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Note

Preparation and purification of cationic solid lipid nanospheres—effects on particle size, physical stability and cell toxicity

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Abstract

Cationic solid lipid nanospheres (SLN) were prepared by the microemulsion technique with polysorbate 80 (Tween 80) and butanol as surfactants. The SLN (diameter 100–500 nm, zetapotential around +15 mV) consisted mainly of stearylamine (SA) and different triglycerides. Three different purification methods, ultrafiltration, ultracentrifugation and dialysis, were investigated and compared with the cellular toxicity and physical stability of the dispersions. The cell toxicity was dependent on both the SLN composition and the purification method. Dialysis was found to easily and efficiently remove excessive surfactant determined by dynamic light scattering (DLS), leading to reduced cell toxicity and increased physical stability of the SLN on storage. The cationic SLN might constitute a promising DNA delivery system.

Keywords: Solid lipid nanospheres; Stearylamine; Polysorbate 80; Cationic; Surfactants; Cell toxicity

Solid lipid nanospheres (SLN) can be produced in a variety of ways using a wide range of chemical ingredients (Müller et al., 2000). All preparation methods make use of surfactants and the resulting particles have either an overall positive, neutral or negative surface charge, determined by the composition, influencing the aggregation tendency in suspension. The microemulsion technique is favourable when working with substances unstable to the high mechanical stress produced by high pressure homogenisation. Using that method, huge amounts of surfactants and co-surfactants including butanol are used (Gasco, 1997). Little has been published about the removal of excessive surfactants by the subsequent purification procedure and the influence of remaining surfactants on particle stability and cell toxicity. Cationic nanoparticles are potential for gene delivery and have shown to be less rapidly cleared from the circulation than negative-charged particles (Gregoriadis and Neerunjun, 1974).

This note describes the physical evaluation of cationic SLN and the effect of stearylamine (SA) and

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polysorbate 80 on cell toxicity as an essential prerequisite for further studies for parenteral administration.

The preparation of the aqueous cationic SLN dispersions consisted of two steps: firstly formulation of an oil-in-water microemulsion and secondly preparation of the SLN by dispersing the warm microemulsion into cold water. The lipid including co-surfactant and the water phase were heated separately, mixed and subsequently titrated with the surfactant until a microemulsion was obtained. The microemulsion was dispersed 1:10 in ice-cold water at a constant speed (2 ml/min) using a syringe with a needle gauge 27 with gentle stirring. The stirring was continued for 30 min at 2–3 °C before purification. Three different ways of removal of surfactants were investigated and compared:

(i) Ultrafiltration (TCF2-Amicon stirred cell) 5 times with Milli-Q water using Diaflo YM 100 membrane (cut-off 100 kDa, 33:1 dilution).
(ii) Ultracentrifugation (Beckman L7-55) in 20% sucrose (41,000 × *g*, 1 h, 5:1 dilution) followed by 4 h dialysis to remove sucrose remnants.
(iii) Dialysis using Spectra/Por® CE (Spectrum) (cut-off 300 kDa, 3 × 8 h, 333:1 dilution).

Particle size was measured by dynamic light scattering, DLS (DynaPro, ProteinSolutions), a mean of 10 measurements, calculated from the regularisation histogram using DYNAMICS version 5.25.44.

The electrophoretic mobility was measured by laser Doppler anemometry (Zeta Master, Malvern Instruments) at 30 V/cm in 0.1 mM NaCl. The zetapotential values were calculated using the Helmholtz–Smoluchowski equation. The cell toxicity was investigated in the different cell lines after 4 h of incubation with SLN or the pure chemicals in a serum free medium and evaluated by trypan blue staining or MTT assay.

It is essential to have a microemulsion before the dispersion step in order to get particles below 300 nm and with an acceptable polydispersity (below 0.3). Cationic SLN-dispersions were produced with different characteristics (Table 1). The sizes were confirmed by scanning electron microscopy showing spherical particles. The zetapotential of formulation D with CTAB decreased significantly after washing, indicating that CTAB is not incorporated into the lipid matrix.

During the preparation, polysorbate 80 was added in concentrations far above the critical micellar concentration (cmc) at 0.012 mM. Polysorbate 80 micelles (size 75,980 Da) are therefore expected and could be observed in the particle size distribution (size 6–10 nm) obtained by DynaPro (Fig. 1a). DynaPro measurements were used to assess the efficiency of the washing method to remove surplus polysorbate 80 (Fig. 1b and c). Micellar polysorbate 80 could not be detected after purification by dialysis. This correlates with the bimodal size distributions observed with unwashed particles and the increase in particle size and decrease in polydispersity index after dialysis (Table 1).

