Development and Evaluation of Atractylodin-Loaded PLGA Nanoparticles

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Abstract

Atractylodin, a natural active compound, has been found to be a potent anti-cholangiocarcinoma activity. However, this compound has poor water solubility and has to be dissolved in organic solvent before use. Recently, nanoparticles are extensively developed as a drug delivery system to increase solubility of hydrophobic drugs and natural active compounds in an aqueous phase. So, the objectives of this study were to develop atractylodin-loaded poly (lactic-co-glycolic acid) (PLGA) nanoparticles and to evaluate nanoparticle formulations. The atractylodin-loaded PLGA nanoparticles were prepared by using a solvent displacement method and characterized size and zeta potential by a dynamic light scattering technique. All nanoparticles had sizes of 160-170 nm in diameter with a narrow size distribution (0.064-0.084) and the effects of surfactant concentration on particle size were not found. Whereas, the encapsulation efficiency (%) was found to be decreased when increased the initial drug amount, the optimal formula exhibited 48% encapsulation efficiency and *in vitro* drug release up to 75% in 72 hours. Moreover, nanoparticles were found to be freely dispersible in water without aggregation. From these findings, atractylodin-loaded PLGA nanoparticles had a potential to be developed as a drug delivery system. However, further studies in stability and anti-cholangiocarcinoma activity are required.

Keywords: *Atractylodin, poly (lactic-co-glycolic acid) (PLGA), nanoparticles, Poloxamer 407, solvent displacement method, Cholangiocarcinoma*

1. Introduction

Cholangiocarcinoma (CCA) or bile duct cancer arises from the epithelial cells of the intrahepatic or extrahepatic bile ducts (Deoliveira et al., 2007; Aljiffry, Walsh, & Molinari, 2009). This cancer is one of the leading causes of morbidity and mortality due to cancer in Thailand, particularly in the northeastern region with the incidence rate of 85 cases *per* 100,000 people *per* year (Kuhlmann, 2012; Bridgewater et al., 2014).

Most of CCA patients presented metastatic stage and require treatment with chemotherapy or chemoradiotherapy (Ramírez-Merino, Aix & Cortés-Funes, 2013). Current chemotherapeutic regimens include 5-fluorouracil (5-FU) based and gemcitabine based, in combination with another chemotherapeutic drugs or targeted therapy (Kuhlmann, 2012; Bridgewater et al, 2014; Ramírez-Merino, Aix, & Cortés-Funes, 2013). However, their clinical efficacy remains unsatisfactory (Ramírez-Merino, Aix, & Cortés-Funes, 2013).

Atractylodin is one of the active constituents of the rhizomes of *Atractylodes lancea* (Thunb.) DC (*A. lancea*). Its promising anti-cholangiocarcinoma activity has been demonstrated in a series of *in vitro* and *in vivo* by our research group (Na-Bangchang, Plengsuriyakarn, & Karbwang, 2017). Recently, there are not any formulations of atractylodin developed. Moreover, one of the limitations of this compound is its poor water solubility which may limit the dissolution in gastrointestinal medium.

Nanoparticles are extensively applied as a drug delivery system (DDS) to increase efficacy and safety of various chemotherapeutic drugs (Vandana & Sahoo, 2010; Rafiei & Haddadi, 2017). They are helpful to enhance water solubility of hydrophobic drugs and improve pharmacokinetic and biodistribution profiles (Vandana & Sahoo, 2010; Joshi, Kumar, & Sawant, 2014; Rafiei and Haddadi, 2017). Nanoparticles are also used as DDS for several traditional medicines or natural active compounds to increase efficacy and solubility in an aqueous solution (Klippstein et al., 2015; Chen et al., 2016; Jiang et

al., 2017). Poly (lactic-co-glycolic acid) (PLGA) is a biocompatible and biodegradable copolymer which is approved to be used in clinical experiments by US FDA (Kerimoglu & Alarcin, 2012). This copolymer is widely used to prepare polymeric nanoparticles to deliver chemotherapeutic drugs and traditional medicines or natural active compounds (Vandana & Sahoo, 2010; Joshi, Kumar, & Sawant, 2014; Klippstein et al., 2015; Tefas et al., 2015; Rafiei & Haddadi, 2017). Moreover, PLGA copolymer has potential to deliver both hydrophobic and hydrophilic drugs and can be administered *via* both oral and parenteral route (Vandana & Sahoo, 2010; Joshi, Kumar, & Sawant, 2014; Rafiei & Haddadi, 2017). Several studies have investigated the potential of nanoparticles as a drug delivery system for chemotherapeutic drugs and targeted therapy for CCA (Kim et al., 2012; Kwak et al., 2015; Ngernyuang et al., 2016). The ability of increasing solubility of hydrophobic compounds by encapsulated in nanoparticles and great properties of PLGA copolymer, development of atractylodin-loaded PLGA nanoparticle would improve solubility and pharmacokinetic properties of this compound.

