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Physical stability of cholesterol derivatives combined with liposomes and their *in vitro* behavior

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***Abstract*—The purpose of this study was to investigate the physical stability and drug release of two cholesterol derivatives (4-cholesterocarbonyl-4'-(*N,N,N*-triethylamine butyloxyl bromide, CTBBA, and 4-cholesterocarbonyl-4'-(*N,N'*-diethylamino-butyloxy, CDBA), when combined with doxorubicin (DOX)-loaded liposomes *in vitro*. CTBBA-liposome revealed a positive charge at a pH between 3 and 10, as indicated by the ζ-potential. DOX-encapsulated CTBBA-liposomes possessed better physical stability both in PBS and in fetal bovine serum (FBS) added to PBS.**

I. Introduction

Liposomes, which are composed of phospholipids, have been widely used as drug delivery systems for decades. [1, 2] Many liposome formulations used for cancer therapy, such as Myocet® and DOXIL® (a stealth liposomal DOX), are approved by the FDA. [3, 4] Liposomes are mobile and could potentially interact with many components in the biological environment; however, the instability of liposomes in aqueous solution during long-term storage could lead to problems, such as aggregation, fusion, or drug leakage due to liposome rupture, which limits the liposome's potential application in drug delivery. [5] Although many methods are available for liposome stabilization, such as lyophilization, freezing, and spray-drying, [6, 7] liposome suspension is still the most direct and convenient method for storage. Size distribution is an important parameter for this purpose. It has been known that intravenous injection of large particles may cause serious problems due to embolism. Thus, particular attention should be paid to size distribution in preparing dispersions. Furthermore, particle size can modulate the capture mechanism by macrophages and influence their biological stability *in vivo*. [8] Another important factor and useful indicator of particle surface charge is the ζ-potential, which can be used to predict and control the stability of liposome storage in suspensions. External parameters, such as temperature, also appear to be important for long-term storage. Generally, the most favorable storage temperature is 4 °C because higher temperatures can lead to changes in the crystalline structure of lipids. [9] Serum-induced drug leakage from liposomes should also be investigated for application in both *in vitro* and *in vivo* situations. [10, 11]

In this study, the parameters, including size distribution and encapsulation efficacy, were investigated to evaluate the long-term stability of two cholesterol derivatives combined with liposomes that were loaded with DOX and were compared to the conventional liposome. The DOX-release profiles were also studied in media with different fetal bovine serum concentrations.

