Tumor delivery of liposomal doxorubicin prepared with poly-L-glutamic acid as a drug-trapping agent

Andang Miatmoko1*, Kumi Kawano1*, Hitomi Yoda1, Etsuo Yonemochi2, and Yoshiyuki Hattori1

1Department of Drug Delivery Research and 2Department of Physical Chemistry, Hoshi University, Ebara, Shinagawa, Tokyo, Japan

Abstract

Context: Poly-L-glutamic acid (PGA) is an anionic polymer with a large number of carboxyl groups that can interact electrostatically with cationic drugs such as doxorubicin (DOX).

Objective: For stable encapsulation of DOX into liposomes, we prepared triethylamine (TEA)-PGA-liposomes using PGA as an internal trapping agent.

Methods: We prepared TEA-PGA-liposomes by remote loading of DOX with a TEA gradient into preformed liposomes prepared with 1, 2, or 4 mg/mL PGA (molecular weights 4800, 9800, and 20 500), and evaluated their biodistribution and antitumor effects on Lewis lung carcinoma (LLC) tumor-bearing mice.

Results: TEA-PGA-liposomes using the higher the molecular weight or concentration of PGA showed a slower release of DOX from the liposomes. TEA-PGA-liposomes prepared with a high concentration of PGA could enhance DOX accumulation in tumors and prolonged DOX circulation in the serum, indicating that DOX may be retained stably in the liposomal interior by interaction with PGA. Furthermore, injection of TEA-PGA-liposomes prepared with 4 mg/mL of PGA4800 or 2 mg/mL PGA9800 strongly inhibited tumor growth in LLC tumor-bearing mice.

Conclusions: PGA may be a potential trapping agent for liposomal DOX for tumor drug delivery.

Keywords

Doxorubicin, liposome, Poly-L-glutamic acid, trapping agent, tumor

Introduction

It is well-known that nanoparticulate carriers have many advantages for the delivery of antitumor drugs. Nanoparticulates can protect against drug degradation, alter tissue disposition, reduce blood clearance, decrease systemic toxicity, and enhance antitumor activity (Cho et al., 2008; Gabizon et al., 2003; Marra et al., 2011; Moghimi & Farhangrazi, 2014). Among many types of nanocarriers, liposomes offer a superior form for drug delivery, with remarkable properties such as offering formulations with desirable composition, size, surface charge, ability to encapsulate both hydrophilic and hydrophobic materials with high efficiency, and the possibility for efficient surface functionalization with specific ligands.

Because nanosize liposomes can passively accumulate in solid tumors via an enhanced permeability and retention (EPR) effect, high and stable drug loading into liposomes has become a critical factor for successful drug delivery to pathological sites. Stable drug encapsulation inside liposomes could be achieved with sturdy bilayer membranes present in the outer layer, which can prevent premature drug leakage during distribution in the body. The use of phospholipids with a high phase-transition temperature ($T_m > 37^\circ$C) and/or a combination with cholesterol has been proven to increase membrane stability and to maintain drugs in the liposomal interior during storage or administration (Du Plessis et al., 1996; Drummond et al., 1999; Kokkona et al., 2000; Kirby et al., 1980). To achieve a level of high drug entrapment in minimum amount of carrier lipids, several remote loading methods have been developed. Depending on the characteristics of drugs, transmembrane gradients of ammonium, pH, or acetate were utilized as driving forces (Hwang et al., 1999; Zucker et al., 2009). Furthermore, after drug loading, intraliposomal stabilization of the drug improves drug retention inside liposomes and suppresses drug release. Intraliposomal trapping agents are useful to retain drugs inside liposomes against diffusion, through the formation of stable drug complexes (Cheung et al., 1998; Hattori et al., 2009; Lasic et al., 1995; Thomas et al., 2011) or physical aggregate-like compounds in water (Drummond et al., 2006; Zhigaltsev et al., 2005; Zucker et al., 2012). In the case of liposomal doxorubicin (DOX), a transmembrane gradient of ammonium sulfate (AS) achieved successful drug loading and retention in liposomes by forming DOX-sulfate fiber-like crystals (Lasic et al., 1995). As a result, Doxil®, an approved liposomal formulation of DOX, showed superior performance in preclinical and clinical treatment by means of an EPR effect.

