Preparation and characterization of solid lipid nanoparticles loaded with doxorubicin

Robhash Kusam Subedia a, Keon Wook Kang a, Hoo-Kyun Choa,b,*

a BK21 Project Team, College of Pharmacy, Chosun University, 375 Seosuk-dong, Dong-gu, Gwangju 501-759, South Korea
b Research Center for Resistant Cells, Chosun University, 375 Seosuk-dong, Dong-gu, Gwangju 501-759, South Korea

ABSTRACT

Solid lipid nanoparticles (SLN) loaded with doxorubicin were prepared by solvent emulsification-diffusion method. Glyceryl caprate (Capmul® MCM C10) was used as lipid core, and curdlan as the shell material. Dimethyl sulfoxide (DMSO) was used to dissolve both lipid and drug. Polyethylene glycol 660 hydroxy-stearate (Solutol®HS15) was employed as surfactant. Major formulation parameters were optimized to obtain high quality nanoparticles. The mean particle size measured by photon correlation spectroscopy (PCS) was 199 nm. The entrapment efficiency (EE) and drug loading capacity (DL), determined with fluorescence spectroscopy, were 67.5 ± 2.4% and 2.8 ± 0.1%, respectively. The drug release behavior was studied by in vitro method. Cell viability assay showed that properties of SLN remain unchanged during the process of freeze-drying. Stability study revealed that lyophilized SLN were equally effective (p < 0.05) after 1 year of storage at 4 °C. In conclusion, SLN with small particle size, high EE, and relatively high DL for doxorubicin can be obtained by this method.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Solid lipid nanoparticles (SLN) offer an attractive means of drug delivery, particularly for poorly water-soluble drugs. They combine the advantages of polymeric nanoparticles, fat emulsions and liposomes (Schwarz et al., 1994). SLN consist of drug trapped in biocompatible lipid core and surfactant at the outer shell, offering a good alternative to polymeric systems in terms of lower toxicity (Dingler et al., 1996; Müller et al., 2000). Moreover, the production process can be modulated for desired drug release, protection of drug degradation and avoidance of organic solvents. This flexibility in large scale may have a paramount importance in commercialization of new products (Wissing et al., 2004). Aforementioned characteristics make SLN an interesting carrier system for optimized delivery of drugs.

Doxorubicin, an anthracycline antibiotic, is a widely used antineoplastic agent. Despite good efficacy of doxorubicin, cardiotoxicity is the serious side effect that follows the treatment. Additionally, anthracyclines are likely to cause alopecia and myelosuppression and oral ulcerations (Zara et al., 1999). These toxicity and non-specific distribution of the drug often results in chemotherapeutic failure (Mehnert and Mäder, 2001). Focus should be made on efforts to kill cancer cells by more specific targeting while sparing normal cells. Several nanoparticulate delivery strategies of doxorubicin have been developed to minimize the exposure of drug to the normal tissues (Pereverzeva et al., 2007). Nanoparticles function as a carrier for entry through fenestrations in tumor vasculature allowing direct cell access. This, enhanced permeability and retention following intravenous injection, shows a great potential to overcome drug resistance (Barraud et al., 2005; Brigger et al., 2002). Therapeutic efficacy of doxorubicin could be improved by using nanoparticles as drug carriers. Doxorubicin has been formulated in different colloidal carriers, for example, poly (butyl cyanoacrylate), poly (isohexyl cyanoacrylate), poly (lactic-coglycolic acid) and gelatin (Dreis et al., 2007). Doxorubicin loaded polymer lipid hybrid nanoparticles demonstrated significant in vivo cytotoxic activity against solid tumors with minimal systemic toxicity (Wong et al., 2007). Despite vast research in the field of nanoparticle based antitumor drug delivery, a need for simple, safe and stable formulation persists.

