Drug Development and Industrial Pharmacy, 2010; 36(7): 751–761

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healthcare

ORIGINAL ARTICLE

Characterization and formulation optimization of solid lipid nanoparticles in vitamin K1 delivery

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Abstract

Background: Solid lipid nanoparticle (SLN) systems have been applied to various drugs and delivery routes. Vitamin K1 is an important cofactor for maintaining hemostasis and preventing hemorrhage. *Method:* Vitamin K1-loaded SLNs are systematically being developed by optimizing triglycerides and lipophilic and hydrophilic surfactants based on the size and stability of the resulting SLNs. Concentrations of the surfactants, Myverol and Pluronic, were optimized by a central composite design and response surface methodology. Vitamin K1 (phylloquinone) was used as a lipophilic drug in the SLN system to evaluate the potential for oral delivery. *Results:* Vitamin K1-loaded SLNs had a mean size of 125 nm and a zeta potential of −23 mV as measured by photon correlation spectroscopy. The prepared SLNs were examined by differential scanning calorimetry and transmission electron microscopy and found to have an imperfect crystalline lattice and a spherical morphology. Effects of ultrasonication duration and drug load on the particle size and entrapment efficiency of the SLNs were also evaluated. *Conclusion:* More than 85% of the vitamin K1 was entrapped in SLNs when the payload was <5%. The vitamin K1 in SLNs was stable for a 54-h duration in simulated gastric and intestinal fluids. The particle size and vitamin K1 entrapped in the SLN were stable after 4 months of storage at 25°C. The results demonstrated that SLNs prepared herein can potentially be exploited as carriers for the oral delivery of vitamin K1.

Key words: *Characterization; oral delivery; response surface methodology; solid lipid nanoparticles; vitamin K1*

Introduction

Solid lipid nanoparticles (SLNs) have several advantages over other drug delivery systems, such as good tolerability, rapid biodegradation, high bioavailability, high drug-loading capacity, good production scalability, and a lack of organic solvents in the preparation process[1]. Factors contributing to the increased rate of drug delivery by SLNs include solubility enhancement, the large surface-to-volume ratio due to the nanosizes, and a permeation enhancer effect[2,3]. However, the choice of lipids and emulsifiers and their concentrations have a great impact on the quality of the SLN dispersions. These formulation ingredients significantly affect the physicochemical properties and the drug-release profiles of the nanoparticles[1]. Lipids such as triglycerides, fatty acids, and waxes, as well as emulsifiers such as lecithin, phosphatidylcholine, poloxamer, and polysorbate, have been used to prepare SLN delivery systems[4,5]. In such studies, all variables except the one being studied are held constant during test runs, that is, one variable is tested at a time. Sometimes, variable interactions exist, which cannot be investigated by the one-variable-at-a-time technique. The response surface methodology (RSM) is useful in simultaneously analyzing process variables when variable interactions are very complicated. RSM has been adopted to optimize the formulation of sustained-release microspheres[6], oral-controlled-release formulations for insulin-lauryl sulfate complex[7], formulations of nanoemulsions[8,9], and penetration rates of meloxicam sodium gel[10]. Those studies demonstrated the value of RSM for formulation optimization in various drug delivery systems. Furthermore, the field of nanoparticle delivery systems for drugs and nutraceuticals with poor water solubility has been rapidly expanding[11].

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(Received 10 Aug 2009; accepted 2 Nov 2009)

ISSN 0363-9045 print/ISSN 1520-5762 online © Informa UK, Ltd.
DOI: 10.3109/03639040903460453

Sufficient data implicate that the bioavailability of hydrophobic drugs can be improved when these drugs are encapsulated in SLNs. For example, the bioavailability of poorly water-soluble drugs such as quercetin[12], vinpocetine[13], all-trans retinoic acid[14], and spironolactone[15] in SLNs was significantly increased compared with that in the solution form after oral administration in rats.

Vitamin K1 is an important cofactor for maintaining hemostasis by aiding the carboxylation of vitamin K-dependent proteins that have great impacts on atherosclerosis and atherogenesis[16]. Vitamin K1 also prevents the hazard of hemorrhage in breastfeeding infants[17]. The deficiency of vitamins K1 and D2 causes reduced bone mineral density in female patients with Alzheimer's disease[18]. Arterial calcification caused by warfarin was alleviated by a vitamin K1-rich diet in rats[19]. In addition, oral vitamin K1 lowers the international normalized ratio more rapidly than subcutaneous vitamin K1 in asymptomatic patients who have supratherapeutic international normalized ratio values while receiving warfarin-associated coagulopathy[20]. Nevertheless, Pereira et al. reported that the intestinal absorption of mixed-micellar vitamin K1 is unreliable in adults with severe acute liver dysfunction and in infants with cholestatic liver disease[21,22]. Determining how to orally deliver vitamin K1 in these patients with high efficiency is an important issue. Particle size is one of the important factors that have a direct impact on the stability, biodistribution, and release kinetics of SLN systems[1]. Small particles with a large surface ensure close contact with tissues. Therefore, nanosized droplets have better chances to adhere to tissue surfaces, penetrate barriers, and transport drugs in a more controlled fashion[23]. Furthermore, small droplet size makes the dispersion stable for a long time against sedimentation, hence offering increased stability. Particle size is also a good indicator of instability, because the size increases before macroscopic changes appear[4]. Reducing the particle size to values below 500 nm improves the bioavailability of carotenoids in the small intestine. This improvement in bioavailability is linked to the direct uptake of the nanoparticles, which is controlled by the size of the nanoparticle system[11]. However, no data on uptake were published for particles smaller than 50 nm. Very recently, two research groups used experimental design to develop the SLN formulation and aimed to obtain SLNs with size range of 100–200 nm[24,25]. High drug payload is the aim of drug delivery. However, the studies discussing effects of drug load on SLN size are few. In this study, different hard lipids and hydrophilic and hydrophobic emulsifiers were systematically screened to choose the best combination for SLN production. SLN with small particle size was developed by using a central composite design and RSM. Vitamin K1 was used as a model drug to be encapsulated in the SLNs. The aim of this work was to optimize the SLN formulations and investigate the influence of surfactants and preparation process on the size, stability, and entrapment efficiency (EE) of the SLNs. The physical characteristics and release profiles of vitamin K1-loaded SLNs under physiological buffers were also investigated. Moreover, the effects of temperature on the stability of vitamin K1-loaded SLNs were studied to evaluate the long-term stability.

