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Design of a zero-order sustained release PLGA microspheres for palonosetron hydrochloride with high encapsulation efficiency

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ABSTRACT

Efficient encapsulation of hydrophilic drugs was substantially challenging when using emulsion solvent evaporation approach. The aim of present study was to design palonosetron hydrochloride-loaded PLGA microspheres (Pal-MS) with high encapsulation efficiency (EE) to sustain drug release for over several days. Pal-MS were prepared using emulsion-solvent evaporation method. Results showed that the pH of external phase could significantly affect the EE and the drug release rate of Pal-MS. By increasing the pH of external phase from 5.0 to 10.0, EE of Pal-MS increased from 55.64% to 94.33%. When the pH of external phase was 7.0, an ideal Pal-MS with EE of 86.51% and a zero-order drug release profile was obtained. The improved EE and drug release performance was proved to be associated with possible PLGA degradation, enhanced drug-PLGA interaction and reduced drug diffusion from organic phase to aqueous phase. After subcutaneous injection, such Pal-MS showed more steady drug plasma concentration (0.207–1.238 ng/ml) over the entire 6-day in comparison with those of multiple-day-dosing intravenous palonosetron hydrochloride solution. It was concluded that Pal-MS were successfully designed by the adjustment of the pH of external phase and could be promising for preventing both acute and delayed chemotherapy-induced nausea and vomiting (CINV).

1. Introduction

Chemotherapy-induced nausea and vomiting (CINV) is a common adverse effect amongst cancer patients, which can not only reduce the quality of life, but also affect the continuous chemotherapy (Hernandez Torres et al., 2015; Sommariva et al., 2016). For moderately emetogenic chemotherapy (MEC), there is a 30%-90% risk of CINV, while the risk of CINV is higher than 90% for the highly emetogenic chemotherapy (HEC) (Nasir and Schwartzberg, 2016; Navari and Aapro, 2016). Typically, CINV occurs in two phases, the acute phase (occurs in the first 24 h after chemotherapy, defined as acute CINV), and the delayed phase (occurs in about 24–120 h after chemotherapy, defined as delayed CINV) (Navari and Aapro, 2016). At present, the acute CINV has been well managed with 5-hydroxytryptamine type 3 (5-HT_3) receptor antagonists (RAs), e.g. ondansetron, dolasetron, granisetron and palonosetron (Hesketh, 2000; Saito et al., 2009). The prevention of delayed CINV, however, is still challenging clinically.

Palonosetron hydrochloride, a second generation 5-HT_3 RA, has been proved to have an approximately 100-fold higher binding affinity to 5-HT_3 receptor and a longer half-time (~40 h) than those of the first generation 5-HT_3 RAs (Rojas et al., 2008). These properties make palonosetron more suitable for the treatment of delayed CINV in comparison with the first generation 5-HT_3 RAs. It was reported from the results of four phase III trials that, compared with the first generation 5-HT_3 RAs (ondansetron, dolasetron and granisetron), palonosetron showed a comparable complete response (CR) for the acute CINV (69% vs 66%), but an increased CR for delayed CINV (57% vs 45%, $P < 0.0001$) in patients received by either MEC or HEC (Schwartzberg et al., 2014). Despite of the improvement in preventing the delayed CINV, the CR rate for delayed CINV was still lower than that for acute CINV after a single dose of palonosetron (Raftopoulos et al., 2015; Schwartzberg et al., 2014). A relatively simple means to improve the therapeutic efficacy for delayed CINV of palonosetron was to apply multiple-day dosing in patients (Einhorn et al., 2007; Mirabile et al., 2014). However, even intravenous administration of palonosetron on day 1, 3 and 5, the CR rates for delayed CINV was only approximately 60% as reported in previous literature (Einhorn et al., 2007; Wu et al., 2012). It was likely that the prevention of delayed CINV required a prolonged and steady blood drug concentration, and this concept was demonstrated by the approval of Sustol® (Granisetron Extended-Release Injection, muscular injection) by FDA for the treatment of CINV, especially for delayed CINV (Inc, 2016). Therefore, given the high

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binding affinity of palonosetron, delayed CINV could be effectively reduced if palonosetron is designed into a sustained release formulation with steady drug concentration level for several days (preferably 5–6 days). To the authors' knowledge, there have been very few researches regarding the injectable sustained release drug delivery systems for palonosetron.

In this study, we intended to design a microsphere drug delivery system for palonosetron using poly(lactide-co-glycolide) (PLGA) to provide a relatively long-period and steady blood drug concentration. PLGA microspheres have been extensively used as sustained release delivery systems for a variety of drugs due to its excellent biocompatibility and tailorable sustained-release period (Lin et al., 2018; Ramazani et al., 2016). An ideal PLGA microspheres should have high drug encapsulation efficiency (EE) and a desirable drug release profile (preferably zero-order) for a certain period of time (Ye et al., 2010). Encapsulation of hydrophilic drugs can be substantially challenging when using emulsion solvent evaporation approach. This was because that hydrophilic drugs tend to diffuse from the organic phase or internal aqueous phase into the aqueous phase (external aqueous phase) during emulsification and solidification, resulting in unacceptable EE value (Ramazani et al., 2016). Our preliminary experiments showed that palonosetron hydrochloride (commercially provided and stable form) had extremely high water solubility, and the EE value was only circa 20% using either O/W or W/O/W emulsion-solvent evaporation method.

Therefore, the overall aim of the present study was to design a palonosetron hydrochloride loaded PLGA microspheres with zero-order drug release profile to potentially improve the therapeutic effect on CINV. Meanwhile, taking palonosetron hydrochloride as a model of typical water-soluble drug, we also investigated the mechanism of increasing the EE value of hydrophilic drugs when preparing PLGA microspheres using emulsion solvent evaporation approach.

2. Materials and methods

2.1. Materials

Palonosetron hydrochloride (Pal) was purchased from Hubei Yuancheng Pharmaceutical Co., LTD. (Wuhan, China). PLGA (Resomer® Select2A, 50:50, IV: 0.15–0.25 dL/g, ended with free carboxylic acid groups; Resomer® RG 502, 50:50, IV: 0.16–0.24 dL/g, ended with ester groups) was kindly donated by Evonik Industries AG (Darmstadt, Germany). Polyvinyl alcohol (PVA 217, 87–89% hydrolyzed, viscosity of 20.5–24.5) was a gift from Kuraray CO., Ltd. (Shanghai, China). Acetonitrile (chromatographic grade) were purchased from Sinopharm Chemical Reagent CO., LTD. (Shanghai, China). All other reagents were of analytical grade.

2.2. Preparation of Pal-loaded PLGA microspheres (Pal-MS)

2.2.1. W/O/W emulsion-solvent evaporation method

Formulation details of Pal-MSs were listed in Table 1. As Pal was freely water soluble (Table S1), initially Pal-MS were prepared by W/O/W emulsion-solvent evaporation method (F1). Briefly, 60 mg Pal was dissolved in 800 μl water to obtain internal aqueous phase. The internal water phase was then added into 4 ml dichloromethane containing 600 mg PLGA and was homogenized at 12000 rpm for 2 min using digital ULTRA-TURRAX® (IKA T18, Germany) at 4 ˚C to obtain W_1/O emulsion. The obtained W_1/O emulsion was subsequently added into 40 ml 1% PVA (w/v) aqueous solution and was homogenized at 5000 rpm for 3 min using IKA T18 digital ULTRA-TURRAX® at 4 ˚C to obtain W/O/W emulsion. The W/O/W emulsion was immediately transferred into 360 ml 1% PVA (w/v) aqueous solution and stirred at 40 ˚C under vacuum to remove the organic solvent. After 15 min, the PLGA microspheres were collected via centrifugation at 1500 rpm for 5 min and washed with purified water for three times. Finally, the microspheres were freeze-dried using freeze dryer (LGJ-15E, China).

2.2.2. O/W emulsion-solvent evaporation method

Since Pal was also soluble in dichloromethane, Pal-MS formulations (F2 ~ F9 in Table 1) was prepared using O/W emulsion-solvent evaporation method. Briefly, Pal and PLGA were dissolved in 4 ml dichloromethane to obtain organic phase. The organic phase was then added into 40 ml external phase and was homogenized at 5000 rpm for 3 min using IKA T18 digital ULTRA-TURRAX® at 4 ˚C to obtain O/W emulsion. The obtained O/W emulsion was immediately transferred into 360 ml 1% PVA (w/v) aqueous solution and stirred at 40 ˚C under vacuum to remove the organic solvent. After 15 min, the PLGA microspheres were collected via centrifugation at 1500 rpm for 5 min and washed with purified water for three times. Finally, the microspheres were freeze-dried using freeze dryer (LGJ-15E, China).