2. Objectives

The objectives of this study were to develop atractylodin-loaded poly (lactic-co-glycolic acid) (PLGA) nanoparticles and to evaluate physicochemical properties of the nanoparticle formulations.

3. Materials and Methods

3.1 Materials

Atractylodin was purchased from WAKO, Osaka, Japan. PLGA (50:50, molecular weight 38,000-54,000: Resomer® RG 504) and D-mannitol were purchased from sigma-aldrich, MO, USA. Acetone was purchased from Fisher Scientific, Co. LLC, USA. Poloxamer 407 (Kolliphor® P 407) was obtained from BASF, USA. Dialysis membrane (MWCO 50,000 Da) was purchased from Spectrum Laboratory Products Inc., Rancho Dominguez, CA, USA. DMSO was purchased from Ameresco, OH, USA.

3.2 Atractylodin-loaded PLGA nanoparticles preparation

Atractylodin-loaded PLGA nanoparticles were prepared by solvent displacement method with slight modification (Martin-Banderas et al., 2012). Briefly, defined amount of atractylodin and 22.5 mg of PLGA were dissolved in 1.5 ml of acetone and thoroughly mixed. The mixture was then added drop-wise by using syringe pump (KD Scientific, USA) into 15 ml of different concentrations of surfactant (poloxamer 407) under magnetic stirring of 405 rpm. The excess surfactant was removed by dialysis (MWCO 50,000 Da) against a 0.2% D-mannitol solution for 2 hours. The nanoparticle suspensions were stored at 4^{o} C for further use. The formulas of atractylodin-loaded PLGA nanoparticles are shown in Table 1 and nanoparticle preparation processes are presented in Figure 1.

Table 1 Formulas of atractylodin-loaded PLGA nanoparticles

Formula	Atractylodin (mg)	PLGA (mg)	Acetone (ml)	Surfactant concentration (%w/v)	Surfactant volume (ml)
Blank F2 NP	-	22.5	1.5	1	15
F1	1	22.5	1.5	0.5	15
F2	1	22.5	1.5	1	15
F3	2	22.5	1.5	1	15

3.3 PLGA nanoparticles characterization

The prepared nanoparticles were examined for particle size, size distribution or polydispersity index (PDI) and zeta potential by dynamic light scattering technique using Zetasizer (Malvern, USA). One hundred microliters of atractylodin-loaded PLGA nanoparticle suspensions were added into 900 µl of ultrapure water. The average size, size distribution and zeta potential were measured using a refractive index of 1.33 for water and 1.460 for PLGA nanoparticles.

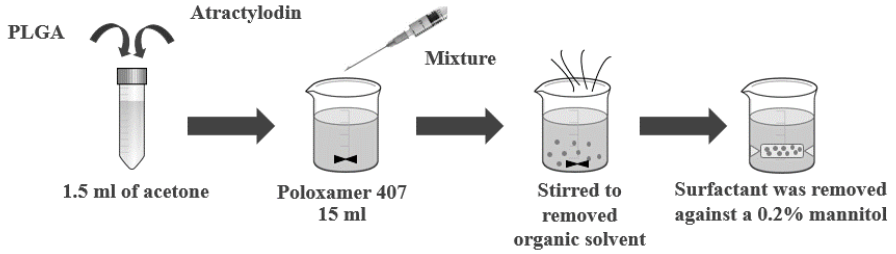

Figure 1 Preparation processes of atractylodin-loaded PLGA nanoparticles

3.4 Determination of drug encapsulation and drug loading efficiency

Five hundred microliters of atractylodin-loaded PLGA nanoparticle suspensions were added into microcentrifuge tube and centrifuged at 13,000 rpm (4 ^{o}C) for 15 minutes. The supernatants were discarded and 500 microliters of DMSO were added to dissolve the drug and polymer. The mixtures were sonicated and the concentration of atractylodin in the solutions were determined spectroscopically using UV absorbance reader (Spectramax microplate reader, Molecular devices, USA) at the wavelength of 340 nm. The amount of atractylodin in the solution was determined from the standard curve. Drug encapsulation efficiency (%EE) and drug loading efficiency (%LE) were calculated by the following equations.

