Article title: Solid lipid nanoparticles loaded with lipoyl-memantine codrug: preparation and characterization
Reference: IJP14710
Journal title: International Journal of Pharmaceutics
Corresponding author: Prof. Antonio Di Stefano
Received at Editorial Office: 19-DEC-2014
Article revised: 27-FEB-2015
Article accepted for publication: 2-MAR-2015
http://dx.doi.org/10.1016/j.ijpharm.2015.03.001

Solid lipid nanoparticles loaded with lipoyl-memantine codrug: preparation and characterization

Sara Laserra,[1] Abdul Basit,[2] Piera Sozio,[1] Lisa Marinelli,[1] Erika Fornasari,[1] Ivana Cacciatore,[1] Michele Ciulla, [1] Hasan Türkez,[3] Fatime Geyikoglu,[4] Antonio Di Stefano[1*]

[1]Department of Pharmacy, "G. d'Annunzio" University of Chieti-Pescara, Via dei Vestini 31, 66100 Chieti, Italy; [2]Department of Pharmaceutics, UCL School of Pharmacy, University College London, WC1N 1AX, London, UK; [3]Department of Molecular Biology and Genetics, Erzurum Technical University, Erzurum 25240, Turkey; [4]Ataturk University, Faculty of Science, Biology Department, 25240, Erzurum, Turkey.

*Corresponding author. Tel. :+39 0871 3554708; fax : +39 0871 3554706. *E-mail address*: adistefano@unich.it.

Abstract

Solid lipid nanoparticles (SLNs) are considered very attractive drug-delivery systems (DDS) able to enhance the efficacy of some therapeutic agents in several pathologies difficult to treat in a conventional way. Starting from these evidences, this study describes the preparation, physicochemical characterization, release, and *in vitro* cytotoxicity of stealth SLNs as innovative approach to improve solubility and absorption through the gastrointestinal tract of Lipoyl-Memantine (LA-MEM), a potential anti-Alzheimer codrug.

Physico-chemical properties of LA-MEM loaded SLNs have been intensively investigated. Differential scanning calorimetry (DSC) was used to clarify the state and crystalline structure of the formulation. The results obtained from particles size analysis, polydispersity (PDI), and zeta potential measurements allowed the identification of the optimized formulation, which was characterized by a drug-lipid ratio 1:5, an average intensity diameter of 170 nm, a PDI of 0.072, a zeta potential of -33.8 mV, and an entrapment efficiency of 88%. Moreover, *in vitro* stability and release studies in both simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), and preliminary *in vitro* cytotoxicity studies revealed that LA-MEM loaded SLNs could represent potential candidate for an *in vivo* investigation as DDS for the brain since it resulted devoid of citotoxicity and able to release the free codrug.

Keywords: Solid lipid nanoparticles, Alzheimer's disease, Memantine, Lipoic acid, Codrug.

1. Introduction

Nanotechnological approaches are often used to improve the pharmacokinetic profile of drugs such as poor gastro-intestinal absorbtion, low drug solubility, and rapid metabolism but also to ensure an efficient CNS delivery of many compounds. Among various colloidal systems, SLNs have roused special interest due to many features that make them particularly intriguing as alternative carriers in the field of drug delivery (Kanwar et al., 2009). In combination with the narrow sizes, sterical properties obtained in stealth SLNs result particularly advantageous to increase the blood circulation time of the particulate, thus reducing RES uptake and extending the contact time between the BBB and the drug, which can therefore be caught in the brain when the target is represented by CNS (Uner and Yener, 2007). Such properties can improve bioavailability of drugs through several mechanisms: 1) augmenting the drug solubility and permeability, 2) overcoming the first-pass effect and the P-gp efflux, and 3) enhancing the stability in the GI tract (Esposito et al., 2008). Several drug-loaded SLN formulations in brain targeting and delivery showed a very low cytotoxicity or resulted to be not-cytotoxic (Patel et al., 2013). However, the biocompatibility assessment seemed to be tough due to the variable composition of SLN formulations that differs in nature, percentage of lipids and loaded molecules (Silva et al., 2012).

