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Evaluation of liposomal doxorubicin as a drug-trapping agent

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1 Introduction

Poly-L-glutamic acid(PGA) is readily degraded by lysosomal enzymes and has non-toxic degradation products. PGA consists of a glutamic acid monomer unit with a large number of carboxyl groups and has an apparent pKa of 5.4. At around pH 7, it is ionized and provides functional binding sites for cationic drugs. It has been reported that the ionic interactions between anionic PGA and DOX produced random colloidal aggregates and sustained DOX release. These characteristics may be favorable for the use of PGA as a trapping agent of DOX in liposomes and useful to achieve desirable antitumor activity via EPR effects. Therefore, in this study, we prepared triethylamine (TEA)-PGA liposomes (TEA-PGA-Ls) using PGA as an internal trapping agent for stable encapsulation of DOX into liposomes. We found that increasing the concentration or molecular weight of PGA in TEA-PGA-Ls could enhance accumulation of DOX in tumors and increased the antitumor effect in Lewis lung carcinoma (LLC) tumor-bearing mice.

2 Experiment

Sample preparation: TEA-PGA-Ls, TEA liposome (TEA-L), and AS liposome (AS-L) were prepared with Hydrogenated soya phosphatidylcholine (HSPC), Cho1, and mPEG-DSPE at a molar ratio of 4.1:2.7:0.4 using the thin-film method. The loading of DOX was then performed by incubating liposomes with DOX solution.

Characterization of samples: The average particle size and zeta-potential of the liposomes were measured by a cumulative method and electrophoretic mobility with a light scattering photometer. We evaluated the small angle x-ray scattering (SAXS) of liposomal DOX using the facility on the BL-6A.The experimental hutch in BL-6A is equipped with a marble table housing the modular-length flight tube and 2D detector (Pilatus3 1M). The liposomes were loaded into a cell using a peristaltic pump, and were set to be put within a 50 cm range of the detector. Data were collected by measuring at an energy of 8.27 keV with an exposure time of 20 s per frame at an x-ray wavelength of 1.5Å. Data processing, and further analysis were performed using ATSAS software.

Evaluation of Antitumor activity: To generate LLC tumors, $1x10^6$ cells suspended in 100 μL PBS, pH7.4 were inoculated subcutaneously into the right rank of female C57BL/6N mice. After the tumor size had reached about 100-200 mm^3, AS-L, TEA-L, and TEA-PGA-Ls were administered via the tail vain at doses equal to 5mg DOX per kg mouse by single-dose injection on day 0. Tumor volume and body weights were measured for individual animals.

3 Results and Discussion

To evaluate the effect of PGA as a liposomal trapping agent, the PGA-TEA system was used to prepare liposomal DOX. TEA can assist in the loading of weakly basic-amphipathic drugs via TEA efflux, accompanying the influx of the drug into liposomes and through the formation of self-perpetuating pH gradient providing a driving force for drug accumulation. These mechanisms may be able to maintain DOX in ionized forms and then increase intra-liposomal stability by electrostatic interactions between DOX and PGA.

TEA-PGA-Ls were prepared by remote loading of DOX with a TEA gradient into pre-formed liposomes prepared with l, 2, or 4 mg/mL PGA (molecular weights 4800, 9800, or 20500) (Table l). However in TEA-PGA_{20500}-Ls was difficult to obtain small, homogenous liposomes prepared with 4 mg/mL PGA_{20500} because of its high viscosity. Moreover in the preliminary study, we evaluated the effect of incubation time and drug-to-lipid ratio on the entrapment of DOX in TEA-PGA-Ls. ln TEA-L and TEA-PGA_{9800}-C2-L, with only 10 min incubation at 60°C, most DOX could be entrapped into liposomes, and extending the incubation period for 30 or 60min decreased entrapment efficiency of DOX. Furthermore, the highest entrapment efficiency was observed in liposomes prepared at a DOX:HSPC ratio of 1:5 (w/w) compared with l:2 or l:1. From these results, a 10-min incubation at 60°C and DOX-to-HSPC ratio of 1:5 (w/w)were chosen for loading of DOX. As shown in Table l, all the liposomes had average particle sizes around 110 nm, with negative charges of particles equivalent to approximately -15 mv.

Table 1: Composition and characteristics of liposomal DOX.

Formulation	Particle size (nm)	Zeta-potential (mV)
AS-L	109.4	-19.4
TEA-L	118.9	-13.9
TEA-PGA_{4800}-C2-L	112.5	-13.5
TEA-PGA_{9800}-C1-L	110.7	-11.9
TEA-PGA_{9800}-C2-L	112.5	-12.9
TEA-PGA_{9800}-C4-L	110.8	-15.6
TEA-PGA_{20500}-C2-L	118.5	-12.1

All the TEA-PGA-Ls showed high entrapment efficiency of DOX (>95%) similar to liposomes using ammonium sulfate (AS-L) and TEA (TEA-L). The molecular weight and concentration of PGA in TEA-PGA-Ls did not affect

the particle size zeta-potential, or entrapment efficiency of DOX.