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II. Materials and Methods

Cholesterol derivatives synthesis

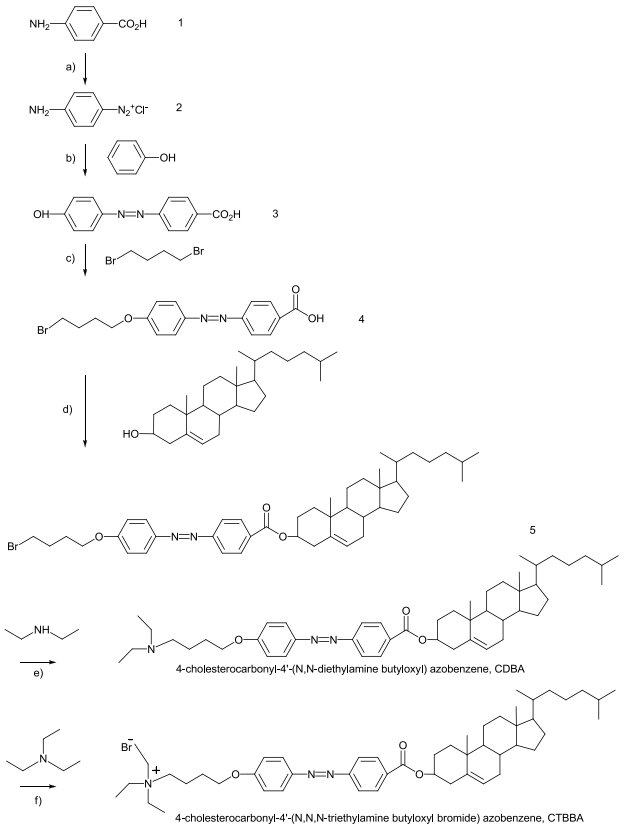
Synthesis of CTBBA and CDBA were followed by these. CTBBA, was synthesized as described by our group previously. [12] CDBA was synthesized as follows: p-aminobenzoic acid (0.2525 g, 0.34 mmol), diethyl amine (0.53 mL) and potassium iodide (0.003 g, 0.017 mmol) were added to chloroform (20 mL) and then stirred at reflux temperature for 3 days. Afterwards, the reaction mixture was cooled to room temperature and filtered. The solvent was removed. The crude product was further purified by passing it through a silica gel column using chloroform – methyl alcohol as the solvent, followed by recrystalization to afford pure CDBA (characterized by IR, MS and 1H NMR).1H NMR (300 MHz, $CDCl_3$, ppm): 8.19-8.16 (2H, d, J = 9Hz), 7.97-7.89 (4H, dd, J = 9Hz), 7.02-6.99 (2H, d, J = 9Hz), 5.44 (1H, m), 4.93-4.83 (1H, m), 4.11-4.07 (2H, t, J = 6Hz), 3.23-3.16 (6H, m), 2.51-0.69 (53H, m).MS (MALDI): 737 (M+), 738 (M++1), 739 (M++2).IR: 2926, 2854, 1713, 1601, 1583, 1502 cm-1. The structure of CTBBA and CDBA were showed in Scheme1.

Liposome preparation

The preparation of liposomes from egg phosphatidylcholine (PC, Sigma, US), cholesterol (Chol, Sigma, US), CTBBA, CDBA was carried out using the standard sonication method under nitrogen. Briefly, lipids in $CHCl_3$ were transferred into flask and dried by evaporation under nitrogen stream. The thin lipid film formed on the wall of flask was hydrated with phosphate buffered saline solution (PBS, pH 7.4) and sonicated under nitrogen for 10 min with a VC 130 probe sonicator (Sonics and Materials, Inc.). Temperature was controlled with an ice water bath. Subsequent centrifugation with 10,000 g was carried out to remove untrapped lipids and titanium. The molar ratio was PC/Chol=1/1 in PC-liposome, PC/Chol/CTBBA=2/1/1 in CTBBA-liposome and PC/Chol/CDBA=2/1/1 in CDBA-liposome. The concentration of total lipid was 8 mmol.

ζ-potentials of blank liposomes

ζ-potentials of blank liposomes at different pH levels were measured by a Zetasizer 2000 (Malvern Instruments Ltd., U.K.). A pH gradient solution was prepared with disodium



Scheme 1. Synthesis of CTBBA and CDBA. Reagents and conditions: (a) HCl, $NaNO_2$, 0 °C; (b) NaOH, 0 °C (70%); (c) K_2CO_3, 18-crown-6, reflux, (90%); (d) dicyclohexylcarbodiimide, 4-dimethylamiopryidine, r.t. (55%); (e) reflux, (50%); (f) KI, $CHCl_3$, 45 °C, (50%).

hydrogen phosphate, citrate, glycine and sodium hydroxide. 200 µl liposome suspension was diluted with 1800 µl PBS and then this sample was measured.

Serum influence on physical properties of liposomes

Liposome suspension was diluted with either PBS or FBS contained PBS solution. The size distribution and ζ-potential were measured by a Zetasizer 3000 HS_A and Zetasizer 2000 (Malvern Instruments Ltd., U.K.). The stability of DOX-liposomes was measured by follows: 200 µl DOX-liposome solution was added to 1800 µl 10% (v/v) serum. These samples were kept at room temperature for 12 hours, and analyzed by Zetasizer 3000 HS_A. Differences between groups were assessed using analysis of variance, followed by multiple comparisons using Bonferroni as post-test.