$$\text{Encapsulation efficiency (\%EE)} = \frac{\text{Amount of drug loaded in NPs}}{\text{Amount of drug added}} \text{ x } 100\%$$

$$\text{Loading efficiency (\%LE)} = \frac{\text{Amount of drug loaded in NPs}}{\text{Amount of drug loaded NPs}} \text{ x } 100\%$$

3.5 Drug releasing study

Four hundred microliters of atractylodin-loaded PLGA nanoparticle suspensions were added into 400 µl of phosphate buffer solution, pH 7.4. The samples were incubated at 37 ^{o}C and collected at each time interval. Samples were centrifuged at 13,000 rpm (4 ^{o}C) for 15 minutes. The supernatants were discarded and 400 µl of DMSO were added to dissolve the drug and polymer. The mixtures were sonicated and the amount of atractylodin in the solutions were determined according to the above mentioned procedure. Percentage of cumulative drug release was calculated by following equation.

$$\text{Cumultive atractylodin release (\%)} = \frac{\text{DL} - \text{DR}}{\text{DL}} \text{ x } 100\%$$

DL: Amount of drug loaded in NPs; DR: Amount of drug remained in NPs

3.6 Statistical analysis

Statistical evaluation of data was performed using the analysis of variance (one-way ANOVA). Bonferroni was used as a post-hoc test to assess the significant difference. Statistical significance level was set at $p = 0.05$.

4. Results and Discussions

The prepared atractylodin-loaded PLGA nanoparticles were kept in form of nanoparticle suspensions and all of them were kept at 4 ^{o}C for further use. All atractylodin-loaded PLGA nanoparticles were investigated in size, size distribution, zeta potential, and drug encapsulation and loading efficiency. The optimal nanoparticle formulation was then selected to investigate for drug release *in vitro* based on that which provided a combination of good characteristics (size, size distribution, and zeta potential), and high drug encapsulation and loading efficiency.

4.1 PLGA nanoparticles characterization

The size, size distribution and zeta potential of nanoparticles measured by dynamic light scattering technique are shown in Table 2. All formulas provided particle size diameter in the range of 160-170 nm with a narrow size distribution (0.064-0.084) which is suitable to be a drug delivery system. There is a study reported that nanoparticles with a size less than 10 nm will be removed by a kidney filtration and a size greater than 200 nm will be removed by a mononuclear phagocyte system (MPS) (Rodzinski et al., 2016). The effect of surfactant concentrations on particle size of the nanoparticles were not observed. Whereas, sizes of the particles increased when increasing initial drug amount in the formulas, in the previous studies, however, decrease in particle sizes were reported with increased surfactant concentrations (Martin-Banderas et al., 2012; Sharma et al., 2014). This might be due to high a stirring rate used for preparation of nanoparticles. For the surface charge of nanoparticles, all formulas provided good zeta potential which would facilitate stability of nanoparticles since all formulations needs to be stored as suspension in an aqueous solution. This result is in agreement with that reported by Honary & Zahir (2013) with regard to improved stability of nanoparticles in suspension or re-suspension if the zeta potential is above ±30 mV due to lower chance of particle aggregation. The final optimal atractylodin-loaded PLGA nanoparticles was formula 2 with a lower particle size and a higher zeta potential value.

Table 2 Particle size, size distribution, and zeta potential of atractylodin-loaded PLGA nanoparticles. Data are presented as mean±SD values from three experiments

Formula	Size (mean±SD)	Polydispersity index (PDI) (mean±SD)	Zeta potential (mean±SD)
Blank F2 NP	159.17±1.91	0.084±0.095	-28.17±0.98
F1	161.97±1.10	0.064±0.010	-29.60±2.82
F2	161.60±1.87	0.069±0.011	-37.40±4.74
F3	169.20±1.11[a,b,c]	0.084±0.025	-29.90±0.90

[a] indicates $p < 0.05$ compared to Blank F2 NP

[b] indicates $p < 0.05$ compared to F1

[c] indicates $p < 0.05$ compared to F2

4.2 Determination of drug encapsulation and drug loading efficiency

Drug encapsulation efficiency (%EE) and drug loading efficiency (%LE) of the three (F1, F2 and F3) atractylodin-loaded PLGA nanoparticle formulations were determined as described above and results are summarized in Table 3.