In a previous study, preparation method of SLN based on the curdlan/cacao butter system was developed (Kim et al., 2005). Verapamil was used as model drug and incorporated in the melted solid core. EE is largely determined by the solubility of drug in the lipoid. Low solubility of doxorubicin in cacao butter limited the use of previous method. In the present work, Capmul® MCM C10 and Solutol® HS 15 were selected as lipid material and surfactant, respectively. These excipients are reported to have better...
bioacceptability and low in vivo toxicity. Recent reports suggested that Capmul® MCM can be employed for parenteral delivery of anticancer agents (Nornoo et al., 2008). The Capmul® MCM based parenteral micro emulsions were well tolerated in vivo. Also, Solutol® HS15 exhibits good parenteral acceptability and is used in current parenteral formulations (Strickley, 2004). Curdlan, a water insoluble polysaccharide that dissolves at higher pH due to conformational change, was used as shell forming material.

The aim of this study was to prepare solid lipid nanoparticles containing doxorubicin to overcome drug resistance and to reduce side effects. This investigation reports the preparation method of SLN loaded with doxorubicin. Drug release from the SLN was studied using dialysis bag method. Stability and cell viability studies were also conducted for the development of SLN based drug delivery system.

2. Materials and methods

2.1. Materials

Capmul® MCM C10 was a generous gift from ABITEC (Columbus, Ohio, USA). Curdlan was obtained from Takeda Chemical Industry Ltd. (Osaka, Japan). Solutol® HS15 was obtained from BASF (Ludwigshafen, Germany). Doxorubicin hydrochloride was obtained from Dong-A Pharmaceuticals (Seoul, Korea). The MCF-7 breast cancer cell line, and its adriamycin-resistant variant, MCF-7/ADR was kindly provided by Professor H.J. Lee (Ewha Womans University, Seoul, Korea). All other chemicals were of analytical grade and used without further purification.

2.2. Preparation of SLN

Doxorubicin hydrochloride was converted into free base form by adding 0.1 N NaOH solution prior to dissolving it in DMSO at 37 °C. Capmul® MCM C10 and Solutol® HS15 were then added to the drug solution. The drug mixture was slowly dropped in curdlan solution (133 mg/l), previously prepared by dissolving curdlan in 2.3 wt% NH4OH aqueous solution, under magnetic stirring. The whole mixture was further stirred overnight, at 900 rpm, to evaporate ammonia. In order to remove large particles, if any, the dispersion was centrifuged at 3000 rpm for 15 min. The pH of the dispersion containing nanoparticles was gradually lowered to 7.4, by slowly adding 0.01 N HCl solution. Finally, dispersion of nanoparticles was purified by dialysis for 12 h to remove the water-soluble impurities. The effects of various processing parameters, such as drug: lipid matrix ratio, Solutol® HS15: Curdlan ratio, aqueous phase volume and DMSO: lipid matrix ratio, on the characteristics of SLN were investigated and those parameters were optimized to obtain high EE and small particle size.

2.3. Photon correlation spectroscopy (PCS)

The mean particle size was measured by PCS at a fixed angle of 90° (Zetasizer 3000HS, Malvern Instrument, UK). Nanodispersion was suitably diluted to measure mean particle size and polydispersity index.

2.4. Differential scanning calorimetry (DSC)

DSC thermograms of doxorubicin:lipid (1:5) were taken to estimate the solubility of doxorubicin in various lipids. Thermal study was done with a PerkinElmer differential calorimeter (Pyris 6 DSC, Netherlands). Temperature scale was calibrated using Indium. Samples were placed in a conventional aluminum pan and heated from 25 °C to 250 °C at a scan speed of 10 °C/min.

