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Preparation and in vitro/in vivo evaluation of doxorubicin-loaded poly [lactic-co-glycol acid] microspheres using electrospray method for sustained drug delivery and potential intratumoral injection

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ABSTRACT

For cancer treatment, intratumoral drug injection has many limitations and not commonly adopted. The poly [lactic-co-glycolic acid] (PLGA) has emerged as a promising vehicle to enhance the in vitro/in vivo characteristic of various drugs. We prepared doxorubicin-PLGA microspheres (DOX-PLGA MSs) using the electrospray method. An in vitro elution method was employed to evaluate the release of DOX from the MSs. We performed an in vivo study on rats, in which we directly injected DOX-PLGA MSs into the liver. We measured liver and plasma DOX concentrations to assess local retention and systemic exposure. The mean diameter of the MSs was 6.74 ± 1.01 μm. The in vitro DOX release from the MSs exhibited a 12.3 % burst release on day 1, and 85.8 % of the drug had been released after 30 days. The in vivo tests revealed a higher local drug concentration at the target lobe of the liver than at the adjacent median lobe. In the first week, the DOX concentration in the peripheral blood of the MS group was lower than that of the direct DOX injection group. Based on the measured intrahepatic concentration and plasma pharmacokinetic profiles, DOX-PLGA MSs could be suitable vectors of chemotoxic agents for intratumoral injection.

1. Introduction

The concept of intratumoral injection has been advocated for decades. However, this technique has been not applied widely due to its disadvantages, including the invasive nature of intratumoral injection, the relatively rapid clearance of topically applied drugs from tumors, and the development of dose-limiting toxicity in tissues surrounding the application site [1]. In the case of unresectable tumors or infiltrating lesions (e.g., unresectable pancreatic cancer, as well as infiltrating cholangiocarcinoma or gastric cancer encasing the celiac trunk, superior mesenteric artery, or porta hepatis), systemic chemotherapy is usually ineffective and local treatment is impossible due to its high-risk nature and anatomical limitations. Patients may experience intolerable pain or obstructive symptoms due to the progression of the disease. Therefore, enhancement of the locoregional therapeutic effect has become an important issue. While conventional local treatment with radiofrequency ablation (RFA) or cryotherapy cannot usually be used in these difficult anatomical regions, intratumoral injection of radioactive holmium-166 microspheres (MSs) has been advocated for local tumor control in the case of recurrent head and neck cancer [2,3]. Brachytherapy, directly implanting iodine-125 into malignant pancreatic tumors, has also been reported [4]. Despite these successes, there have been difficulties regarding the widespread acceptance of the manipulation of radioisotopes by patients.

Doxorubicin hydrochloride (DOX) is a representative chemotherapeutic agent for treating various cancers, such as head and neck cancer, breast cancer, gastric cancer, hepatocellular carcinoma, ovarian cancer, bladder tumors, leukemia, bone and soft tissue sarcoma, etc [5]. Intratumoral DOX injection has been shown to increase necrosis in combination with percutaneous RFA in a rat breast tumor model [6]. Furthermore, DOX can act as a radiosensitizing agent, which is used to increase the effectiveness of radiotherapy. Pegylated liposome-

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encapsulated DOX has been used to deliver target drugs directly to tumors [7]. DOX has also been found to synergize with cancer immunotherapies, enhancing antitumor responses in syngeneic mouse models [8]. This is due to its ability to boost the antitumor responses of different cancer immunotherapies.

Poly[lactic-co-glycolic acid] (PLGA) has been demonstrated to be an appropriate vector for drug targeting and delivery, and has advantages such as a longer release time, biodegradability, lack of toxicity, and antigenicity. PLGA hydrolysis produces lactic and glycolic acids, which are metabolized into carbon dioxide and water via the Krebs cycle [9]. Although PLGA has been studied extensively as a material for use in drug carriers [10], its feasibility as an intratumoral chemotoxic agent has not been evaluated in depth by in vitro and in vivo studies. In this study, we manufactured DOX-embedded PLGA MSs, and tested them as a chemotoxic agent in a rat model. It is difficult to efficiently encapsulate a hydrophilic drug (such as DOX) in MSs composed of hydrophobic polymer matrices (such as PLGA) using the water-in-oil emulsification method, and the size of the resulting MSs is difficult to control. Electrospray is a promising method for efficiently and eff ;ectively encapsulating proteins, drugs, and contrast agents whilst maintaining the stability of the hydrophilic therapeutic agents, minimizing the loss of biological viability, and allowing for outstanding control of the MS architecture [11–13]. The process allows DOX, as a representative chemotoxic agent, and many other drugs or even vulnerable proteins be integrated into PLGA MSs, which expands the choice of therapeutic agents.

