Formulation of curcumin-loaded solid lipid nanoparticles produced by fatty acids coacervation technique

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Abstract
Curcumin (CU) loaded solid lipid nanoparticles (SLNs) of fatty acids (FA) were prepared with a coacervation technique based on FA precipitation from their sodium salt micelles in the presence of polymeric non-ionic surfactants. Myristic, palmitic, stearic, and behenic acids, and different polymers with various molecular weights and hydrolysis grades were employed as lipid matrixes and stabilisers, respectively. Generally, spherical-shaped nanoparticles with mean diameters below 500 nm were obtained, and using only middle-high hydrolysis grade-polymer SLNs with diameters lower than 300 nm were produced. CU encapsulation efficiency was in the range 28–81% and highly influenced by both FA and polymer type. Chitosan hydrochloride was added to FA SLN formulations to produce bioadhesive, positively charged nanoparticles. A CU-chitosan complex formation could be hypothesised by DSC analysis, UV–vis spectra and chitosan surface tension determination. A preliminary study on HCT-116 colon cancer cells was developed to evaluate the influence of CU-loaded FA SLNs on cell viability.

Keywords: curcumin, SLN, fatty acids, coacervation

Introduction
Curcumin (diferuloylmethane; CU) is a yellow pigment derived from the rhizome of the plant Curcuma longa, with phenolic groups and conjugated double bonds, which is unstable at light and basic pH, degrading within 30 min (Tonnesen et al., 1986; Wang et al., 1997).

Although CU has shown a wide range of pharmacological activities (chemosensitising, radiosensitising, wound-healing, antimicrobial, antiviral, antifungal, immunomodulatory, antioxidant and anti-inflammatory), its anticancer properties have attracted a great interest (Villegas et al., 2008). The anticancer activity of CU has been the subject of hundreds of papers and has been reviewed in several recent articles (Chauhan, 2002; Aggarwal et al., 2003; Thangapazham et al., 2006). Numerous in vitro assays using colon cancer cell lines have been performed showing the anticancer activity of CU, but the underlying mechanisms remain largely to be defined. Moreover, animal model systems have revealed that diferuloylmethane prevents tumours induced by a diversity of chemical carcinogens in tissues such as skin, breast and liver (Huang et al., 1991; Lopez-Lazaro et al., 2007). In the gastrointestinal tract, in particular stomach and duodenum, anticancer properties of CU have also been documented (Strick et al., 2000). Moreover, epidemiological data suggest that the incidence of several common cancers (i.e., colon, breast, prostate and lung) is higher in Western countries than in countries such as India, where CU is highly consumed with foods (Aggarwal et al., 2003).

The antitumorigenic property of CU has been attributed partly to its ability to inhibit the processes of initiation and promotion in carcinogenesis. Jaiswal et al. (2002) demonstrated that CU treatment caused p53- and p21-independent G(2)/M phase arrest and apoptosis in human colon cancer HCT-116(p53(+/-)),...
HCT-116(p53(−/−)) and HCT-116(p21(−/−)) cell lines. A G2/M cell cycle arrest by CU was also confirmed in HT-29 human colon cancer cells (Van Erk et al., 2004).

Unfortunately, these studies have revealed that CU has poor oral bioavailability due to its low water solubility under acidic or neutral conditions. Results from pharmacokinetic assays in animals showed low serum levels of the polyphenol following a single-dose administration (Cheng et al., 2001; Sharma et al., 2001): about 75% of ingested CU was excreted unaltered in the faeces and negligible amounts appeared in the urine. Several human studies have revealed that, after oral administration, the levels of CU in plasma are very low (generally in the nanomolar range) while they are higher in colorectal tissue (low micromolar) (López-Lázaro, 2008). This suggests that, outside the gastrointestinal tract, most of the reported cancer-related effects of CU may not be achieved in vivo.

