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Cisplatin-loaded SLN produced by coacervation technique

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Abstract: 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.

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] emul- sions 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 litera- ture 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

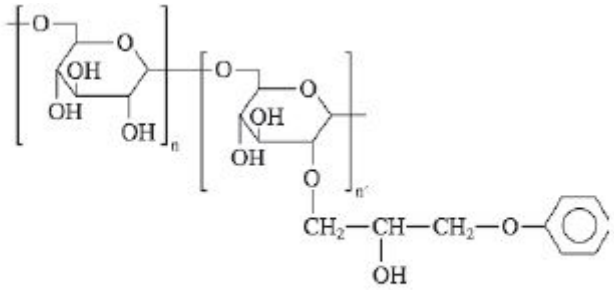
Lactic acid was from ACEF (Fiorenzuola d'Arda, Italy), 80 % hydrolysed PVA 9000 MW (PVA), Pluronic F68 (PF68), Pluronic F127 (PF127), dextran 60000-90000 MW (dextran) and cisplatin (cisPt) were from Sigma (Dorset, United Kingdom); sodium stearate (SS) and diethyldithiocarbamate (DDTC) were from Fluka (Buchs, Switzerland); 2,3-epoxypropylphenylether was fromAldrich (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); AgPF6 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.



$$\tau = 100 * n'/(n + n')$$

Figure 1 dexP formula and substitution degree (t).

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 (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 drop- wise 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 (Papp) of cisPt-AOT was determined between CH2Cl2, 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 CH3CN 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 (Beck- mann 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 ob- tain 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 (coacer- vating 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.

	SLN 1 % PVA	SLN 1 % dexP (τ=7 %)	SLN 0.8 % dexP (τ=11 %)	SLN 0.5 % dexP (τ=15 %)	SLN 0.2 % dexP (τ=22 %)	SLN 1 % PF127	SLN 1 % PF68
SS	215 mg*	215 mg*	215 mg*	215 mg*	215 mg*	215 mg*	215 mg*
PVA	200 mg						
dexP (τ=7 %)		200 mg					
dexP (τ=11 %)			160 mg				
dexP (τ=15 %)				100 mg			
dexP (τ=22 %)					40 mg		
PF 127						200 mg	
PF 68							200 mg
Mean size (nm)	285 ± 11	525 ± 50	500 ± 48	380 ± 33	334 ± 32	324 ± 29	275 ± 15
Polydispersity	0.010	0.205	0.184	0.139	0.085	0.135	0.015
T_{peak}	52.5	53.2	51.6	51.6	53.4	51.6	52.0
$\Delta H_{melting}$ (J/g)	46.7	137.0	136.0	119.5	123.4	87.7	127.5

Each system contained 19 mL water was and 1 mL 1M lactic acid (coacervating solution). *Corresponding to 200 mg SA.

Table 1 Composition, mean size, polydispersity, melting temperatures and enthalpies of empty 1 % SA SLN.

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. Ac- cording 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 tion on SA-SLN with X-rays [9] confirmed that, in SLN, SA was in the low melting B form, which is characterised by monoclinic lattice [26].

Successively, it was pointed out that SA polymorphism is typi- cal of coacervation process regardless the presence of a stabiliser. B form of SA was also obtained after acidification of Na-S solution with lactic acid in the absence of PVA. B-form showed a distinct DSC pattern compared to C-form (*Figure 3*) and proved to be stable upon re-crystallisation.

This confirms that the formation of polymorph B is typical of the coacervation process, regardless of the polymer used as stabiliser [9]. Small differences among transition temperatures (T_{peak}) and melting enthalpies ($\Delta H_{melting}$) might probably be ascribed to polymer-lipid interactions, depending on both polymer grade and concentration (*Table I*).