Recently, our research has been focused on the development of innovative therapeutic strategies to reduce the disease progression and improve the quality of life of patients suffering from Parkinson's (PD) (Di Stefano et al., 2009) and Alzheimer's (AD) Diseases (Cacciatore et al., 2012, 2013; Sozio et al., 2009, 2013a). To date, AD treatments mainly exploit a symptomatic approach based on the use of cholinesterase inhibitors for patients with mild to moderate AD, and N-methyl-D-aspartate (NMDA) receptor antagonists, such as memantine (3,5-dimethyl-1-adamantanamine, MEM), for patients with moderate to severe AD (Anand et al., 2014; Parihar et al., 2004). According to the multifactorial hypothesis of AD, glutamate exitocitotoxicity, oxidative stress (OS), depletion of antioxidant defence systems, metal dyshomeostasis, and protein misfolding and aggregation are considered the main causes of neuronal loss in selected brain regions (Di Stefano et al., 2011). Taking into account the role played by OS and over-stimulation of glutamate NMDA receptors in causing neuronal damage, we previously reported the synthesis of Lipoyl-Memantine (LA-MEM) (Figure 1), a new potential anti-Alzheimer codrug obtained by joining MEM with a natural neuroprotective agent, (R)-α-lipoic acid (LA) (Sozio et al., 2013b). Despite of its good capabilities

to inhibit Aβ(1–42) aggregation and scavenge free radicals, LA-MEM showed poor water solubility (0.010 mg/mL) to permit an oral administration.

This paper is focused on the preparation and characterization of a new SLN formulation containing LA-MEM codrug to increase its solubility in g.i. fluids and favor its intestinal absorption. High lipophilic compounds, such as LA-MEM (LogP = 4.20), have often been selected to be incorporated into SLNs due to their high drug loading and entrapment efficiency (Al Haj et al., 2008). The new formulation was characterized evaluating particle size, zeta potential, surface morphology, drug loading capacity, drug encapsulation efficiency, and stability in simulated gastric (SGF) and intestinal (SIF) fluids. Few studies are reported in literature about the potential toxicity of SLN formulations (Nassimi et al., 2010), thus *in vitro* citotoxicity of LA-MEM loaded SLNs was assessed against mouse N2a neuroblastoma (NB) and primary human whole blood (PHWB) cells using MTT and lactate dehydrogenase (LDH) assays, respectively. Total antioxidant capacity (TAC) and total oxidative stress (TOS) levels were also determined to valuate oxidative alterations.

2. Material and methods

2.1. Chemicals

LA-MEM was synthesized as we previously reported (Sozio et al., 2013b). Stearic acid, Brij 78 and Acetone were purchased by Sigma Aldrich (St. Louis, MO, USA). Sucrose was obtained from Alfa Aesar (MA, USA), Cremophor® ELP was obtained from BASF (Ludwigshafen, Germany). NaCl, NaOH, KH_2PO_4 and all other solvents of chromatographic grade were obtained from Fisher (Loughborough, UK). MilliQ water was produced by a MilliQ Direct-Q UV3 Millipore system (Merck, Darmstadt, Germany). Chromafil® PET-120/25 filters (Duren, Germany) were used for all the formulations.

2.2. Preparation of empty and LA-MEM loaded stealth Solid Lipid Nanoparticles

SLNs were prepared according to a slight modification of the emulsification-evaporation-solidifying method previously reported (Sjostrom et al., 1995; Shahgaldian et al., 2003). For the blank SLN, stearic acid (SA) (33.36 mg) was completely dissolved in 8 mL of acetone under mild magnetic stirring at 40 ± 2 °C. Concurrently, a solution of surfactant (Brij 78) in deionized water, with different lipid:surfactant ratio

(1:0.4 and 1:1), was prepared and heated at 75 ± 2 °C. The organic phase was added dropwise to the aqueous phase under mechanical stirring (1000 rpm) to obtain the lipid emulsion which was subsequently concentrated to 11.2 mL evaporating the solvents mixture at 75 ± 2 °C. The translucent emulsion system was quickly added to 11.2 mL of cold deionized water on ice bath under mechanical stirring (1000 rpm). The obtained solidified nanoparticles suspension was adjusted to total 25 mL with cold distilled water. The drug-loaded nanoparticles were prepared with the same procedure adding LA-MEM to the organic phase with different drug:lipid ratio (1:2.5, 1:5, 1:10, 1:20). The final suspension was filtered with 1.20 μm filters to remove the unentrapped drug.