2.3. Preparation of Pal solution (Pal-sol) for intravenous administration

Pal-sol was prepared according to the formulation of commercial palonosetron hydrochloride injection (ALOXI®). Briefly, 0.1 g disodium EDTA, 0.028 g Pal and 20.75 g mannitol were respectively dissolved in 480 ml sterilized water. The pH of the solution was adjusted to 5.0 using citric acid aqueous solution (0.2 M) and the volume was then made up to 500 ml with sterilized water. Finally, the preparation was sterilized by filtration through a filter with pore size of 0.22 μm.

2.4. Preparation of blank PLGA microspheres

In order to thoroughly investigate the effect of external phase pH on the properties of Pal-MS, blank PLGA microspheres, which were termed as B-F4 ~ B-F9, were prepared using the same procedure of formulation (F4 ~ F9) in Table 1 without the addition of Pal.

2.5. Characterization of Pal-MS

2.5.1. Determination of drug loading (DL) and encapsulation efficiency (EE)

Approximately 10 mg Pal-MS were dissolved in 10 ml acetonitrile containing 1% trimethylamine. 2 ml of the obtained solution was diluted to 10 ml using methanol–water (7:3, v/v) solvents. The concentration of Pal was determined using high performance liquid chromatography (HPLC, Agilent 1260, Germany) equipped with a UV detector at 210 nm. The chromatographic separation was performed on a ZORBAX SB-C18 column (150 mm × 4.6 mm, 5 μm) using acetonitrile–water- trifluoroacetic acid (28:72:0.67) as mobile phase at a flow rate of 1.0 ml/min. DL and EE of Pal-MS were respectively calculated using the following two equations:

$$DL = (\text{the determined mass of Pal/the mass of Pal}-\text{MS}) \times 100\%$$

$$EE = (\text{the calculated DL of Pal}-\text{MS/the theoretical DL of Pal}-\text{MS}) \times 100\%$$

All the tests were performed in triplicate.

2.5.2. Particle size distribution of Pal-MS

The particle size distribution of Pal-MS was determined using a laser diffraction particle size analyzer (Microtrac S3500, USA). 10 mg of Pal-MS were dispersed in 1 ml distilled water containing 0.1% (w/v) polysorbate 80 and was then added into the particle size analyzer for analysis.

2.5.3. Morphology of Pal-MS

The surface morphology of Pal-MS and blank PLGA microspheres was observed using scanning electron microscopy (SEM, Hitachi VP-SEM SU1510, Japan). The cross-sectional morphology of Pal-MS was also observed using SEM. The cross-sections of microspheres were

Table 1
Formulations and properties of Pal-loaded PLGA microspheres (Pal-MSs).

Formulation	Pal(mg)	PLGA (mg)	Drug/PLGA (w/w)	pH of external phase	Preparation method	External phase	DL[a] (%)	EE[b] (%)	BR[c] (%)	Particle size		
										d_{10}	d_{50}	d_{90}
F1	60	600	1:10	Deionized	W/O/W	1%PVA, deionized	1.61	17.93	25.76	4.29	8.40	12.43
F2	60	600	1:10	Deionized	O/W	1%PVA, deionized	2.03	22.57	11.81	11.13	22.90	34.55
F3	30	600	0.5:10	Deionized	O/W	1%PVA, deionized	1.62	34.32	9.26	12.21	23.06	38.45
F4	15	600	0.25:10	Deionized	O/W	1%PVA, deionized	1.40	53.95	7.26	14.60	25.69	41.35
F5	15	600	0.25:10	5.0	O/W	1%PVA, pH = 5.0	1.37	55.64	7.06	15.78	27.01	62.71
F6	15	600	0.25:10	6.0	O/W	1%PVA, pH = 6.0	1.81	71.98	10.65	9.09	20.16	44.36
F7	15	600	0.25:10	7.0	O/W	1%PVA, pH = 7.0	2.15	86.51	13.89	12.62	22.66	46.76
F8	15	600	0.25:10	8.0	O/W	1%PVA, pH = 8.0	2.23	90.16	28.11	13.06	23.09	55.54
F9	15	600	0.25:10	10.0	O/W	1%PVA, pH = 10.0	2.33	94.33	28.18	12.73	19.97	40.83

[a] DL represents drug loading of Pal-MS.
[b] EE represents encapsulation efficiency of Pal-MS.
[c] BR represents burst release of Pal-MS.

obtained via cutting with freezing microtome (Leica CM 1950, Germany). The intact microspheres and cross-sections of microspheres were spread on conductive adhesive tape and were glued on copper stub. The samples were then coated with a layer of gold and observed using SEM.

2.5.4. Molecular weight (Mw) of PLGA

5 mg freeze-dried blank PLGA microspheres prepared with different external phase pH values were respectively dissolved in 5 ml N,N-dimethyl formamide. The samples were analyzed using Waters 1515 gel permeation chromatography (GPC, Waters Corp., USA) equipped with a refractive index detector (Waters 2414, Waters Corp., USA). The mobile phase was N,N-dimethyl formamide at a flow rate of 1.0 ml/min.

2.5.5. Fourier infrared spectrum (FT-IR)

FT-IR spectrums of Pal, Pal-MS, blank microspheres and PLGA raw materials were recorded within the range of 3500–500 cm^{-1} on a FT-IR spectrometer (Bruker Tensor II, Germany) using attenuated total reflection (ATR) method.

2.6. In vitro drug release studies

Sample-and-separate method was used to evaluate the *in vitro* drug release of Pal-MS (Jiamian et al., 2019; Xia et al., 2018). Briefly, approximately 10 mg of Pal-MS were dispersed into 10 ml EP tubes containing 2 ml dissolution media (10 mM phosphate buffered saline (PBS) solution containing 0.1% (w/v) polysorbate 80 with pH of 7.4). The tubes were then incubated in a reciprocal shaking water bath (SHZ-B, China) at 37 °C with a shaking speed of 100 rpm. At each predetermined time point (0.083, 0.5, 1, 2, 3, 4, 5, 6 and 7 d), all the dissolution media were removed and replaced with 3 ml fresh dissolution media. The concentration of Pal in each sample was analyzed using HPLC. All experiments were carried out in triplicate.

2.7. Diffusion of Pal from organic phase into external phase

22.5 mg Pal and 900 mg PLGA were dissolved in 6 ml dichloromethane to obtain organic phase. 1 ml of the organic phase was added to EP tubes containing 10 ml 1% PVA (w/v) aqueous solution (external phase) with different pH values, including deionized, pH = 5.0, 6.0, 7.0, 8.0 and 10.0. The tubes were then incubated in a reciprocal shaking water bath (SHZ-B, China) at 40 °C with a shaking speed of 100 rpm. At each predetermined time point (5, 10, 15 and 30 min), 200 μl solution in the lower layer (external phases) were removed and replaced with 200 μl fresh external phase. The concentration of Pal in each sample was analyzed using HPLC. All experiments were carried out in triplicate.

The diffusion of Pal from dichloromethane without PLGA into different external phases was also carried out using the above method.

2.8. Pharmacokinetics studies

2.8.1. Experimental design

The pharmacokinetics of optimal Pal-MS was evaluated after subcutaneously injected to rats in comparison with intravenous palonosetron hydrochloride solution (Pal-sol). Twelve male Sprague-Dawley rats (Joinn laboratories CO., Ltd., China, with weight of 180–220 g) were randomly divided into two groups. All SD rats were fasted for 12 h with access to water ad libitum prior to experiments. For one group, Pal-sol was intravenously injected to each rat through the caudal vein at a dose of 0.5 mg/kg every other day for three doses. For another group, Pal-MS was suspended in 0.9% (w/v) NaCl aqueous solution containing 0.1% (w/v) polysorbate 80 and 1.0% (w/v) sodium carboxymethyl cellulose. The obtained Pal-MS suspension was subcutaneously injected into the nuchal midline of each rat at a single dose of 1.5 mg/kg. At each predetermined time point, approximately 0.3 ml blood samples were collected to heparinized tubes via the retro-orbital puncture. The collected blood samples were then centrifuged at 4000 rpm for 10 min. The supernatant plasma samples were collected and stored at −20 °C until analysis.

The protocol of the study was approved by Animal Ethics Committee Jiangnan University (approval number JN.No20181130W0361230[259]). The European Community guidelines as accepted principles for the care and use of experimental animals were adhered to.

2.8.2. Preparation of plasma samples

100 μl plasma, 20 μl ondansetron methanol solution (internal standard, IS, 100 ng/ml) and 100 μl NaOH solution (1 M) were added to 10 ml EP tube, and were vortexed for 2 min. The mixture was then extracted with 3 ml methyl tertiary butyl ether by vortexing for 10 min. After centrifugation at 4000 rpm for 10 min, 2.5 ml supernatant was collected into a 5 ml EP tube and evaporated to dry at 40 °C under the stream of nitrogen. The dried sample was reconstituted with 120 μl methanol and centrifuged at 15000 rpm for 10 min. 5 μl of the supernatant were injected into an ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) system for analysis.