Table 3 Drug encapsulation efficiency (%) and drug loading efficiency (%) of atractylodin-loaded PLGA nanoparticles. Data are presented as mean±SD values from three experiments

Formula	% Encapsulation efficiency (%) (mean±SD)	% Loading efficiency (%) (mean±SD)
F1	43.84±2.30	1.96±0.10
F2	48.31±0.83	2.15±0.04
F3	33.10±2.03[a,b]	2.95±0.18 [a,b]

[a] indicates $p < 0.05$ compared to F1

[b] indicates $p < 0.05$ compared to F2

All PLGA nanoparticle formulations had the ability to encapsulate atractylodin in nanoparticles. The highest and lowest %EE were observed with formula 2 and 3, respectively. This indicates that the %EE was significantly decreased when the initial drug amount was increased ($p < 0.05$). This observation supports previous studies of hydrophobic drug-loaded PLGA nanoparticles. This could be due to the limitation of the drug amount that can be encapsulated in the nanoparticles (Martin-Banderas et al., 2012). The %LE was, however, found to be increased with an increased initial drug amount. Higher drug amount could be in loaded in nanoparticles compared to the constant amount of polymer.

4.3 Drug releasing study

As formula 2 provided optimal characteristics, encapsulation and loading efficiency, the nanoparticles prepared from this formula was selected to further investigate for drug release *in vitro*. The nanoparticles were incubated in phosphate buffer solution, pH 7.4 at 37 ^{o} C and collected at each time interval until 72 hours. Drug released from nanoparticles followed Higuchi kinetic model (R^2=0.9247) and occurred in biphasic manner with initially burst release by drug diffusion through the polymer matrix followed by sustained release by drug diffusion and polymer degradation. During the first 4 hours, drug burst released from nanoparticles was 30%, reaching 70% in 24 hours, with maximum value of 75% (Figure 2). The burst release might be from releasing drug adsorbed on the surface of nanoparticles (Mukerjee & Vishwanatha, 2009) and from large surface to volume ratio of the nanoparticles (Chittasupho et al., 2009). According to reported in the study of doxorubicin-loaded dextran-*b*-PLGA nanoparticles that the drug-loaded nanoparticles exhibited higher *in vitro* cytotoxicity on doxorubicin-resistant cholangiocarcinoma cell line, HUCCT-1, with 80% drug release from nanoparticles in 72 hours (Jeong et al., 2011). As well as, the study in this research group found that the cytotoxicity activity (half maximum inhibitory concentration: IC50) of atractylodin against cholangiocarcinoma cell line, CL-6, was 41.66 ± 2.51 µg/ml (Na-Bangchang, Plengsuriyakarn, & Karbwang, 2017). Thus, 75% of drug released from nanoparticles could affect the cholangiocarcinoma cells.

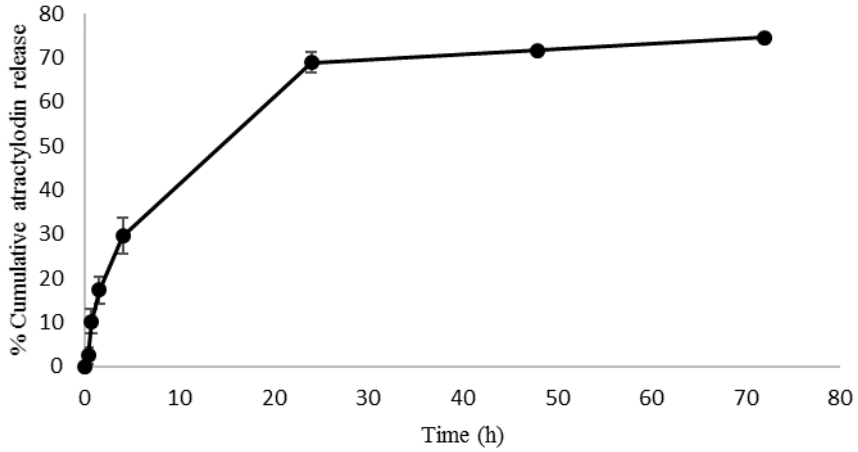

Figure 2 Drug releasing profile of atractylodin-loaded PLGA nanoparticles.
Data are presented as mean±SD values from three experiments

Apart from atractylodin-loaded PLGA nanoparticles provided a small size with a narrow size distribution and suitable zeta potential value for nanoparticle suspensions as well as high drug encapsulation and loading efficiency and drug release from nanoparticles, atractylodin-loaded PLGA nanoparticles were found to be freely dispersible in distilled water without aggregation. Whereas atractylodin pure compound was insoluble in distilled water as shown in Figure 3.

Figure 3 Atractylodin-loaded PLGA nanoparticles (left) and atractylodin pure compound (right) in distilled water

5. Conclusions

Atractylodin-loaded PLGA nanoparticles were successfully developed with good characteristics, drug encapsulation and loading efficiency, and drug releasing profile which are suitable to be developed as a drug delivery system. Further studies on their stability and anti-cholangiocarcinoma activity are ongoing.

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