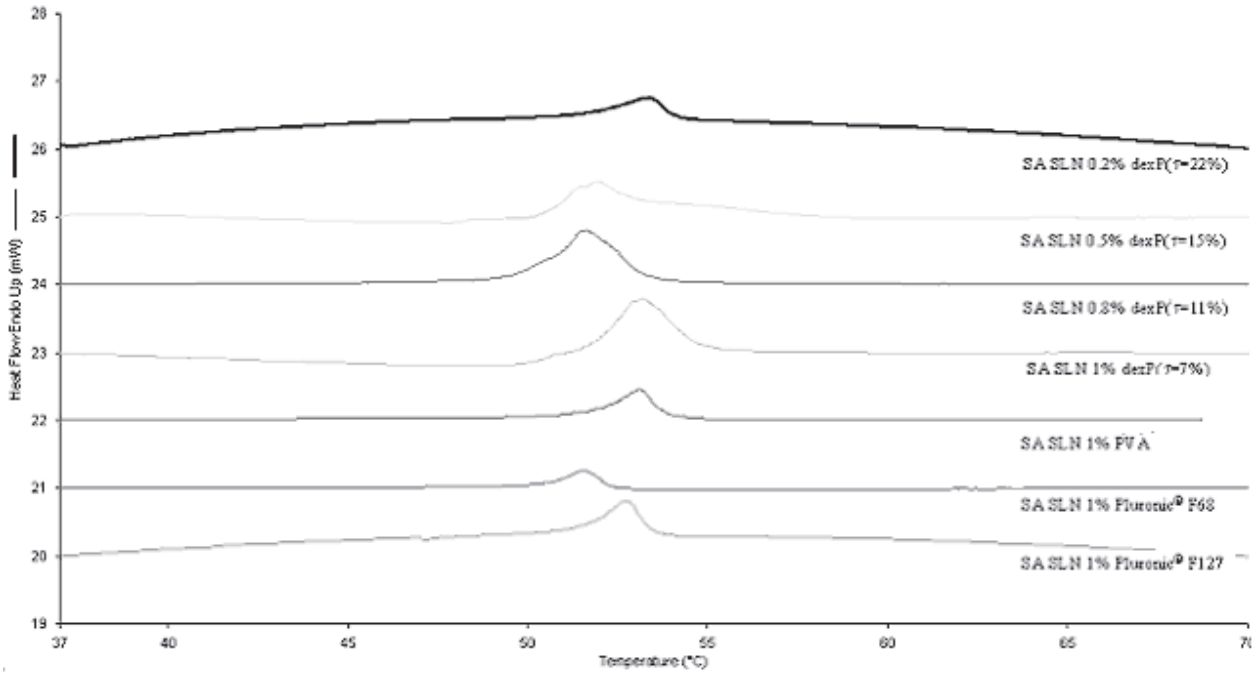


Figure 2 DSC patterns of empty 1 % SA-SLN obtained with different stabilisers.

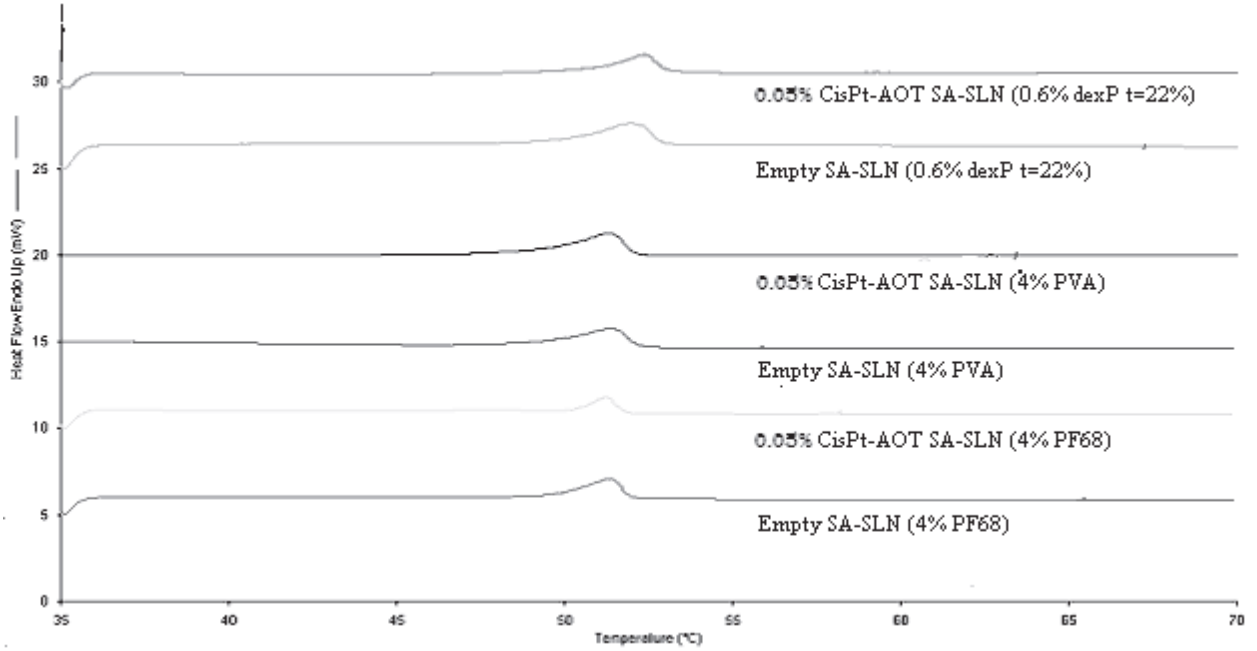


Figure 3 DSC patterns of 4 % SA-SLN.