The antitumor activity of TEA-PGA-Ls was evaluated in LLC tumor-bearing mice (Fig. 1).

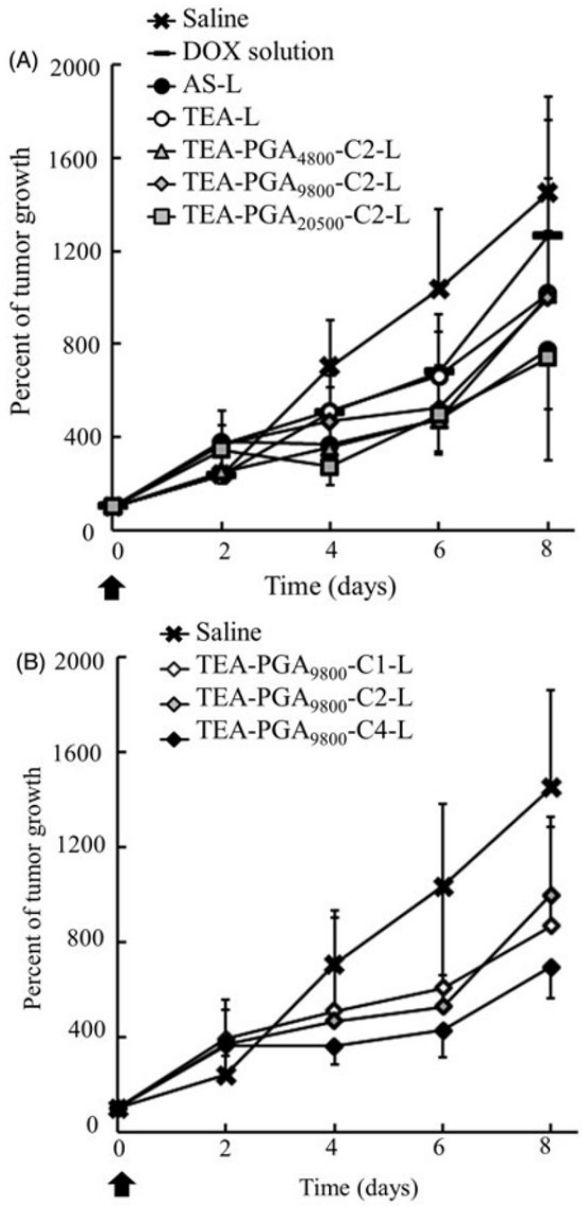

Fig. 1: The antitumor activity of triethylamine-poly-L-glutamic acid liposomes (TEA-PGA-Ls) on Lewis lung carcinoma (LLC) tumor-bearing mice. TEA-PGA-Ls were prepared with PGA with molecular weights of 4800, 9800 , or 20500 at 2 mg/mL (A) or at various concentrations of PGA9800 (1, 2, or 4 mg/mL) (B) were administered by a single intravenous injection of 5 mg DOX per kg on day 0 (as indicated by black arrows).

Compared with the injection of saline or DOX solution, a single injection of liposomal DOX could inhibit tumor growth up to day 8. Among the TEA-PGA-Ls prepared with different molecular weights of PGA (Fig. 1A) or concentrations of PGA (Fig.1B), TEA-PGA_{20500}-C2-L or TEA-PGA_{9800}-C4-L strongly inhibited tumor growth similar to AS-L. No body weight change was observed during the period of the experiment.

Preparation of liposomes with high and stable drug loading is a promising strategy to enhance the antitumor effect by an enhance permeability and retention (EPR) effect. ln this study, we prepared TEA-PGA-Ls using anionic PGA as an intra-liposomal trapping agent that electrostatically interacted with cationic DOX inside liposomes. As a result, TEA-PGA-Ls prepared with high molecular weight or high concentration of PGA could efficiently accumulate DOX in tumors and strongly inhibit tumor growth in LLC tumor-bearing mice, which were similar to those of AS-L prepared using an AS gradient.

PGA has many carboxyl groups on its polymeric structure, which can be ionized at around pH7 and provide useful sites for interactions with cationic drugs, such as DOX. When the solution of PGA was mixed with DOX solution, water insoluble-like aggregates of different sizes and densities of aggregates were observed depending on the molar ratio of DOX to PGA. Furthermore, reduction of DOX release from liposomes was observed in TEA-PGA-Ls compared with TEA-L that did not contain PGA. Although the details of DOX association with PGA in liposomes could not be clarified, we speculated that the physicochemical interaction between PGA and DOX may occur in more stable conditions by raising the number of carboxyl group of L-glutamic acid either by increasing the concentration or length of PGA. lt has been reported that the interaction of PGA and DOX does not only involve ionic interactions between the amine group of DOX and carboxyl group of PGA, but also the hydrophobic interaction between the anthracycline ring of DOX and the hydrophobic domains of the polymer. Furthermore, we observed a pH-dependent release of DOX from PGA/DOX aggregates. lt has been reported that the pKa value of PGA was 5.4. Therefore, protonation of the carboxyl group in PGA at pH5.5 might result in the dissociation of DOX from DOX/PGA aggregates.