2.5. Entrapment efficiency and drug loading

For the quantitative determination of doxorubicin, a spectrofluorometric method was used. The amount of doxorubicin loaded into the SLN was determined as follows: 1.5 ml of 0.1 M hydrochloric acid was added to 0.3 ml of freshly prepared doxorubicin loaded SLN suspension and the mixture was stirred at 37 °C for 24 h. Then, the mixture was centrifuged to separate the undissolved components, and supernatant containing the drug extracted from SLN was analyzed using a fluorescence spectrophotometer (RF-5301 PC, Shimadzu, Kyoto, Japan). A stock solution of doxorubicin was prepared by dissolving a known amount of doxorubicin hydrochloride in distilled water. The excitation and emission wavelengths were fixed at 470 nm and 590 nm, respectively. The EE of doxorubicin in SLN was determined, as the ratio between actual and theoretical loading, using the following equation:

\[ \text{EE}(\%) = \frac{\text{amount of drug in SLN}}{\text{amount of drug added}} \times 100 \]

Drug loading capacity (DL) was calculated as drug analyzed in the nanoparticles versus the total amount of the drug and the excipients added (Capmul® MCM C10, curdlan and Solutol® HS15) during preparation, according to the following equation:

\[ \text{DL}(\%) = \frac{\text{amount of drug loaded in SLN}}{\text{amount of drug added + amount of excipients added}} \times 100 \]

2.6. In vitro drug release

In vitro release study was performed on suspension of nanoparticles within 24 h of preparation. 1 ml of dispersion was transferred to a dialysis tube (molecular weight cutoff 12000, Membra-Cel®, Viskase, USA), and the sealed tube was introduced into a vial containing 10 ml of a phosphate buffer solution (pH 7.4 and pH 5). Samples were shaken horizontally in a shaker (Lab tech, Korea) at 37 ± 1 °C and 50 strokes per minute. At predetermined time intervals, 2 ml sample of the medium was taken and replaced with the same amount of fresh medium. The amount of doxorubicin released from the SLN was quantified by fluorescence spectrophotometer, at excitation and emission wavelengths of 470 nm and 590 nm, respectively.

2.7. Lyophilization

The SLN were lyophilized using a programmable freeze-dryer (Shin PVTFD10R, Shinil Lab, Korea). Cryoprotectant was added to the SLN dispersion before freezing. Trehalose, mannosil, sucrose and fructose were screened at the level of 5% and 10% w/v for their cryoprotectant efficiency. Slow freezing was carried out on the shelves in the freeze dryer (shelf temperature –40 °C). The samples were lyophilized for 24 h from –40 °C to 25 °C at an increasing rate of 5 °C/h. Lyophilized products were reconstituted by sonication (2 min, 500 W, Power Sonic 510, Korea).

2.8. Cell viability assay

Adriamycin resistant and non-resistant breast cancer cell lines (MCF7/ADR & MCF7) were used in this study. Cells were seeded on 96 well plates (SPL Lifescience, Korea) and treated with SLN without drug, doxorubicin solution, doxorubicin loaded SLN, freshly freeze dried doxorubicin loaded SLN, freeze dried doxorubicin loaded SLN after 1 month and 1 year of storage at 4 °C. Cell viability was determined by crystal violet staining (Shin et al., 2005). Briefly, cells were incubated with 0.4% crystal violet in methanol for 30 min at room temperature and washed with water three times. Stained cells were
extracted with 50% methanol, and dye extracts were measured for changes in optical density at 540 nm using spectrophotometric microtiter plate reader (Tristar LB 941, Berthold Tech., Bad Wildbad, Germany).

2.9. Statistical analysis
Statistical evaluation of data was performed using one-way analysis of variance (ANOVA). Tukey–Kramer multiple comparison test was used to compare the significance of the difference between the groups, a p-value < 0.05 was accepted as significant. Data were expressed as mean and standard deviation of separate experiments (n = 10).