2.3. Dynamic Light Scattering and Zeta Potential measurement

Intensity mean hydrodynamic size, polydispersity index (PDI), and Zeta potential of the particles were measured on a Malvern Zetasizer-NanoZS with a He-Ne laser with a wavelength of 632.8 nm. The measurements were carried out at a scattering angle of 173° using disposable sizing cuvette and keeping the temperature at 25 °C throughout the experiments. All measurements were repeated three times (n = 3) and the results are given as the effective diameter, also called *Z*-average diameter, and the PDI as a parameter of the particle size distribution. The measurements were performed at 25 °C, in triplicate (n = 3) and the average values were calculated. The electrophoretic mobility was determined in an aqueous medium with a Smoluchowski approximation.

2.4. Lyophilization of SLNs

SLNs dispersed in water were subjected to lyophilization to obtain dried formulations. Two different cryoprotectants were tested to avoid coalescence of SLNs (Shwarz et al., 1997; Cavalli et al., 1997), 0.8 mL of sucrose (5% w/v, 10% w/v) and 0.8 mL of trehalose (5% w/v, 10% w/v) aqueous solutions were mixed with 2 mL of SLNs water suspension; the mixture was then frozen at -20 °C overnight. The lyophilization process was carried out using a VirTis Advantage Freeze Dryer. The freeze-drying protocol employed was the following:

Freezing: Shelf temperature -38 °C for 120 minutes.

Primary drying (sublimation): Step 1: Shelf temperature -30 °C, vacuum 200 mTorr for 120 minutes; Step 2: Shelf temperature -10 °C, vacuum 200 mTorr, for 120 minutes. Secondary Drying (desorption): Shelf temperature: 0 °C, vacuum 200 mTorr, duration 20 hours. Post heat: Shelf temperature: 10 °C, vacuum: 200 mTorr, duration 240 minutes.

2.5. Morphology evaluation by transmission electron microscopy

Morphological examination of the prepared SLNs was performed using a transmission electron microscope (TEM). A drop of SLNs suspension was placed on a copper grid and stained with 1% (w/v) of uranyl acetate. The sample was viewed under TEM (FEI CM120 BioTwin instrument). Images were recorded using an AMT 5 Mp Digital camera.

2.6. Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) was performed on DSC Q2000 (TA instruments, LLC, USA). The instrument was calibrated with indium (Tm = 156.6, ΔHf = 28.71 J/g) according to the manufacturer instructions. Nitrogen was used as purge gas with a flow rate of 50 mL/min for all the experiments. TA aluminium pans and lids were used. Data were analysed using TA Instruments Universal analysis 2000. A heating rate in the range of 10 °C/min and 100 °C/min was employed. The samples analysed were stearic acid, LA-MEM, Brij 78, a physical mixture of LA-MEM and stearic acid, blank, and drug-loaded SLNs.

2.7. Drug loading capacity, encapsulation efficiency, and yield of production

The amount of encapsulated LA-MEM was quantified by dissolving an aliquot of the nanoparticles suspension (100 µL) in 900 µL of methanol. The obtained solution was analyzed by high performance liquid chromatography (HPLC). Chromatographic analyses were performed on an HPLC system equipped with a quaternary pump, an auto sampler, and a diode array detector. Analyses were carried out using a Waters, X-Terra RP18 column (4.5 x 150 mm, 5 µm pore size). The injection volume was 20 µL and the analyte was eluted at a flow rate of 1 mL/min for an isocratic elution period of 13 min. The mobile phase was composed of 79% v/v ACN and 21% v/v Water with 0.1% v/v TFA. The column was thermostated at

25 °C and the detection was carried out by monitoring the absorbance signals at 215 nm. HPLC was calibrated with standard solutions of LA-MEM ranging from 7.85 to 250 μg/mL.