Materials and methods

Materials

Precirol ATO5, a glyceryl palmitostearate, was kindly donated by Gattefossé Co. (Paramus, NJ, USA). Surfactants such as Myverol 18–04K (palmitic acid monoglycerides) and Centrol 2F-SB were obtained from Quest International (Vernier, the Netherlands) and Central Soya Co. (Fort Wayne, IN, USA), respectively. Estasan 3575 (caprylic/capric triglyceride) was provided by Uniqema (Bromborough, UK). Tween 20, Tween 80, Span 60, Pre-7070, lauric acid (C12), myristic acid (C14), palmitic acid (C16), and stearic acid (C18) were purchased from IL-Shin Emulsifier Co. (Seoul, Korea). All reagents were used without further purification. Vitamin K1 was purchased from Roche (Basel, Switzerland) and Pluronic® F68 was provided by Sigma-Aldrich (St. Louis, MO, USA). The water used in this study was freshly purified by Milli-Q Gradient A10 system (Millipore, Molsheim, France). Ethanol, methanol, isopropanol, and other chemicals were analytical reagent grade.

Preparation of SLN

SLNs were prepared according to previous articles with some modification[26,27]. In brief, aqueous and oil phases were separately prepared. Drug, solid lipid, and emulsifier were melted at 85°C to prevent the recrystallization of lipids during the process. Hydrophilic surfactants and water were mixed at 85°C and added to the melted oil phase. The mixture was then dispersed with an ultrasonic probe (XL2000, Misonix, Farmingdale, NY, USA) for 3 minutes. Finally, the SLN dispersion was obtained after cooling down at room temperature for 12 hours. The SLNs prepared were analyzed by the following methods. In the storage test, the vitamin K1-loaded SLN was stored at different temperatures for 4 months. The average size and EE were determined at a fixed interval[28].

Measurement of physicochemical properties of solid lipid nanoparticles

The average particle size, polydispersity index (PI), and zeta potential in different formulations were characterized

by using Zetasizer Nano ZS 90 (Malvern, Worcestershire, UK) at a fixed angle of 90° and a temperature of 25°C. The smaller the PI, the more uniform the size distribution of dispersion. Zeta potential characterizes the surface charge of particles, which is an indicator of the long-term stability. Zeta potential values of ±30 mV and above represent a stable formulation. Samples were diluted with water to a suitable concentration before the analysis of size distribution.

In vitro release studies of vitamin K1 from SLN

The release studies were performed with static Franz diffusion cells (diffusion area: 0.195 cm^2, Chi-Fa Co., Hsinchu, Taiwan). The cells consist of donor and receptor chambers between which a membrane is positioned. Polycarbonate membranes (Millipore, Schwalbach, Germany) with an average pore size of 50 nm were used as a barrier to prevent the entrance of SLN to the receptor liquid. A volume of 0.5 mL of SLN dispersion (containing 0.25% vitamin K1) was applied to the donor compartment. The volume of receptor fluid was 5 mL. The receptor medium consisted of a solution of phosphate buffered saline:ethanol (4:1, v/v). The composition of the receptor medium was chosen because of the insufficient solubility of vitamin K1 in the aqueous saline. Because the receptor buffer was not intended to mimic in vivo conditions, it is suitable for the in vitro study. Each receptor chamber contained a stirring magnetic bar to maintain the homogeneity of buffer during the experiment. Simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 7.5) were prepared according to USP with pepsin (0.32%, w/v) and pancreatin (1%, w/v), respectively[29]. The temperature of receptor chamber was controlled at 37°C by a water circulator. The whole buffer (5 mL) in the receptor chamber was taken and fresh buffer was replenished at fixed intervals.

Samples collected were analyzed by high-performance liquid chromatography (HPLC) using the following protocol as modified from the previous report[30]. The analysis condition included a Microsorb-C18 column (250 × 4.6 mm, Varian, Palo Alto, CA, USA), a mobile phase of methanol and isopropanol (80:20, v/v), and a flow rate of 1 mL/min. Vitamin K extracted from SLN was diluted with acetone and analyzed using an UV detector at 270 nm.

Entrapment efficiency

The free vitamin K1, which is not entrapped in the SLN, was determined by using the following protocol. Aqueous SLN dispersion (250 μL) and hexane (750 μL) are mixed in an eppendorf tube by a Disruptor-Vertex (Scientific Industries, Bohemia, NY, USA) for 30 minutes. Hexane served as the extracting solvent to extract the free vitamin K1, whereas the SLNs and the entrapped vitamin K1 remain intact in this solvent at the operation condition for 30 minutes. The amount of incorporated vitamin K1 was determined as a result of the initial vitamin K1 minus the free vitamin K1. The EE could be calculated by the following equation:

$$\text{Entrapment efficiency}\,(\%) = \frac{(W_{\text{initial drug}} - W_{\text{free drug}})}{W_{\text{initial drug}}} \times 100\%$$

where '$W_{\text{initial drug}}$' is the mass of initial drug used for the assay and the '$W_{\text{free drug}}$' is the mass of free drug detected in the supernatant after centrifugation of the aqueous dispersion[27].