Poly-L-glutamic acid (PGA) is a synthetic polypeptide that has many practical applications in various fields,
including tissue engineering, food products, drug and gene delivery systems (Buescher & Margaritis, 2007; Dekie et al.,
2000; Haag & Kratz, 2006; Otani et al., 1996). PGA is readily
degraded by lysosomal enzymes and has nontoxic degradation
products (Nickels et al., 2015; Otani et al., 1996; Tansey et al.,
2004). PGA consists of a glutamic acid monomer unit with a
large number of carboxyl groups and has an apparent pKa of
5.4 (Abbruzzetti et al., 2000). 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 sus-
tained DOX release (Manocha & Margaritis, 2010). 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.

**Materials and methods**

**Materials**

Hydrogenated soya phosphatidylcholine (HSPC) and meth-
oxy-(polyethylene-glycol)-distearoylphosphatidyl-ethanola-
mine (mPEG-DSPe, PEG mean molecular weight, 2000)
were purchased from NOF Inc. (Tokyo, Japan). Cholesterol
(Chol) and TEA were purchased from Wako Pure Chemical
Industries Inc. (Osaka, Japan). DOX hydrochloride was
obtained from LC Laboratories (Woburn, MA). PGA
sodium salt was purchased from Sigma (Tokyo, Japan) and
has three types with mean molecular weight of 4800, 9800,
and 20500. All other chemicals and solvents used in this
study were of the highest grade available.

**Preparation of liposomes**

TEA-PGA-Ls, TEA liposome (TEA-L), and AS liposome
(AS-L) were prepared with HSPC, Chol, and mPEG-DSPe at
a molar ratio of 4.1:2.7:0.4 using the thin-film method (Dicko
et al., 2007). First, all the lipids were dissolved in chloroform
and placed in a round-bottomed flask. The thin films formed
following the removal of chloroform using a rotary vacuum
evaporator in a water bath at 60 °C. In AS-L and TEA-L,
lipids were hydrated with 0.25 M of AS solution and 0.65 M
of TEA solution, respectively. In TEA-PGA-Ls, lipids were
hydrated with 0.65 M of TEA solution containing PGA, as
shown in Table 1. After hydration, the lipids were vortexed to
prepare liposomes, followed by heating in a water bath at
60 °C for 5 min. The liposomes were sonicated for 30–60 min
to produce a final size about 100 nm. For replacement of the
external phase, liposomes were passed through a gel filtration
column (Sephadex G100) with phosphate-buffered saline
(PBS, pH 7.4). The loading of DOX was then performed by
incubating liposomes with DOX solution at weight ratio of
DOX:HSPC of 1:5 in a water bath at 60 °C for 10 min. Free
DOX was separated from AS-L, TEA-L, and TEA-PGA-Ls
to a column packed with Sephadex G50. The concentra-
tion of phospholipids was quantified using a fluoromet-
ric method at λex= 485 nm and λem= 590 nm after lysing
with Triton X-100 (final concentration 5% v/v). Drug loading
was calculated as follows:

\[
\text{Percent of DOX loading} = \frac{\text{amount of DOX entrapped inside liposome}}{\text{total amount of DOX}} \times 100%
\]

**Measurement of particle size and ζ-potential
of liposomes**

The average particle size and ζ-potential of the liposomes
were measured by a cumulative method and electrophoretic
mobility with a light scattering photometer (ELS-Z2, Otsuka
Electronics Co., Ltd., Osaka, Japan) at 25 °C after dilution in
an appropriate volume of deionized water.

**Evaluation of doxorubicin-poly-L-glutamic acid
complex formation**

By assuming that liposomes with diameter of 120 nm
contains about 4.8 μL of total entrapped vesicular volume
per 1 mg of liposomal lipids, we estimated the ratios of PGA
and DOX inside liposomes, as shown in Table 1. A mole of
PGA was calculated as L-glutamic acid monosodium salt.
To evaluate complex formation, the DOX solution was mixed
with PGA solution in 0.65 M TEA at the indicated ratios
and then left at room temperature for at least 30 min. As a control,
DOX solution was mixed with 0.25 M of AS solution, L-glutamic acid solution, and 0.65 M of TEA solution, respectively. Photomicrographs of the mixtures were taken using an optical microscope (Nikon Eclipse TS100-F, Nikon Inc., Tokyo, Japan).