2.8.3. Determination of plasma sample by UPLC-MS/MS

The concentration of Pal in plasma sample was determined using an UPLC-MS/MS method.

Liquid chromatography was performed on an ACQUITY™ UPLC system (Waters Corp., Milford, MA, USA). The separation of Pal was performed on an ACQUITY™ UPLC BEH C18 column (50 mm × 2.1 mm i.d., 1.7 μm; Waters Corp., USA) with the column temperature maintained at 35 °C. The sample was eluted gradiently

with a mobile phase consisting of 0.1% formic acid aqueous solution (A) and acetonitrile (B) at a flow rate of 0.2 ml/min. The gradient program was as follows: 30%→80% B (0–0.8 min), 80% B (0.8–2.0 min), 80%→30% B (2.0–2.5 min), 30% B (2.5–3.0 min).

A waters ACQUITY™ triple quadrupole (TQD) mass spectrometer (Waters Corp., Manchester, UK) equipped with electrospray ionization (ESI) source in positive ionization mode was used for mass analysis. The source parameters were as follows: capillary 1.0 kV, extractor 3.0 V, source temperature 150 °C, desolvation temperature 400 °C, desolvation gas 550 l/h, cone gas 50 l/h and collision gas 0.2 ml/min. Quantification of Pal and IS were operated in the multiple reaction monitoring (MRM) mode. The MRM transitions for Pal and IS were *m/z* 297.13 → 110.08 and *m/z* 294.14 → 170.08, respectively. The scan time for each MRM transition was set to 0.02 s. All data was acquired and processed using MassLynx™ V4.1 software (Waters Corp.).

Validation of analytical method for Pal showed that the method showed a linear response over the range of 0.05–100 ng/ml with a lower limit of quantification (LLOQ) of 0.05 ng/ml. The relative recovery of Pal from the plasma samples at three concentrations (0.1, 2.0 and 80 ng/ml) were in the range of 65.43%-89.4%. The inter-day precision and intra-day precision at three concentrations (0.1, 2.0 and 80 ng/ml) were all less than 8.93%, while the accuracy was −12.74% ~13.86%. The precision and accuracy were all within the limits (< 15%), showing acceptable precision and accuracy.

2.8.4. Data analysis

The pharmacokinetic parameters were calculated by non-compartmental method using Drug and Statistics software (DAS, version 3.0, Mathematical Pharmacology Professional Committee of China, Shanghai, China).

3. Results

3.1. Effects of preparation methods on properties of Pal-MS

W/O/W and O/W emulsion-solvent evaporation methods were commonly used for the preparation of PLGA microspheres. Since Pal could be both soluble in water and in dichloromethane, two kinds of Pal-MS were respectively prepared using W/O/W and O/W emulsion-solvent evaporation method. Therefore the effect of preparation methods on the EE and *in vitro* drug release of Pal-MS could be understood.

As can be seen in Table 1, Pal-MSs of F1 (by W/O/W method) and F2 (by O/W) both showed low EE values that were both below 25%. Comparing F1 and F2, it was found that F2 showed relative higher EE and larger particle size. Results of *in vitro* drug release showed that F2 had lower burst release (BR, defined as the cumulative drug release on day 1) than that of F1 as seen in Table 1 and Fig. 1. In addition, over the entire 7 day time period, F2 showed slower drug release than that of F1 (Fig. 1). This was likely to be attributed to the large particle size of F2, leading to increased drug diffusion length and decreased drug release surface area, and thus a lower burst release and slower drug release rate was observed for F2.

Although Pal-MS prepared by O/W method showed a higher EE than that of microspheres by W/O/W method, the EE value was only 22.57% which was far below the required value for micro-particulate medicines. This was because that Pal was freely water soluble (Table S1), and therefore a great level of drug migration from organic phase to aqueous phase would occur during the emulsification process, resulting in low EE value. The results suggested that conventional O/W approach should be optimized in order to improve the EE value of Pal-MS.

3.2. Effects of drug/PLGA ratio on properties of Pal-MS

It has been reported that the EE of hydrophilic drug in PLGA microspheres could be significantly affected by the drug/PLGA ratio

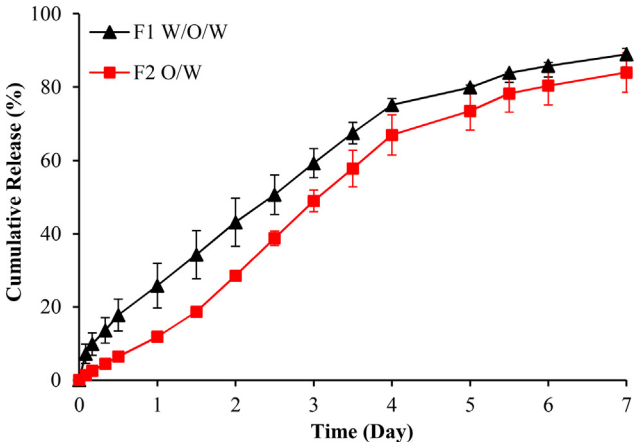

Fig. 1. The *in vitro* drug release profiles of Pal-MSs prepared using W/O/W and O/W emulsion-solvent evaporation method (F1 and F2) in 10 mM PBS solution (pH 7.4, 0.1% polysorbate 80) at 37 °C. Each point represents the mean ± SD; n = 3.

(Chigumira et al., 2015; Espanol et al., 2016). Pal-MSs with different drug/PLGA ratios (1:10, 0.5:10 and 0.25:10, F2, F3 and F4 in Table 1) were prepared and compared to investigate the effects of drug/PLGA ratio on the properties of Pal-MS.

It can be seen in Table 1 that that the EE values of Pal-MS increased from 22.57% to 53.95% by decreasing the drug/PLGA ratio from 1:10 to 0.25:10. This result was consistent with the research reported by Chigumira, *et al.* and Pathak, *et al.* (Chigumira et al., 2015; Pathak et al., 2016). The reason could be explained that the drug concentration gradient between the organic phase and the external phase decreased with decreasing the drug/PLGA ratio, which reduced the diffusion rate of drug from emulsion droplets to the external phase and thus increasing the EE of Pal-MS.

Drug release profiles of F2 to F4 were shown in Fig. 2, and it can be seen that the drug release rate of F3 and F4 were both slower than that of F2, indicating that decreasing the drug/PLGA ratio could reduce drug release rate of Pal-MS. In addition, drug release profiles of Pal-MS with drug to PLGA ratio of 0.5:10 and 0.25:10 (F3 and F4) were both well fitted to the zero-order kinetic equation as reflected by the acceptable regression coefficients (Table 2), while the drug release profile of F2 was poorly fitted to the zero-order equation. In short summary, these results suggested that when using O/W approach, Pal-MS with the drug to polymer ratio of 0.25:10 presented the highest EE value and the slowest drug release rate amongst the formulations. Therefore, in this

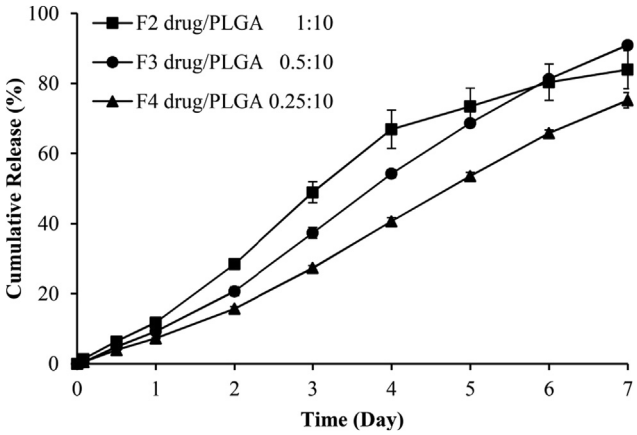

Fig. 2. The *in vitro* drug release profiles of Pal-MSs with different drug/PLGA ratios (F2, F3 and F4) in 10 mM PBS solution (pH 7.4, 0.1% polysorbate 80) at 37 °C. Each point represents the mean ± SD; n = 3.

Table 2
Evaluation of drug release kinetics of different Pal-MSs according to the zero-order release kinetics equation.

Formulation	F2	F3	F4	F5	F6	F7	F8	F9
Slope	13.25	13.64	11.03	9.858	11.72	12.29	12.40	12.01
Intercept	2.319	−2.202	−2.507	−1.042	0.546	1.906	12.15	11.12
R^2	0.9660	0.9953	0.9937	0.9934	0.9886	0.9957	0.9415	0.9312

study the following Pal microspheres were designed and investigated with this fixed drug/PLGA ratio (0.25:10).

