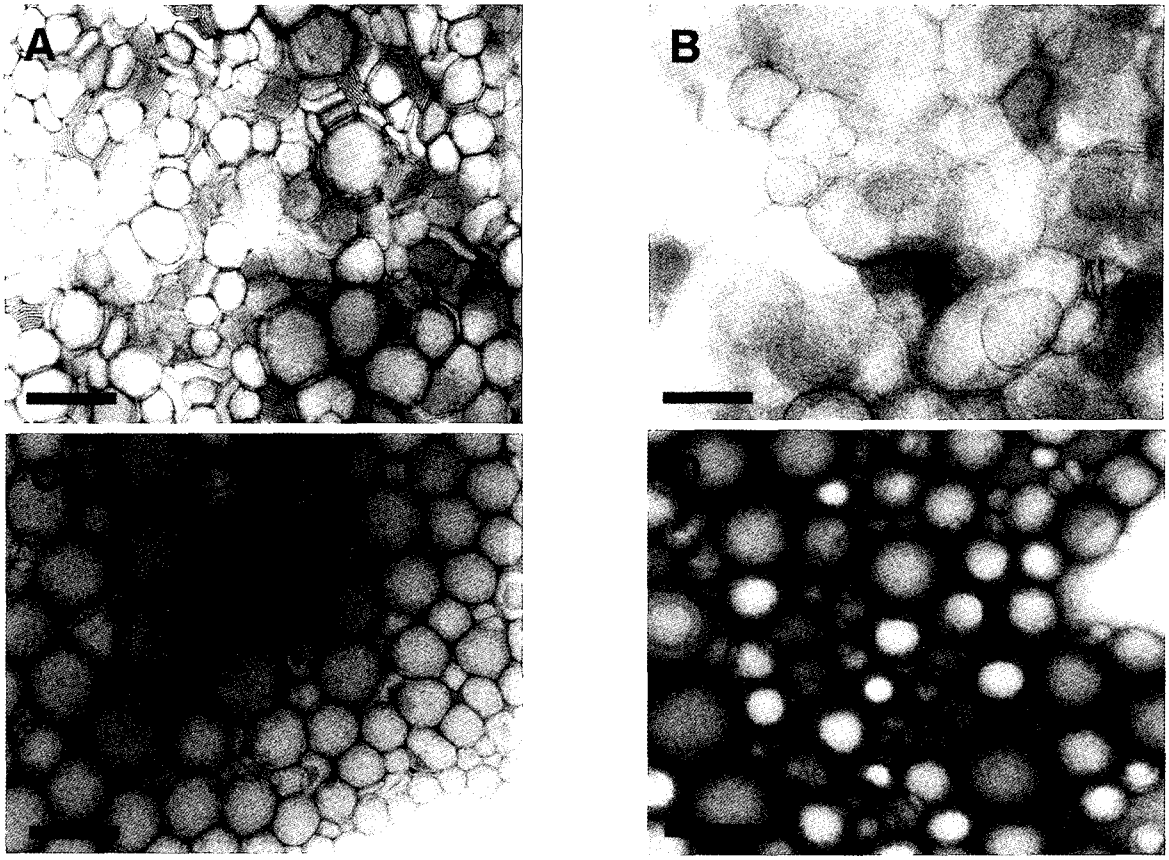

Figure 3. TEM images of PC-Chol liposomes and polymer-associated liposomes containing Rh-HPβCD complexes: (A) L-HPβ-Rh1, (B) L-HPβ-Rh2, (C) PL-HPβ-Rh1, (D) PL-HPβ-Rh2. Scale bar is 200 nm.

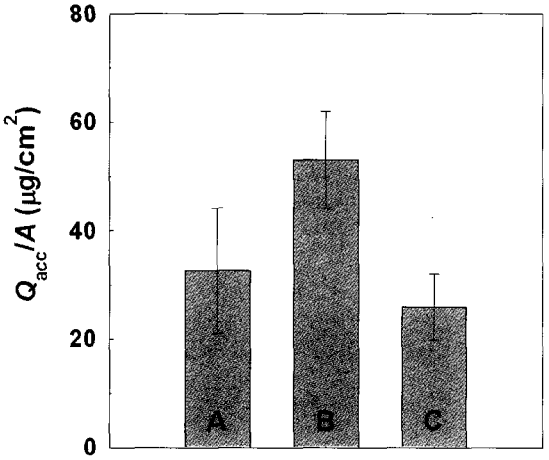

Figure 4. Accumulated amount of Rh molecules that pass through the skin when using (A) Rh (2 wt%)-HPβCD (11.7 wt%) complex aqueous solution, (B) PL-HPβ-Rh2, and (C) L-HPβ-Rh2. The concentration of Rh was 2 wt%, respectively.

3.3. *In vitro* Skin Permeation Test

In *in vitro* skin permeation test, we found that the stability of vesicles against Rh-HPβCD influences the delivery efficiency of Rh into the skin. We measured the accumulated amounts of Rh that had passed through the skin of guinea pig as a function of time. As shown in Figure 4, the highest amount of Rh was obtained after 18 h using PL-HPβ-Rh2, and the amount of Rh decreased in the order of PL-HPβ-Rh2, Rh-HPβCD, and L-HPβ-Rh2. The vesicle structure of L-HPβ-Rh2 was collapsed as shown in Figure 2 and Figure 3, which might cause a loss of the enhancing Rh delivery function which PC-Chol liposomes inherently possess. In contrast, the delivery efficiency of Rh was much improved through polymer-associated liposomes. Combining with previous results, the stability of vesicle structure should be considered as one of the factors influencing drug delivery function.

4. Conclusion

We investigated the influence of βCD, HPβCD, and Rh-HPβCD on the vesicle stability of PC-Chol liposomes and polymer-associated liposomes in aqueous media. We found that polymer-associated liposomes show much more improved vesicle stability than PC-Chol liposomes in the presence of CDs. Furthermore, polymer-associated liposomes form more stable vesicle formation in the presence of high concentration of Rh-HPβCD than PC-Chol liposomes, which might partly attribute to a higher skin delivery efficiency of Rh. The results obtained in this study strongly suggest the potential of polymer-associated liposomes with improved long-term vesicle stability in the presence of CDs or drug-CD complexes for the new application of drugs-in-CDs-in-liposomes system.

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