Small angle X-ray diffraction of liposomal doxorubicin
We evaluated the small angle X-ray diffraction (SAXRD) of liposomal DOX using the facility on the BL-6A beam line at the National Laboratory for High Energy Physics (KEK, Tsukuba, Japan). The experimental hutch in BL-6A is equipped with a marble table housing the modular-length flight tube and 2D detector. The liposomes were loaded into a 1.8-mm diameter quartz capillary 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 1.5 Å using the dedicated beam line software PILATUS Measurement Control Software at Photon Factory. Data processing and further analysis were performed using ATSAS software (http://www.embl-hamburg.de/biosaxs/software.html, Hamburg, Germany) (Konarev et al., 2003).

In vitro doxorubicin release from doxorubicin/ poly-L-glutamic acid aggregate and liposomal doxorubicin
DOX/PGA aggregate was prepared by mixing DOX with PGA20500 at molar ratio of DOX/PGA of 5.8 in 0.65 M of TEA solution. One mole of PGA was calculated based on L-glutamic acid monosodium salt. As a model of aggregate entrapped in AS-L, DOX/AS aggregates were prepared by mixing DOX in 0.25 M of AS. The aggregates were left at room temperature for 30 min after mixing. The study of DOX release from DOX/PGA or DOX/AS aggregates was performed by placing 200 μL of aggregate suspension in dialysis tubing Spectra Por®7 with molecular weight cutoff (MWCO) of 3500 (Spectrum Laboratories Inc., Rancho Dominguez, CA). The dialysis tubing was immersed in a 50 mL of PBS pH 5.5 or 7.4 with continuous stirring in a water bath at 37°C.

The release studies of DOX from liposomes were performed by placing 200 μL of liposome solution into dialysis tubing Spectra Por®7 with MWCO 3500 (Spectrum Laboratories Inc.). The liposomes were then immersed in 50 mL of PBS, pH 7.4, with continuous stirring in a water bath at 37°C.

At various time points, 200 μL aliquots were withdrawn from the outer aqueous solution and replaced by 200 μL of PBS. The DOX concentration was measured fluorometrically at λex=485 nm and λem=590 nm. Wurster correction was used for calculating the cumulative amount of DOX released.

In vitro cytotoxic assay of liposomes
Murine LLC cells were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Miayagi, Japan). LLC cells were cultured in RPMI-1640 medium with 10% heat-inactivated fetal bovine serum and kanamycin (100 μg/ml) in a humidified atmosphere containing 5% CO2 at 37°C. For the in vitro cytotoxic assay, LLC cells were seeded separately at a density of 1 × 10⁴ cells per well in 96-well plates and maintained in the medium for 24 h before treatment.

To examine the cytotoxicity for DOX, the cells were treated with medium containing various concentrations of DOX in AS-L, TEA-L, or TEA-PGA-Ls, and they were then incubated for 48 h. After treatment, the cell number was determined using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). Cell viability was expressed relative to absorbance at 450 nm in untreated cells, and the concentration leading to 50% cell viability (IC₅₀) was calculated.

Antitumor activity of liposomal doxorubicin
All animal experiments were performed with approval from the Institutional Animal Care and Use Committee of Hoshi University. To generate LLC tumors, 1 × 10⁶ cells suspended in 100 μL of PBS, pH 7.4, were inoculated subcutaneously into the right flank of female C57BL/6N mice (7-weeks old, Sankyo Lab. Service Corp., Tokyo, Japan). After the tumor size had reached about 100–200 mm³, AS-L, TEA-L, and TEA-PGA-Ls were administered via the tail vein at a dose equal to 5 mg DOX per kg mouse by single-dose injection on day 0. Tumor volume and body weights were measured for individual animals. The tumor volume was calculated using the following formula: tumor volume = 0.5 × a × b², where a and b are the larger and smaller diameters, respectively.

Biodistribution of liposomal doxorubicin
To generate LLC tumors, 1 × 10⁶ cells were inoculated subcutaneously into the flank of female C57BL/6N mice (female, 7-weeks old). After the tumor size had reached 200 mm³, AS-L, TEA-L, and TEA-PGA-Ls were administered intravenously via the tail vein at a dose equivalent to 5 mg DOX per kg. Twenty-four hours after injection, blood was collected from the inferior vena cava and centrifuged to obtain serum. The tumor, liver, spleen, kidney, and lung were collected from the inferior vena cava and centrifuged to obtain serum. The tumor, liver, spleen, kidney, and lung were excised and homogenized in 0.1 M of NH₄Cl/NH₃ buffer, and DOX was extracted with chloroform-methanol (2:1 v/v). The DOX concentration was further quantified using HPLC method as previously described (Taniguchi et al., 2010).

