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Cisplatin-loaded SLN produced by coacervation technique

M. Gallarate*, M. Trotta, L. Battaglia, D. Chirio

Dipartimento di Scienza e Tecnologia del Farmaco, via Pietro Giuria 9, 10125 Torino, Italy
*Correspondence: marina.gallarate@unito.it

Coacervation technique, a solvent-free, feasible and versatile method, based on a phase transformation from soap micellar solution into fatty acid solid particles by acid addition, was used to prepare cisplatin loaded solid lipid nanoparticles (SLN) of stearic acid. Different polymers were tested to stabilise SLN suspensions. Solubilisation of cisplatin, a hydrophilic antitumor agent, within sodium stearate micelles was possible by the formation of a hydrophobic ion-pair with sodium dioctylsulfosuccinate. Several SLN were produced, whose particle size were in the 275-525 nm range. Cisplatin encapsulation efficiency up to 90 % was obtained, depending on both stearic acid concentration and stabilisers type and concentration. The in vitro cisplatin release showed a burst effect of about 10-20 %, corresponding to the non-encapsulated drug, and then a complete drug release was reached after 24 h.

Key words: Cisplatin – Ion pairs – SLN – Coacervation.

Solid lipid nanoparticles (SLN) are disperse systems widely used in literature for drug delivery [1]. Several methods are described in literature to produce SLN, such as cold and hot homogenisation [2], microemulsion dilution [3], microemulsion cooling [4], solvent evaporation [5], solvent injection [6], o/w [7] and w/o/w [8] emulsions solvent dilution. Each of the mentioned techniques presents some disadvantages, such as the high operative temperatures, the use of toxic solvents, the requirement of sophisticated apparatus.

Recently [9,10], a new, solvent-free SLN production technique was developed. Briefly, when the pH of a fatty acid alkaline salt micellar solution is lowered by acidification, fatty acid precipitates owing to proton exchange between the acid solution and the soap: this process was defined as "coacervation". Myristic, palmitic, stearic, arachidic and behenic acid were used as lipid matrixes and various molecular mass partially hydrolysed polyvinyl alcohols and hydroxypropylmethyl cellulose were investigated as stabilisers.

In the present work, commercial polymers such as Pluronics and polymers such as modified dextrans, synthesised according to a literature method [11], are tested as new stabilisers for drug-loaded stearic acid SLN production. As a model drug to be encapsulated within SLN, cisplatin (cisPt), a well-known cytotoxic drug, used to treat various kinds of solid tumours, was chosen. Its limitations in clinical use are the short half-life and the high nefrotoxicity after prolonged treatment [12]: cisPt-loaded SLN might act as a sustained release system, aimed to protract drug effect and reduce its toxicity, as already described in literature for polymeric nanoparticles [13-18].

The primary requirement to obtain fatty acid SLN with high drug encapsulation efficiency by the coacervation technique is to dissolve the drug in the hydrophobic micellar core of sodium stearate micelles present in the initial alkaline solution. Since cisPt is a hydrophilic molecule, drug encapsulation can be enhanced through hydrophobic ion pairing. In the present work sodium dioctyl sulfosuccinate was used as counter ion [19]. This ion pair acts like a prodrug, since in normal saline chloride ions can substitute dioctyl sulfosuccinate giving cisPt [19].

I. MATERIALS AND METHODS

1. Materials

Lactic acid was from ACEF (Fiorenzuola d'Arda, Italy), 80 % hydrolysed PVA 9000 MW (PVA), Pluronic F68 (PF68), Pluronic F127 (PF127), dextran 60000-90000 MW (dextran) and cisplatin (cisPt) were from Sigma (Dorset, United Kingdom); sodium stearate (SS) and diethyldithiocarbamate (DDTC) were from Fluka (Buchs, Switzerland); 2,3-epoxypropylphenylether was from Aldrich (St Louis, United States); sodium chloride, sodium citrate, sodium lactate, sodium acetate, sodium nitrate, di-sodium phosphate and dioctyl sulfosuccinate (AOT) were from Merck (Darmstadt, Germany); $AgPF_6$ was from Aldrich (St Louis, United States); deionised water was obtained by a MilliQ system (Millipore, Bedford, United States); all other chemicals were of analytical grade and used without any further purification.

2. Methods

2.1. Dextran 3-phenoxy-2hydroxypropane (DexP) synthesis

DexP was synthesised according to a literature method [10]: 1 g dextran was allowed to react with the required amount of 1,2-epoxy-3-phenoxypropane in 10 mL 0.1 M NaOH at 25 ± 0.1 °C for 48 h. The crude products were then precipitated with ethanol and dried under vacuum overnight. Substitution degrees (τ) were calculated as reported in *Figure 1* and were measured through spectrophotometric detection at 278 nm.