To overcome these bioavailability problems, various strategies have been tried to enhance CU delivery. Takahashi et al. (2009) prepared CU-loaded liposomes and administered them orally to Sprague-Dawley rats at a dose of 100 mg CU/kg body weight. Pharmacokinetic parameters after oral administration of liposomes evidenced higher bioavailability, a faster rate and better absorption of CU compared to other dosage forms. Mukerjee and Vishwanatha (2009) vehicled CU into poly(lactic-co-glycolic acid) (PLGA) nanoparticles and investigated intracellular uptake of CU-loaded PLGA nanoparticles in DU145, PC3 and LnCaP cell lines. These nanoparticles were actively taken up by all the cancer cells. Further, to investigate the therapeutic potential of formulation, prostate cancer cell lines, DU145, PC3 and LnCaP were treated with free CU, CU-loaded PLGA nanoparticles and blank nanoparticles at different concentrations (0–30 μM) for 72 h and cell proliferation was measured. CU-loaded PLGA nanoparticles exhibited a lower IC50 value in comparison to free CU in all cancer cell lines studied. Tønnesen et al. (2002) prepared complexes between CU and cyclodextrin (CD) in order to increase its water solubility and then its bioavailability. CU had the highest affinity for randomly methylated β-CD that increased CU solubility at pH 5 of 104 factor.

It is well-known that CU undergoes rapid hydrolysis under alkaline conditions to yield ferulic acid, its methyl ester and vanillin (Lin et al., 2000); it is also very susceptible to photochemical degradation (Pfeiffer et al., 2003).

CU inclusion improves the hydrolytic stability dramatically while it decreases its photostability. In a recent study, we (Chirio et al., 2009) vehicled CU in solid lipid nanoparticles (SLNs) prepared also with α- and γ-lipophilic CDs in order to combine the advantages of lipid nanoparticles and CDs. CU fits better the α-CU cavity, which is larger than that of α-CU forming a 2:1 CD:CU molar ratio complex. A significant reduction in CU photodegradation was noted when the drug was vehicled in tristearin-SLN, which became less pronounced in the presence of CD derivatives. The hydrolytic stability of CU was highly improved by drug loading in tristearin-SLN and this seemed not to be influenced by the presence of CD derivatives. The presence of CDs into nanoparticles improved up to 20 folds the CU skin uptake compared to drug solution and up to threefolds compared to nanoparticles in the absence of CDs.

In this study, a new, solvent-free technique is used to produce CU-loaded SLN of fatty acids (FAs) by acidification of a micellar solution of their alkaline salts (Battaglia et al., 2010).

CU-loaded SLNs were developed to obtain a model drug delivery system based on physiological lipid matter as FAs. In literature, several liposomal (Takahashi et al., 2009) and polymeric nanoparticulate (Mukerjee and Vishwanatha, 2009) systems were proposed as CU vehicles, but stability, as well as bio-compatibility problems are related to the use of the former and the latter formulations, respectively. In a previous work (Chirio et al., 2009), hydrolytic and oxidative CU stability enhancement was noted when the drug was loaded in triglyceride-based SLN.

Using coacervation method, SLN can be produced overcoming some disadvantages of previous techniques, such as the need of complex machines in high-pressure homogenisation, the toxicity of most solvents employed in solvent-based methods and the requirement of high temperatures to melt the lipid matrix in solvent-free methods. Moreover, the need to overcome patented methods leads to the development of potential alternative techniques for SLN (Battaglia et al., 2010).

The use of myristic, palmitic, stearic and behenic acids as lipid matrices and of different non-ionic polymeric surfactants as stabilisers is investigated. The aim is to individuate the best lipid and stabiliser composition to optimise SLN mean diameters and CU entrapment efficiency. After characterising SLN by determining their physico-chemical properties, a preliminary in vitro study is developed to test SLN in HCT-116 human colon adenocarcinoma cells to evaluate the influence of CU-loaded SLN on cell viability.