Characterization by differential scanning calorimetry

Differential scanning calorimetry (DSC) analysis was performed using TA Instruments DSC 2010 (New Castle, DE, USA). A heating rate of 5°C/min was used in the range of 10–90°C. Analysis was performed under a nitrogen purge (50 mL/min) and standard aluminum sample pans (40 μL) were used. About 5–10 mg sample was taken for analysis. An empty pan was used as reference. Thermal analysis of SLN was performed according to the paper of Venkateswarlu and Manjunath[31].

SLN observation by transmission electron microscopy

SLN samples were diluted 1:25 with Milli-Q water and dried on carbon film (CF200-Cu, Electron Microscopy Science, Washington, PA, USA) for 12 hours. After stained with a 1% solution of phosphotungstic acid (Merck, Darmstadt, Germany) for 30 seconds, samples were then analyzed by transmission electron microscopy (TEM) (JEOL JEM 2000 EXII, Tokyo, Japan).

Experimental design and statistical analysis

In this study, all data are reported as mean ± standard deviation (SD, $n = 3$). In the optimization experiment, a second-order function (response surface) is used to characterize the effects of surfactants on particle size in this study. Data obtained from the experiments designed on the central composite design were regressed to obtain the coefficients in the second-order equation. Regression analysis can be facilitated by coding the factor so that the lower and upper levels of each ingredient are −1 and +1, respectively. The response surface regression procedure in SAS software (Cary, NC, USA) was used to find the coefficients (k_{1-5}) in the quadratic equation as demonstrated in the following equation:

$$\begin{aligned}\text{Nanoparticle size} &= \text{function (Myverol 18-04K, Pluronic F68)} \\ &= \text{constant} + k_1 \times x_1 + k_2 \times x_2 \\ &\quad + k_3 \times x_1 \times x_2 + k_4 \times x_1^2 + k_5 \times x_2^2\end{aligned} \quad (1)$$

where x_1 and x_2 represent the coded levels of Myverol 18-04K and Pluronic F68, respectively. The response surface can be further analyzed to determine the optimum concentrations of the ingredients. Sigmaplot package (Systat, San Jose, CA, USA) was used to generate the contour plot.

Results and discussion

Screening experiments

Different hard lipids and hydrophilic and hydrophobic emulsifiers were systematically screened to choose the best combination for SLN production. The lipids tested in this study were lauric acid (number of carbons: 12, melting point (MP): 45°C), myristic acid (number of carbons: 14, MP: 59°C), palmitic acid (number of carbons: 16, MP: 63°C), stearic acid (number of carbons: 18, MP: 70°C), and Precirol ATO5 (glyceryl palmitostearate, MP: 57°C). The formulations consisted of 10% lipid, 1.5% Tween 80, 0.5% Myverol 18-04K, and 88% water. When the carbon number in the fatty acid was <14, that is, lauric acid and myristic acid, the SLNs were of the gelation type. The observed gelation of SLNs is reported to be associated with the polymorphic transition of a solid lipid[32]. When SLN samples were in gelation, their particle size could not be determined. SLNs containing stearic acid were previously used to study the oral adsorption of octadecylamine[33]. However, the particle size of SLNs composed of stearic acid (914 nm) was significantly larger than that of SLNs composed of Precirol ATO5 (298 nm) as indicated in Table 1. Precirol ATO5, a glyceryl palmitostearate, is a good solid lipid for sustained release[34]. Precirol ATO5 was chosen from the five tested hard lipids based on its ability to form stable small-sized SLNs. The rationale for developing small-sized SLNs is that small particles with a large surface ensure close contact with tissues. Therefore, nanosized droplets have better chances to adhere to tissue surfaces, penetrate barriers, and transport drugs in a more controlled fashion[23]. Consequently, the SLN with the minimal particle size was the target of this study.

Table 1. Effects of lipids on the particle size of SLNs.

Lipids	Particle size (nm)
Lauric acid (C12)	ND (gelling)
Myristic acid (C14)	ND (gelling)
Palmitic acid (C16)	6516
Stearic acid (C18)	914
Precirol ATO5	298

The formulation contained 0.5% of Myverol 18-04K, 1.5% of Tween 80, and 10% lipid.

The hydrophilic–lipophilic balance (HLB) system for selecting suitable emulsifiers to stabilize emulsions has been used for more than 50 years. The HLB value is a function of the weight percentage of the hydrophilic portion of a nonionic surfactant for determining the HLB of emulsifier blends. The stability of an emulsion depends on the balance of the emulsifier at the oil–water interface, the nature of the oil phase, the additives in the aqueous and oil phases, the viscosity, and the electrokinetic properties of the emulsion[35]. Blends of hydrophilic and hydrophobic emulsifiers can increase an emulsion's stability and reduce the size of the oil droplets by reducing interfacial tension between the oil and water phases[36]. Additionally, several nonionic emulsifiers offer additional steric stabilization effects by avoiding aggregation of the nanoparticles in the colloidal system[37]. In this study, different binary mixtures of emulsifiers were tested to evaluate their effects on SLNs. Seven surfactants were screened including three hydrophilic surfactants (Tween 80, Tween 20, and Pluronic F68) and four hydrophobic surfactants (Centrol 2F-SB, Myverol 18-04K, Span 60, and Pre-7070). All surfactants screened in this study are generally recognized as safe for the oral delivery use. In addition, hydrophilic or hydrophobic surfactants are classified according to the value of the HLB[38]. The hydrophilic emulsifiers have HLB values > 10, whereas the lipophilic emulsifiers have HLB values from 1 to 10[39]. Combinations of hydrophilic and lipophilic emulsifiers are usually used to achieve a stable dispersion by forming a film around the dispersed droplets and maintaining the droplet stability[40]. Therefore, several combinations of surfactants were tested in the study. HLBs for Centrol 2F-SB, Myverol 18-04K, Span 60, Pre-7070, Tween 80, Tween 20, and Pluronic F68 are 4, 7, 4.7, 3.8, 15, 16.7, and 19, respectively. The concentrations of hydrophilic and hydrophobic surfactants in the screening test were maintained at 1.5% and 0.5%, respectively. Results of particle size analyses for these 12 combinations are given in Table 2. The SLNs were in gelation when combinations of Span 60 plus Tween 20 and Span 60 plus Pluronic F68 were used. Among these surfactant blends, the combination of Myverol 18-04K and Pluronic F68 had the smallest particle size for the SLNs containing Precirol ATO5. Therefore, Myverol 18-04K and Pluronic F68 were chosen from the seven surfactants based on their small particle size and stability.