Dextrans with 4 different τ (8, 11, 15 and 22 %) were prepared.

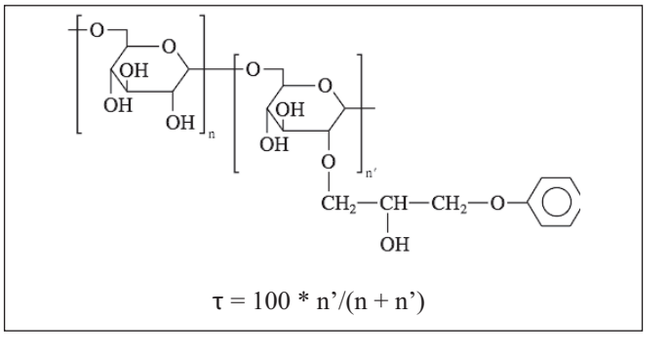

Figure 1 - dexP formula and substitution degree (τ).

2.2. CisPt-AOT ion pair preparation.

Hydrophobic cisPt-AOT ion-pair was prepared according to a literature method [19]. CisPt solution (2 mg/mL) was added to $AgPF_6$ (10 mg/mL) at a cisPt:$AgPF_6$ 1:2 molar ratio. After mixing, the solution was left to settle at 4 °C overnight and then centrifuged at 1,500 g for 10 min (AllegraTM R64 centrifuge, Beckmann Coulter) to remove the precipitated silver chloride. The resulting water-soluble hydrolysed cis-diamine platinum intermediate was then added dropwise to 20 mM AOT at cisPt:AOT 1:2 molar ratio. The light-yellow

precipitate formed was allowed to settle for 2 h at room temperature, centrifuged at 55,000 g, washed three times with deionised water, and then freeze dried with a Modulyo freeze dryer (Edwards, Yardley, United States). Conditions were as follows:
- freezing: - 40 °C, 2 h,
- primary drying: at 1.0 mbar, -30 °C, 12 h,
- secondary drying: 0.1 mbar, 30 °C, 3 h.

2.3. CisPt-AOT apparent partition coefficient

Apparent partition coefficient (P_{app}) of cisPt-AOT was determined between CH_2Cl_2, chosen as a model of the lipophilic core of SS micelles, and water or 0.1 M sodium citrate, sodium acetate, sodium lactate, di-sodium phosphate and sodium chloride solutions. One millilitre of the organic solution containing 0.25 mg cisPt-AOT was added to an equal volume of aqueous phase. The system was shaken for 30 min. After phase separation, drug concentration was determined in the aqueous phase by HPLC and the apparent partition coefficient was calculated, according to the reported formula:

$$P_{app} = [cisPt]_{solvent}/[cisPt]_{buffer}$$

where $[cisPt]_{solvent} = [cisPt]_{buffer} - [cisPt]_{0\ buffer}$, $[cisPt]_{buffer}$ is the cisPt molar concentration in buffer phase after partition, and $[cisPt]_{0\ buffer}$ the cisPt molar concentration in buffer phase before partition.

HPLC analysis was performed modifying a literature method [19]. 0.8 mL sample was added to 0.1 mL 0.5 % DDTC solution and 0.1 mL saturated sodium nitrate solution. The resulting solution was heated to 60 °C for 1 h and after cooling to room temperature and centrifuging at 10,000 g, the obtained precipitate (cisPt-DDTC) was dissolved in 0.2 mL CH_3CN and analysed by HPLC.

Experimental conditions were as follows: LC9 pump equipped with SPD10AV UV-visible lamp and C-R5A integrator (Shimadzu, Kyoto, Japan); column: Ultrasphere C18 250 mm × 4.6 mm (Beckmann Coulter); mobile phase: CH_3CN-water 75:25; flow: 1 mL/min; λ_{max}: 340 nm; retention time: 6.0 min. The linearity of the calibration graph was demonstrated by the value (0.9993) of R^2 coefficient of the regression equation: y = 53118x + 206475. The LOQ was 20 μg/mL; the LOD was 10 μg/mL.

2.4. SLN preparation

Stearic acid (SA)-SLN was prepared according to the coacervation method described in a previous paper [9]. Briefly, SS was dispersed in 19 mL of an aqueous solution of the polymeric stabiliser and the mixture was then heated under stirring (300 rpm) up to 48 °C to obtain a clear solution. A known amount of cisPt-AOT ethanol solution (25 mg/mL) was then added and kept under stirring until complete dissolution. One millilitre of a selected acidifying solution (coacervating solution) was then added drop-wise until pH 4.0 was reached. The obtained suspension was then cooled to 15 °C under stirring at 300 rpm. Empty nanoparticles were produced in the same manner, but without adding cisPt-AOT ethanol solution.