Combining the FDA-approved PLGA based particles and intra-tumoral delivery access, the treatment would be much closer to the clinical practice than other studies and could be a simple alternative or complementary to upgrade the effect of present standard care. To prove this concept, we chose the combination of PLGA, DOX and electrospray process, apply the MSs in the liver model and find it feasible with anticipated effect. Most previously developed microparticles were designed for transarterial delivery and the particle size was the most important issue rather than the loaded drug to avoid nontarget organ injury. Therefore, we applied the electrospray method to fabricate DOX-PLGA MSs.

The objective of this study was to prepare DOX-PLGA MSs for potential intratumoral injection and target drugs at unresectable tumors or tumors that respond poorly to conventional treatment, and to achieve sustained drug release and prolonged chemotherapeutic effects. The MSs were prepared via an electrospray method, and evaluated in vivo and in vitro in terms of drug loading, size distribution, and drug release. In the in vivo tests using a rat model, we evaluated the diffusion of the drug from the injection site into the peripheral blood to obtain an indirect metric of drug stagnation. We also monitored the drug concentration at the injection site to validate the effectiveness of drug targeting. This study explores a new intratumoral administration method for DOX, thus advancing the current state of research on intratumoral delivery systems. This result could serve as the base for further studies on tumor treatment models.

2. Experimental section

2.1. Materials

PLGA was purchased from a commercial source (Resomer RG 503; Boehringer, Ingelheim am Rhein, Germany), and had a 50:50 lactide-to-glycolide ratio and a molecular weight of 33,000 Da. DOX was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). The electrospray apparatus included a needle and syringe, an aluminum collection plate, a grounding electrode, and a high-voltage direct current power supply. 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) solvent was also obtained from Sigma-Aldrich Co.

2.2. Preparation and manufacture of DOX-PLGA MSs

The DOX-PLGA MSs were fabricated using the electrospray method. The electrospraying parameters were chosen based on previous studies of the optimization of electrosprayed particles [14]. The temperature and relative humidity of the environment used for the electrospray experiments ranged from 22 to 24 °C, and 44–49%, respectively. The collectors were made from standard aluminum foil (20 × 20 cm2). Approximately 300 mg of PLGA and 50 mg of DOX were pre-mixed with 2 mL of HFIP. After combining thoroughly, the mixture was loaded into a 2.5-mL glass syringe fitted with a 26-gauge stainless steel nozzle (Terumo, Tokyo, Japan and Becton Dickinson, Franklin Lakes, NJ, USA), and then extruded through the nozzle at a constant rate of 0.5 mL/h using a syringe pump (World Precision Instruments, Sarasota, FL, USA). The tip-to-collector distance was set to 20 cm. A voltage of 18 kV was applied between the needle and the collector. After electrospraying, the collectors were placed in a vacuum for 72 h to remove any HFIP residue from the MSs. The MSs were then transferred into glass vials and further evacuated for storage.

2.3. Characterization of the DOX-PLGA MSs

We evaluated the surface morphologies of the MSs by coating them with gold then imaging them using a scanning electron microscope (SEM; S3000 N; Hitachi, Tokyo, Japan). We used the ImageJ software application to calculate the diameter distribution and average diameter of the MSs based on the SEM images. To confirm that the drugs have been successfully loaded in the MSs, the Fourier transform infrared spectroscopy (FTIR) assay was conducted on a Bruker Tensor 27 spectrometer in the absorption mode at a resolution of 4 cm^{-1} for 32 scans. Samples of MSs were pressed as KBr discs, and the absorption spectra were recorded over the 700 to 4000 cm^{-1} range.

2.4. In vitro release study

We used in vitro elution to monitor the release of DOX from the MSs. Phosphate-buffered saline (PBS, pH 7.4) was used as the dissolution medium. The MSs were placed in glass test tubes with 1 mL of PBS. All tubes were incubated at 37 °C. The dissolution medium was collected and analyzed every 24 h. Fresh PBS (1 mL) was then added for the next 24 h. The appropriate concentrations of DOX in the buffer for the elution studies were determined using high-performance liquid chromatography (HPLC). The HPLC analyses were conducted on a Waters 600 Multisolvent Delivery System (Waters, Milford, MA, USA), along with a SYMMETRY C8 column (3.9 cm × 150 mm; Waters) to separate the DOX. The mobile phase contained Trichloroacetic Acid BioXtra (≥ 99.0 %; Sigma-Aldrich Co.) and acetonitrile (Mallinckrodt, St. Louis, MO, USA) (40/60, v/v). The absorbance was set to 480 nm, and the flow rate was 1.0 mL/min. All analyses were repeated three times for 30 days.