Myverol 18-04K, a hydrogenated palm oil glyceride, is often used in margarine and ice cream as an emulsifier.

Table 2. Effects of surfactants on the particle size of SLNs.

Lipid phase emulsifier (0.5%)	Aqueous phase emulsifier (1.5%)	Particle size (nm)
Centrol 2F-SB	Tween 80	299.02
	Tween 20	294.95
	Pluronic F68	276.01
Myverol 18-04K	Tween 80	298
	Tween 20	275.91
	Pluronic F68	225.03
Pre-7070	Tween 80	268.02
	Tween 20	276.42
	Pluronic F68	260.45
Span 60	Tween 80	307.43
	Tween 20	ND (gelling)
	Pluronic F68	ND (gelling)

The lipid used in this study is 10% Precirol ATO5.

Pluronic F68 (also called Poloxamer 188) is a nonionic surfactant that is widely used in pharmaceutical formulations[41]. Recently, Pluronic F68 was used to constitute a nanostructured lipid carrier system because of its steric stabilization ability[37]. The combination of the chosen ingredients (Precirol ATO5, Myverol 18-04K, and Pluronic F68) for the SLN preparations has not been investigated before. Concentrations of surfactants in the SLNs were optimized using a central composite design and RSM. It is reported that Pluronic F68 moderately inhibits the P-glycoprotein efflux system and intestinal lipoprotein secretion, leading to the improved oral absorption of cyclosporin A[42]. The inhibition of P-glycoprotein and cytochrome P450 by F68 can improve the oral absorption of hydrophobic drugs[43]. Therefore, the ability of F68 to manipulate P-glycoprotein efflux system is a good surfactant as an excipient for oral drug delivery systems. In the next section, factorial design based on response surface method was adopted to minimize SLN for encapsulation of a model drug—vitamin K1. A full factorial design was used to evaluate the combined effect of the surfactant concentration on the size of the prepared SLN.

Optimization of SLN preparation

Precirol ATO5, Myverol 18-04K, and Pluronic F68 were found to be key ingredients for stable SLN production based on our previous screening experiments. Optimal concentrations for the binary blend of Myverol 18-04K and Pluronic F68 were developed by fixing the concentration of the lipid (Precirol ATO5) at 10%. Central composite design provides the minimal experimental number to investigate the effects of a tested variable. The matrix of the central composite design and experimental results are shown in Table 3. The tested ranges of surfactants were 0.15–0.85% for Myverol 18-04K and 0.44–2.56% for Pluronic F68. Three repeats at the center point were used to calculate the pure error; therefore, the total number of trials for this design was 13. The full second-order polynomial model for SLN size obtained by the response surface regression procedure using SAS software is given by

Table 3. The matrix designed by the 2^2 factorial experiment and particle size of SLNs.

	Myverol 18-04K (%, w/w)	Pluronic F68 (%, w/w)	
1	−1 (0.25)	−1 (0.75)	305.63 ± 0.52
2	+1 (0.75)	−1 (0.75)	293.25 ± 0.02
3	−1 (0.25)	+1 (2.25)	232.40 ± 3.82
4	Run	Factors	Particle size (nm)
5	−1.41 (0.15)	0 (1.5)	224.72 ± 3.80
6	+1.41 (0.85)	0 (1.5)	259.05 ± 6.06
7	0 (0.5)	−1.41 (0.44)	331.72 ± 0.45
8	0 (0.5)	+1.41 (2.56)	214.87 ± 1.04
9	0 (0.5)	0 (1.5)	233.92 ± 3.75

The lipid used in this study is 10% Precirol ATO5 ($n = 3$).

$$size = 233.81 + 5.08 \times x_A - 36.90 \times x_B + 6.40 \times x_A^2 + 4.22 \times x_A x_B + 22.20 \times x_B^2; \quad (2)$$

where x_A = Myverol 18-04K and x_B = Pluronic F68 in coded values.

By analyzing these coefficients in the above second-order polynomial mode, the increase of Pluronic could efficiently reduce the particle size of SLNs than Myverol for its negative and large coefficient (−36.9). The increase of Myverol concentration would lead to the increase of size. The cross-interaction of Pluronic and Myverol on size of SLN was not significant because the coefficient of $x_A x_B$ was very small. Furthermore, the *F*-value for the full quadratic equation for the SLN particle size was 10.5, indicating that the second-order response surface model was significant at the 5% level. The determination coefficient (R^2) for the quadratic equation was 0.9459, which indicates a good fit for the model (particle size) and two surfactants. Accordingly, the relation between SLN size and two surfactants was well represented by the second-order model. The canonical analysis in the SAS software is a mathematical procedure for simplifying a second-order polynomial model and finding the extreme (maximum or minimum) value of the response surface model. The results of the canonical analysis demonstrated that the response surface of the SLN had a minimal particle size. The effects of Myverol 18-04K and Pluronic F68 on SLN size were visualized from a contour plot based on Equation 2 (Figure 1). The SLN's composition with a minimal particle size was clearly observed in the center of the plot. Moreover, a new set of experiments with