Statistical analysis
All data were produced in replicates and presented as the mean ± SD. To evaluate the significance of the difference, data were analyzed by one-way ANOVA, followed by Tukey’s HSD post-hoc test, with p values less than 0.05 considered as statistically significant.

Results
Preparation and characterization of liposomal doxorubicin using poly-L-glutamic acid
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 (Drummond et al., 2006, 2010). These mechanisms may be
able to maintain DOX in ionized forms and then increase intraliposomal stability by electrostatic interactions between DOX and PGA.

TEA-PGA-Ls were prepared by remote loading of DOX with a TEA gradient into preformed liposomes prepared with 1, 2, or 4 mg/mL PGA (molecular weights 4800, 9800, or 20500; Table 1). However, in TEA-PGA20500-L, it was difficult to obtain small, homogenous liposomes prepared with 4 mg/mL of PGA20500 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. In TEA-L and TEA-PGA9800-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 60 min decreased entrapment efficiency of DOX (data not shown). Furthermore, the highest entrapment efficiency was observed in liposomes prepared at a DOX:HSPC ratio of 1:5 (w/w) compared with 1:2 or 1:1 (data not shown). 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 1, all the liposomes had average particle sizes around 110 nm, with negative charges of particles equivalent to approximately −15 mV. 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, ζ-potential, or entrapment efficiency of DOX.

Evaluation of doxorubicin-poly-L-glutamic acid complex formation

To evaluate the interaction of PGA with DOX, we mixed DOX solution with PGA and then observed the mixture by microscopy. To estimate the intraliposomal conditions, the molar ratio of PGA and DOX inside the liposomes was calculated based on the concentration of DOX and total vesicular volume. When PGA and DOX were mixed at estimated molar ratios of DOX/PGA as listed in Table 1, aggregate-like substances were observed (Figure 1A–E), which had different structures compared with aggregates formed by mixing of DOX and AS solution (Figure 1F). Furthermore, an increase in molecular weight or concentration of PGA in the mixture of PGA and DOX resulted in larger aggregates. On the other hand, no aggregates were observed with the mixture of DOX and L-glutamic acid (Figure 1G) or DOX in TEA solution (Figure 1H). These results indicated that PGA may electrostatically interact with DOX inside liposomes and facilitate the encapsulation of DOX in TEA-PGA-Ls.

Evaluation of in vitro doxorubicin released from doxorubicin-poly-L-glutamic acid aggregates

To examine the effect of the interaction between DOX and PGA inside liposomes, we measured the amount of DOX released from DOX/PGA aggregates into PBS at pH 5.5 or 7.4. Here, we used PGA with molecular weight of 20500. In DOX/AS aggregates, regardless of pH, most of the DOX was released from the aggregate over 23 h (Figure 2). In contrast, in PGA20500/DOX aggregates, the amount of DOX released at pH 7.4 was only 28% over 23 h. On the other hand, at pH 5.5, the amount released was significantly increased 2-fold than that at pH 7.4 (Figure 2). This finding suggested that PGA might have an advantage in the pH-sensitive release of DOX from PGA/DOX aggregates. Furthermore, in PGA with molecular weight of 9800, the amount of DOX released from PGA9800/DOX aggregates (a molar ratio of DOX/PGA9800 of 5:8) at pH 7.4 and 5.5 was 55% and 80%, respectively, over 23 h (data not shown), indicating that PGA9800/DOX aggregates released DOX faster than PGA20500/DOX aggregates.

The effect of poly-L-glutamic acid on drug release from liposomes

The in vitro profiles of DOX release from liposomes were evaluated by immersing liposomes in PBS, pH 7.4 (Figure 3). Compared with TEA-L, TEA-PGA-Ls showed reduced DOX release. Among the TEA-PGA-Ls prepared with 2 mg/mL of PGA, TEA-PGA20500-C2-L, which were prepared with the highest molecular weight PGA, showed the lowest release of DOX from liposomes of approximately 13% of the cumulative dose over 52 h, which was similar to AS-L. Furthermore, among TEA-PGA-Ls prepared at 1, 2, and 4 mg/mL PGA9800, the TEA-PGA9800-C4-L, which were prepared with 4 mg/mL PGA9800, yielded slower release of DOX than TEA-PGA9800-C1-L and TEA-PGA9800-C2-L that were prepared with 1 and 2 mg/mL of PGA9800, respectively.

