One-step preparation method for multiple drug-loaded lipid-based calcium carbonate nanoparticles

（複数の薬物を搭載した脂質・炭酸カルシウムナノ粒子を一段階で調製する方法に関する研究）

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［Purpose］

In cancer treatments, a variety of combinations composed of small molecular drugs, genes and proteins have been developed in clinical treatments and scientific researches. The application of combination therapies aims at increasing therapeutic effects, decreasing side effects and realizing reversion of multidrug resistance. To achieve synergistic effects of combination therapies, functional drug delivery systems (DDSs) are promising. The DDSs for combination therapies require several characteristics, including simplified preparation process for encapsulation of drugs, co-delivery to diseased regions, stimuli-responsive release of cargos after uptake by target cells, and sufficient stability of DDSs *in vivo*. However, it is a big challenge for DDSs to meet these requirements and widely apply to various drug combinations. Here, I developed a nanoplatform consisted of calcium carbonate ($CaCO_3$), lipids, polyelectrolytes, targeting moieties and multiple cargos to satisfy above requirements, and applied to co-delivery of drug/drug and protein/drug combinations.

［ Methods ］

An ethanol injection method was applied in this study. In preparations, lipids, inorganic salts, polyelectrolytes, drugs were dissolved in ethanol and water phases, separately. The components were added to the different phases in optimized order and amounts. After pre-stirring, the ethanol phase was injected into the water phase dropwise and stirred for sufficient time to form the nanoparticles. Rotary evaporation or ultrafiltration were used to remove the organic solvents.

［ Results ］

1. One-step formation of nanoparticles for drug/drug combination

1.1. Preparation and optimization of nanoparticles

I designed a one-step formation method of a novel nanoparticles (LPCCD) which consisted of lipids, poly acrylic acids (PAA) and $CaCO_3$ for encapsulation of curcumin (Cur) and doxorubicin (Dox). After optimization on the ratios among components, the received nanoparticles were about 100 nm with low polydispersity index (PDI) and possessed high encapsulation efficiency of both Cur and Dox (about 80%). The characteristics of LPCCD were suitable for efficient cellular uptake and *in vivo* drug delivery.

1.2. pH sensitivity and stability of nanoparticles

LPCCD possessed a pH-sensitive drug release behavior in both Dox and Cur, especially Dox (**Fig. 1A**). It was reasonable to attribute the obvious sensitive release of Dox to electrostatic interactions between Dox and PAA, which was directly and comprehensively

affected by destruction of $CaCO_3$ in acidic environments. Besides, LPCCD also have sufficient stability in serum-containing medium (**Fig. 1B**).

1.3. *In vitro* and *in vivo* performance of nanoparticles

LPCCD achieved simultaneous cellular uptake of both Dox and Cur by HepG2 cells. Despite the significantly reduced cellular uptake of Dox, LPCCD enhanced the cytotoxicity in comparison of free Dox group (**Fig. 2A**). This may be explained by increased cellular uptake of Cur from the LPCCD, indicating the synergistic effect of Dox and Cur. Blank carrier LPC possessed no cytotoxicity (**Fig. 2B**), indicating the usefulness of the nanoparticles. Furthermore, LPCCD increased plasma concentration of both Dox and Cur and decreased partition of drugs to heart that ameliorated the Dox-induced cardiac toxicity.

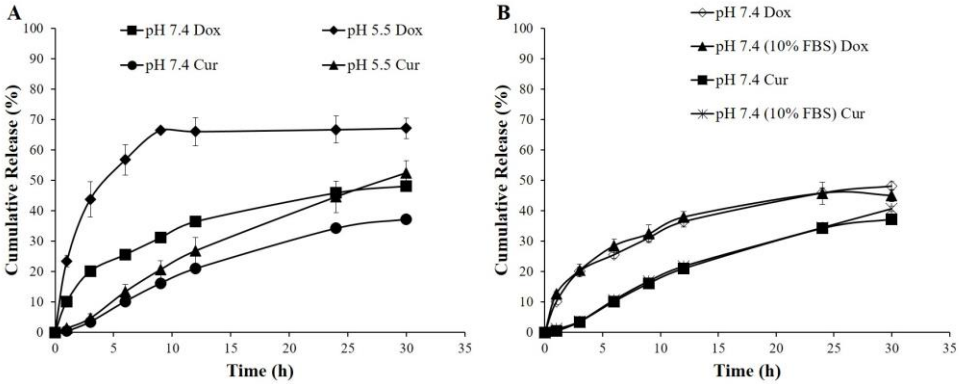

Fig. 1 Drug release from LPCCD. (A) pH sensitivity and (B) stability in the presence of FBS.