2.5. Preparation of DOX-PLGA-MSs before in vivo injection

Before injection into the liver of the rat model, 7 mg of MSs were placed in 2 mL of distilled water for 5 min to prevent an initial burst and subsequent fluctuations in drug concentration. Afterwards, the MSs suspension was centrifuged at 1000 rpm for 10 min to separate the MSs from the fluid. We then aspirated the supernatant as much as possible using a 25-gauge needle, and then evaluated the remainder using HPLC to determine the percentage of DOX released by the burst. Finally, PBS was added to the residual MSs to a total volume of 0.4 mL for further intrahepatic injection in the test group.

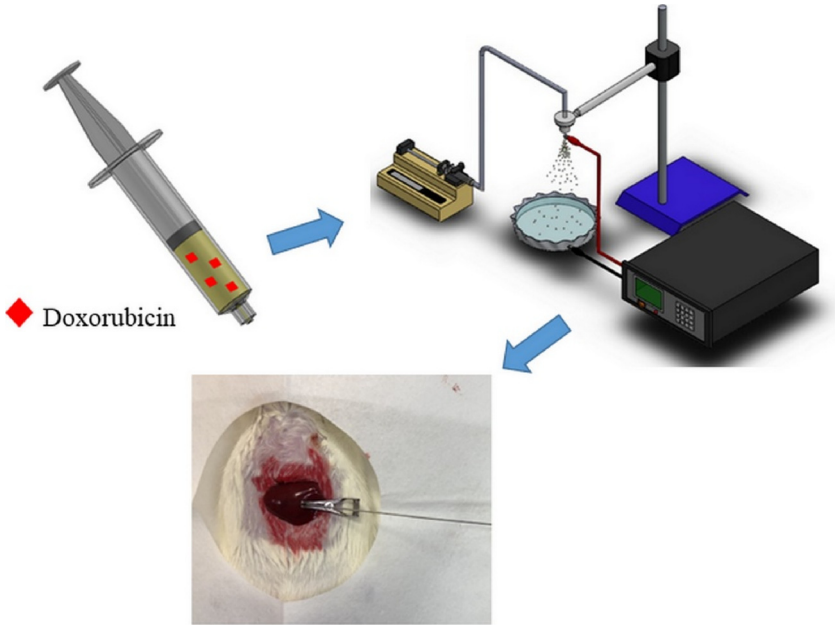


Fig. 1. Fabrication process of DOX-PLGA MSs and injection of DOX-PLGA MSs into left lateral lobe of liver.

2.6. In vivo study

2.6.1. Animal study

This study was approved by the institutional animal care and use committee and performed in accordance with institutional guidelines. In total, 28 male Sprague Dawley rats, weighing approximately 250 g each, were used for the pharmacokinetic studies; 25 rats were given DOX-PLGA MSs injections and 3 rats receiving direct DOX injection were used as the control. The rats were anesthetized by general anesthesia with isoflurane (Sigma-Aldrich Co.) (1.5 % mixture with O2, 1.5 L/min flow through a nose cone; VetEquip Inc., Pleasanton, CA, USA). Fig. 1 shows the experimental setup. After shaving and sterilization, we made a laparotomy incision about 2 cm in length. The left lateral lobe of the liver was identified and exposed. A 21-gauge needle was used to puncture the liver, followed by an injection of 7 mg of DOX-PLGA MSs in 0.4 mL of PBS solution, corresponding to an equivalent dose of 1 mg pure DOX. After the MS injection, a 5-mm sterile stainless steel wire was placed in the liver through the needle. This served as a localization marker for further tissue sampling. Blood and tissue samples were taken after 3, 7, 14, 28, and 42 days, and the specimens collected were then analyzed with HPLC. After 3, 7, and 42 days, the liver tissues were extirpated for histological analysis.

In the control group, we calculated the equivalent amount of DOX administered by deducting the amount released by the initial burst. We then injected the appropriate dose into the left hepatic lobe using the same method. We monitored the systemic drug concentrations by taking blood samples after 3, 7, 14, 28, and 42 days.