In vitro cytotoxic assay of liposomes

Next, we evaluated the cytotoxicity of TEA-PGA-Ls in LLC cells by 48-h exposure (Figure 4). TEA-L and AS-L showed lower cytotoxicity (0.6 and 0.9 µg/mL, respectively) than DOX solution (0.05 µg/mL; Figure 4A). Irrespective of the PGA molecular weight (Figure 4B) or PGA concentrations (Figure 4C), the IC50 values of TEA-PGA-Ls (0.6–0.9 µg/mL) were almost the same as those of AS-L and TEA-L, which were approximately 18-fold lower than that of DOX solution. These results indicated that exposure of cells to dosage forms such as liposomes or solution was more influenced by the in vitro cytotoxicity of DOX than the intraliposomal conditions.

Antitumor activity of liposomal doxorubicin

The antitumor activity of TEA-PGA-Ls was evaluated in LLC tumor-bearing mice (Figure 5). Compared with the injection of saline or DOX solution, a single injection of liposomal DOX could inhibit tumor growth until day 8. Among the TEA-PGA-Ls prepared with different molecular weights of PGA (Figure 5A) or concentrations of PGA (Figure 5B), TEA-PGA20500-C2-L or TEA-PGA9800-C4-L strongly inhibited tumor growth similar to AS-L. No body weight change was observed during the period of the experiment (data not shown).

Biodistribution of liposomal doxorubicin

Finally, the biodistribution of liposomal DOX at 24 h after intravenous injection was evaluated in LLC tumor-bearing mice (Figure 6). Compared with DOX solution, which was
almost cleared from the systemic circulation (0.6% of the injected dose remained at 24 h), TEA-PGA-Ls remained in serum at 10–15% of the injected dose at 24 h (Figure 6A). Higher tumor accumulation of DOX was observed in TEA-PGA9800-C4-L and AS-L than TEA-PGA4800-C2-L or TEA-PGA9800-C2-L (Figure 6B). This suggested that TEA-PGA-Ls prepared with a high concentration of PGA could improve tumor accumulation of DOX by prolonged

Figure 1. Microscopic observation of mixtures of doxorubicin (DOX) and poly-L-glutamic acid (PGA) (A–E), DOX in 0.25 M of ammonium sulfate (AS) solution (F), mixture of DOX and L-glutamic acid solution (DOX/L-glutamic acid at molar ratio of 5) (G), and DOX in 0.65 M of triethylamine (TEA) solution (H). The number in parentheses represents the estimated molar ratio of DOX and PGA inside liposomes, as listed in Table 1. A mole of PGA was calculated as L-glutamic acid monosodium salt. Scale bar = 100 μm.

Figure 2. Profiles of doxorubicin (DOX) release from DOX/poly-L-glutamic acid (PGA) and DOX/ammonium sulfate (AS) aggregates in phosphate-buffered saline (PBS), pH 5.5, and 7.4. DOX/PAGA20500 aggregates were prepared at a molar ratio of DOX/PAGA of 5.8. A mole of PGA was calculated as L-glutamic acid monosodium salt. Each value represents mean ± SD (n = 3). *p < 0.05.
circulation in the bloodstream (EPR effect). The accumulation
of DOX in the liver and spleen after injection of TEA-PGA-
Ls and AS-L was higher than that of DOX solution (Figure 6C
and D). In contrast, the accumulation of DOX in kidneys,

lung, and heart after injection of DOX solution, AS-L, or
TEA-PGA-Ls was low and did not show significantly
differences between them (Figure 6E–G).

Discussion

Preparation of liposomes with high and stable drug loading is
a promising strategy to enhance the antitumor effect by EPR.
In this study, we prepared TEA-PGA-Ls using anionic PGA
as an intraliposomal 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 pH 7 and provide useful sites
for interactions with cationic drugs, such as DOX
(Abbruzzetti et al., 2000). 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 (Figure 1).
Furthermore, reduction of DOX release from liposomes was
observed in TEA-PGA-Ls compared with TEA-L that did not

Figure 3. Profiles of release of doxorubicin (DOX) from triethylamine-
poly-L-glutamic acid liposomes (TEA-PGA-Ls) in phosphate-buffered
saline (PBS), pH 7.4 at 37 °C. Each value represents mean ± SD (n = 3).
*p < 0.05.