Methods

Materials

Citric acid, phosphoric acid, lactic acid, sodium phosphate dibasic and sodium phosphate monobasic were purchased from A.C.E.F. (Fiorenzuola d’Arda, Italy), 98% hydrolysed PVA 14 000–21 000 Mw (PVA 14 000) from BDH Chemicals (Poole, UK); 80% hydrolysed PVA 9000–10 000 Mw (PVA 9000), 89% hydrolysed PVA 85 000–124 000 Mw (PVA 85 000), sodium myristate (Na-M), CU, Pluronic F127 (PLF127), Pluronic F68 (PLF68), dextran and trehalose (TRH) from Sigma (Dorset, UK); sodium stearate (Na-S), sodium palmitate (Na-P), myristic acid (MA), palmitic acid (PA), behenic acid (BA), polyvinylpyrrolidone K15 (10 000 Mw – PVP 10 000) and polyvinylpyrrolidone K25 (24 000 Mw – PVP 24 000) from Fluka (Buchs, Switzerland); stearic acid (SA) and Arabic gum from Merck (Darmstadt, Germany); polyvinylpyrrolidone K12 (3500 Mw – PVP 3500) from Acros Organics (Geel, Belgium).
Poval PVA L10, Poval PVA L508, L polymer L9-78 and LM polymer LM20 were kind gifts from Seppic (Milan, Italy), HPMC 2910 (hydroxypropylmethyl cellulose, 28–30% methyl substitution degree, 7–12% isopropyl substitution degree) and 15Cp (Beneccel E15—14 000 Mw), 50Cp (Beneccel E50—21 000 Mw) were kind gifts from Eigenmann & Veronelli (Rho, Italy).

Sodium behenate (Na-B) was obtained by adding a stoichiometric amount of NaOH ethanolic solution to the ethanolic solution of BA: the soap was purified by re-crystallisation and stored in a desiccator at room temperature. Also, 22% hydrolysed dextran (DexP) was obtained by adding 10 mL 0.1 M NaOH and 200 mL 2.3 epoxypropyl-phenylether to 1 g dextran: the mixture was stirred up to have clear solution. Deionised water was obtained by a MilliQ system (Millipore, Bedford, MO). All other chemicals were of analytical grade and used without any further purification.

**SLN preparation**

SLNs were prepared by the coacervation method (Battaglia et al., 2010). Different operative conditions were used for SLN preparation according to the FAs under study. Stock solutions of each polymeric stabiliser (PVA 9000, PVA 14 000, PVA 85 000, PVA L508, PVA L10, PVA LM20, PVA L9-78, PL F68, PL F127, DexP, chitosan hydrochloride, PVP 3500, PVP 10 000, PVP 24 000, Arabic gum) at appropriate concentrations were prepared by heating the polymer in water and then cooling at room temperature. Briefly, 1% w/w each FA sodium salt was dispersed in 9.9 mL water brought to pH 4; 100 mL 0.1 M NaH2PO4 and then 1 M HCl to obtain a clear solution. A fixed amount of 8.3 mg/mL ethanol CU solution was added to precipitate the lipid matrix and the supernatant obtained was injected in HPLC for CU quantification. The morphology of SLN and CU localisation were determined using optical microscopy equipped with a fluorescent lamp (Leica DM 2500, Germany).

<table>
<thead>
<tr>
<th>Sodium salt</th>
<th>Coacervating solution</th>
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<tr>
<td>Na-M</td>
<td>1 M NaH2PO4 + 1 M citric acid</td>
</tr>
<tr>
<td>Na-P</td>
<td>1 M Citric acid</td>
</tr>
<tr>
<td>Na-S</td>
<td>1 M Lactic acid</td>
</tr>
<tr>
<td>Na-B</td>
<td>1 M NaH2PO4 and then 1 M HCl</td>
</tr>
</tbody>
</table>

SLN particle sizes and polydispersity indexes were determined using laser light scattering (LLS) technique (Brookhaven, USA). Measurements were obtained at an angle of 90°. The dispersions were diluted with water for size determination or with 0.01 M KCl for Zeta-potential determination, in order to achieve the prescribed conductivity.

Differential scanning calorimetry (DSC) analysis were performed with a DSC 7 Perkin Elmer differential calorimeter (Norwalk, CT, USA). Lipid bulk material and SLN suspensions were placed into conventional aluminium pans and heated from 30°C to 70°C at 2°C per minute. The degree of crystallinity of SLN was estimated by calculating the ratio between the melting enthalpy/g lipid in SLN dispersion and the melting enthalpy/g of the bulk material (Siekmann and Westesen, 1994; Freitas and Mülller, 1999).