2.6.2. Pharmacokinetic study of DOX

First, 7 mg of DOX-PLGA MSs containing 1 mg of DOX, corresponding to an equivalent dose of 4 mg/kg, was administered to each rat in the DOX-PLGA MS group. The rats were euthanized via intraperitoneal injection of sodium pentobarbital (70 mg/kg). We collected blood samples by cardiac puncture using syringes. We sampled 0.05 *g* of liver tissue from the left lateral lobe of each rat. We also took samples from the liver tissue in the median lobe for comparison. Blood and tissue samples were collected after 3, 7, 14, 28, and 42 days and centrifuged. Samples was collected and stored at −20 °C until analysis. The tissue sample of liver was homogenized (Homogenizer Prep-CB24, MedClub, Tao-Yuan, Taiwan) with 1 mL of PBS at the speed of 6 m/s for 30 s. The process was repeated for three times, followed by filtration with PVDF syringe filter (0.22 μm pore size). After centrifugation for 10 min at 10,000 rpm, the supernatant was collected and 20 μL of the supernatant was injected into the HPLC system for further analysis. The DOX concentrations in the plasma were also characterized quantitatively using HPLC.

2.7. Statistical analysis

We conducted a Mann–Whitney *U* test to compare the pharmacokinetic parameters and DOX tissue concentrations between the control and DOX-PLGA MS groups. All statistical analyses were performed using the commercially available SPSS software package (ver. 15.0; SPSS Inc., Chicago, IL, USA). The results were considered to be statistically significant when $p < 0.05$.

3. Results and discussion

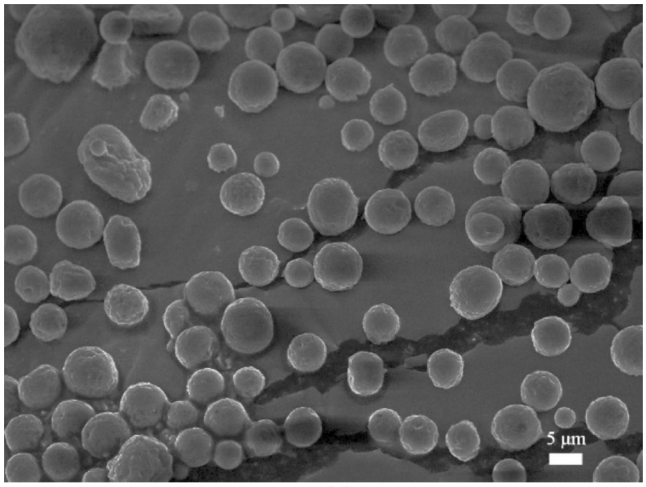
3.1. Preparation and characterization of DOX-PLGA MSs

The drug loading of the fabricated DOX-PLGA MSs was 14.3 %. The initial burst when the DOX-PLGA-MSs were placed in distilled water for 5 min was calculated to be 9.7 %. Therefore, 0.9 mg of DOX was used for each injection in the control group.

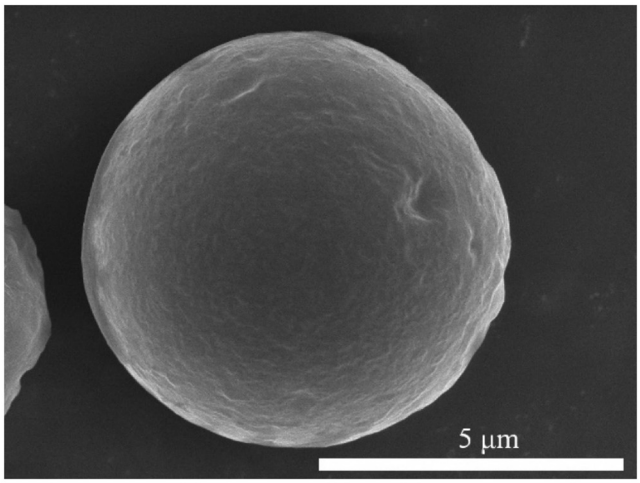
3.2. Scanning electron microscopy

Fig. 2A and B show the SEM images of the DOX-PLGA MSs. The DOX-PLGA MSs had a mean diameter of 6.74 ± 1.01 μm, a uniform shape, and relatively smooth outer surfaces.

Biodegradable DOX-eluting MSs made of alginate, chitosan, cellulose, and PLGA have been used as embolic particles for local drug delivery [15–18]. These have mainly been fabricated using the ion exchange and oil-in-water emulsion/solvent evaporation methods [10,15–18]. Although the emulsification method is a relatively simple procedure to fabricate drug-loaded particles, its encapsulation efficiency is usually low [19,20]. Besides, to precisely control the size of particles by emulsification could be challenging [21,22]. The advantage of electrospraying is that different morphologies and sizes of drug-loaded microparticles can be fabricated by manipulating the operating



(A)