The previously reported method, washing SLN by ultrafiltration after the microemulsion preparation

Table 1
Characteristics of investigated SLN formulations ($n = 3$)

SLN formulation	Average diameter (nm)	Polydispersity index	Zetapotential (mV)	Tolerated dose (μg/ml)
A unwashed	151.2[a]	0.38	13.7	n.d.
A ultrafiltrated	146.2[a]	0.31	10.2	42
A ultracentrifuged	287.1	0.25	13.1	340
A dialysed	226.9	0.21	12.7	255
B unwashed	159.1[a]	0.29	17.1	n.d.
B dialysed	191.4	0.19	18.4	510
C unwashed	92.6[a]	0.26	14.0	n.d.
C dialysed	102.8	0.17	14.9	42
D unwashed	189.5[a]	0.21	17.9	n.d.
D dialysed	211.7	0.25	−10.1	128

A: 0.7% SA, 2.5% trimyristin, 6.2% butanol, 28.9% polysorbate 80 and 100% water; B: 0.7% SA, 2.6% glycerol behenate, 6.5% butanol, 25.8% polysorbate 80 and 100% water; C: 4.0% SA, 4.0% butanol, 11.0% polysorbate 80 and 100% water; D: 1.8% SA, 1.2% cetyltriammonium bromide (CTAB), 1.8% trimyristin, 4.8% butanol, 19.0% polysorbate 80 and 100% water. n.d. not determined.
[a] Average of a bimodal size distribution.

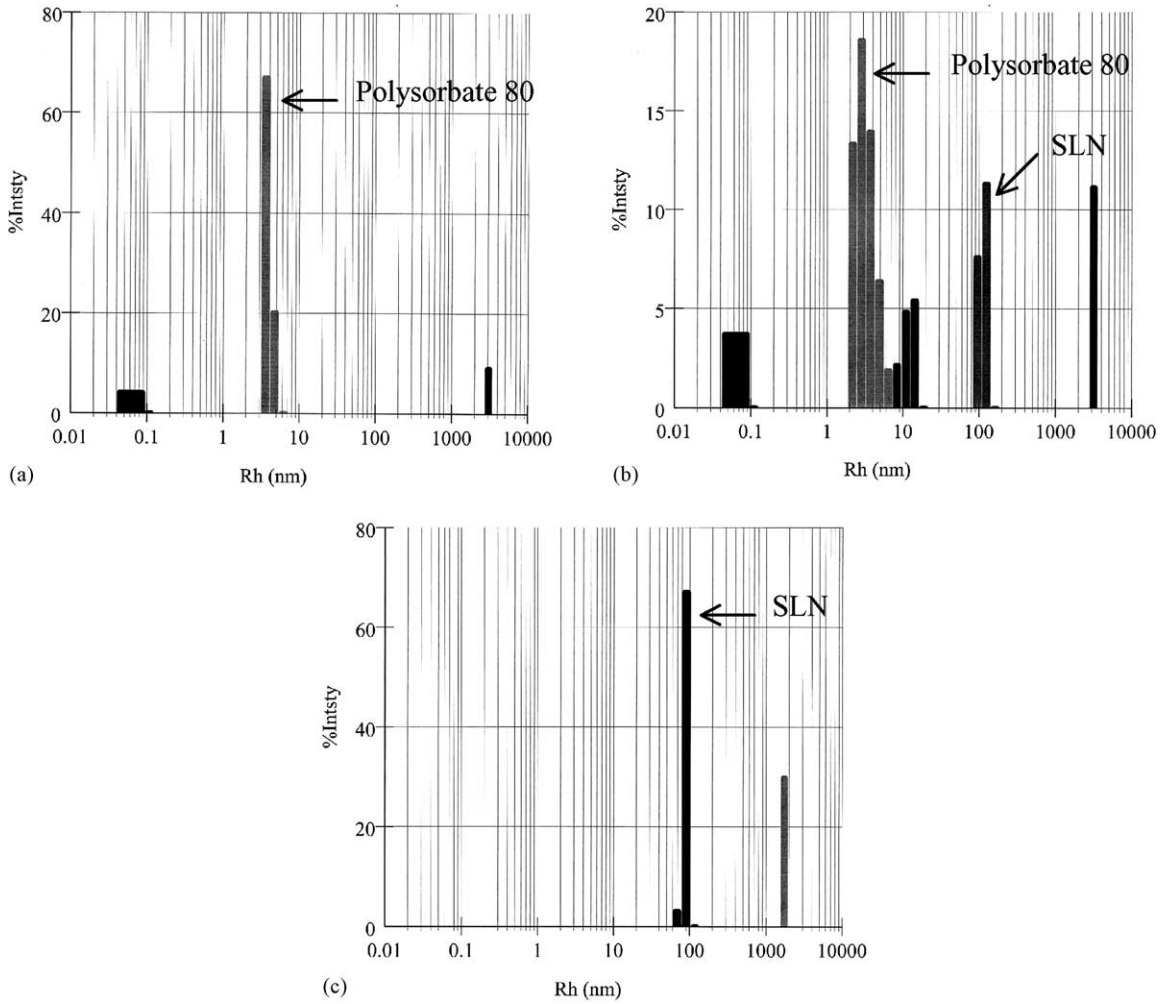

Fig. 1. Particle size distribution (regularisation histogram) from DynaPro used as a mean to assess the removal of surplus surfactants (a) solution of polysorbate 80 above the cmc in water, i.e. polysorbate 80 micelles; (b) formulation A before washing; (c) formulation A after dialysis (cut-off 300 kDa, 3 × 8 h).

method, used by the Gasco group (Miglietta et al., 2000) was found unsatisfactory for removal of polysorbate 80.