CU entrapment efficiency (EE%) was calculated as the ratio between CU amount in SLN and that in the starting micellar solution × 100. CU analysis was performed as follows: 1 mL SLN suspension was centrifuged for 30 min at 24 000 rpm and the precipitate was washed twice with 1 mL ethanol/water 20/80 to eliminate adsorbed CU. The solid residue was dissolved in 1 mL ethanol; 2 mL water was then added to precipitate the lipid matrix and the supernatant obtained was injected in HPLC for CU quantification. HPLC analysis was performed using a LC9 pump (Shimadzu, Japan) with an Allsphere ODS-2 5 μ 150 × 4.6 mm column and a C-R5A integrator (Shimadzu, Japan); mobile phase: CH3OH/H2O/CH3COOH 70/30/1 (flow rate 1 mL/min); detector: UV $\lambda = 450$ nm (Shimadzu, Japan). Retention time was 5.5 min.

The limit of quantification, defined as the lowest CU concentration in the curve that can be measured routinely with acceptable precision and accuracy, was 0.7 μmol/mL; the limit of determination, defined as the lowest detection limit, was 0.3 μmol/mL (signal to noise > 2.0).

**Chitosan surface tension determination**

All measurements were performed at 25.0 ± 0.1°C using a ring tensiometer (Krüss, K10, Germany). Surface tension measurements were executed on chitosan water solution and on chitosan 1% w/w PVA 9000 aqueous solution. Briefly, 6 mg/mL chitosan water solution and 6 mg/mL chitosan 1% w/w PVA 9000 aqueous solution were prepared and utilised as starting solutions. Each solution was then diluted with water or 1% PVA 9000 aqueous solution in order to determine chitosan surface tension in the 0.5–6 mg/mL concentration range. The pH of each solution was 5.0.

**CU-chitosan complex characterisation**

To verify the formation of CU-chitosan complex, CU UV–vis absorption spectra in the presence of different chitosan concentrations were carried out. Briefly, 3.75, 5, 7.5, 10 and 15 mg chitosan were dissolved in 9.9 mL water brought to pH 4; 100 μL 3 mg/mL CU
ethanol solution were added to each sample. Suspensions were stirred for 4 h and then centrifuged 10 min at 25 000 rpm. A spectrum of the supernatant was registered by UV–vis spectrophotometer from 200 to 600 nm.

**SLN and CU stability**

Sizes and CU EE% of PA-SLN stabilised with PVA 9000 or PVA L508 or chitosan + PVA 9000 were monitored for 3 months to study SLN physical stability and CU chemical stability. Mean sizes were determined by LLS and CU EE% overtime was obtained by HPLC.

The same SLN suspensions were freeze-dried without adding any cryoprotectant (FD-SLN) and in the presence of 2% w/v trehalose (FD-TRH-SLN) using a Modulyo Freeze Dryer (Edwards Alto Vuoto, Italy). The resulting samples were characterised by LLS analysis.

**Preliminary in vitro cytotoxicity studies**

In vitro cytotoxicity studies were set up with HCT-116 colon cancer cells

Approximately 2500 cells were seeded in 96-well plates and allowed to attach and proliferate for a period of 24 h. The cells were then incubated with various dosages of different SLN types. Cell cytotoxicity was determined after incubation of 24 and 48 h. SLNs were removed and cells treated with 1:10 diluted WST-1 medium (Roche, Italy). Plates were incubated 2 h at 37°C to form crystals of formazan by the metabolically active cells. Finally, plates were read spectrophotometrically at 450 nm. Cell viability was calculated as a percentage of the control. All experiments were carried out three times, each condition being performed eightfold.