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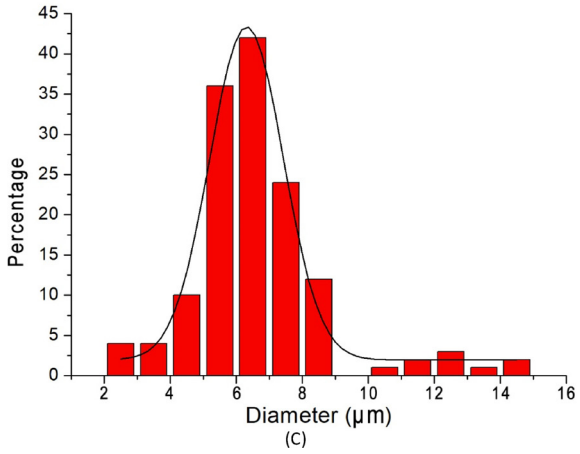

(C)

Fig. 2. (A, B) Scanning electron microscopy images of microspheres. (C) size distribution with the mean diameter of 6.74 ± 1.01 μm.

parameters such as the external voltage, flow rate, and polymer concentration, etc. with a high encapsulation efficiency up to 100 % theoretically [19]. Furthermore, advanced coaxial electrospray techniques mean that double-layered microparticles can also be produced to encapsulate proteins, drugs, and contrast agents with high efficiency and controllable shell thicknesses [12].

DOX-loaded PLGA MSs made by emulsification have been used previously for intraarterial embolization in rats with the mean diameter of 26.36 ± 6.39 μm and drug encapsulation efficiency of 50.25 % [15]. The ideal size for achieving therapeutic embolization effects in rats should allow the particles to penetrate into the terminal arterioles (10–50 μm) but not pass through the capillaries (8–10 μm), so as to avoid systemic circulation [23]. In addition to the considerations regarding embolization, particles with small diameters and large surface areas per unit volume are associated with an increased rate of drug release than larger particles. This is due to water permeation and matrix degradation [12,24]. This makes small microparticles ideal for intratumoral injection due to the increase in the amount of drug released locally.

One of the major concerns relating to intratumoral injection is its invasive nature, the safety of the puncture route and the potential risk of tract tumor seeding. Sometimes, the deep location of the tumor may be obscured by an internal organ or abutting major vessels. Several studies have shown the safety and feasibility of percutaneous needle puncture through the intra-abdominal solid organ or bowel loop using either fine needles or core needles [25–27]. In this study, the DOX-PLGA MSs were injected using fine needles with gauges up to 21 to minimize the risk of organ injury. Furthermore, due to their small size, the DOX-PLGA MSs may also be delivered to the target sites using endoscopic ultrasound-guided puncture for gastrointestinal tract tumors, or endobronchial ultrasound-guided transbronchial puncture in the case of original or metastatic pulmonary tumors.

3.3. Fourier transform infrared spectroscopy

Fig. 3 shows the FTIR spectra of the analyzed samples. The new absorptions at wavenumbers 1600 cm^{-1}, 1620 cm^{-1} and 3320 cm^{-1} may corresponded to the amine NH_2 bending, aromatic C=C stretch, and the alcohol OH stretch of DOX, respectively. The results of FTIR assay demonstrated the successful loading of DOX into the PLGA polymers.

3.4. In vitro drug release

Fig. 4 shows the results of the in vitro DOX release from the MSs. We observed a 12.3 % burst release on the first day, followed by a sustained and constant drug release, and 85.8 % of the drug was released after 30 days.

Drug release from a biodegradable carrier generally occurs in three phases: primary burst, diffusion-dominated release, and degradation-dominated elution [13]. After electrospraying, most drugs are dispersed into the volume of DOX-PLGA MSs; however, some drug formulations on the particle surface may result in an initial drug release burst, followed by controlled drug release by diffusion and other factors. The agglomeration of particles, leading to a smaller total surface area, may also affect the release pattern. Relatively constant slow elution of the anticancer agents was thus observed.

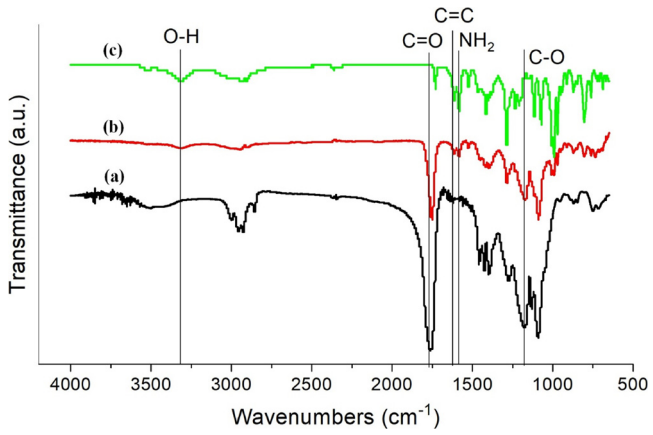

Fig. 3. FTIR spectra of PLGA (a), DOX-PLGA MSs (b) and pure DOX (c).