2. CisPt-aot - loaded SLN

During SLN preparation, AOT could be substituted by less hy- drophobic anions, like those formed in the acidification step during the coacervation process, leading to extraction of cisPt from micelles to the water phase, reducing the drug encapsulation within the lipid matrix. Several solutions were therefore tested, in order to select the acid whose corresponding anion was able to minimise cisPt extraction from the micellar core.

CisPt-AOT apparent partition coefficients between CH_2Cl_2 and water, sodium citrate, sodium acetate, sodium lactate and di-sodium phosphate aqueous solutions are shown in *Table II*. The highest partition coefficient was obtained with lactate ion, indicating the lowest ability of lactate, among the examined anions, to extract the drug from the micellar core. As a consequence, lactic acid was chosen as coacervating solution to optimise drug loading.

	Partition coefficient
Water	16.3
0.1 M citrate	0.02
0.1 M lactate	5.7
0.1 M acetate	1.1
0.1 M phosphate	0.04
0.1 M chloride	0.03

Table 2 CisPt-AOT CH_2Cl_2/water solutions apparent partition coefficient.

Drug-loaded SLN were prepared with different lipid concentra- tions (from 1 to 4 % w/w) using PVA as stabiliser and cisPt-AOT at a 0.025 and 0.05 % w/w concentration, and increasing proportionally the molar concentration of acidifying solution. From *Figure 4a*, it can be noticed that EE % increased by increasing the lipid concentration. Indeed, in 4 % w/w SA-SLN, EE % was about 90 %. When drug concentration was increased to 0.05 % w/w, no further significant increase in EE % was obtained. Different polymer were then tested as stabilisers to evaluate their possible influence on EE % of 0.05 % w/w cisPt-AOT: DexP (τ = 22 %) and PF68 were used. As it can be noticed from *Figure 4b*, EE % was highly influenced by the polymer type. A dramatic decrease of EE % was noted with PF68.

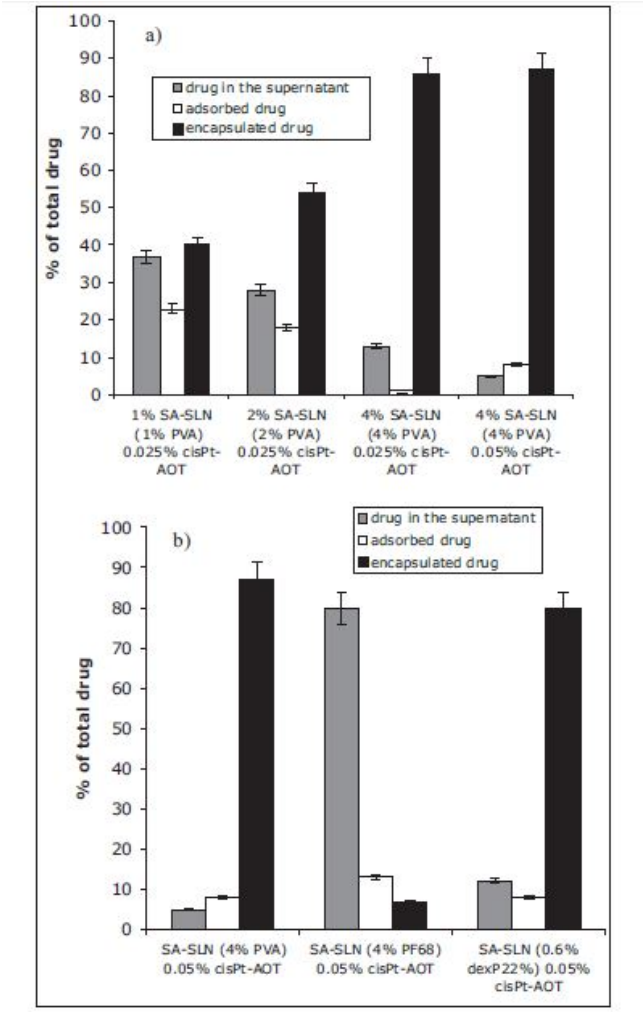


Figure 4 EE % of cisPt-AOT within SLN: **a)** with different SA and PVA 9000 concentrations, **b)** with 4 % SA and different polymeric stabilisers.