3. Results and discussion
3.1. Screening of lipid phase
In order to optimize preparation of doxorubicin loaded SLN by solvent emulsification–diffusion method, several formulation variables were studied. For the selection of lipid core, solubility of doxorubicin in lipids was evaluated by measuring DSC thermogram of doxorubicin/lipid (1/5) mixture and is shown in Fig. 1. The melting peak of doxorubicin base at 218 °C completely disappeared when pentadecanoic acid or Capmul® MCM C10 was used as a lipid core. Although the physical state of the drug was not confirmed by other analytical means, the absence of the melting peak suggested that doxorubicin had better solubility in both pentadecanoic acid and Capmul® MCM C10 as compared to the other lipids screened. Solubility of the drug in the lipid melt is known to be an important precondition to obtain sufficient EE (Battaglia et al., 2007). Capmul® MCM C10 was selected as lipid core because: it melts at 37 °C (body temperature), as compared to pentadecanoic acid that has a melting point of 52 °C, possesses lower toxicity (Cho et al., 2004) and is listed as generally recognized as safe (GRAS) according to 21 CFR §184.1505. Additionally, both doxorubicin and Capmul® MCM C10 are soluble in DMSO. Drug and lipid phase should be dissolved in the solvent for the formation of nanoparticles by solvent emulsification-diffusion method (Trotta et al., 2001).

3.2. Influence of curdlan
Curdlan forms shell around the lipid core during the formation of nanoparticles. In a previous study it was reported that, insufficient curdlan available to coat the surface of lipid droplets could result in aggregation of particles and decrease in EE (Kim et al., 2005). As evident in Fig. 2, increasing curdlan concentration above 0.01% of the external phase significantly increases the particle size with little effect on the EE. This contrasting result may due to the change in the surfactant employed in current study. The present system used Solutol® HS15 instead of Tween 80® as used in the previous study. Emulsification of the oil phase was found to be inadequate when Tween 80® was evaluated in the present study. Interestingly, a two-fold increase in EE was obtained by replacing Tween 80® with Solutol® HS15 (data not shown). Some studies have shown that Solutol® HS15 has better emulsifying ability than Tween 80® (Date and Nagarsenker, 2007). However, emulsification efficiency of surfactant is dictated by the physicochemical properties of the dispersion. Solutol® HS15, which is commonly used in parenteral formulations (Jumaa and Müller, 2002), acted as an efficient surfactant in the present system. This leaves a limited role for curdlan to act as co-surfactant, rather it acted as shell forming material through surface deposition. When curdlan was not used, nanoparticles with smaller particles could be formed without any change in the EE. The observed decrease in particle size may due to the absence of curdlan coat on the nanoparticles. Since, Capmul® MCM C10 has melting point around 37 °C, coat of curdlan was essential for the robustness of the nanoparticles. Besides imparting strength to the nanoparticles, curdlan could modulate the release of doxorubicin from nanoparticles. Several studies suggested the use of curdlan as controlled drug delivery vehicle (Kanke et al., 1992, 1995a,b; Kim et al., 2005). In the absence of curdlan, faster release of doxorubicin could be anticipated. This prompt release could lead to reduction in efficacy for overcoming multi drug resistance (Némáti et al., 1996). In the study, nanoparticles that allowed too rapid doxorubicin release were not efficient against P388 sensitive and resistant cell lines.

3.3. Influence of Solutol® HS15/curdlan ratio
The influence of Solutol® HS15/curdlan ratio on the EE and average size of doxorubicin loaded SLN are shown in Fig. 3. Unless the ratio of Solutol® HS15 was 3 times or higher than that of curdlan, EE was quite low and particle size was larger. Further increase in Solutol® HS15 did not have much influence on the particle size and the EE. Higher concentration of surfactants is expected to reduce surface tension resulting in reduction of particle
size. But increase in surfactant concentration may not significantly affect particle size once optimal packing is reached (Liu et al., 2007).