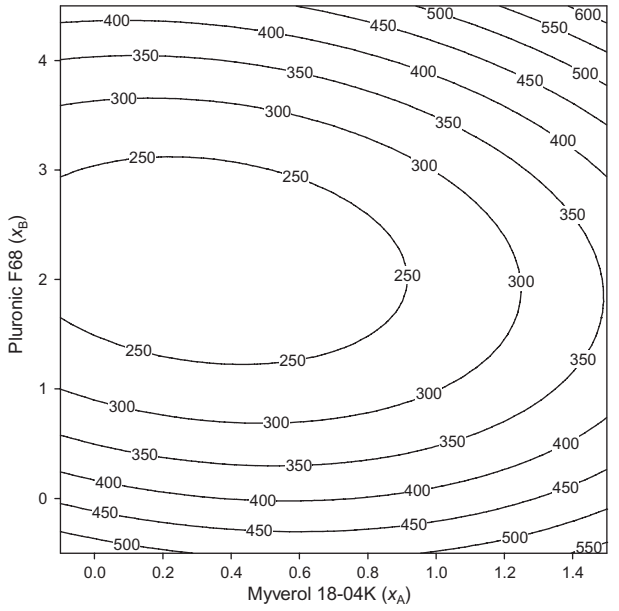

Figure 1. Contour plot for the average diameter (nm) of SLNs versus the surfactants (Myverol 18-04K (x_A) and Pluronic F68 (x_B)based on the response surface model (Equation 2).

different concentrations of Myverol 18-04K and Pluronic F68 were performed to evaluate whether the response surface fits the experimental data. As shown in Table 3, a minimal size of 208 nm was experimentally obtained at a composition of 0.27% Myverol 18-04K and 2.54% Pluronic F68, which were slightly shifted from the predictions of the model (0.33% Myverol 18-04K and 2.17% Pluronic F68). In addition, a small deviation exists between the model predicted by Equation (2) and the experimental results shown in Table 4. The closeness in the values of prediction and experimental data indicates the validity of the generated mathematical model for the prediction of particle size of SLN. In summary, the optimized composition (0.27% Myverol 18-04K, 2.54% Pluronic F68, 10% Precirol ATO5, and 92.19% water) can produce nanosized SLNs.

The characterization of pH, PI, and zeta potential of the SLNs prepared by the optimal composition was investigated. The PI and zeta potential for the prepared SLNs were 0.18 ± 0.04 and −31.20 ± 3.08, respectively. The PI is an indicator of the homogeneity of the size distribution. A PI value of >0.5 indicates a broad size distribution and a value of 0 indicates a monodispersed population. The PI value of 0.18 indicated that the optimal composition could be used to produce a stable SLN dispersion with a uniform size distribution. The zeta potential provides electrostatic repulsion to prevent the coagulation of particles in dispersion systems. Generally, particle aggregation is less likely to occur for particles with surface charges exceeding 30 mV. Because Pluronic F68 is a nonionic surfactant, the negative charge of SLNs may be due to fatty acids released from the hydrolysis of Myverol (palmitic acid monoglycerides) or Precirol (glyceryl palmitostearate). Previously, a combination of hydrophilic and lipophilic emulsifiers has been used to maintain a stable dispersion. In such a system, the hydrophilic and lipophilic emulsifiers are thought to align alongside each other imparting more rigidity and strength to the emulsifier film through hydrogen bonding[40]. Our results also showed that mixed surfactants with appropriate concentrations could prepare the SLNs with the smallest particle size.

Effects of sonication

Ultrasonic emulsification was used to provide the energy to disperse the lipid phase and prepare the SLNs in this study. The ultrasonic energy intensity has great impacts on the droplet size of the nanoemulsions[44]. The effects of dispersion energy provided by ultrasonication on particle size and EE of the prepared SLNs were investigated by using the optimal formulation plus 0.25% vitamin K1. The size of the SLNs decreased from 239 to 127 nm as the time of ultrasonication increased from 2 to 8 minutes (Figure 2). However, more than an 8-minute ultrasonic time did not further significantly reduce the particle size. In contrast, a 25-minute ultrasonication was needed to prepare SLNs containing trimyristin (2%), soylecithin (0.2%), poloxamer 188 (1%), and stearylamine (0.05%)[31] by using a hot homogenization method. Ultrasonic emulsification has been

Table 4. The formulations designed by the steepest descent method.

Factors	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	Step 7	Step 8	Step 9
Myverol (%)	0.53	0.48	0.43	0.38	0.33	0.28	0.23	0.18	0.13
Pluronic F68 (%)	0.72	1.08	1.45	1.81	2.17	2.54	2.90	3.3	3.62
Predicted size (nm)	296.14	260.86	235.66	220.54	215.50	220.55	235.68	260.89	296.18
Real size (nm)	369.68 ± 8.57	253.54 ± 6.23	239.69 ± 5.92	225.0 ± 5.65	215.91 ± 3.73	208.31 ± 3.68	215.49 ± 1.80	212.73 ± 5.12	213.76 ± 2.60

The formulation contained 10% Precirol ATO5 and 0.25% vitamin K1. The mixture was dispersed with an ultrasonic probe for 3 minutes ($n = 3$).