**Samples under study**

SLN: MA-PVA L508, PA-PVA L508 and SA-PVA L508 1:50 diluted (CU = 2.7 × 10⁻⁵ M, MA = 8.8 × 10⁻⁴ M, PA = 7.8 × 10⁻⁴ M, SA = 7.0 × 10⁻⁴ M); SA-PVA L508, SA-PVA L508-CU, SA-PVA 9000, SA-PVA 9000-CU, 1:100 (CU = 1.4 × 10⁻⁵ M, SA = 3.5 × 10⁻⁴ M), 1:200 (CU = 6.8 × 10⁻⁶ M, SA = 1.7 × 10⁻⁴ M), 1:1000 (CU = 1.4 × 10⁻⁶ M, SA = 3.5 × 10⁻⁵ M) diluted.

Suspensions: CU-PVA L508, CU-PVA 9000 1:100 (CU = 1.4 × 10⁻⁵ M), 1:200 (CU = 6.8 × 10⁻⁶ M), 1:1000 (CU = 1.4 × 10⁻⁶ M) diluted.

**Statistical analysis**

Statistical analysis of differences between varying SLN samples and the control was performed using one-way analysis of variance. A 0.05 level of probability was taken as the minimal level of significance.

**Results**

**SLN characterisation**

By FA coacervation method, CU-loaded SLNs were obtained with all FA tested. CU did not undergo chemical degradation even if exposed at high pH values of sodium salt FA micellar solutions, as pH was immediately lowered by addition of the appropriate acidifying solution and the temperature cooled by an ice-bath. Several polymeric stabilisers at different molecular weights and various hydrolysis grades were used. Both FA and stabilisers were used at 1% w/w, just as in previous works. In Table 2, mean diameters and polydispersion indexes (PI) of SLN prepared with different FAs and stabilisers are reported. As it can be noted, several SLN had diameters below 500 nm; particularly, using PVA with middle-high hydrolysis grade (PVA 9000, PVA L508, PVA L-10), SLN with size lower than 300 nm were obtained. PVA type, therefore, seems to influence SLN mean diameters and polydispersity, probably due to different interactions between the lipid matrix and the stabiliser, which might depend both on hydrolysis degree and polymer molecular weight. The influence of PVA type on size is also described in literature: Hong et al. (2006) reported that in PVA-stabilised emulsions, low hydrolysis degree-polymers determine a reduction of emulsion droplet sizes.

Also, FA type seemed to affect SLN sizes: the smallest CU-loaded SLNs (mean diameter lower than 200 nm) were obtained using PA, as also reported in a previous work (Battaglia et al., 2010). The differences in mean sizes among different FA-SLNs can probably be related to a number of factors, such as the operating temperature (Methods section), FA chain length and stabiliser concentration, which varied in the production process of the different FA-SLN.

Other polymeric stabilisers were used as an alternative to PVA: PLF produced SLN in the range 400–1300 nm, PVP in the range 300–4600 nm, different viscosity–HPMC in the range 400–5000 nm. Enhancing the viscosity of the medium, as in HPMC samples, an increase in the mean size of resulting nanoparticles was noted; also, in literature, it is reported that an increase of aqueous medium viscosity may cause an increase of SLN particle size (Schubert and Müller-Goymann, 2003).

Using DexP as stabiliser, SLNs in the range 290–500 nm were obtained. Chitosan hydrochloride was added to FA-PVA 9000 SLN formulations to produce bioadhesive, positively charged nanoparticles. Using MA and SA as matrices, SLNs of about 350–400 nm were obtained.

The observation of SLN nanosuspension under microscope light confirmed both the spherical shape of the particles and the dimensional range. The use of fluorescent light allowed us to locate CU in SLN nanosuspensions: homogeneous dispersions of fluorescent nanoparticles were observed with middle-high hydrolysis degree PVA (Figure 1); non-loaded CU yellow crystals were detectable in other formulations (data not shown).

In Table 3, Zeta potential values are reported. Almost all formulations showed slight negative charge with values in the range −2.7 to −6.0. Probably charges are due to carboxyl groups of FA sodium salts present in traces on the particles surface. On the contrary, positive charges are shown by chitosan nanoparticles indeed, at final pH of SLN suspension, chitosan is positively charged and can interact with...
carboxyl groups on nanoparticles surface conferring them a positive charge. Particularly, Zeta potential of CU-loaded SLN value was higher than +20 mV. It seems very difficult to explain such a dramatic increase between blank and drug-loaded chitosan-coated SLN; probably, the interaction between CU and chitosan (see below) was responsible for the major adsorption of the polymer on nanoparticle surface.