3.4. Influence of aqueous phase volume

The ratio of oil phase and aqueous phase showed great impact on the EE of SLN. Fig. 4 clearly shows that increasing aqueous phase volume results in an increase in EE. This could be due to lesser aggregation of the particles in a larger space. Different studies have shown that the aqueous phase volume has paramount effect on the formation of nanoparticles. In a recent study, with increased volume of aqueous phase, increase in drug content of particles prepared by homogenization and sonication was observed (Budhian et al., 2007). Aqueous phase volume beyond 15 ml was not advantageous, because of lower concentration of nanodispersion without increase in EE.

3.5. Influence of drug/lipid matrix ratio

Increase in matrix content is expected to raise the EE by providing more space to incorporate the drug. Increment of the lipid content also reduces the escaping of drug into the external phase, which accounts for an increase in EE (Shah et al., 2007). EE significantly increased at drug:lipid ratio smaller than 1/5. An increase in particle size was observed as the drug:lipid ratio decreased from 1:1 to 1:15 (Table 1). Numerous studies have reported that increasing lipid content results in larger particles and broader particle size distribution (Trotta et al., 2003; Mehnert and Mäder, 2001; Battaglia et al., 2007). Larger particle size with increase in lipid content could be attributed to decrease in emulsifying efficiency and increase in particle agglomeration.

3.6. Influence of DMSO/lipid ratio

In the absence of DMSO, the lipid rapidly crystallized when placed in the aqueous phase. This resulted in the precipitation of the drug in the aqueous phase. To minimize this loss of the drug in the external phase, solvents are commonly employed (Trotta et al., 2003). When DMSO was used, after the oil phase containing drug and surfactant was transferred to aqueous phase containing shell forming material, nanoparticles were formed due to diffusion of the organic solvent from the droplets to the continuous phase with the consequent solidification of lipid. As can be seen in Table 2, when the ratio of DMSO to lipid was less than 5:1, both EE and drug loading decreased. Further increasing the amount did not have significant effect on the EE and DL.

3.7. In vitro drug release

Due to high glycolytic rate, high lactic acid production and insufficient drainage by convective transport, H+ ions accumulate in the tumor tissue. As a result, pH is shifted towards more acidic values, especially in bulky and/or low-flow tumors (Vaupel et al., 1989). Furthermore, extracellular acidosis in solid growing tumors leads to a chemoresistant phenotype due to increased p-glycoprotein activity (Sauvant et al., 2008). Ideal anti cancer drug delivery system should be able to kill cancer cells by more specific targeting while sparing normal cells. To exploit the pH difference, between normal and cancer cells, various investigations on fabricating pH-sensitive drug delivery system are reported (Kang et al., 2003; Liu et al., 2006). Since, cancer cells develop more acidic microenvironment, delivery system having distinct release profile close to the physiological pH would be an invaluable approach for anticancer chemotherapy. As clearly seen in Fig. 5, higher release rate of doxorubicin was achieved at lower pH, with the present system. Because of the basic nature of doxorubicin (pKₐ=8.3), it has higher solubility at lower pH. Therefore, the entrapped doxorubicin in the SLN has a greater tendency to go into the release medium of lower pH. The favored release in acidic medium would result in higher release rate of doxorubicin in tumor cells, even more in the resistant cell lines, adding therapeutic efficiency to the delivery system.

Table 1

<table>
<thead>
<tr>
<th>Drug: lipid</th>
<th>Mean particle size (nm)</th>
<th>EE (%)</th>
<th>DL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:15</td>
<td>306</td>
<td>712 ± 1.7</td>
<td>2.95 ± 0.07</td>
</tr>
<tr>
<td>1:10</td>
<td>263</td>
<td>64.8 ± 2.6</td>
<td>3.39 ± 0.14</td>
</tr>
<tr>
<td>1:5</td>
<td>240</td>
<td>48.4 ± 2.4</td>
<td>3.44 ± 0.17</td>
</tr>
<tr>
<td>1:3</td>
<td>229</td>
<td>43.4 ± 0.9</td>
<td>3.59 ± 0.08</td>
</tr>
<tr>
<td>1:1</td>
<td>206</td>
<td>35.6 ± 7.6</td>
<td>3.54 ± 0.75</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>DMSO: lipid</th>
<th>EE (%)</th>
<th>DL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>22.9 ± 2.9</td>
<td>1.58 ± 0.25</td>
</tr>
<tr>
<td>3:1</td>
<td>50.3 ± 5.9</td>
<td>1.78 ± 0.09</td>
</tr>
<tr>
<td>5:1</td>
<td>75.5 ± 0.8</td>
<td>2.69 ± 0.23</td>
</tr>
<tr>
<td>7:1</td>
<td>74.5 ± 1.6</td>
<td>2.58 ± 0.61</td>
</tr>
</tbody>
</table>

