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Colistin-entrapped liposomes driven by the electrostatic interaction: Mechanism of drug loading and *in vivo* characterization

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ABSTRACT

The potential *in vivo* application of liposome for polycationic colistin has been hindered by the poor entrapment efficiency (EE) due to their phospholipid membrane permeability. The objective of this study is to investigate the loading mechanism and validity of applying electrostatic attraction for the colistin entrapment and delivery in liposomes. Anionic lipids with various structures were used for colistin entrapment, and the properties of resulting liposomes (*i.e.* zeta-potential, EE and release rate) were highly dependent on the structure of anionic lipids. Based on consideration of intermolecular interactions, the retention of electrostatically entrapped colistin is essentially determined by the balance of interfacial hydrophobic attraction and electrostatic repulsion. The liposomal colistin showed the reduced bacterial killing rate, but did not compromise the *in vitro* antibacterial activity. Specially, the PEGylated liposomal colistin of sodium cholesteryl sulfate (Chol-$SO4^{-}$) showed the best drug retention, resulting in the significantly increased maximum-tolerated dose, prolonged blood circulation and decreased colistin distribution in kidney after intravenous administration in mice. These results highlight the potential utility of electrostatically entrapped liposome for polycationic colistin delivery.

1. Introduction

Emergence of drug resistant microorganism has caused severe constraints to the choice of antibiotics. Fortunately, antimicrobial peptides have been found as an exciting class of antimicrobial agents with the great potential of overcoming the antibiotics-resistant infections. However, the clinical application of antimicrobial peptides has been severely limited by their poor stability, unknown toxicology and pharmacokinetics (Marr et al., 2006). Colistin (also known as polymyxin E, Fig. 1) is one of the two antimicrobial peptides currently used clinically (Kwa et al., 2007). Colistin has the strong positive charges and a hydrophobic acyl chain. The amphipathic structure of colistin facilitates its insertion into the cytoplasmic membrane, resulting to a loss of membrane integrity and to the bacterial death (Yu et al., 2015). Due to the presence of a cyclic heptapeptide and unnatural amino acids (diaminobutyric acid) in the structure (Northfield et al., 2014), colistin shows an excellent enzymatic stability which enables its intravenous use for the treatment of severe infections (Huang et al., 2010). However, the direct intravenous colistin is rarely used due to its inherent toxicity such as neurotoxicity, nephrotoxicity and painful irritation at subcutaneous or intramuscular injection (Li et al., 2006).

Entrapment of antimicrobial agents in liposome has been shown to decrease or completely abolish toxicity by improving the pharmacokinetics and tissue distribution of antibiotics (Drulis-Kawa and Dorotkiewicz-Jach, 2010; Alhariri et al., 2013). However, the development of the liposomal formulation of colistin remains a challenging task probably due to its low encapsulation efficiency (EE) (Wallace et al., 2012). This is likely due to the phospholipid membrane permeability of colistin, enabling itself and other ions to transfer freely through the liposomal bilayer (Wallace et al., 2012, 2013). Although colistin shows inherent affinity towards phospholipid membrane, the efficient loading within the liposomal bilayer cannot be achieved due to its highly hydrophilic nature.

In an effort to develop the liposomal colistin with high EE for *in vivo* application, colistin was electrostatically entrapped in the liposomes modified with various anionic lipids. As shown in Fig. 1, these anionic lipids mainly differed in the anionic head and hydrophobic moiety. Influences of anionic lipids on the properties of liposomes (*i.e.* zeta-potential, EE and drug release behavior) were investigated in detail in order to explain the mechanism of colistin entrapment in anionic liposome. Biodistribution study in

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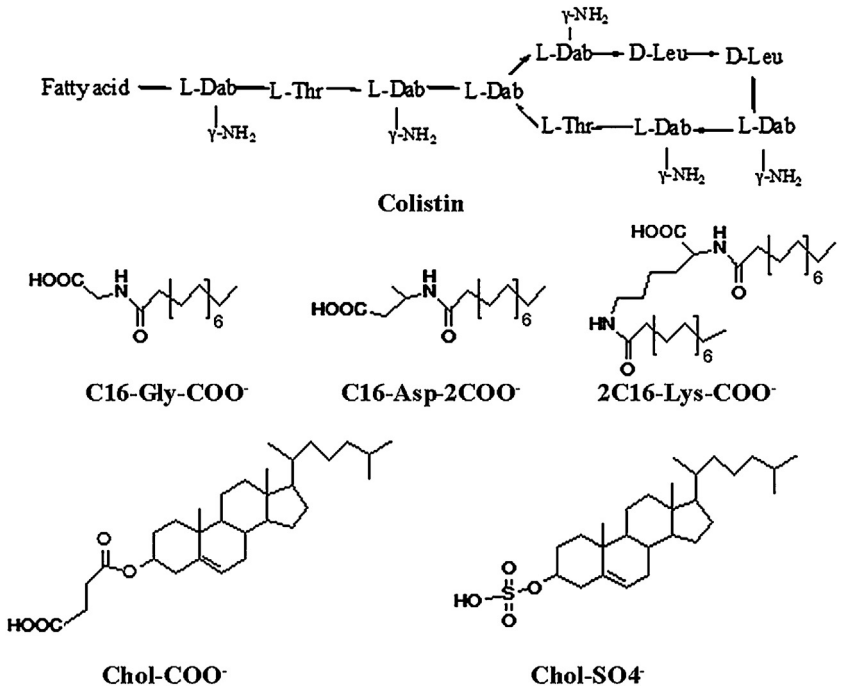