In Table 4, the values of CU EE% in small-sized SLN prepared with different FAs are reported. As it can
be noticed, EE% was in the range 28–81% and was highly influenced by both FA and polymer type; probably CU EE% depends on the preparation conditions used for various FAs and on the solubilising properties of different polymeric stabilisers, which could auto-assembly in the aqueous medium modifying CU solubility in the dispersing medium. Generally, the highest CU EE% was scored in PA-SLN; among the studied stabilisers, the highest CU EE% was observed in the presence of PVA 9000, which had the lowest efficiency in improving CU water solubility.

The solid state of SLN after coacervation was verified by DSC. DSC thermograms of PA and SA SLN in the presence and in the absence of CU were depicted in Figures 2–5. In Figure 2, PA-SLN thermograms in the absence of CU are reported. As already noted in a previous work (Battaglia et al., 2010), DSC thermograms of SLN showed only small differences between the melting point of raw lipid and that of the corresponding SLN. According to Siekmann and Westesen (1994), 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 the re-crystallisation of the lipid matrices in a metastable polymorph. The presence of impurities, surfactants and stabilisers could also affect this phenomenon (Hou et al., 2003; Liu et al., 2007). An evident decrease of enthalpy with respect to raw FA (from 201 to 77 J/g) was noted in the PA-PVA 9000-chitosan SLN thermogram. This result could indicate a different interaction between lipid and chitosan; probably, as supposed from Zeta potential measurements, the polymer might interact with carboxyl groups of the lipid present on particle surface.

In Figure 3, SA-SLN thermograms in the absence of CU are reported. In this case, a significant SLN transition temperature decrease compared to raw SA was noted. This difference can be ascribed to polymorphism. In fact, SA can exist in three crystalline forms, A-B-C (Raghavan et al., 2000), with three different melting points (43°C, 54°C and 69°C, respectively). Previous investigation on SA-SLN with X-rays (Battaglia et al., 2010) confirmed that, in SLN, SA was in the low melting B form, which is characterised by monoclinic lattice (Goto and Asada, 1978). SA polymorphism is typical of coacervation process regardless of the presence of a stabiliser (Battaglia et al., 2010). B form of SA was also obtained after acidification of Na-S solution with lactic acid in the absence of PVA. Also, in this case, chitosan-SLNs present the lowest transition enthalpy, indicating a stronger lipid–polymer interaction.

In Figures 4 and 5, DSC thermograms of CU-loaded SLN are reported. In PA-PVA 9000 and PA-PVA L508 SLN, the presence of CU influences neither temperature nor