3.3. Effects of the pH of external phase during preparation processing on the properties of Pal-MS

It has been reported that alkaline drugs could interact with PLGA chains that were ended with free carboxylic acid groups (Sanchez-Lopez et al., 2018). Such interactions could be influenced by the pH of aqueous phase (external phase) during the preparation processing possibly due to the deionization or ionization of the alkaline drugs when using O/W approach. These interactions could eventually affect the EE of PLGA microspheres. As Pal was a typical alkaline drug, in this study the effects of external phase pH on the properties of Pal-MS were accordingly investigated.

3.3.1. Particle size distribution, DL and EE of Pal-MS

As shown in Table 1, the Pal-MSs prepared with different external phase pH values showed similar particle size distributions. The DL and EE of Pal-MSs, however, both significantly increased with increasing the external phase pH values. When the external phase pH value was over 7.0, the EE of Pal-MSs (F7, F8, and F9) were all higher than 80%, which were significantly higher than the EE of F4 (53.95%) that was prepared using deionized external phase.

The above results may indicate that the interaction between Pal and the free carboxylic acid groups in PLGA might be influenced by the pH of external phase, which subsequently could affect the DL and EE of Pal-MS. To further confirm such interaction, PLGA that was ended with ester groups (Resomer® RG502, with similar Mw distribution to that of Resomer® Select2A) was used to prepare Pal-MS using the same formulation and preparation method as that of F7. Results showed that the drug loading of the obtained Pal-MS was only 0.17%, and the EE was only 6.74%, which were both significantly lower than those of F7. These results may demonstrate that the interactions between Pal and free carboxylic acid groups in PLGA played an important role in improving the DL and EE of Pal-MS.

3.3.2. Surface and cross-section morphology of Pal-MS

The surface and cross-section morphology of Pal-MSs prepared with different external phase pH values was observed using SEM.

As shown in Fig. 3, significant differences in the surface morphology were observed between the Pal-MSs prepared with different external phase pH values. It can be seen that F5 (Fig. 3B), F6 (Fig. 3C) and F7 (Fig. 3D) prepared with external phase pH values of 5.0, 6.0 and 7.0 all presented smooth surface, which was similar to F4 using deionized external phase (Fig. 3A). With increasing the external phase pH values to 8.0 (F8) and 10.0 (F9), evident porous surface was observed as shown in Fig. 3E and Fig. 3F.

Pal-MSs (F5 ~ F7) prepared using external phase with pH lower than or equal to 7.0 all showed dense internal structure with little pores, which were similar to F4 that was prepared using deionized external phase. When the pH of external phase was 8.0, the surface of the prepared Pal-MS (F8) became porous, and the Pal-MS started to present the internal structure with observed pores (Fig. 3E). With increasing the pH of external phase to 10.0, porous internal structure could be clearly seen in F10 (Fig. 3F).

The above results indicated that the external phase that had the pH values lower than or equal to 7.0 showed little influence on the surface and internal structure of Pal-MS. By increasing the pH of external phase higher than 8.0, the degradation of PLGA possibly occurred during the preparation process of microspheres, leading to a porous surface and internal structure of microspheres.

3.3.3. FT-IR spectra of Pal-MS

FT-IR spectra of Pal, PLGA raw materials and Pal-MSs prepared with different external phase pH values were analyzed, and the results were shown in Fig. 4.

As shown in Fig. 4, all Pal-MSs showed a strong peak at 1748 cm^{-1} and a weak peak at 1644 cm^{-1}, which were respectively ascribed to the C═O stretching vibration of the carbonyl groups from PLGA and the C═O stretching vibration of acylamino groups from Pal. For the Pal-MSs (F7 ~ F9) prepared with the external phase pH values higher than or equal to 7.0, a peak at 1622 cm^{-1} was observed and the intensity of this peak increased with increasing pH values of external phase (as pointed by red arrows in Fig. 4). The peak at 1622 cm^{-1} might be ascribed to the antisymmetric C═O stretching vibrations of carboxylate groups in PLGA. Detailed discussion of FT-IR results was in following sections.

3.3.4. In vitro drug release of Pal-MS

In vitro drug release profiles of Pal-MSs prepared with different external phase pH values were summarized in Fig. 5. The results showed that drug release rate of Pal-MS was associated with the external phase pH values. The burst release and drug release rate from Pal-MS both increased with increasing the external phase pH values. For instance, when the external phase pH was lower than or equal to 7.0, the obtained Pal-MSs (F4 ~ F7) all showed low level of burst release. The drug release profiles of these formulations were all well fitted to zero-order kinetic equation as shown in Table 2. By increasing the external phase pH value higher than or equal to 8.0, the corresponding Pal-MSs (F8 and F9) showed significantly increased burst release of approximately 28%. In addition, the drug release profiles of F8 and F9 were both poorly fitted to zero-order equation.

The above results demonstrated that the external phase pH values could substantially affect the key properties of Pal-MS, including EE, drug release profiles and morphology. Interestingly, although observed with porous structure, Pal-MSs that were prepared with high external phase pH values (F8 and F9) had high EE values compared with other formulations. According to the standard procedure of manufacturing microspheres using O/W approach, microspheres that have porous structure normally had low EE values (Lee et al., 2017; Xia et al., 2015). However, this was not the case in this study. An incubation study of microspheres with smooth surface was carried out. Formulations of F4 to F7 were respectively incubated in dissolution media at 37 °C for 2 h and the morphology was then observed using SEM (Fig. 6). After 2-hours incubation in dissolution media, the surface of F4 and F5 remained smooth, whereas the surface of F6 and F7 both became porous, indicating that F6 and F7 had faster PLGA degradation rate than that of F3 and F4. The results of incubation study also explained the reason why F6 and F7 showed faster drug release rate than F4 and F5, though they were all observed with smooth surface. All these results suggested that the adjustment of external phase pH values may have more influence on PLGA rather than on drugs.

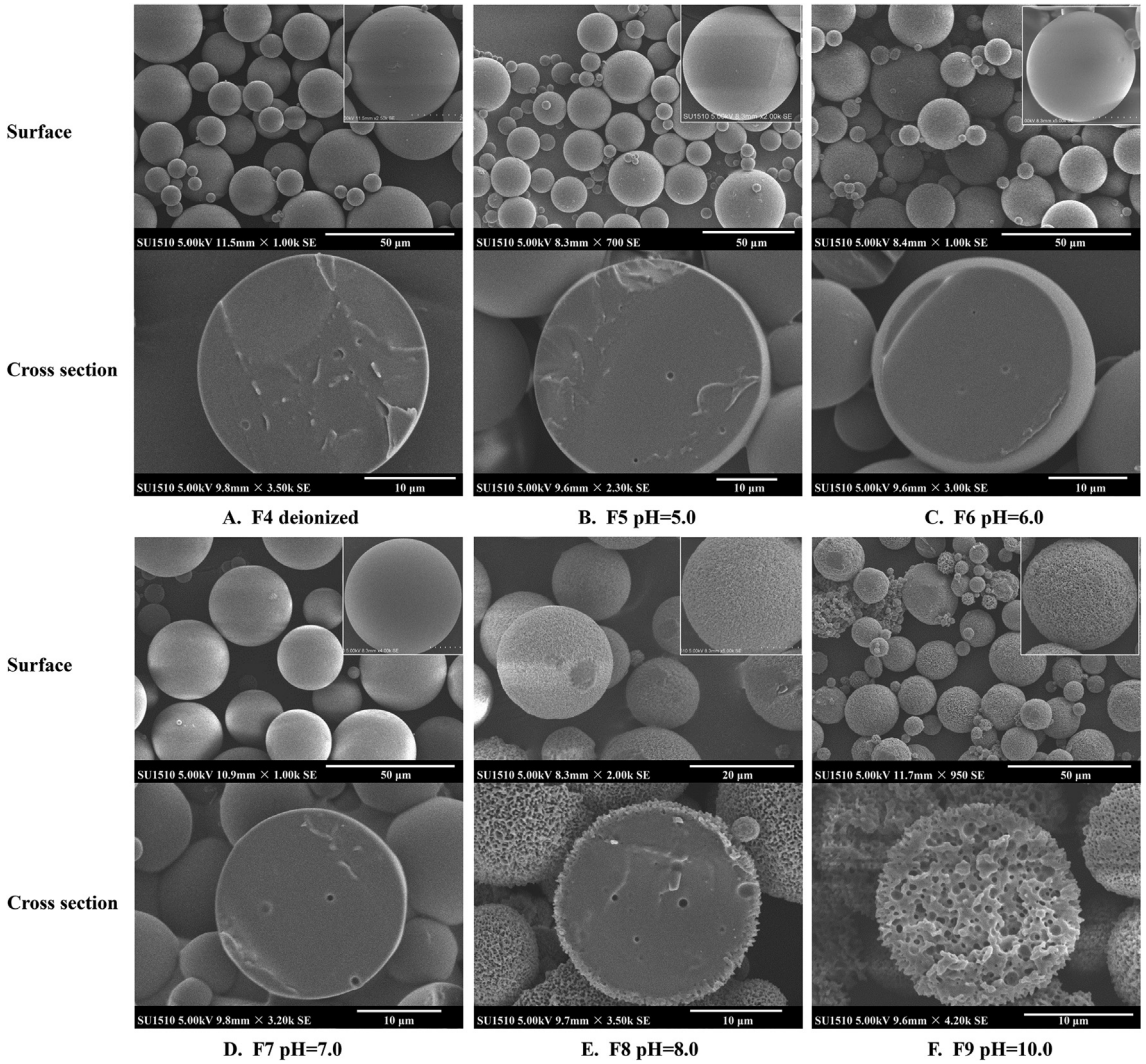

Fig. 3. SEM micrographs of surface and cross-sectional morphology of Pal-MSs (F4 ~ F9) prepared with different external phase pH values.