Fig. 1. Chemical structures of the colistin and anionic lipids including N-palmitoylglycine (C_{16}-Gly-COO^-), N-palmitoylaspartate (C_{16}-Asp-$2COO^-$), N-palmitoyllysine ($2C_{16}$-Lys-COO^-), cholesteryl hemisuccinate (Chol-COO^-) and sodium cholesteryl sulfate (Chol-$SO4^-$).

mice was investigated to demonstrate the validity of electrostatically entrapped liposome for colistin delivery.

2. Material and methods

2.1. Material

Colistin sulfate was obtained from MEILUN Biology Technology Co., LTD. (Dalian, China). Lipoid® S75 was obtained from Toshisun Biology Technology Co., LTD. (Shanghai, China). Chol-$SO4^-$ were obtained from Sinopharm Chemical Reagent Co., LTD. (China). Methoxy poly(ethylene glycol) 2000 ($mPEG_{2000}$) was purchased from Sigma-Aldrich Shanghai Trading Co., Ltd. (Shanghai, China). Amino acid based anionic lipid, Chol-COO^- and $mPEG_{2000}$-cholesteryl hemisuccinate (Chol-$mPEG_{2000}$) were synthesized in our laboratory (seeing Fig.S1 in the Supporting information). All other chemicals were analytical reagent grade and used without further purification.

2.2. Animal

Kunming (KM) and BALB/c nude mice were provided by the Experimental Animal Center of Shenyang Pharmaceutical University. The animals had free access to water and mouse chow. All experimental procedures were carried out in accordance with the guidelines of the Experimental Animal Care and Use Committee of Shenyang Pharmaceutical University.

2.3. Preparation of colistin-entrapped liposome

Liposomal colistins were prepared using a reversed-evaporation method (New, 1990), where the colistin could interact sufficiently with anionic lipids at the water/oil interface. For this, 12.0 mg colistin sulfate was dissolved in 1 ml phosphate buffer (pH 7.4, 10 mM), 150 mg Lipoid® S75 and anionic lipids (at a charge ratio of 2, anionic lipids/colistin) were dissolved in 5 ml diethyl ether. After making water-in-oil emulsions, the diethyl ether was evaporated under pressure to obtain a semi-solid gel at the room temperature. Then, 5 ml phosphate buffer was added enabling the collapse of the gel to form a liposomal suspension, which was dried for another 10 min at 40 °C to remove the residual diethyl ether. The liposomal suspension was sonicated subsequently to decrease size using a probe sonicator (Jy92-2D, Scientz, China). PEGylated liposome was prepared by the same procedure with Chol-$mPEG_{2000}$ (5%, Chol-$mPEG_{2000}$/Lipoid® S75, mol/mol) dissolved in diethyl ether.

2.4. Microbiological assay for the measurement of colistin

Concentration of colistin was measured by a microbiological assay employing *Escherichia coli* (*E. coli*) (CMCC 44103) as an indicator organism. This organism was grown overnight in Luria-Bertani (LB) broth at 37 °C, and the bacterial suspension was adjusted and mixed with the autoclaved molten LB agar (2%) medium at 50 °C to give a final bacterial concentration of approximately 1×10^6 colony forming units (CFU)/ml. After solidification, wells were created in the plate using a puncher device (6.5 mm) and filled with 30 μl sample or standards. The glass plate was covered and incubated overnight at 37 °C. The zone inhibition diameters were measured to determine the concentration of unknown samples by comparison with a series of standards. Standard curves were made with known quantities of colistin solution in saline. The range of linearity for colistin was from 1 to 80 μg/ml with a correlation coefficient of at least 0.99.

2.5. Size and zeta potential analysis

The mean diameter and zeta potential of the liposome were measured using a dynamic light scattering (DLS) instrument (Nano-ZS90, Malvern, England) at 25 °C. Liposomal colistins were diluted 10-foldedly by the distilled water to give a final colistin concentration of 240 μg/ml. The measurements were performed at a fixed angle of 90° to the incident light in disposable plastic cuvettes (size) and clear disposable zeta cell (zeta potential). DTS software (version 6.32) was