<table>
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<tr>
<th>MA-SLN</th>
<th>PA-SLN</th>
<th>SA-SLN</th>
<th>BA-SLN</th>
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<tbody>
<tr>
<td><strong>Value</strong></td>
<td><strong>SD</strong></td>
<td><strong>Value</strong></td>
<td><strong>SD</strong></td>
</tr>
<tr>
<td>PVA 9000 50 mg</td>
<td>48.4</td>
<td>2.4</td>
<td>81.4</td>
</tr>
<tr>
<td>PVA 9000 50 mg - chitosan hydrochloride 7.5 mg</td>
<td>26.2</td>
<td>1.3</td>
<td>64.6</td>
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<tr>
<td></td>
<td>14.1</td>
<td>0.7</td>
<td>11.2</td>
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<tr>
<td>PVA 85000-124000 50 mg</td>
<td>49.9</td>
<td>2.5</td>
<td>60.7</td>
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<tr>
<td>PVA L10 50 mg</td>
<td>39.6</td>
<td>2.0</td>
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<td></td>
<td>45.9</td>
<td>2.3</td>
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<td>14.5</td>
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<td>40.3</td>
<td>2.0</td>
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<td>37.0</td>
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<td>54.5</td>
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<td>36.0</td>
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<td>9.5</td>
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<td></td>
<td>5.1</td>
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<tr>
<td>DEXP 22% 15 mg</td>
<td>59.9</td>
<td>3.0</td>
<td>43.8</td>
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<td></td>
<td>0.2</td>
<td>0.0</td>
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<tr>
<td></td>
<td>39.9</td>
<td>2.0</td>
<td>56.0</td>
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<td>35.4</td>
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<td>PVP 24000 50 mg</td>
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<td>2.5</td>
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Note: SD, standard deviation. Formulations were referred to 50 mg FA and 2.5 mg CU into 5 mL suspension.
transition enthalpy. A different behaviour can be observed in PA-PVA 9000-chitosan SLN: the transition peak was 3°C shifted to higher temperatures and the transition enthalpy halved. These results might probably be due to different CU localisations, as in PVA 9000 and PVA L508 SLNs it could be uniformly dispersed throughout the lipid matrix, while in PVA 9000-chitosan SLNs, it could be more concentrated on the outer surface. In all SA-SLN formulations, a significant influence of CU on transition peak was noted: with PVA 9000 and PVA L508 in the presence of CU, a marked temperature drop was noted, while with PVA 9000-chitosan, an enthalpy transition decrease was also observed. Probably, in SA-SLN, CU is mostly located on the surface when compared with PA-SLNs, minimising the effect due to the presence of chitosan on the surface.

From these observations, a CU-chitosan complex formation could therefore be hypothesised, which was further investigated by CU UV-vis absorption studies in the presence of different chitosan concentrations and by chitosan surface tension determination.
UV–vis absorption spectra of 30 μg/mL (8.14 × 10⁻⁵ M) CU aqueous solutions showed a progressive increase of CU absorbance at 420 nm as a function of chitosan concentration (Figure 6); 1:12 to 1:50 CU:chitosan w/w ratios were examined. The marked increase in CU aqueous solubility was not ascribable to a possible micellisation in chitosan micelles, as evidenced by surface tension measurements. Indeed, a chitosan critical association concentration (CAC) was evidenced both in water and in PVA 9000 aqueous solution at pH 5.0 (Figures 7 and 8). Chitosan CACs were quite higher than those used in solubility studies and in SLN preparation process.

The lower CU EE% observed in chitosan-stabilised SLN could therefore be ascribed to an enhanced CU water solubility, due to the formation of a hydrophilic complex with chitosan. In fact, in literature, the formation of complexes between chitosan and polyphenols is documented (Popa et al., 2000).

As CU water solubility is generally considered one of the most important hurdles to CU administration, the possibility of using a hydrophilic complex could represent a pre-amble to further development of CU dosage forms, even if it may be undesirable in SLN production.

**SLN and CU stability**

In order to evaluate the stability of CU-loaded SLN suspensions, LLS measurements and EE% determinations were performed for 12 weeks. In Figure 9, SLN sizes are reported: all analysed formulations were stable for 12 weeks, as only very low increases in SLN sizes were noted.

In Table 5, CU EE% over time are shown. After 12 weeks, a 15% CU loss was noted in all samples; a 6.5% and 13.6% decrease of CU EE% was noted in PA-PVA 9000 and PA-PVA L508 SLN, respectively, while a mild increase of non-loaded and adsorbed CU was observed. These results can suggest that CU was slowly released from SLN, partially solubilised in polymeric stabilisers solution and partially adsorbed onto nanoparticle surface. At the same time, a more evident CU EE% decrease and adsorbed-CU increase were observed in PA-PVA 9000-chitosan SLN, confirming the formation of a CU-chitosan complex, that, mainly located on SLN surface, contributes to enhance the amount of adsorbed CU. Moreover, the presence of chitosan in the bulk PVA solution could further improve CU aqueous solubilisation.