3.4. Effects of external phase pH on the properties of blank PLGA microspheres

The above results suggested that adjusting the external phase pH may have caused changes to PLGA, leading to improved EE values, increased drug release rate and porous surface. Therefore, in order to thoroughly understand the underpinning mechanism, blank PLGA microspheres, (named as B-F4 ~ B-F9 in this study), were prepared using the same procedure of formulation (F4 ~ F9) in Table 1 without the addition of Pal. The surface morphology, Mw of polymer and FT-IR spectrums of blank PLGA microspheres were investigated.

3.4.1. Surface morphology of blank PLGA microspheres

The surface morphology of blank PLGA microspheres prepared with different external phase pH values was shown in Fig. 7. It can be seen that the blank PLGA microspheres prepared with external phase pH lower than 7.0 (B-F4 ~ B-F7) all exhibited smooth surface, while the blank PLGA microspheres prepared with external phase pH of 8.0 and 10.0 (B-F8 and B-F9) both showed porous surface. This result was consistent with the Pal-loaded PLGA microspheres. These results demonstrated that the porous surface of microspheres prepared with external phase pH higher than 8.0 was not caused by drug migration from the emulsion droplets into the external phase during preparation process, but might be due to the degradation of PLGA in basic environment.

3.4.2. Polymer Mw of blank PLGA microspheres

Polymer Mw of different blank PLGA microspheres was determined using GPC to check whether or not the porous surface could cause the change of Mw. The results were shown in Fig. S1. It was found that although different blank PLGA microspheres exhibited significant different surface morphology, all the blank PLGA microspheres (B-F4 ~ B-F9) showed similar molecular weight distribution to that of PLGA raw material, as reflected by the similar chromatograms (Fig. S1). This might be attributed to the following two reasons. Firstly, only a little part of PLGA underwent degradation in basic external phase since the whole preparation time was less than 1 h. The degraded oligomers concentration might be too low to be detected. Secondly, the degraded oligomers might be soluble in basic environment and thus diffused from the emulsion droplets into the external phase, leaving few degraded oligomers to be encapsulated in the microspheres and thus leading to little change in the molecular weight distribution.

3.4.3. FT-IR spectra of blank PLGA microspheres

FT-IR spectra of blank PLGA microspheres were taken to further analyze the change of PLGA by the external phase pH values, and the results were summarized in Fig. 8.

As shown in Fig. 8, all the blank PLGA microspheres and PLGA raw materials showed strong bands at 1748 cm^{-1}, which was ascribed to the C=O stretching vibration of the ester carbonyl groups and carboxylic acid groups of PLGA (Gurpreet et al., 2014). Similar to the FT-IR

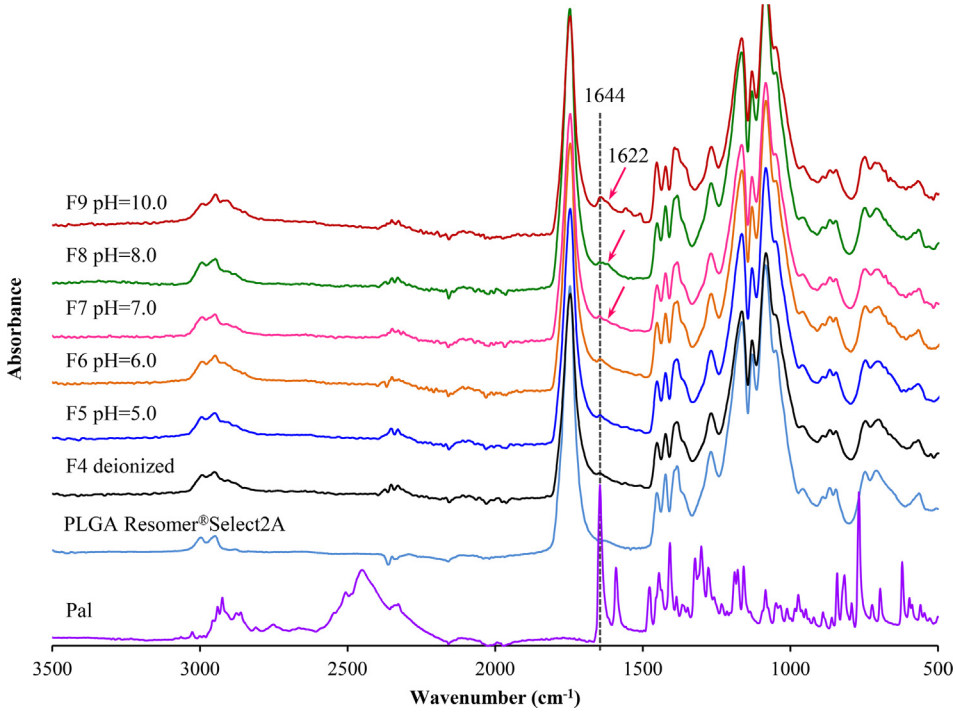

Fig. 4. FT-IR spectrums of PLGA raw materials (PLGA Resomer® Select 2A), Pal powder and Pal-MSs (F4 ~ F9) prepared with different external phase pH values.

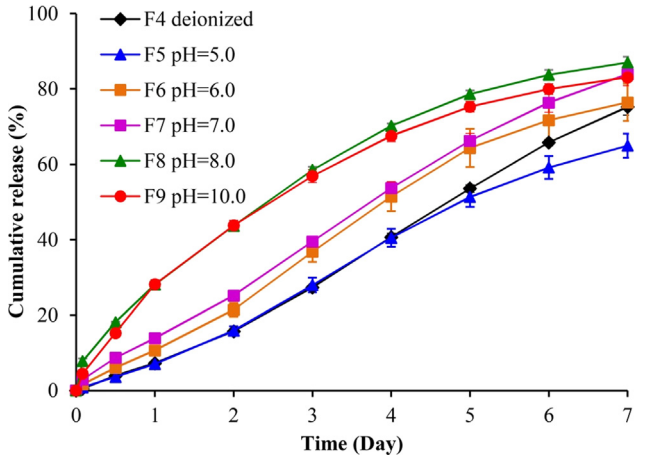

Fig. 5. The *in vitro* drug release profiles of Pal-MSs (F4 ~ F9) prepared with different external phase pH values in 10 mM PBS solution (pH 7.4, 0.1% polysorbate 80) at 37 °C (mean ± SD; n = 3).

spectra of Pal-MSs, another typical carbonyl group of PLGA was at the wavelength of 1622 cm^{-1} (as pointed in Fig. 8). It can be observed that when microspheres prepared with the external phase pH value higher than 7.0, the intensity of the peak at 1622 cm^{-1} significantly increased compared with the peak of other microspheres. This result conformed that the IR band at 1622 cm^{-1} was ascribed to the antisymmetric C═O stretching vibrations of carboxylate groups (Mesu et al., 2005; Oomens and Steill, 2008). The appearance of peak at 1622 cm^{-1} for samples prepared with high external phase pH values (8.0 and 10.0) could be attributed to the following two reasons. Firstly, since the pKa of PLGA was 3.85 (Yoo and Mitragotri, 2010), the conversion from carboxylic acid groups of PLGA into carboxylate groups thus could occur when the pH was higher than the pKa of PLGA, leading to the appearance of the peak in IR spectrum. Secondly, PLGA could be driven to go through ester bond hydrolysis when being treated in basic environment (Baiti et al., 2015), which produced more carboxylate groups and thus leading to the appearance of the peak in IR spectrum. As evident porous surface were observed in Pal-MS of F8 and F9 (external pH = 8.0 and 10.0), the appearance of the peak of 1622 cm^{-1} was likely to be contributed by the carboxylate groups which were generated by the degradation of PLGA.

The above results of different blank PLGA microspheres indicated that degradation of PLGA may occur when the external phase pH was higher than 7.0, generating carboxylic groups. Since the alkaline drug could interact with free carboxylic acid groups in PLGA, more interactions between Pal and PLGA would take place, which could effectively increase the EE values. On the other hand, some degraded oligomers might be soluble in basic environment and thus can be diffused from the emulsion droplets into the external phase, leading to the porous surface, i.e. formulations of F8 and F9. The produced porosity could be used as diffusion pathway of drug. As a result, the drug release rate increased with increasing the pH of external phase.