The drug loading capacity (DLC%), drug entrapment efficiency (EE%), and yield of nanoparticles (Recovery%), were calculated by the following equations, respectively:

DLC% = (Weight of Codrug in SLNs/weight of SLNs) x 100

EE% = (Actual Codrug content/Theoretical Codrug content) x 100

Recovery% = (Weight of SLNs Recovered/Weight of Lipid, Surfactant and Codrug Used Initially) x 100.

2.8. In vitro release studies in Simulated Gastric and Intestinal Fluids

In vitro release studies were performed using the dialysis bag method (Verger et al., 1998). Blank SGF at pH 1.2 with 0.1% (v/v) Cremophor ELP and blank SIF at pH 6.8 with 0.1% (v/v) Cremophor ELP were used as dissolution media. In Tables A.1-2 (see appendices) are reported the materials used to prepare the fluids according to the USP. The dialysis bag (molecular weight cutoff 12400 Da) could retain nanoparticles and allow the diffusion of free drug into dissolution media (Luo et al., 2006). The bag was soaked in deionized water for 12 h before use. In detail, 10 mL of drug-loaded SLNs were poured into the dialysis bag, then placed in 400 mL flasks where 200 mL of dissolution medium was added. The ratio drug:fluids used was 1:20. The flasks were placed into a thermostatic shaker (Gallenkamp shaking incubator) at 37 °C and 100 rpm. At specific time intervals (0, 15, 30, 45, 60, 75, 90, and 120 min for SGF, and 0, 15, 30, 60, 90, 120, 180, and 240 min for SIF) aliquots (100 μL) of the suspension were collected from the bags and assayed for the codrug content; 100 μL of the suspension containing SLNs were dissolved in 900 μL of MeOH and analyzed by the HPLC method mentioned above. The percentage of codrug released from the SLNs at each time point was calculated by the following equation:

% released codrug = 100 – (%remaining codrug).

Sink conditions were maintained throughout the experiment. All the operations were carried out in triplicate.

2.9. In vitro stability of SLNs in Simulated Gastric and Intestinal Fluids

Stability studies were carried out in blank SGF pH 1.2, blank SIF pH 6.8, and in both fluids with pepsin and pancreatin, respectively. 0.1 mL of SLNs suspension were incubated with 1.9 mL of fluids in a thermostatic shaker at 37 °C and 100 rpm. Samples were withdrawn at 0, 15, 30, 60, 90, 120 min for SGF and at 0, 15, 30, 60, 90, 120, 180, 240 for SIF. The samples in the presence of enzymes were centrifuged for 5 min at 10000 rpm and 15 °C to remove pancreatin aggregates from the medium. The particles size and PDI after incubation were determined by DLS on a Malvern Zetasizer-NanoZS, as reported above.

A one month preliminary stability study was conducted comparing 10% (w/v) Sucrose blank and drug-loaded SLNs with those stored as aqueous suspensions. The vials were stoppered and crimped for storage. The SLNs were stored at 4 ± 2 °C. Lyophilized SLNs were reconstituted with deionized water and all the samples were tested for changes in the particles size, PDI, and entrapment efficiency. The free drug was removed by filtration with 1.20 μm polyester filter.

2.10 Citotoxicity studies

2.10.1. Blood samples cell lines

Blood samples were obtained by vein puncture from three healthy, man, non-smoking donors. PHWB cultures were set up according to a slight modification of the protocol described by Evans and O'Riordan (1975). The heparinized blood (0.5 mL) was cultured in 6 mL culture medium (Chromosome Medium B, Biochrom®) with 5 μg/L of phytohemagglutinin (Biochrom®). The mouse N2a-neuroblastoma cell line was obtained from Turkey FMD Institute, Ankara, Turkey. Prior to the experiments, cells were thawed and grown in tissue culture flasks as a monolayer in Dulbecco modified eagles medium (DMEM; Sigma-Aldrich®), supplemented with 1% glutamine, 0.5% penicillin/streptomycin, and 10% fetal bovine serum (PAN Biotech®) at 37 °C in a humidified (95%) incubator with CO_2 (5%). The cultured cells were trypsinised with trypsin/EDTA for a maximum of 5 min and seeded with a sub cultivation ratio of 1:3-1:8. Different concentrations (10 and 100 μM) of SLNs formulations (SLNs, SLNs-LA, SLNs-MEM and SLNs-LA-MEM) were added to the cells. After applications of SLNs formulations, the cultures were incubated for 24 h at 37 °C to adjust body conditions. Untreated cells were used along with H_2O_2 (50 μM, Merck®) as a positive cytotoxic control in all assays.