To verify the possibility of decreasing both CU degradation and CU release from SLN occurring in an aqueous medium, PA-PVA 9000, PA-PVA L508 and PA-PVA 9000-chitosan SLN-suspensions were freeze-dried in the absence of cryoprotectants (FD-SLN) and in the presence of 2% w/v trehalose (FD-TRH-SLN). SLN mean diameters and polydispersities before and after freeze-drying are reported in Table 6. Similar values in mean diameters after freeze-drying were obtained without any cryoprotectant only for PA-PVA 9000 SLN; mean diameters of PA-PVA 9000 and PA-PVA 9000-chitosan SLNs could be maintained only in the presence of TRH.

**In vitro cytotoxicity studies**

Since current treatment options have only limited efficacy on colon cancer and the human colorectal carcinoma cell line HCT-116 is an *in vitro* model extensively used to gain better understanding of CU action (Nautiyal et al., 2011), in this study, a preliminary *in vitro* study of anticancer efficacy of CU-loaded SLN was introduced.

The preliminary study on HCT-116 colon cancer cells was developed to evaluate the influence of CU-loaded SA-SLN on cell viability. Among different FA-SLNs prepared, SA-SLNs were chosen for cell survival studies, as, in preliminary text, SA revealed the lowest cytotoxicity on
HCT-116 colon cancer cell compared with MA and PA; BA was not tested, as BA-SLN did not present physico-chemical characteristics suitable to CU entrapment.

Figures 10 and 11 show the survival histograms of HCT-116 after 24 and 48 h exposures to the different CU-loaded and unloaded SLNs at various dilutions. CU aqueous PVA 9000 and PVA L508 suspensions were chosen as blanks.

After 24 h incubation, at the highest CU concentration (1:100 dilution), both CU suspensions showed cytotoxicity, as a decrease of cell growth of 35% (PVA 9000) and of 30% (PVA L508) was noted: this effect was quite dampened at the lowest CU concentrations (1:200, 1:1000 dilutions) with a residual cell survival of almost 80%. At 1:100 and 1:200 dilutions, no significant difference was noted between CU-loaded and unloaded SLN, probably being the cytotoxicity of SLN not negligible. At 1:1000 dilution, a flattening of the survival histogram occurred, probably as CU concentration was too low to exert an effect by itself.

At 48 h, at each dilution, CU PVA 9000 suspension lost its cytotoxicity, probably owing to its very low CU solvent efficiency, which probably allowed a renewed cell viability: this effect was not noted for CU PVA L508 suspension, having PVA L508 a better CU solvent efficiency. Cytotoxicities of all the CU-loaded and unloaded SLN were quite similar, more pronounced at 1:200 and 1:100 than at 1:1000 dilution.

These rather unsuccessful results indicate the necessity to develop CU colloidal carriers able to entrap higher CU amounts, in order to treat cell cultures with higher CU concentrations in the presence of lipid matrices showing a as low as possible cytotoxicity.

Conclusions

The use of FA coacervation technique permitted us to obtain CU-FA-SLN with sizes below 300 nm and with
Table 5. CU EE% of PA-SLN determined over 12 weeks.

<table>
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<tr>
<th></th>
<th>PVA 9000</th>
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<tr>
<td></td>
<td>CU Amount (%)</td>
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<td>CU Amount (%)</td>
<td>CU EE% (%)</td>
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Note: Formulations were referred to 50 mg PA, 50 mg stabiliser and 2.5 mg CU into 5 mL suspension.
EE% in the range 28–81%, depending on FA and polymer type employed; the formulation study showed the employment of middle-high hydrolysis grade-polymers as more suitable. The introduction of chitosan hydrochloride in FA formulation produced bioadhesive, positively charged nanoparticles and evidenced a water-soluble CU-chitosan complex probable formation, as confirmed by DSC analysis, UV–vis spectra and chitosan surface tension. SLN-suspension sizes were stable until 12 weeks while CU delivery decreased until 85% at the same time; the freeze-drying process in the presence or in the absence of cryoprotectant could enhance the CU stability.

The preliminary study on HCT-116 colon cancer cells evidenced the necessity to develop CU colloidal carriers able to entrap higher CU amounts, in order to treat cell cultures with higher CU concentrations in the presence of lipid matrices showing an as low as possible cytoxicity.

### Acknowledgement

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References