2.5. SLN characterisation

SLN particle size and size distribution were determined by the laser light scattering technique LLS (Brookhaven, New York, United States). The dispersions were diluted with water and measurements were done at an angle of 90°.

DSC was performed with a Perkin-Elmer differential calorimeter (Norwalk, CT, United States). Lipid bulk material and SLN suspensions were placed in conventional aluminium pans. Experimental conditions were as follows: scan speed 2 °C min^{-1}; temperature range 30-80 °C. Drug encapsulation efficiency (EE %) was calculated as the ratio between the amount of drug encapsulated within lipid matrix and the amount of drug used to prepare nanoparticles. For the determination of the encapsulated drug, a known amount of SLN suspension was centrifuged at 55,000 g and the precipitate was washed with normal saline in order to remove drug adsorbed on nanoparticles surface. In fact, in normal saline chloride ions can substitute AOT giving cisPt [19]; moreover normal saline increases cisPt water solubility. In this way ion pair, which is not encapsulated within the lipid core, can be removed by simple washing with normal saline. Then the precipitate was dried and dissolved in CH_2Cl_2. CisPt was extracted with normal saline and analysed by HPLC.

2.6. *In vitro* drug release

Drug release studies from freshly prepared 4 % SA-SLN with 0.6 % dexP(τ = 22 %) and 4 % SA-SLN with 4 % PVA, loaded with 0.05 % cisPt-AOT, were done by diluting nanoparticles suspension with normal saline as receiving phase. Normal saline is a good dissolving medium for cisPt, as discussed above. Experiments were performed under 50 rpm stirring at 37°C. At scheduled times a small amount of the receiving phase was centrifuged at 55,000 g for 15 min and the supernatant analysed by HPLC for cisPt determination.

II. RESULTS AND DISCUSSION

1. Empty SLN

A series of preliminary experiments were performed to optimise coacervation process.

Various stabilisers were used to produce empty 1 % SA-SLN to investigate the influence on particle size. PVA-stabilised SLN, already characterised in a previous study [9], were used as reference system, while dexP (τ = 7, 11, 15, 22 %), PF68 and PF127 were tested for comparative purposes. In *Table I* the final compositions used to prepare 20 mL of empty 1 % SA-SLN are shown.

Submicron sized SLN were obtained with each polymer (*Table I*). For SLN stabilised with dexP, it should be noticed that, as τ increased, particle size decreased and a lower polymer concentration was required. Phase separation was noted using a higher dexP concentration.

In *Figure 2*, DSC patterns are depicted. DSC thermograms of SLN revealed sharp melting peaks and no supercooled melt was revealed. SLN melting point was always detected nearly at 53 °C (polymorph B) [21]. In a previous work [9] we noted that DSC thermograms of SLN prepared with different fatty acids, showed only small differences between melting point of pure lipid and of corresponding SLN. According to Siekmann and Westesen [22], the melting point decrease of SLN colloidal systems can be due to the colloidal dimensions of the particles, in particular to their high surface to volume ratio, and not to recrystallisation of the lipid matrices in a metastable polymorph. If the bulk matrix material is turned into SLN, the melting point is depressed [23]; the presence of impurities, surfactants and stabilisers could also affect this phenomenon [24, 25]. SA-SLN melting point, quite lower than raw SA (69 °C) is ascribed to polymorphism. In fact SA can exist in three crystalline forms, A-B-C [20], with three different melting points (43, 54 and 69 °C, respectively). Previous investiga-

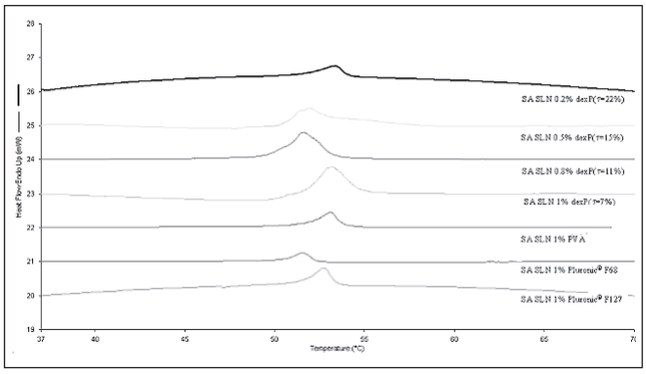

Figure 2 - DSC patterns of empty 1 % SA-SLN obtained with different stabilisers.