3.5. Distribution of Pal from organic phase into external phase

It has been reported that the protein encapsulation of PLGA microspheres could be affected by the pH of external aqueous phase which could affect the distribution coefficient of protein between the organic solution and external aqueous phase (Leo et al., 1998). Since the predicted pKa of palonosetron was 7.97 (Drug Bank, 2019), the pH of external phase might also affect the distribution of Pal from organic phase into different external phases, which subsequently could influence the EE of Pal-MS.

Pal distribution between organic phase and external phase was carried out with and without the addition of PLGA. Without the existence of PLGA, it can be seen in Fig. 9 that the drug distribution rate was substantially decreased with increasing the pH values of external phase. Similarly, with the addition of PLGA in organic phase, the distribution rate of Pal from organic phase into the external phase was also decreased with increasing the pH of external phase (Fig. 9B). These results indicated that Pal tended to be more distributed in organic phase with increasing the pH values of external phase, leading to the increased EE of Pal-MS. In addition, for a given pH value of external phase, the distribution rate of Pal from the organic phase containing

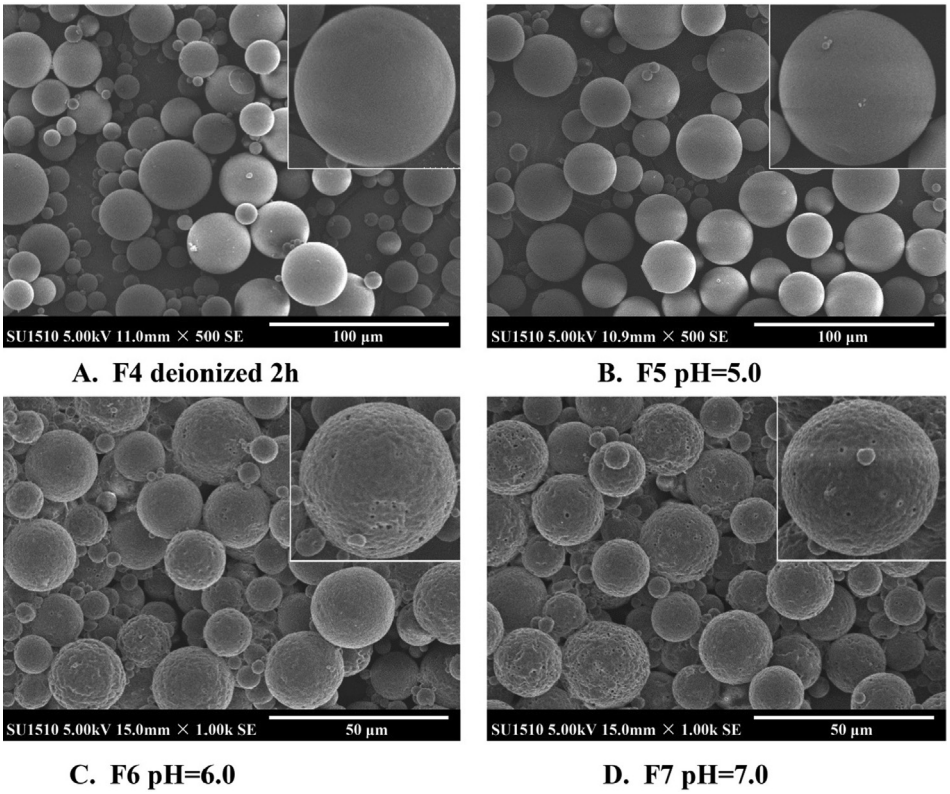

Fig. 6. SEM micrographs of surface morphology of Pal-MSs (F4 ~ F7) after 2-hours incubation in dissolution media (10 mM PBS solution containing 0.1% (w/v) polysorbate 80 with pH of 7.4) at 37 °C.

PLGA was slower than that from the organic phase without PLGA. This was mainly because that the interactions between drug and PLGA could further delay the diffusion of Pal from organic phase to the external phase and thus improve the EE of Pal-MS.

3.6. Pharmacokinetics of Pal-MS

Results of the *in vitro* study showed that Pal-MS prepared using external phase with pH of 7.0 (F-7) showed a high EE (86.51%) and exhibited a zero-order drug release behavior. Therefore in this work, the pharmacokinetics of Pal-MS (F-7) was studied after subcutaneously injected to rats at a single dose of 1.5 mg/kg to evaluate the *in vivo* drug release behavior. As a comparison experiment, the pharmacokinetics of Pal-sol that was intravenously administered by multiple-day dosing was also studied. In addition, clinically the commercial palonosetron hydrochloride injection was administrated via intravenous injection for the treatment of CINV. Pal-sol was injected to rats at a dose of 0.5 mg/kg every other day for three doses. The plasma concentration–time profiles and main pharmacokinetic parameters of Pal-MS and Pal-sol were shown in Fig. 10 and Table 3.

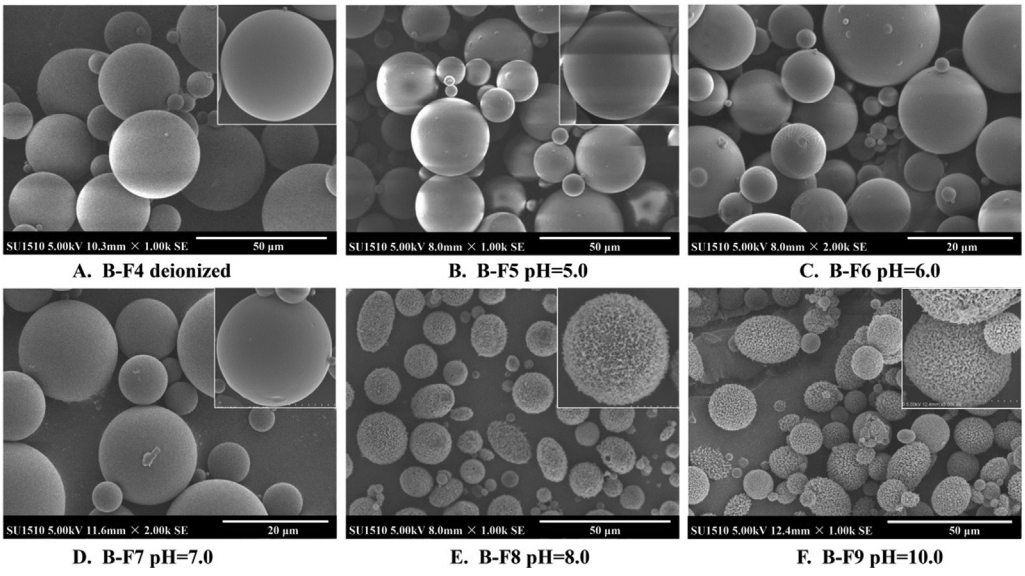

Fig. 7. SEM micrographs of surface morphology of blank PLGA microspheres (B-F4 ~ B-F9) prepared with different external phase pH values.

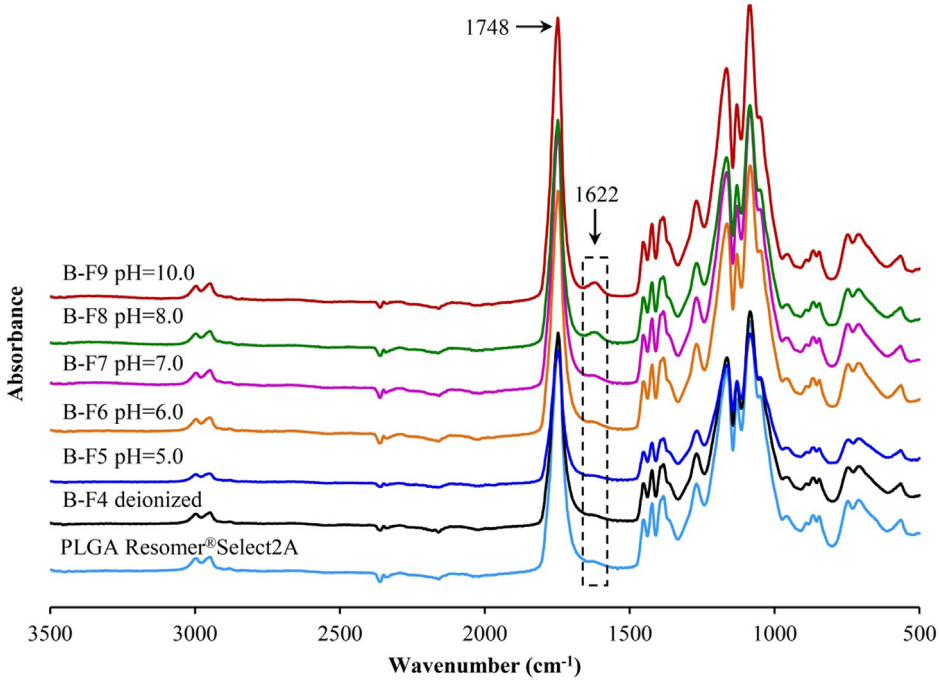

Fig. 8. FT-IR spectrums of PLGA raw materials (PLGA Resomer® Select 2A) and blank PLGA microspheres (B-F4 ~ B-F9) prepared with different external phase pH values.