The TEA-PGA-Ls prepared with high molecular weight or high concentration of PGA showed similar profiles of cytotoxicity, bio-distribution, and antitumor activity, compared with those of AS-L. However, the aggregates of PGA/DOX had different physical characteristics from the aggregates of DOX produced by addition of AS. Therefore, to investigate the physical characteristics of their aggregates, we measured small angle x-ray scattering (SAXS) for AS-L, TEA-L and TEA-PGA-Ls after loading with DOX at a weight ratio of DOX/HSPC of 1:5. As the result, PGA-TEA-L and AS-L had almost identical patterns of scattering profile at scattering vector (q) between 0.06 - 0.6 angstrom. (Fig. 2), indicating that their liposomes exist as vesicles. However, at q below 0.05 angstrom, the difference of scattering intensities was observed between TEA-PGA-Ls and AS-L, revealed that DOX/PGA aggregates filled in inner phase of TEA-PGA-Ls were dissimilar in shape with DOX aggregates in AS-L. It has been reported that interaction of DOX with sulfate produced aggregation inside liposomes in the form of one-dimensional rods,

which forced the vesicle shape to change from spherical to non-spherical. In TEA-PGA-Ls, the addition of PGA into TEA-L increased the scattering intensity indicated that TEA-PGA-Ls had DOX/PGA aggregates in the inner phase; however, the difference of scattering profiles of TEA-PGA_{4800}-C2-L and TEA-PGA_{20500}-C2-L was negligible. These findings might suggest that TEA-PGA-Ls are spherical vesicles with DOX/PGA aggregates in inner phase. However, further study must be performed to investigate the physical characteristics of PGA/DOX aggregates in liposomes.

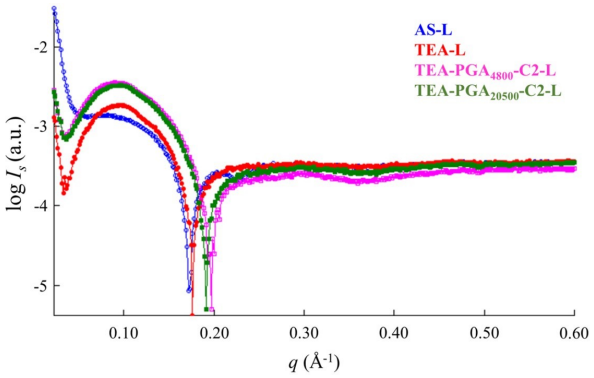

Fig. 2: The profiles of SAXS of ammonium sulfate liposomes (AS-L), triethylamine liposomes (TEA-L), and triethylamine-poly-L-glutamic acid liposomes (TEA-PGA-Ls) prepared at a DOX: HSPC weight ratio of l:5. Data are plotted as scattering intensity (Is) in arbitrary units as a function of the scattering vector (q) in angstrom.

In this study, all TEA-PGA-Ls could enhance the antitumor activity of DOX in LLC tumor-bearing mice until day 8 after only a single drug injection, compared with DOX solution. This could be due to the successfull delivery of TEA-PGA-Ls to the tumor tissue by EPR effects. In EPR effects, maintaining a high drug concentration in the blood can have substantial impact on drug exposure of tumor tissues. DOX concentration in the serum at 24 h after administration of TEA-PGA-Ls was approximately 16-25-fold higher than that of DOX solution, but a high accumulation of DOX in tumors was observed after injection of TEA-PGA_{9800}-C4-L. Unstable drug entrapment can cause premature drug release from liposomes in systemic circulation, resulting lower amounts of liposomal drug accumulated in tumor tissue. These findings indicated that DOX stably entrapped in TEA-PGA-Ls circulated for a prolonged time in the systemic blood and accumulated in tumors.

The use of PGA as an intra-liposomal trapping agent could improve tumor delivery of liposomal DOX. Unstable drug entrapment in liposomes causes rapid release of the drug, thus reducing the benefits of liposomal formulation. Excessively slow drug release will compromise therapeutic activity of the entrapped drug, because it will produce an inadequate drug concentration. It is important to tailor drug delivery for deliberate release of the drug in an appropriate manner in order to achieve high antitumor activity. However, further investigation is still required to evaluate the physicochemical properties of the aggregates of DOX and PGA for enhancing the therapeutic outcomes of TEA-PGA-Ls.

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Reference

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