DOX release profile in PBS or FBS contained PBS

The drug release behavior *in vitro* was studied in PBS or FBS contained PBS solution at 37 °C. Briefly, the 100 µl DOX-liposome suspension was diluted by 4.9 mL PBS or FBS contained PBS. At various intervals of time, the drug encapsulation efficacy was calculated. The amount of DOX encapsulated by liposomes was calculated by the equation: Encapsulation Efficiency (%) = $100 \times (I_{max}-I_o)/(I_{max})$, where I_o is the fluorescence intensity of the liposome suspension containing DOX at the initial time, and I_{max} is the maximum fluorescence intensity after the addition of 0.5% Triton X-100.

Data analysis

The results were expressed as mean±standard deviations (SD). Differences between groups were assessed using analysis of variance, followed by multiple comparisons using Bonferroni as post-test. Value of $P<0.05$ was considered significant. All analyses were conducted using SPSS 13.0 (IBM Corporation, Somers, NY, US).

III. Results and Discussion

ζ-potentials of blank liposomes

Considering the difference between the structures of CDBA and CTBBA, the surface charge of their liposomes should be different, which can be evaluated by ζ-potential analysis. Fig. 1 shows that the ζ-potential value varied with pH. The PC-liposome revealed a small negative charge at low pH (-1.60±0.52 mV at pH 3.13), a neutral charge at neutral pH (0.67±0.21 mV) and a sharp decrease to a negative charge at high pH (-52.90±0.83 mV). The CDBA-liposome had a value of 31.93±6.18 mV at pH 3.13, -3.63±0.92 mV at neutral pH (pH 6.95) and -39.97±5.52 mV at pH 9.53. The ζ-potential of the CTBBA-liposome was 35.90±1.65 mV at pH 3.13, then decreased to 25.43±7.66 mV at neutral pH (pH 6.95), and finally changed to 2.23±0.23 mV at pH 9.53, still maintaining some positive charge. The CTBBA had a triethylamine moiety, which possessed a positive charge in its side chain. This positive charge was revealed when it combined with the liposome, as proved by the ζ-potential. The CDBA had a diethylamine moiety, and the ζ-potential of the CDBA-liposome declined with rising pH. Normally, the electrostatic force creates a repulsive barrier to prevent the membranes from coming into close contact, which maintains the vesicles' stability in suspension. [13]

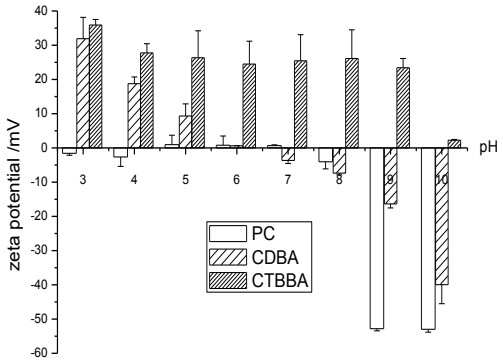


Figure 1. ζ-potential values of blank liposomes at different pH (n=3).

Serum influence on physical properties of liposomes

Since serum components can decrease drug delivery into the cells by changing the size and surface properties of the delivery system, [14] it is necessary to understand the interaction between the serum and liposomes. Fig. 2A shows the size of the DOX-liposome influenced by the serum. In PBS, the size of DOX-PC, DOX-CDBA and DOX-CTBBA was 190.40±12.31 nm, 108.47±5.95 nm and 133.20±4.60 nm, respectively. After adding serum to the PBS, the size