Mean particle sizes of drug loaded-SLN, compared to empty ones, are shown in *Table III*. Nanoparticles in the submicron range are obtained for each formulation. For PVA-stabilised SLN, the increase in lipid concentration and in drug loading caused an increase of nanoparticles mean size. Moreover a proportional increase of polymer (PVA, PF68) concentration was needed to stabilise nanoparticles suspension when the lipid concentration was increased; for dexP(τ = 22 %), nanoparticles stabilisation was achieved using 0.6 % w/w polymer.

	Empty SLN		0.025 % cisPt-AOT loaded SLN		0.05 % cisPt-AOT loaded SLN	
	Mean size (nm)	Poly.	Mean size (nm)	Poly.	Mean size (nm)	Poly.
1 % SA SLN 1 % PVA	285 ± 11	0.010	353 ± 15	0.206		
2 % SA SLN 2 % PVA	373 ± 35	0.079	385 ± 23	0.076		
4 % SA SLN 4 % PVA	413 ± 36	0.105	453 ± 34	0.176	494 ± 48	0.137
4 % SA SLN 4 % PF68	294 ± 28	0.051			334 ± 30	0.089
4 % SA SLN 0.6 % dexP (τ=22 %)	422 ± 43	0.097			454 ± 46	0.148

Table 3 Mean sizes of cisPt-AOT loaded SLN.

It should also be noted that, drug-loaded SLN melting point was always detected nearly at 53° C (*Figure 3*), indicating that the forma- tion of polymorph B of SA is influenced neither by drug encapsulation, nor by drug concentration within the lipid matrix (data for 0.025 % cisPt-AOT-loaded SLN not shown).

In vitro drug release study from 4 % SA-SLN stabilised with 0.6 % dexP(τ = 22 %) or 4 % SA-SLN stabilised with 4 % PVA are depicted in *Figure 5*. Freshly prepared nanoparticles were used for release studies, without elimination of the free (not encapsulated) drug, since encapsulation efficiency for this formulation was high and the amount of free drug relatively small. The initial amount of the drug recovered at the beginning of the experiment in the release medium could be probably ascribed both to the free drug and to the burst release of the adsorbed drug. However most of the drug was slowly released within 24 h for both the formulations; on the contrary pure cisPt-AOT dissolution in normal saline was complete within few minutes (data not shown).

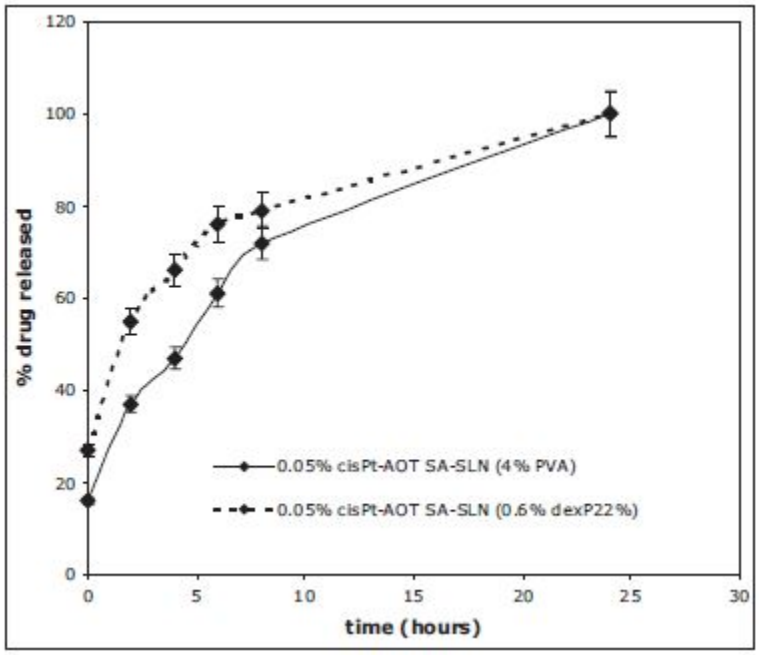


Figure 5 *In vitro* drug release from 4 % SA-SLN.

Drug-loaded SLN were prepared according to the coacervation technique, by using different polymers as stabilisers. CisPt, chosen as hydrophilic model drug, was encapsulated in SLN through hydrophobic ion pairing. Depending on the polymer type and lipid concentration, different drug encapsulation efficiencies were obtained. *In vitro* drug release from SLN was complete within 24 h. This makes them promising as a sustained release system for cisPt.

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