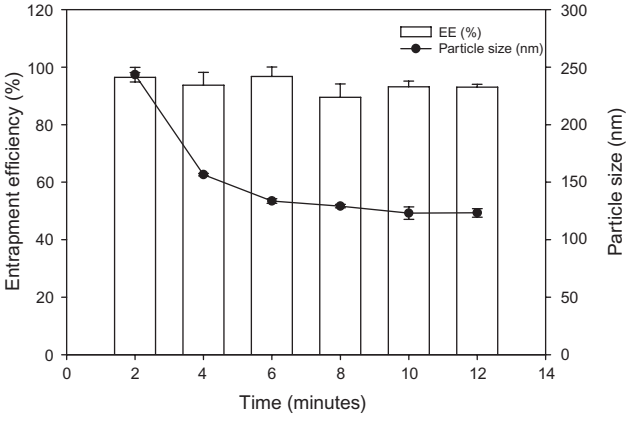

Figure 2. Influence of ultrasonication time on the particle size and entrapment efficiency (EE) of SLN loaded with 0.25% vitamin K1. Error bar stands for standard deviation ($n = 3$).

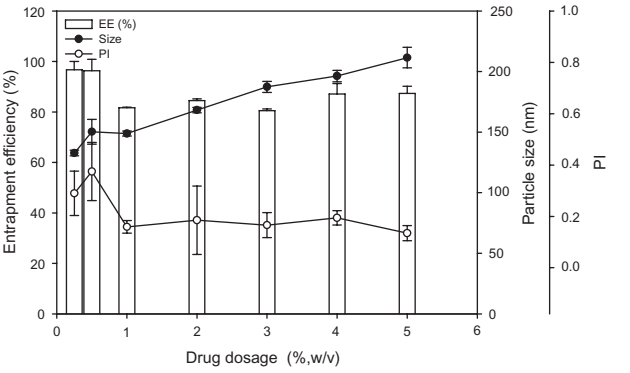

Figure 3. Effects of vitamin K1 load on the particle size, entrapment efficiency (EE), and polydispersion index (PI). The mixture was dispersed with an ultrasonic probe for 8 minutes. Error bar stands for standard deviation ($n = 3$).

reported to be superior in terms of droplet size and energy efficiency when compared with classical rotor-stator dispersion. It is also more practicable with respect to production cost, equipment contamination, and aseptic processing than a microfluidization approach[44]. This research group also reported that excess energy input leads to an increase in droplet size of emulsion by increasing the collision frequency of the formed particles and inducing reaggregation. However, the increase in ultrasonication time did not influence the EE of vitamin K1 at the concentration of a 0.25% drug load in the optimized formulation. The SLN samples were prepared using an ultrasonic probe for 8 minutes for the following experiments.

Effects of drug load on size and EE

Effects of an increase in the vitamin K1 load on the particle size and EE in the developed formulation were evaluated. When the load of vitamin K1 increased from 0.25% to 5.0%, the size of the SLNs increased from 132 to 212 nm and the PI decreased from 0.30 to 0.14 as shown in Figure 3. The incorporation of more drugs in the SLNs increased the particle size, which might be due to entrapment of the drug in the nanoparticles. In addition, the EE of vitamin K1 slightly dropped from 98% to ~85% when the drug load increased from 0.25% to 5%. The lipophilic solubility of SLNs makes them good carriers for lipophilic drugs with high loading capacities[45]. More than 90% lipophilic clozapine entrapped in SLNs was reported[31]. The high EE of vitamin K1 in SLNs may be attributed by its limited water solubility and high lipophilicity. In addition, the high drug load in SLNs increased the concentration gradient toward the target. Hence, vitamin K1 permeation from the lipid core to the aqueous phase was enhanced. Sequentially, the payload of vitamin K1 was set to 0.25% for further experiments. The properties of vitamin K1-loaded SLNs were also characterized. The PI and zeta potential for the 0.25% vitamin K1-loaded SLNs were 0.17 ± 0.02 and −26.83 ± 2.83, respectively. The payload of 0.25% vitamin K1 slightly changed the zeta potential (−26.83 mV) compared to that (−31.2 mV) of the drug-free SLN. The high loading capacity and the small and uniform particle size demonstrated the superiority of the formulation developed herein.

Release experiments

One of our aims was to evaluate the gastrointestinal stability of SLNs with vitamin K1 load. When SLNs are given orally, they pass through different gastrointestinal regions where they are exposed to different pH and different enzymatic conditions, which can influence their drug-release behavior and stability. To test this hypothesis, vitamin K1-loaded SLNs were subjected to different fluids with different ionic strengths and enzymatic conditions, and the drug-release behavior was elucidated by using the modified Franz diffusion cells. Diffusion cells with polycarbonate membranes (with a pore size of 100 nm) were adapted to investigate the release of vitamin K1 from the SLNs according to the paper of Truong Cong et al.[46] Figure 4 shows the percentage release of vitamin K1 from the SLNs in SGF and SIF. In SIF, about 4.4% of the entrapped vitamin K1 was released in the initial 6 hours, and only about 5% of the entrapped drug was further released during the subsequent 50 hours of incubation. The amount of vitamin K1 released from the SLNs increased in the order of SIF > SGF > pH 1.2 buffer > pH 7.5 buffer. The released vitamin K1 after 54 hours was only 10% and 5% of the entrapped drug in SGF and SIF, respectively. These results indicated that the vitamin K1-loaded SLNs were

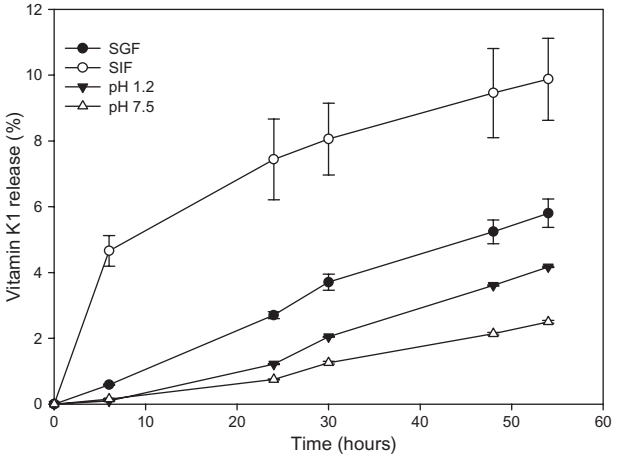

Figure 4. In vitro release profile of vitamin K1 from SLNs after incubated in different buffers (mean ± SD; $n = 3$). Donor buffer was composed by SLN and buffer (1:1, v/v). The pore size of membrane was 100 nm.