Fig. 3. Effect of Solutol®HS15/curdlan ratio on the mean particle size and EE of nanoparticles. Values are expressed as mean ± standard deviation (n = 3).

Fig. 4. Effect of aqueous phase volume on the EE of nanoparticles. Values are expressed as mean ± standard deviation (n = 3).
3.8. Lyophilization

Usually, SLN dispersion shows an increase in particle size in a short period of time during the storage. Particle size of prepared SLN crossed the nanometric range within a week (data not shown). Lyophilization offers chemical and physical stability by preventing Ostwald ripening and hydrolysis reactions (Mehnert and Mäder, 2001). Cryoprotectants have been used to decrease SLN aggregation due to the stress during the process of freeze-drying (Shahgaldian et al., 2003). Various cryoprotectants were screened at the concentrations of 5 and 10% w/v. All the compounds tested, significantly prevented particle size growth as compared to control (Fig. 6). Trehalose was slightly better than other cryoprotectants in reducing particle size. Furthermore, based on the morphology of the freeze-dried cake, trehalose was chosen for further experiments. The observation was in agreement with the results obtained from previous studies on lyophilization of SLN (Schwarz and Mehnert, 1997; Cavalli et al., 1997; Pozo-Rodríguez et al., 2008).

3.9. Stability and cytotoxicity of SLN

Fig. 7 shows percentage survival of MCF7 and MCF7/ADR cells after exposure to SLN. No cytotoxicity of the unloaded SLN was observed, as cell viability remained constant. Resistant cells were more sensitive to doxorubicin loaded SLN when compared to doxorubicin solution ($p < 0.001$). Doxorubicin solution, equimolarly tested, did not affect cell growth in both cell lines. Other studies also have demonstrated higher cytotoxic properties of doxorubicin loaded SLN than that of doxorubicin solution (Serpe et al., 2004; Fundarò et al., 2000). Freshly prepared SLN and freeze dried SLN showed similar efficacy implying that the drying process did not alter the efficacy of SLN. Although there was an increase in particle size by 31.5% and decrease in EE by 10.4% after 1 year of storage at 4°C, it still maintained the nanometric range and nanoparticles were found equally effective in reducing the cell viability. Additionally, drug loaded SLN exhibited more toxicity towards the resistant cell line. Drug resistance has been a major issue in the arena of medical science. The present mode of drug delivery could play a beneficial role in overcoming this challenge.

4. Conclusions

In this study, SLN using doxorubicin as model drug were successfully prepared using Capmul® MCM C10 as lipid core and curdlan as the shell material, by solvent emulsification–diffusion method. Different formulation parameters, found to influence fabrication of drug into nanoparticles, were optimized for high EE and DL. The most important parameters were drug:lipid ratio, Solufo® HS15:curdlan ratio, and DMSO:lipid ratio. Higher in vitro drug release was observed in pH 5 than in pH 7.4. Cytotoxicity results showed that lyophilized SLN were equally effective after 1 year of storage at 4°C. These observations suggest that present system offers an exciting mode of delivery to the lipophilic anticancer drugs.

Acknowledgements

This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MEST) through the Research Center for Resistant Cells (R13–2003–009).

References