decreased as the concentration of serum increased from 1% to 10% for both DOX-PC and DOX-CDBA. However, the serum addition caused an initial sharp increase in the size of DOX-CTBBA, which then decreased with the addition of more serum. Fig. 2B shows the ζ-potential of the DOX-liposome influenced by the serum. In the absence of serum, the ζ-potential of DOX-PC, DOX-CDBA and DOX-CTBBA was -16.90±4.18 mV, -9.43±0.31 mV and 11.0±4.0 mV, respectively. With the addition of serum, the ζ-potentials of both DOX-PC and DOX-CDBA increased slightly. For DOX-CTBBA, a positive charge was still present in 1% serum. However, it became negative when 5% or 10% serum was added. These results indicate that the serum components had a greater effect on the positive liposome than on the negative ones. For the DOX-PC and DOX-CDBA, which were negative in PBS, the serum reduced the particle size. Some serum components, such as HDL (high density lipoprotein), can disrupt the structure of the liposome and induce a decrease in the size with increasing serum concentration. [15] Other works by Jones and Nicholas have shown that those liposomes that remained intact in the presence of serum were smaller, and that the size of the intact liposomes decreased with serum concentration. [16] The results from Figure 2A were similar to the work by Foradada et al. [17] They hypothesized that serum protein could promote either the breakdown of the particles or the adsorption to the particles' surface. The ζ-potential of DOX-PC and DOX-CDBA declined from a strong negative to weak negative, and the value corresponded to that of serum proteins. This suggests that the particles acquired a coating of serum proteins and were disrupted. For positive DOX-CTBBA, the result was different. The size increased immediately when DOX-CTBBA was mixed with serum, which denoted an aggregation process. This also could be explained by the adsorption of serum protein onto the liposome surface through electrostatic attraction. Then its positive charge was neutralized, as shown by the increase in particle size and the decrease in ζ-potential. The increase in the size of positive liposomes after exposure to serum has been noted previously. [18] After the charge of the positive CTBBA-liposome was neutralized by serum, ζ-potential became negative (Fig. 2B), and the size of the DOX-CTBBA decreased slightly with increasing serum concentration, similar to the DOX-PC or DOX-CDBA. However, some other factors also affected the interaction between the serum and liposomes, such as the oxygen content. [19]

Then the change of size and ζ-potential of DOX-liposomes according to incubation time after adding serum to PBS were studied. The results were shown in Fig. 3. The size of DOX-loaded PC-liposome increased from 162 nm to 198 nm. The change in the size of our samples is smaller than what has been reported by Han et al. [20] We thought that the concentration of lipid and serum could also be an important factor to influence physicochemical properties. In Han's work, the concentration of lipid in 50 % (v/v) serum was about 5 mmol. In our work, it was about 0.8 mmol in 10 % (v/v) serum. In conclusion, the ζ-potential of all DOX-liposomes didn't change much in 10 % (v/v) serum after 12 hours (data not shown).

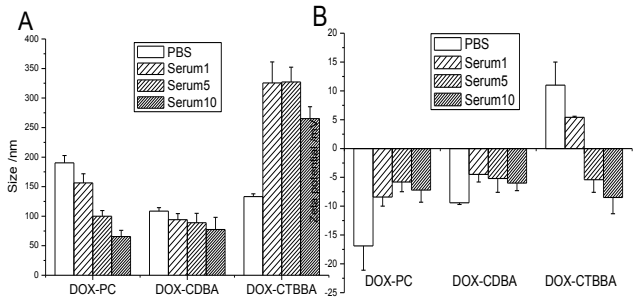


Figure 2. The size and ζ-potential of DOX-liposomes influenced by serum concentration (n=3). (A) The size of DOX-liposomes. (B) The ζ-potential of DOX-liposomes. FBS 1 indicates 1% FBS in PBS, FBS 5 indicates 5% FBS in PBS, FBS 10 indicates 10% FBS in PBS.

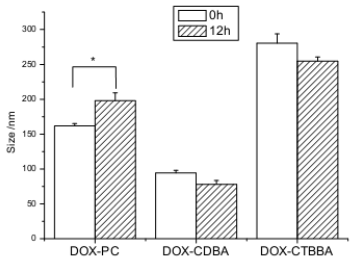


Figure 3. Change of liposome size during incubation in 10% (v/v) serum at room temperature. (n=3) Statistical analysis was performed using Bonferroni as post-test. *P<0.01