Fig. 2 shows the influence of the purification methods on the particle size after storage. The particle size and the polydispersity of unwashed or ultrafiltrated SLN rapidly increased after 1-week storage at 5 °C in Milli-Q water, while no increase was observed after ultracentrifugation or dialysis. As expected, additional experiments showed that the particle size of all formulations increased after storage in media with increasing ionic strength and at elevated temperatures.

Because all formulations showed similar properties in terms of size and zetapotential, the influence of composition and purification on toxicity can be evaluated. The toxicity of the formulations, evaluated in the CPH54A cell line (Engelholm et al., 1986), is strongly dependent upon both the composition and the purification procedure (Fig. 3). Similar result were observed with the NR6 (Batra et al., 1995) and the HEK293 cell lines and by performing a MTT test. Earlier the cationic lipid SA (pK_a 10.6) has been reported to be toxic in liposomes, both in cell culture systems and in vivo (Klang et al., 1994). As expected,

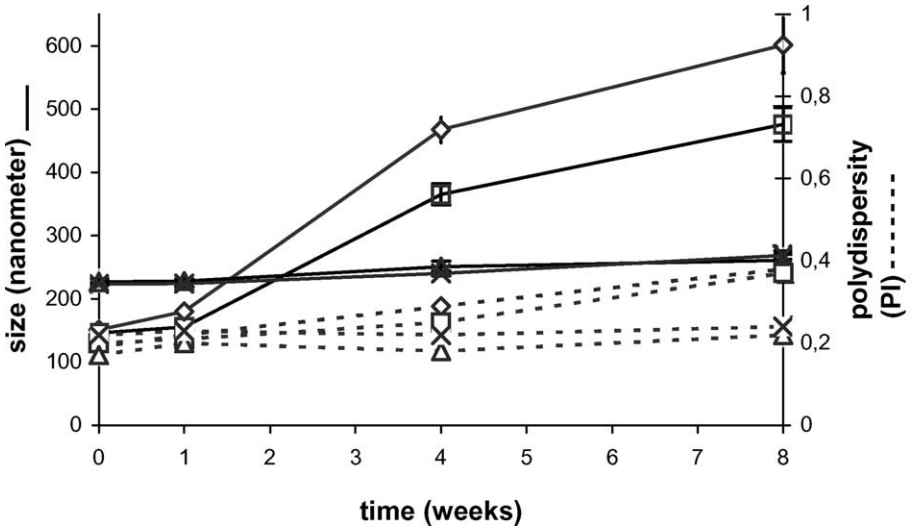

Fig. 2. DynaPro diameter (solid line) and PCS polydispersity index (PI) (dashed line, secondary axis) of formulation A vs. time, stored in Milli-Q water at 5 °C, after different purification methods; (◇) unwashed, (□) ultrafiltrated (cut-off 100 kDa), (Δ) ultracentrifuged (40,000 × *g*) and (×) dialysed (cut-off 300 kDa).

the formulation C containing only SA showed the highest toxicity. Surprisingly however, decreased amounts of SA in the formulations led to higher acceptable doses in total, indicating that the other lipids might alter the toxicity possible by changing some surface characteristics. Previous studies of different SA formulations (liposomes, emulsions, etc.) have not shown any correlation between SA concentration and cell viability. The toxicity is likely to be dependent on other factors such as size, charge and formulation.

SLN prepared from hot microemulsions and washed by ultrafiltration contained no residual butanol as detected by gas chromatography (Gasco, 1997).

When comparing the purification methods, it is observed that the removal of excessive polysorbate 80 decreased the toxicity of the formulations 10-fold. This agrees with earlier studies showing that surfactant toxicity is markedly reduced in SLN formulations compared to free surfactant (Müller et al., 1997). Dialysis was found to be the preferred

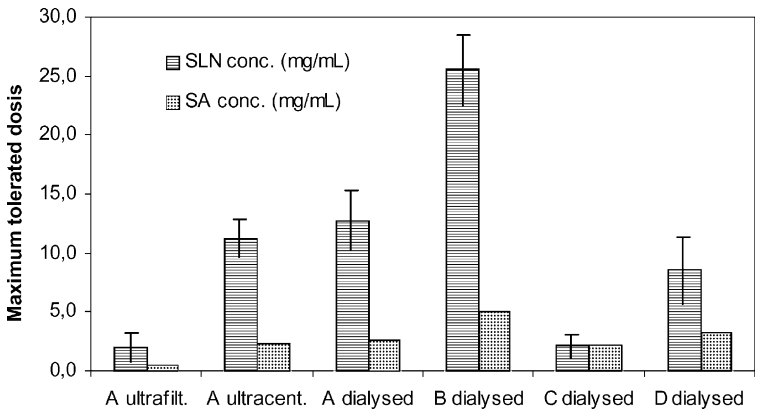

Fig. 3. Cytotoxicity of different SLN formulations measured by maximum tolerated dose or amount of SA after 4 h incubation with CPH54A cells.

purification method, washing several samples at a time with minimal handling and leading to acceptable cell toxicity.

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