As shown in Fig. 10A, the plasma concentration of palonosetron after 5 min-administration of each individual dose of Pal-sol was ranging from 58.56 ± 20.75 ng/ml to 89.91 ± 35.62 ng/ml. After 4 h-administration, the plasma concentration of palonosetron rapidly decreased to below 1 ng/ml. The mean $MRT_{0\text{-}t}$ of Pal-sol was only about 3.38 h. The rapid elimination of drug after intravenous administration of Pal-sol might result in a poor efficiency for preventing delayed CINV.

By encapsulation of Pal into PLGA microspheres, the elimination of palonosetron was significantly delayed, which was reflected by the significantly increased $MRT_{0\text{-}t}$ (49.01 ± 10.92 h) after subcutaneous administration of Pal-MS in comparison with that of Pal-sol (mean $MRT_{0\text{-}t}$ = 3.38 h). The C_{max} of Pal-MS was 1.238 ± 0.314 ng/ml, while the plasma concentration of palonosetron was still larger than 0.2 ng/ml after 6 days (Fig. 10B). Such prolonged elimination of drug may assist the prevention of delayed-phase CINV.

Moreover, Pal-MS exhibited a much steadier plasma concentration level over the entire 6-days study period in comparison with Pal-sol. The plasma concentration of palonosetron after subcutaneous administration of Pal-MS from 0 to 6 days was in the range of 0.207–1.238 ng/ml, while that of Pal-sol was in the range of 0.05–89.91 ng/ml. The $C_{max}/C_{min(0\text{-}6d)}$ was calculated to further evaluate the plasma-concentration fluctuation, where C_{max} was the maximum plasma concentration, $C_{min(0\text{-}6d)}$ represented the minimum plasma concentration over the 6-days study period. A low $C_{max}/C_{min(0\text{-}6d)}$ value represented a small plasma-concentration fluctuation. As shown in Table 3, the $C_{max}/C_{min(0\text{-}6d)}$ of Pal-MS was significantly reduced from 817.4 (Pal-Sol) to 5.8 (Pal-MS), which also confirmed the extremely low plasma-concentration fluctuation of Pal-MS. The results of pharmacokinetic studies showed that Pal-MS could not only prolong the blood drug concentration, but also significantly reduce the drug concentration fluctuation *in vivo*. It has been well accepted that steady plasma drug concentration could enhance the therapeutic effect and reduce the side effects (Bai et al., 2007). The prepared Pal-MS with low plasma-concentration fluctuation in this study, therefore, might protect the patients from some undesired effects of palonosetron hydrochloride.

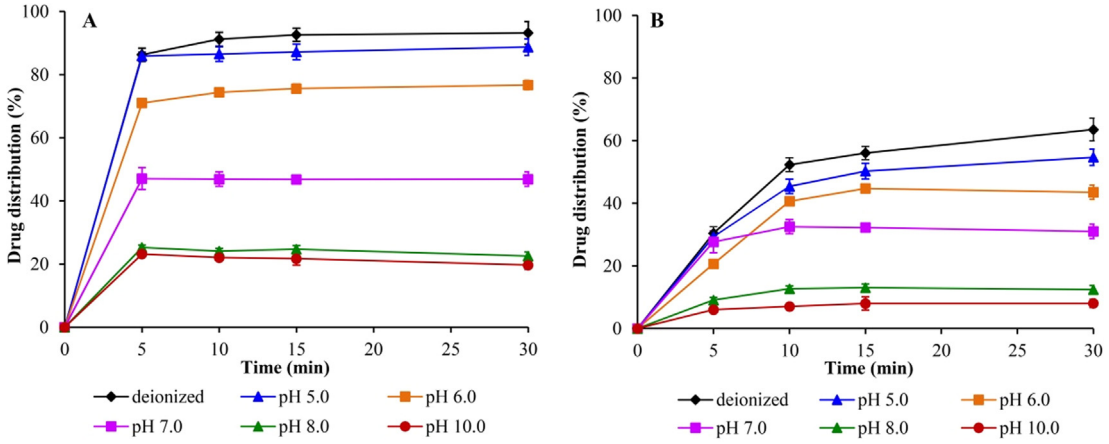

Fig. 9. Distribution profiles of Pal from organic phase (A. without PLGA; B. containing PLGA) to external phases with different pH values (mean ± SD; n = 3).

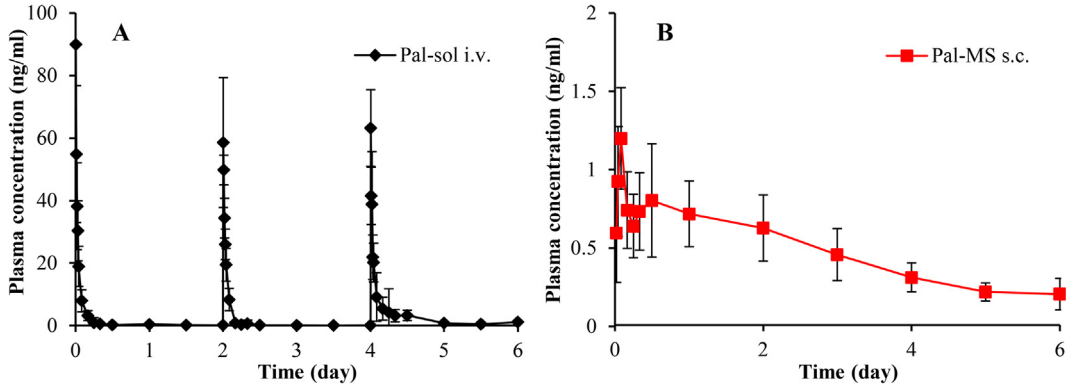

Fig. 10. The plasma concentration–time profiles of palonosetron after intravenous administration of Pal-sol (Pal-sol i.v.) to rats at a dose of 0.5 mg/kg every other day for three doses (A) and after subcutaneous administration of Pal-MS (Pal-MS s.c.) to rats at a single dose of 1.5 mg/kg (B) (mean ± SD; n = 6).

4. Discussions

The results of preliminary study by us showed that Pal was freely soluble in water with the equilibrium solubility higher than 0.35 g/ml. Highly water soluble drugs may have low encapsulation efficiency (lower than 50%) of drug-loaded PLGA microspheres when using traditional W/O/W or O/W emulsion-solvent evaporation methods (Chaisri et al., 2009; Ramazani et al., 2015). Therefore, from the view of formulation development, there is an imperative need to develop a preparation method which could significantly improve the EE of Pal-MS.

The *in vitro* results of the present work showed that the pH of external phase could significantly affect the EE of Pal-MS as well as the drug release behavior of Pal-MS. For a given drug/PLGA ratio (0.25:10), the EE and the drug release rate of Pal-MS both increased with increasing the pH of external phase.

The effect of external phase pH values on EE and drug release of Pal-MSs was summarized on Fig. 11. As illustrated in Fig. 11, when the external phase was deionized water, drug migration occurred at a great level during the emulsification, resulting in low EE (53.95%) of Pal-MS. When increasing the external pH values, PLGA started to undergo ester bond hydrolysis progressively, generating more carbonyl groups. In addition, the adjustment of external phase pH values may also affect the distribution of Pal. Therefore, overall there may be two main reasons accounting for the achieved results. Firstly, since palonosetron was an alkaline drug and exhibited poor affinity to basic condition, the distribution rate of Pal from organic phase into external phase was significantly decreased when increasing the pH of external phase. Consequently, most of drugs were left in the droplets during the solidification of Pal-MS, resulting in higher EE of Pal-MS. Secondly, in basic external aqueous phase (pH ranging from 6.0 to 10.0), the PLGA underwent partial degradation, resulting in increased amount of free carboxylic groups. The newly created carboxylic groups could attract more Pal molecules due to the enhanced interactions between Pal and PLGA. Therefore, the drug diffusion rate was further slowed down, resulting in high EE of Pal-MS. The above two mechanisms both contributed to the increased EE of Pal-MS. It should be noted that when increasing the pH of external phase higher than 8.0, although the EE of Pal-MS (F-8 and F-9) was higher than 90%, the drug release rate, especially the initial drug release rate, was also dramatically increased. Such burst release was likely to be caused by the porous surface, which in turn could be an evident of the degradation of PLGA when using high pH external phases. The *in vitro* study indicated that, for the alkaline drug, such as Pal, PLGA microspheres with high EE and steady drug release behavior could be prepared by adjusting the pH values of external phase.