DOX release profile in PBS or FBS contained PBS

Drug leakage from liposomes induced by serum is another important factor that limits their applications; therefore, it is necessary to estimate the drug-release profile of the liposomes in serum added to solution before being used *in vivo*. The *in vitro* release behaviors of DOX from DOX-liposomes were studied both in PBS and in FBS added PBS (Fig. 4). In the first hour in PBS, approximately 22.13±0.52% of DOX was released from the DOX-CTBBA, while this value was 31.35±4.32% and 34.57±0.24% for DOX-CDBA and DOX-PC, respectively. After 9 hours, 35.42±8.43% remained in the DOX-CTBBA, but these values were only 6.58±3.12% and 11.19±0.88% for DOX-CDBA and DOX-PC, respectively (Fig. 4A). On the other hand, the presence of FBS in high concentrations induced significant leakage of the encapsulated drug, but there was more DOX remaining in the DOX-CTBBA than that remaining in the DOX-PC or DOX-CDBA after 3 hours. In the later period, only 5% to 10% of DOX remained in the different DOX-liposomes after 9 hours in FBS added to PBS (Fig. 4B-D). A comparison of the DOX release profiles of DOX-liposomes between the PBS and FBS added to PBS indicated that the DOX release behavior from the DOX-liposomes was influenced by the type of compound and the FBS concentration. Cholesterol derivatives combined with the liposomes prevented the DOX release, and the positively charged CTBBA had better DOX release profiles than the neutral CDBA in PBS. The serum components accelerated the DOX release from several different kinds of DOX-liposomes. Normally, the interaction of the liposomes with serum components caused leakage of the contents entrapped in the liposome. [21, 22] These studies indicated that the serum-induced drug leakage varied greatly

with cholesterol level, which had great effects on the stability of the bilayer membrane. According to the results of the physical characteristics affected by the serum, the presence of serum proteins adsorbed onto the surface of the liposomes reduced the size of the DOX-PC and DOX-CDBA, which destabilized larger particles and led to a rapid release of the internal contents. While in the DOX-CTBBA, serum addition significantly increased the size of the liposome. These protein-coated particles prevented the DOX release in the early period, but their instability caused the release of more DOX in the later stage.

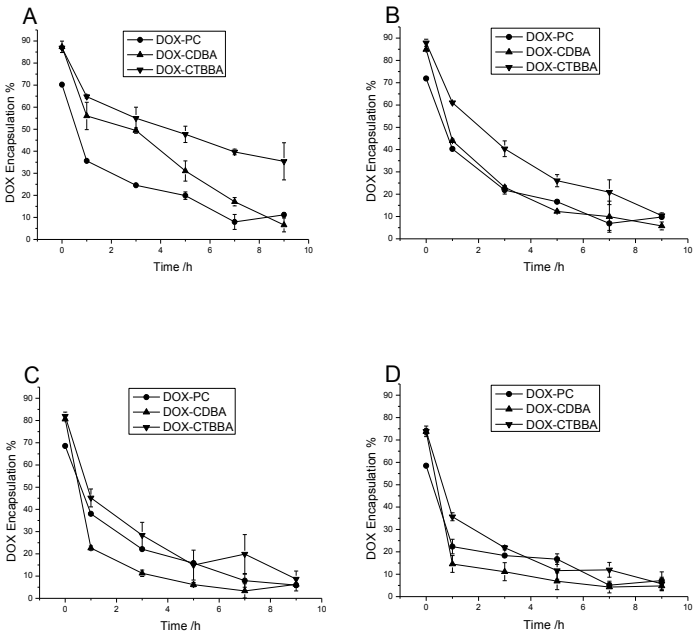


Figure 4. Effects of FBS on DOX release from DOX-liposomes at 37 °C (n=3). (A) PBS without FBS. (B) PBS with 1% FBS. (C) PBS with 5% FBS. (D) PBS with 10% FBS.

IV. CONCLUSIONS

In conclusion, the positively charged CTBBA liposomes had a better characteristic size distribution and drug encapsulation efficacy. The DOX release profiles also indicated the advantage of these two liposomes in PBS. In particular, the positively charged DOX-CTBBA could sustain the release of DOX even in the presence of FBS.

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