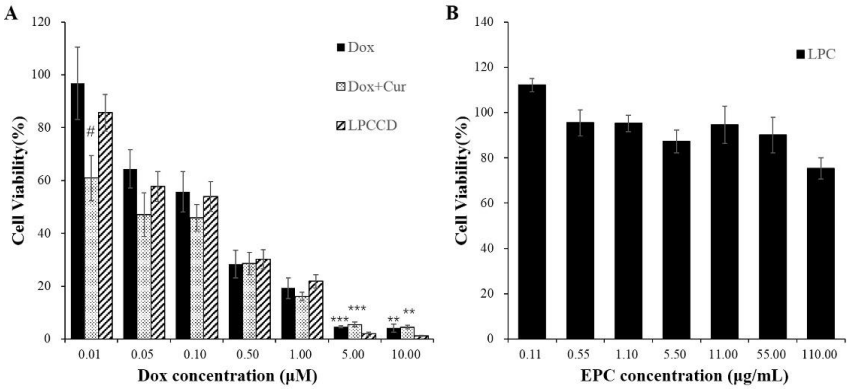

Fig. 2 Cytotoxicity of (A) free Dox, free Dox + Cur, LPCCD and (B) LPC.
Significant differences from LPCCD and free Dox are represented as **p<0.01, ***p<0.001 and #p<0.05, respectively. (LPC: blank nanoparticles without drug encapsulation, EPC: egg lecithin)

2. Co-delivery of protein/drug combinations by nanoparticles

2.1. Preparation and optimization of nanoparticles

I further applied the nanoplatform to protein/drug combinations (i.e. BSA-FITC/lipophilic carbocyanine dye (DiD) and superoxide dismutase (SOD)/paclitaxel (PTX) combinations) with arginylglycylaspartic acid (RGD) modification, and prepared the RGD/lipid/BSA-FITC/DiD/$CaCO_3$ (RLBDC) and RGD/lipid/SOD/PTX/$CaCO_3$ (RLSPC). Sizes of nanoparticles were less than 140 nm with low PDI. Although encapsulation efficiency was varied in terms of the cargos, the efficiency of both proteins (30-60%) and drugs (about 45%) were enough high for further studies.

2.2. pH sensitivity of nanoparticles

pH sensitivities of nanoparticles were analyzed by the variation in size and PDI. By decreasing pH from 7.4 to 5.5, the nanoparticles swelled, while aggregation did not occur. The nanoparticles possessed similar pH-sensitive drug release behavior with LPCCD. For proteins that interacted with lipids mainly via electrostatic interactions showed much more sensitive release behavior than that of small-molecular lipophilic drug PTX.

2.3. *In vitro* evaluation

RLBDC improved cellular uptake and intracellular disposition of BSA-FITC and DiD in Colon 26 cells compared with free combination group. It was proved that the RLBDC was taken up via integrin receptor, verified by competitive inhibition of cellular uptake through co-incubation with RGD-modified liposomes. The efficient co-delivery of cargos contributed to the improved cytotoxicity of RLSPC, which was the highest cytotoxicity among all preparations.

2.4. Synchronized bio-distribution *in vivo*

The nanoparticles prolonged circulation time of BSA-FITC and DiD, especially the lipophilic molecule DiD (**Fig. 3**). Most importantly, the nanoparticles have successfully achieved synchronized distribution of BSA and DiD *in vivo* for enough long time to expect synergistic anticancer efficacy.

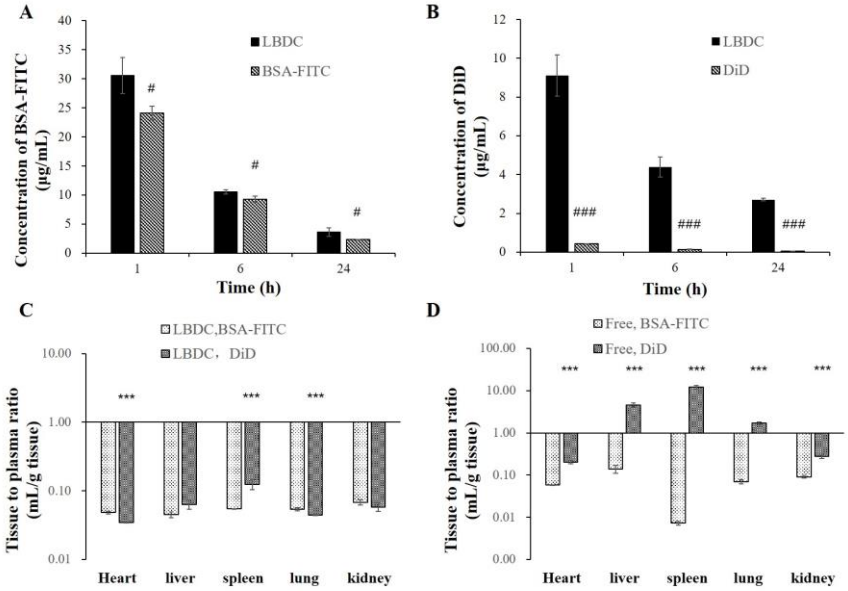

Fig. 3 Bio-distribution of BSA-FITC and DiD from the free combination and LBDC in mice.
(A, B) Plasma concentrations of BSA-FITC and DiD after i.v. injection. $^{\#}p<0.05$, $^{\#\#\#}p<0.001$.
(C, D) Tissue-to-plasma ratios of BSA-FITC and DiD 6 h after i.v. injection. $^{***}p<0.001$.

[Conclusion]

I have successfully developed the nanoplatform and achieved encapsulation of multiple drug combinations by simple preparation method. Both nanoparticles encapsulating drug/drug and protein/drug combinations exhibited pH-sensitive drug release, improved delivery to cytoplasm and synchronized distribution of cargos, which enhanced cytotoxicity. These achievements would be valuable in development of DDSs for combination therapies.

［基礎となった学術論文］

Peng Jianqing, Fumoto S., Miyamoto, H., Chen, Y., Kuroda, N. & Nishida, K., 2017. One-step formation of lipid-polyacrylic acid-calcium carbonate nanoparticles for co-delivery of doxorubicin and curcumin. *J. Drug Target.*, 25, 704-714.