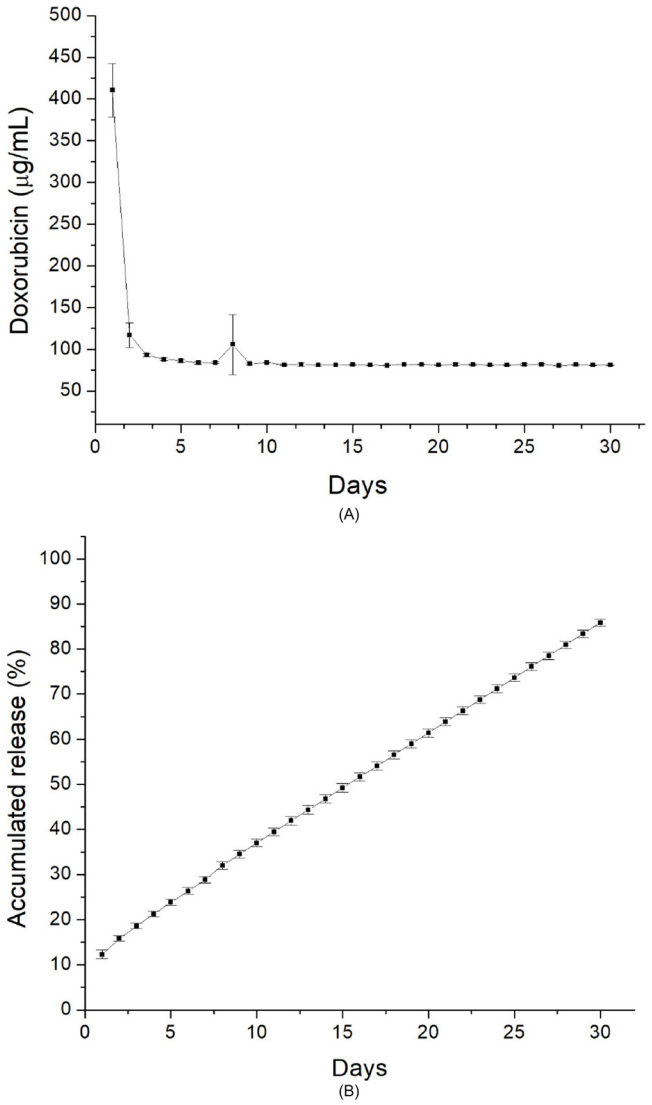

Fig. 4. In vitro drug release test. The daily accumulated release kinetics of PLGA microspheres loaded with doxorubicin (DOX-PLGA MSs). In vitro release of DOX in a static elution model. (A) Daily and (B) accumulated DOX release curves of the MSs. An initial release burst occurred on the first day, followed by stable release for 30 days.

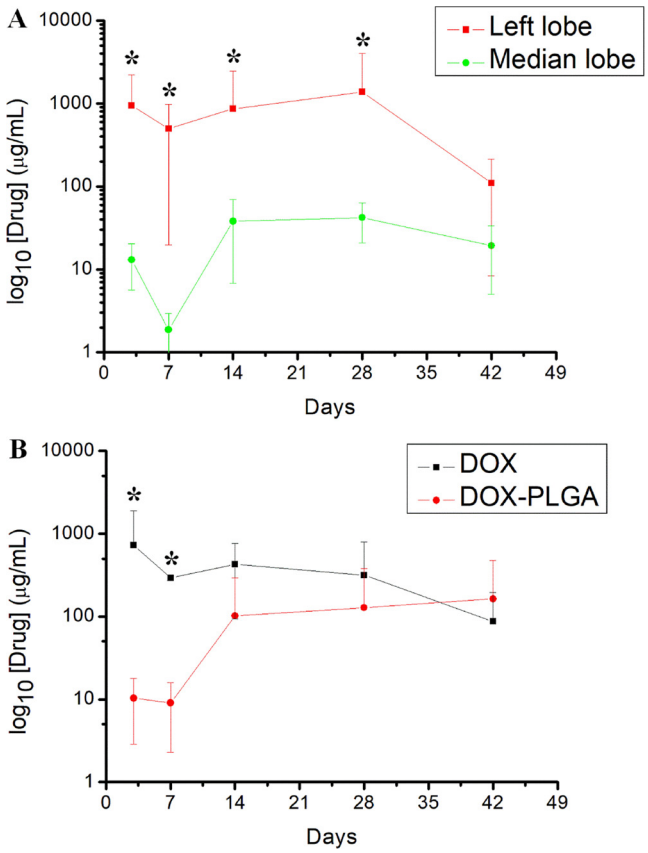

Fig. 5. (A) Doxorubicin (DOX) concentrations in the left and median hepatic lobes of the rats. The concentration in the left lobe was much higher than that in the median lobe, and the diff ;erence was significant between day 3 and day 28. (B) Plasma DOX concentrations after injection of DOX-PLGA microspheres (MSs) and free DOX in rats. In the first week, the concentration of DOX in the blood of the control group was significantly higher than that of the DOX-PLGA MS group. Each point is represented as the mean ± standard deviation. *, $P < 0.05$.