Figure 4. In vitro cytotoxicities of ammonium sulfate liposomes (AS-L) and triethylamine liposomes (TEA-L) (A), and triethylamine-poly-L-glutamic
acid liposomes (TEA-PGA-Ls) (B and C) on LLC cells. AS-L and TEA-L (A), TEA-PGA-Ls prepared with PGA with molecular weights of 4800,
9800, or 20 500 (B) or at various concentrations of PGA9800 (1, 2, or 4 mg/mL) (C) were added to the cells at various concentrations of DOX and then
incubated for 48 h. Each value represents mean ± SD (n = 3).
contain PGA (Figure 3). 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 by increasing either the concentration or length of PGA. It has been reported that the interaction of PGA and DOX does involve not only 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 (Manocha & Margaritis, 2010). Furthermore, we observed a pH-dependent release of DOX from PGA/DOX aggregates (Figure 2). It has been reported that the \( pKa \) value of PGA was 5.4 (Abbruzzetti

Figure 5. The antitumor activity of triethylamine-poly-L-glutamic acid liposomes (TEA-PGA-Ls) on LLC tumor-bearing mice. TEA-PGA-Ls were prepared with PGA with molecular weights of 4800, 9800, or 20 500 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). Each value represents mean ± SD (\( n = 3–4 \)).

Figure 6. The biodistribution of doxorubicin (DOX) in LLC tumor-bearing mice at 24 h after a single intravenous injection of DOX solution, ammonium sulfate liposomes (AS-L) and triethylamine-poly-L-glutamic acid liposomes (TEA-PGA-Ls) at a dose equal to 5-mg DOX per kg. Each value represents mean ± SD (\( n = 4 \)). *\( p < 0.05 \).
et al., 2000). Therefore, protonation of the carboxyl group in PGA at pH 5.5 might result in the dissociation of DOX from DOX/PGA aggregates.

In the case of the AS gradient method, beside DOX precipitation occurring as the result of over-solubility limits, the formation of DOX-sulfate gel increased drug retention inside the liposomes (Haran et al., 1993; Lasic et al., 1995). However, it has been reported that these precipitates of DOX are bioavailable, which means the DOX can be released from liposomes (Horowitz et al., 1992). From the results of in vitro cytotoxicity assays against LLC cells, the cytotoxicity of TEA-PGA-Ls was similar to that of AS-L (Figure 3), indicating that DOX entrapped in TEA-PGA-Ls could also be bioavailable to produce toxic effects on tumor cells.

The TEA-PGA-Ls prepared with high molecular weight or high concentration of PGA showed similar profiles of cytotoxicity, biodistribution, 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 (Figure 1). Therefore, to investigate the physical characteristics of their aggregates, we measured small angle X-ray diffraction (SAXRD) 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 and 0.6 Å⁻¹ (Supplemental Figure S1), indicating that their liposomes exist as vesicles (Brzustowicz & Brunger, 2004; Kucerka et al., 2005). However, at q below 0.05 Å⁻¹, 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 (Hirai et al., 2013). 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 nonspherical (Barenholz, 2012). 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-PGA₄₈₀₀₀-C₂-L and TEA-PGA₉₈₀₀₀-C₂-L was negligible (Supplemental Figure S1). 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.

DOX release from AS-L was slower than those of TEA-PGA₄₈₀₀₀-C₂-L and TEA-PGA₉₈₀₀₀-C₂-L, although it was similar with that of TEA-PGA₂₀₅₀₀-C₂-L (Figure 3). As shown in Figure 1(F), AS/DOX aggregates were larger in size than PGA/DOX aggregates. Especially, PGA with low molecular weight (PGA₄₈₀₀₀ or PGA₉₈₀₀₀) produced smaller aggregates in size than PGA with high molecular weight (PGA₂₀₅₀₀) or AS (Figure 1), and TEA-PGA₄₈₀₀₀-C₂-L and TEA-PGA₉₈₀₀₀-C₂-L exhibited faster release of DOX than TEA-PGA₂₀₅₀₀-C₂-L or AS-L (Figure 3). These findings indicated that the size of aggregate inside the liposome might affect release rate of DOX. Therefore, the molecular weight of PGA in TEA-PGA-L can adjust release rate of DOX, and combination of PGAs with low and high molecular weights might be able to produce TEA-PGA-Ls having both properties of immediate and delayed releases of DOX.