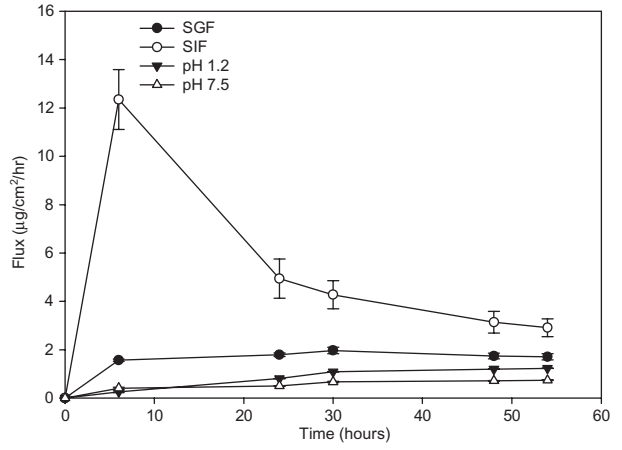

Figure 5. Flux profile of vitamin K1 from SLNs after incubated in different buffers (mean ± SD; $n = 3$). Donor buffer was composed by SLN and buffer (1:1, v/v). The pore size of membrane was 100 nm.

stable under the tested solutions. The enzyme, pancreatin, in the SIF helped to digest the lipids in the SLNs and release the vitamin K1. Therefore, SLNs in the SIF had the highest diffusion flux of 12 μg/cm^2/h (Figure 5). Vitamin K1-loaded SLNs showed maximal flux in the first 5 hours in the SIF, which was due to a burst release. Pepsin, an enzyme in the stomach, also contributed to the release of vitamin K1 in the SGF. In addition, the release rate of vitamin K1 was really slow in the pH 1.2 and pH 6.8 buffers without digestive enzymes (Figure 5). The flux of vitamin K1 increased in the order of SIF >SGF >pH 1.2 buffer >pH 7.5 buffer. The SLNs developed could retain most entrapped drug (>85%) for 56 hours in SGF and SIF, indicating the good gastrointestinal stability of SLNs. Previously, the uptake of particles by the Peyer's patches in the small intestine was reported to increase with decreasing particle size[47]. The nanoscale and stability of SLN might help the particles permeate the Peyer's patches and increase the bioavailability. Nevertheless, further in vivo studies for vitamin K1-loaded SLNs are needed to validate its oral delivery efficacy.

Storage experiments

Chemical transformation of solid lipids during storage is reported to change the structure of SLNs, the load and/or release capacity, the interfacial properties, and their in vivo fate[4]. Size change and drug degradation during storage are important considerations in designing formulations. The effects of storage temperature and duration of storage on the stability of vitamin K1-loaded SLN were studied at different storage temperature (4°, 25°, and 37°C). Previously, the stability of vitamin K1 (phylloquinone) in total parenteral nutrition at-home admixtures was studied[48]. Vitamin K1 showed good stability for 20 days at a room temperature in the presence or absence of lipids and trace elements in admixtures. Similarly, our storage results indicated that vitamin K1 loaded in SLN was stable over 30 days (Figure 6). However, a significant increase in the size of the SLNs was observed when they were stored at 37°C for 60 days. The size of the SLNs stored at 37°C increased from 125 to 361 nm after 120 days of storage. Some precipitate was also observed in samples stored at 37°C for 90 days. The particle sizes increased before macroscopic changes appeared. Therefore, the increase in particle size is a good indicator of instability[4]. SLNs did not aggregate or precipitate when stored at a temperature below 25°C for 120 days. Therefore, vitamin K-loaded SLNs are stable using the formulations

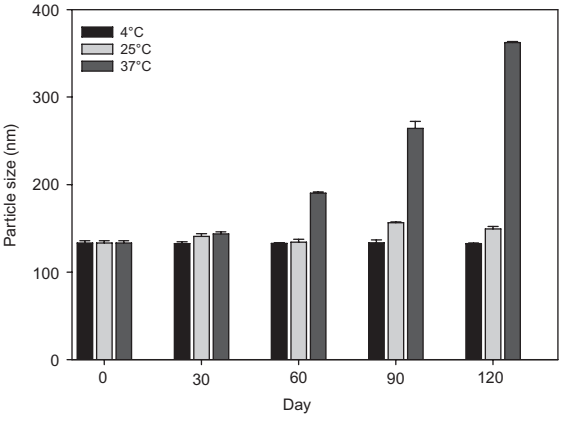

Figure 6. Effects of storage temperature on the particle size of vitamin K1-loaded SLNs. Error bar stands for standard deviation. *$P < 0.05$ ($n = 3$).

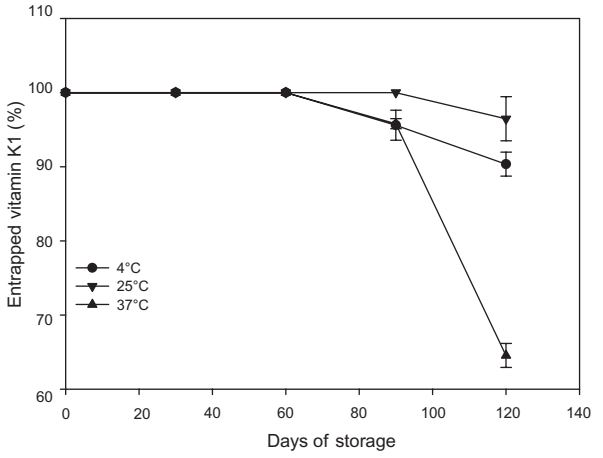

Figure 7. Effects of storage temperature on the stability of vitamin K1-loaded SLNs. Error bar stands for standard deviation. *$P < 0.05$. ($n = 3$).