3.5. In vivo pharmacokinetics

We evaluated the in vivo DOX levels after 3, 7, 14, 28, and 42 days using five rats for each test. The measured DOX concentrations in the liver tissues and peripheral blood are shown in Fig. 5. There were two peaks in mean DOX concentration of the left hepatic lobe, after 3 and 28 days, with values of 944.99 ± 1257.85 μg/mL and 1375.40 ± 2634.28 μg/mL, respectively. The concentration then dropped to 110.36 ± 101.98 μg/mL at day 42. The concentration of DOX in the median lobe was consistently lower than that of the left lateral lobe (Fig. 5A). Meanwhile, the concentration of DOX in the left lateral lobe remained much higher than that of the blood. In the control group, which received direct drug injections, the concentration of DOX in the blood was higher than that of the DOX-PLGA MS group in the first week (Fig. 5B). This indicated that the drug was redistributed rapidly from the liver tissue, leading to systemic circulation after the DOX had been injected directly into the liver.

The results of our study showed that the DOX-PLGA MSs sustainably released high concentrations of DOX to the local area for at least 28 days, while the DOX concentration of the neighboring median lobe and peripheral blood remained consistently low. The biodegradable MSs provide continuously high concentrations of DOX, thus potentially enabling local tumor control and minimizing systemic toxicity and side effects.

3.6. Histology

Fig. 6 presents the results of the histological examination of normal liver, presence of MSs after 3 days and absence of MSs at 42 days.

Raising the locoregional drug concentration is critical for cancer treatment, especially when chemotherapy is ineffective or has limited results due to dose-related systemic toxicity. Hepatic arterial infusion of oxaliplatin with systemic chemotherapy offers an improved survival outlook for patients with unresectable hepatic colorectal cancer, after previous systemic chemotherapy has failed [28]. Commercialized drug-eluting beads are mostly made of polyvinyl alcohol-based hydrogel (PVA), Polyzene®-F-coated hydrogel, and tris-acryl gelatin, and are classified as permanent embolizers for transarterial chemoembolization [10,29]. Doxorubicin, epirubicin, and irinotecan, which are positively charged, are the only chemotherapies that can be readily used in clinical practice [30]. The total drug release from DOX-loaded PVA MSs has been reported to be less than 30 % [31]. By contrast, the biodegradable DOX-PLGA MSs developed in this study allow for the sustained and complete release of chemotherapy drugs into lesions, thereby achieving a prolonged effect on tumor cells while minimizing systemic exposure. The intratumoral injection can be performed alone or combined with