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 successful delivery of TEA-PGA-Ls to the tumor tissue by EPR effects. In EPR effects, maintaining a high drug concentrations in the blood can have substantial impact on drug exposure of tumor tissues (Bertrand et al., 2014; Dawidczyk et al., 2014). As shown in Figure 6, 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-PGA₉₈₀₀₀-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 (Iyer et al., 2006; Fang et al., 2011). 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 intraliposomal 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.

Conclusions

In this study, for stable drug encapsulation into liposomes, we prepared liposomal DOX using PGA as an internal trapping agent, and evaluated its biodistribution and antitumor effect on LLC tumor-bearing mice. Tumor growth suppression and DOX accumulation were achieved in TEA-PGA-Ls prepared with high molecular weight or high concentration PGA. This finding suggested that PGA can act as an excellent trapping agent for liposomal carriers.

Acknowledgements

The authors would like to thank Mr. Takuto Kikuchi for his assistance and kind help in this experimental work. We are also grateful to Dr. Hideaki Takagi and Dr. Nobutaka Shimizu for kind help and technical assistance in setting up the SAXRD experiments.

Declaration of interest

The authors declare no conflicts of interest or financial interests in any product or service mentioned in this article, including grants, employment, gifts, stock holdings, honoraria, consultancies, expert testimony, patents, and royalties.

The experiments involving SAXRD analysis requiring synchrotron beam time were performed with approval from
the Photon Factory Program Advisory Committee (Proposal No. 2014P012) of the National Laboratory for High Energy Physics (KEK), Tsukuba, Japan.

References

Abbruzzetti S, Viappiani C, Small JR, et al. (2000). Kinetics of local helix formation in poly-l-glutamic acid studied by time-resolved photoacoustics: neutralization reactions of carboxylates in aqueous solutions and their relevance to the problem of protein folding. Biophys J 79:2714–21.

Barenholz Y. (2012). Doxil® - the first FDA-approved nano-drug: lessons learned. J Control Release 160:117–34.

Bertrand N, Wu J, Xu X, et al. (2014). Cancer nanotechnology: the impact of passive and active targeting in the era of modern cancer biology. Adv Drug Deliv Rev 66:22–25.

Brzustowicz MR, Brunger AT. (2004). X-ray scattering from unilamellar lipid vesicles. J Appl Cryst 38:126–31.

Buescher JM, Margaritis A. (2007). Microbial biosynthesis of polyglutamic acid biopolymer and applications in the biopharmaceutical, biomedical and food industries. Crit Rev Biotechnol 27:1–19.

Cheung BC, Sun TH, Leenhouw JM, Cullis PR. (1998). Loading of doxorubicin into liposomes by forming Mn2+-drug complexes. Biochim Biophys Acta 1414:205–16.

Cho K, Wang X, Nie S, et al. (2008). Therapeutic nanoparticles for drug delivery in cancer. Clin Cancer Res 14:1310–16.

Dawidczyk CM, Kim C, Park JH, et al. (2014). State-of-the-art in design rules for drug delivery platforms: lessons learned from FDA-approved nanomedicines. J Control Release 187:133–44.

Dickie L, Toncheva V, Dubruel P, et al. (2000). Poly-l-glutamic acid derivatives as vectors for gene therapy. J Control Release 65:187–202.

Dicko A, Tardi P, Xie X, Mayer L. (2007). Role of copper gluconate/ammonium sulfate gradients in liposomes produce efficient and stable physical stability of liposomes upon storage. Int J Pharm 127:273–8.

Drummond DC, Meyer O, Hong K, et al. (1999). Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors. Pharmacol Rev 51:691–743.

Drummond DC, Noble CO, Guo Z, et al. (2010). Development of a highly stable and targetable nanoliposomal formulation of topotecan. J Control Release 141:13–21.

Drummond DC, Noble CO, Guo Z, et al. (2006). Development of a highly active nanoliposomal irinotecan using a novel intraliposomal stabilization strategy. Cancer Res 66:3271–7.

Du Plessis J, Ramachandran C, Weiner N, Muller DG. (1996). The influence of lipid composition and lamellarity of liposomes on the physical stability of liposomes upon storage. Int J Pharm 127:273–8.

Fang J, Nakamura H, Maeda H. (2011). The EPR effect: unique features of tumor blood vessels for drug delivery, factors involved, and limitations and augmentation of the effect. Adv Drug Deliv Rev 63:136–51.

Gabizon A, Shmeeda H, Barenholz Y. (2003). Pharmacokinetics of pegylated liposomal Doxorubicin: review of animal and human studies. Clin Pharmacokinet 42:419–36.