In present study, an optimal Pal-MS (F-7), with high EE (EE = 86.5%, DL = 2.15%) and zero-order drug release profile, was successfully prepared using external phase with pH of 7.0. In our preliminary studies, it was difficult to increase the DL by modifying the formulation and process parameters when the pH of external phase was fixed at 7.0. The DLs of different formulations prepared using external phase with pH 7.0 were all close to 2% when using Resomer® Select2A. This was likely to be attributed to the interaction between Pal and PLGA. Pal molecules could only be loaded in microspheres when using PLGA ended with free carboxylic acid groups. This was proved by the extremely low DL value (only 0.17%) of Pal-MS prepared by using PLGA ended with ester groups. Therefore, the maximum DL value was dependent on the amount of free carboxylic acid groups of PLGA in the formulation. Clinically, the commercial palonosetron hydrochloride injection (ALOXI®) was administered with a single dose of 0.25 mg for the prevention of CINV, and it was recommended to be used every other day for up to 5 days to prevent acute and delayed CINV associated with multiple-day HEC (Einhorn et al., 2007; Wu et al., 2012; Mirabile et al., 2014). Therefore, for Pal-MS, the predetermined clinical dose was set as about 1 mg. Accordingly, the total weight of the formulation was circa 46.5 mg when the DL of Pal-MS was 2.15%, which could meet the clinical requirements and may not affect the patient compliance.

After subcutaneous injection into rats, the optimal Pal-MS formulation (F-7) displayed steady plasma concentration level with a range of 0.207–1.238 ng/ml over the entire 6-day study period, while Pal-sol showed a wide plasma concentration range (0.05–89.91 ng/ml). These results demonstrated that the optimal Pal-MS had a steady *in vivo*

Table 3
The main non-compartmental model pharmacokinetic parameters of palonosetron after subcutaneous administration of Pal-MS (Pal-MS s.c.) to rats at a single dose of 1.5 mg/kg and after intravenous administration of Pal-sol (Pal-sol i.v.) to rats at a dose of 0.5 mg/kg every other day for three doses (mean ± SD; n = 6).

Parameters	Units	Pal-sol i.v.			Pal-MS s.c.
		1st dose	2nd dose	3rd dose	
$AUC_{(0-t)}$	ug/L*h	87.46 ± 18.38	73.94 ± 32.09	83.93 ± 17.57	63.02 ± 9.62
$AUC_{(0-\infty)}$	ug/L*h	90.91 ± 20.45	74.45 ± 31.97	84.53 ± 17.45	83.88 ± 17.57
$MRT_{(0-t)}$	h	3.552 ± 1.848	2.448 ± 0.600	4.152 ± 1.992	49.01 ± 10.92
C_{max}	ug/L	89.91 ± 35.62	58.56 ± 20.75	85.71 ± 34.39	1.238 ± 0.314
$C_{max}/C_{min(0-6d)}$		817.4			5.8

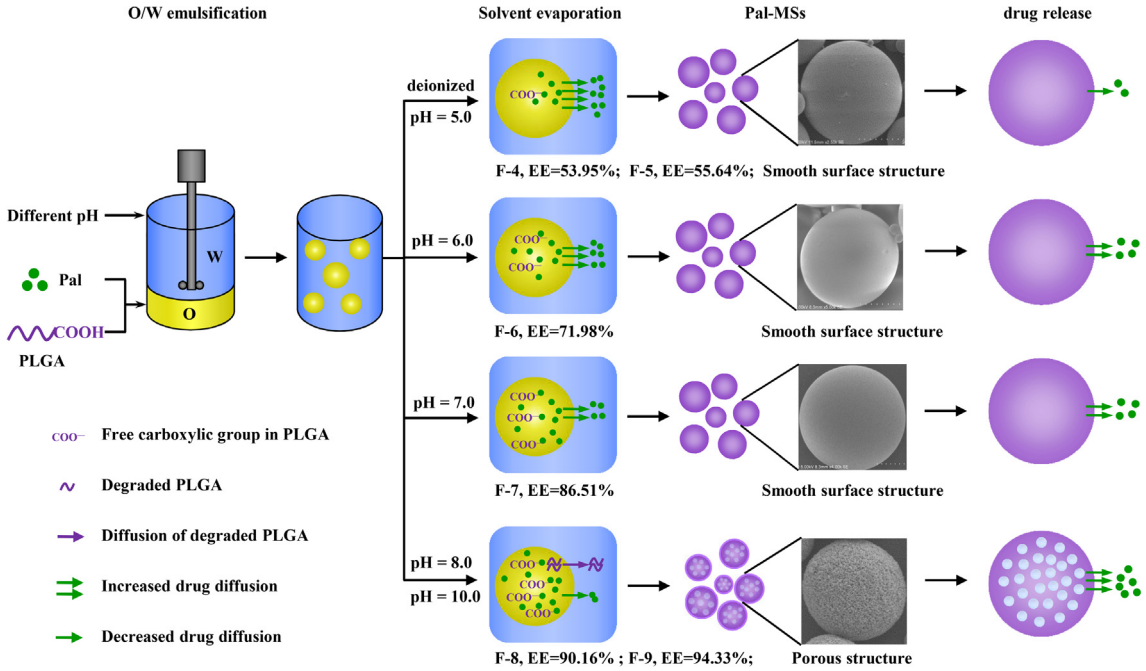

Fig. 11. This figure illustrates the effect of external phase pH values on the properties of Pal-MS.

drug release profile. In addition, the low $C_{max}/C_{min(0\text{-}6d)}$ value ($C_{max}/C_{min(0\text{-}6d)}$ = 5.8) of Pal-MS also confirmed the steady *in vivo* drug release of Pal-MS. Despite of superior efficiency of palonosetron in preventing the delayed CINV than the first generation 5-HT_3 RAs, palonosetron was not indicated for the prevention of delayed CINV after HEC (Helsinn Hlthcare, 2014). Notably, an extended-release injection of the first generation 5-HT_3 RA (Sustol®), which could maintain the therapeutic concentrations of granisetron for 7 days, displayed maintained efficacy in preventing acute and delayed CINV over multiple MEC or HEC cycles (Boccia et al., 2013). In addition, Sustol® has been indicated for the prevention of both acute and delayed CINV associated with MEC or HEC in adults by FDA (Heron Therapeutics Inc., 2016). This suggested that a steady plasma concentration of an antiemetic for a certain time period would be in favor of the prevention in delayed CINV associated with MEC or HEC (especially multiple MEC or HEC cycles). In this study, the pharmacokinetics results showed that Pal-MS designed in this study was capable to maintain the drug plasma concentrations at steady level for approximately 6 days. Therefore it could be expected that Pal-MS may be clinically useful for the prevention of delayed CINV. In addition, it has been reported that palonosetron showed significant difference in plasma half-life among different species. The plasma half-life of palonosetron in rats, dogs and monkeys was 1.49 h, 1.87 h and 4.44 h, respectively, while that in human was 37.4 h (European Medicines Agency Science Medicines Health, 2006). Drug release from Pal-MS developed in this study was in the manner of zero-order *in vitro*, and the pharmacokinetics results demonstrated a highly steady drug plasma concentration. Therefore, due to the capability in controlling drug release, Pal-MS were still likely to be able to prolong drug existence *in vivo* across species.

5. Conclusions

In the present study, by adjusting the pH of external phase, Pal-MS with high encapsulation efficiency was successfully developed, and the drug can be released in zero-order manner for up to 7 days. It was found that the pH of external phase could significantly affect the EE and the drug release rate of Pal-MS via affecting the drug diffusion rate, the interaction between drug and PLGA, the degradation of PLGA and the structure of microspheres. The investigation results indicated that the EE and the drug release rate of Pal-MS both increased with increasing the pH of external phase. When the pH of external phase was 7.0, about 86% of the drug was encapsulated in PLGA microspheres, and the *in vitro* drug release profile was well fitted to zero-order model ($y = 12.29x + 1.906$, $R^2 = 0.9957$) over the 7-days period time. After a single subcutaneous injection of the optimal Pal-MS, a much steadier plasma concentration level (0.207–1.238 ng/ml) was observed in comparison with Pal-sol. The developed Pal-MS therefore may be a promising delivery system for preventing both the acute CINV and delayed CINV.

CRediT authorship contribution statement

Ziyi Yang: Conceptualization, Methodology, Validation, Formal analysis, Writing - original draft, Writing - review & editing. **Lu Liu:** Methodology, Investigation, Visualization, Data curation. **Lili Su:** Methodology, Investigation, Visualization, Data curation, Writing - original draft. **Xueqing Wu:** Investigation, Visualization. **Yicheng Wang:** Methodology, Investigation. **Lei Liu:** Software, Formal analysis. **Xia Lin:** Conceptualization, Methodology, Supervision, Funding acquisition, Project administration, Writing - original draft, Writing - review & editing.

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.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://

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