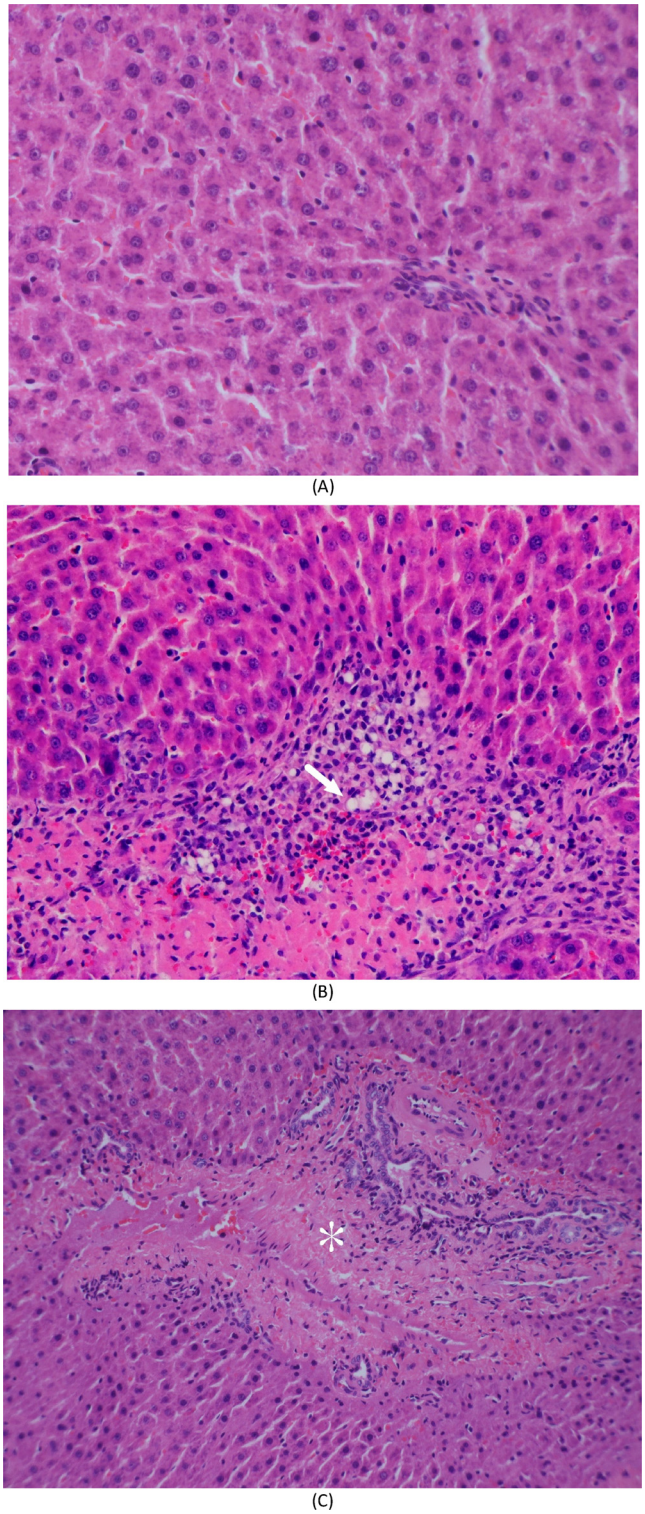

Fig. 6. Histological analysis of the liver tissue (hematoxylin and eosin staining, ×20). (A) normal liver, (B) 3 days, which showing DOX-PLGA MSs (arrow) surrounded by macrophages. (C) 42 days, which showing the absence of DOX-loaded MSs with residual fibrosis (star) in the liver parenchyma.

RFA, systemic chemotherapy, radiotherapy, or immunotherapy to obtain a synergistic treatment protocol. Furthermore, the electrospraying process could potentially be used to manufacture MSs containing a single chemotherapeutic agent or multiple drugs to achieve a better therapeutic effect.

There are some limitations to this study. First, the sample size was small, which could introduce prominent biases. Second, we used blood sampling to assess the systemic concentration of DOX. Nevertheless, we did not monitor any solid organs other than the liver, so we did not make a full assessment of systemic exposure. Third, we aimed to evaluate the feasibility and potential of DOX-PLGA-MSs for intratumoral injection. We did not consider the influence of the manufacturing parameters on the MS diameter, drug loading, and release pattern. Fourth, we used a healthy liver model rather than a diseased tumor model because the effect of DOX is not limited to a single type of tumor and the pharmacokinetics of the drug release can be preliminarily evaluated in healthy rat models. Finally, the MSs were immersed in distilled water to induce the initial burst release prior to the intrahepatic injection. Approximately 9.7 % of the drug was lost, thus reducing the effective drug loading. A coaxial electrospray method may be used to minimize drug loss due to the initial burst. All of these topics will be addressed in future studies.

4. Conclusions

We applied the electrospray method to fabricate PLGA MSs loaded with DOX. The DOX-PLGA MSs were uniform in shape and had relatively smooth outer surfaces with a mean diameter of 6.74 ± 1.01 μm. We detected a 12.3 % burst release of the drug on the first day, while about 85.8 % of DOX had been eluted from the PLGA MSs after 30 days.

The in vivo results suggested that the biodegradable MSs released high concentrations of DOX to the target tissue for more than 4 weeks. The results of the assessments of the neighboring median hepatic lobe and peripheral blood suggested that systemic exposure was consistently low. Therefore, DOX-PLGA MSs are good candidates as sustainable drug delivery vehicles to targeted lesions, and may enhance the therapeutic efficacy of various treatments for tumors.

CRediT authorship contribution statement

Ming-Yi Hsu: Conceptualization, Formal analysis, Writing - original draft. **Yu-Ting Huang:** Conceptualization, Writing - original draft. **Chun-Jui Weng:** Data curation. **Chien-Ming Chen:** Formal analysis. **Yong-Fong Su:** Methodology. **Sung-Yu Chu:** Methodology, Resources. **Jeng-Hwei Tseng:** Resources. **Ren-Chin Wu:** Resources. **Shih-Jung Liu:** Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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