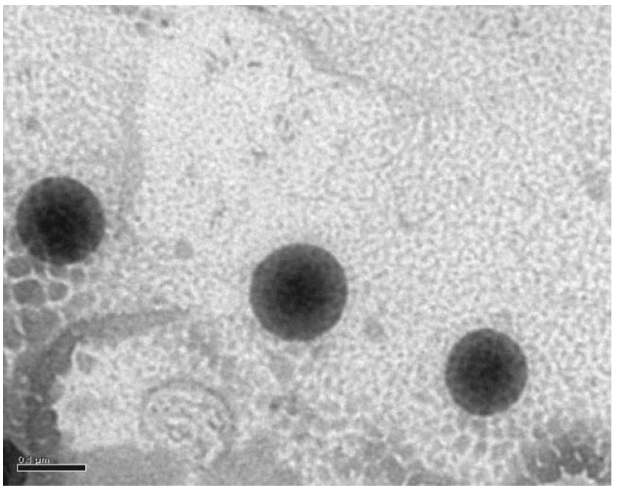

Figure 8. TEM photograph of vitamin K1-loaded SLNs (bar, 100 nm).

developed when stored at a temperature below 25°C. Effects of storage on the entrapped drug were also investigated using HPLC. From the results of Figure 7, vitamin K1 in SLNs was stable at 37°C (or lower temperatures) for only 2 months. In addition, vitamin K1 in the SLNs deteriorated quickly at 37°C after 90 days as indicated in Figure 8. In contrast, vitamin K1 in SLNs was stable after being stored at 25°C for 120 days. Previously, Venkateswarlu and Manjunath[31] found that clozapine-loaded SLNs increased from 40 to 79 nm after 6 months of storage at 25°C. The fact that transitions of dispersed lipids from metastable forms to a stable form might slowly occur during storage may lead to drug expulsion from SLNs. The degradation of vitamin K1 observed at 37°C may have been due to drug expulsion during lipid transformation. Our storage test showed that vitamin K1-loaded SLNs were stable below a temperature of 25°C with 4 months of storage.

Characterization of vitamin K1-loaded SLNs

The morphology of vitamin K1-loaded SLNs was observed using TEM. Samples for TEM were stained according to a previous report[39]. The mean size of the SLNs was 125 ± 8 nm in the TEM image (Figure 8), which was consistent with the size (124 nm) measured by photon correlation spectroscopy. It is evident that the particles prepared in this study are spherical ones, and the formulation developed can efficiently control the size of nanoparticles. Previously, SLNs were reported to have a spherical morphology by García-Fuentes et al.[29] The morphology of SLNs might be influenced by the use of different dispersing devices, SLN compositions, or photographic devices.

DSC analysis was performed to obtain the thermograms and evaluate the degree of lipid crystallinity. The enthalpy of the SLNs was calculated on the basis of the total weight used. The incorporation of 0.25% vitamin K1 into the SLNs did not influence the crystallinity of SLNs (Table 5). In this study, values of crystallinity for SLNs with and without vitamin K1 were 88% and 89%, respectively. This suggests that the loading of vitamin K1 in the SLN did not significantly affect lattice arrangement in the lipid cores. Previously, values of 64–98% crystallinity for SLNs indicated that the crystals of the solid lipid are in a β-form[31]. Therefore, the SLNs prepared in this study might be the β-form.

Conclusions

Among the 12 lipids and surfactants screened in this study, Precirol ATO5, Myverol 18-04K, and Pluronic F68 formed stable SLN dispersions. The optimal concentrations of Myverol 18-04K and Pluronic F68 were 0.33% and 2.17% using a central composite design and RSM. The prepared SLNs were found to have an imperfect crystalline lattice and a spherical morphology. In addition, ultrasonication for 8 minutes could efficiently reduce the particle size to 125 nm. More than 85% vitamin K1 was entrapped in SLNs when the payload was less than 5%. The developed SLNs could keep the drug entrapped in the presence of SGF and release the drug in SIF in the in vitro study. SLNs were demonstrated to be potential carriers for the oral delivery of vitamin K1 based on our results. However, in vivo studies for vitamin K1-loaded SLNs should be performed to determine its oral delivery efficacy.

Table 5. Enthalpies and crystallinity of bulk ingredients and SLN.

	Sample	Enthalpy (J/g)	Crystallinity (%)
Material	Precirol ATO5	197.25	100.00
	Myverol 18-04K	187.4	100.00
	Pluronic F68	131	100.00
	Vitamin K1	0	0
PM[a]	Material (*a*, *b*, *c*)	170.4	97.16
	Material (*a*, *b*, *c*) + vitamin K1	169.3	99.62
SM[a]	Material (*a*, *b*, *c*)	173.05	98.67
	Material (*a*, *b*, *c*) + vitamin K1	160.75	94.59
Lyophilized SLN	SLN (blank)	154.4	88.04
	SLN (vitamin K1)	152.2	89.56

[a]Physical mixture (PM) and solvent evaporated mixture (SM) of *a* and *b* contain 95.24% triglyceride; lyophilized SLN (*a*), (*b*), and (*c*) contains 63.96%, 63.61%, and 61.98% triglyceride, respectively. Degree of crystallinity of PM, SM, and lyophilized SLN was calculated by comparing their enthalpy with enthalpy of bulk ingredients. Enthalpy of bulk lipid is being taken as 100%. Enthalpy of SLN was calculated on the basis of total weight taken.

Acknowledgment

This work was supported by grants NSC96-2628-E-182-039-MY2 and CMRPD 170081.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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