Haag R, Kratz F. (2006). Polymer therapeutics: concepts and applications. Angew Chem Int Ed Engl 45:1198–215.

Haran G, Cohen R, Bar LK, Barenholz Y. (1993). Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphiphatic weak bases. Biochim Biophys Acta 1151:201–15.

Hattori Y, Shi L, Ding W, et al. (2009). Novel irinotecan-loaded liposome using phytic acid with high therapeutic efficacy for colon tumors. J Control Release 136:30–7.

Hiromi M, Kimura R, Takeuchi K, et al. (2013). Structure of liposome encapsulating proteins characterized by X-ray scattering and shell-modeling. J Synchrotron Radiat 20:869–74.

Horowitz AT, Barenholz Y, Gabizon AA. (1992). In vitro cytotoxicity of liposome-encapsulated doxorubicin: dependence on liposome composition and drug release. Biochim Biophys Acta 1109:203–9.

Hwang SH, Maitani Y, Qi XR, et al. (1999). Remote loading of diclofenac, insulin and fluorescein isothiocyanate labeled insulin into liposomes by pH and acetate gradient methods. Int J Pharm 179:181–8.

Iyer AK, Khaled G, Fang J, Maeda H. (2006). Exploiting the enhanced permeability and retention effect for tumor targeting. Drug Discov Today 11:812–18.

Kirby C, Clarke J, Gregoriadis G. (1980). Effect of the cholesterol content of small unilamellar liposomes on their stability in vivo and in vitro. Biochem J 186:591–8.

Kokkonen M, Kallinteri P, Fatouros D, Antimisiaris SG. (2000). Stability of SUV liposomes in the presence of cholate salts and pancreatic lipases: effect of lipid composition. Eur J Pharm Sci 9:245–52.

Konarev PV, Volkov VV, Sokolova AV, et al. (2003). PRIMUS: a windows PC-based system for small-angle scattering data analysis. J Appl Cryst 36:1277–82.

Kucerka N, Trisram-Nagle S, Nagle JF. (2005). Structure of fully hydrated fluid phase lipid bilayers with monounsaturated chains. J Membr Biol 208:193–202.

Lasic DD, Ceh B, Stuart MC, et al. (1995). Transmembrane gradient driven phase transitions within vesicles: lessons for drug delivery. Biochim Biophys Acta 1239:145–56.

Manoche B, Margaritis A. (2010). Controlled release of doxorubicin/γ-glutamic acid ionic complex. J Nanomater 2010:780171.

Mats M, Salzano G, Leonetti C, et al. (2011). Nanotechnologies to use bisphosphonates as potent anticancer agents: the effects of zolodronic acid encapsulated into liposomes. Nanomedicine 7:955–64.

Moghimi SM, Farhangrazi ZS. (2014). Just so stories: the random acts of anti-cancer nanomedicine performance. Nanomedicine 10:1661–6.

Nickels JD, Perticaroli S, Ehlers G, et al. (2015). Rigidity of poly-l-glutamic acid scaffolds: influence of secondary and supramolecular structure. J Biomed Mater Res A 103:2909–18.

Otani Y, Tabata Y, Ikada Y. (1996). A new biological glue from gelatin and poly(ε-glutamic acid). J Biomed Mater Res 31:58–66.

Taniguchi Y, Kawano K, Minowa T, et al. (2010). Enhanced antitumor efficacy of folate-linked liposomal doxorubicin with TGF-β type I receptor inhibitor. Cancer Sci 101:2207–13.

Tansey W, Ke S, Cao XY, et al. (2004). Synthesis and characterization of branched poly(ε-glutamic acid) as a biodegradable drug carrier. J Control Release 94:39–51.

Thomas AM, Kapanen AI, Hare JI, et al. (2011). Development of a liposomal nanoparticle formulation of 5-fluorouracil for parenteral administration: formulation design, pharmacokinetics and efficacy. J Control Release 150:212–19.

Zhigaltsev IV, Maurer N, Akhong QF, et al. (2005). Liposome-encapsulated vincristine, vinblastine and vinorelbine: a comparative study of drug loading and retention. J Control Release 104:103–11.

Zucker D, Marcus D, Barenholz Y, Goldblum A. (2009). Liposome drugs’ loading efficiency: a working model based on loading conditions and drug’s physicochemical properties. J Control Release 139:73–80.

Supplementary material available online
Supplemental Figure S1