2.10.2. MTT assay

Viability of cells was spectrophotometrically assessed by measuring the formation of formazan crystals via MTT commercial kits (Cayman Chemical®). At the end of the experiment, the neurons were incubated with 0.7 mg/mL MTT for 30 min at 37 °C. After washing, the blue formazan was extracted from cells with isopropanol/formic acid (95:5) and photometrically determined at 560 nm. The density of formazan formed in untreated cells was taken as 100% viability.

2.10.3. LDH assay

Lactate dehydrogenase (LDH) released from damaged cells in culture medium was quantified by using LDH assay kit (Cayman Chemical®). A total of 100 µL of cell medium was used for LDH analysis. Released LDH catalyzed the oxidation of lactate to pyruvate with simultaneous reduction of NAD^+ to NADH. The rate of NAD^+ reduction was measured as an increase in absorbance at 490 nm, and it resulted directly proportional to LDH activity in the cell medium.

2.10.4. TAC and TOS analyses

Following homogenization, intracellular levels of total antioxidant capacity (TAC) and total oxidant status (TOS) were determined by commercially available kits (Rel Assay Diagnostics®). In TAC assay, antioxidants in the sample reduce dark blue-green colored ABTS radical to colorless reduced ABTS form. The change of absorbance at 660 nm is related to the total antioxidant level of the sample. The assay is calibrated with a stable antioxidant standard solution, Trolox Equivalent, that is a vitamin E analog. Since the separate measurement of different oxidant molecules is not practical and their oxidant effects are additive, the TOS of a sample is measured and is named total peroxide (TP), serum oxidation activity (SOA), reactive oxygen metabolites (ROM) or some other synonyms. In TOC assay, oxidants present in the sample oxidize the ferrous ion-chelator complex to ferric ion. The oxidation reaction is prolonged by enhancing molecules, which are abundant in the reaction medium. The ferric ion makes a colored complex with chromogen in an acidic medium. The color intensity, which can be spectrophotometrically measured, is related to the total amount of oxidant molecules present in the sample (Erel, 2004, 2005).

2.11. Statistics and pharmacokinetics analysis

Data are expressed as the mean ± standard deviation (SD) of three repetitions. One-way analysis of variance (ANOVA) was used to determine the significant differences between the groups followed by a Dunnett post-hoc test for multiple comparisons. Probabilities < 0.05 and < 0.1 were considered as significant. All analyses were performed using SPSS version 15.0 (SPSS Inc®).

3. Results and discussion

3.1. Preparation and characterization of SLNs LA-MEM

The solid lipid nanoparticles were made of stearic acid as lipid, Brij 78 (polyoxyethylen(20) stearyl ether) as surfactant, and LA-MEM as drug (Figure 1). In this formulation the stealth character is due to the presence of Brij 78; it was derived from stearic acid molecules covalently grafted with PEG 1000 to form a hydrophilic steric shell around the SLNs. The presence of this hydrophilic PEG chain is expected to protect SLNs from interaction with human serum albumin, to prolong the half-life of SLNs in blood circulation, leading to higher and protracted drug plasma levels *in vivo* (Zhang et al., 2007).

LA-MEM loaded SLNs were prepared according to the emulsification-evaporation-solidifying method as described above. The properties, such as intensity average diameter, polydispersity indices, zeta potential, yield, and entrapment efficiency are reported in Table 1. To define the best formulation, LA-MEM loaded SLNs were formulated with different drug:lipid ratio, 1:20, 1:10, 1:5, and 1:2.5, respectively. It was found that the size of LA-MEM loaded SLNs was slightly greater than that of the drug-free ones, due to the incorporation of drug into the particles matrix. The different formulations did not show significant changes in yield, zeta potential, and entrapment efficiency. The entrapment efficiency was evaluated after filtration of the particles with 1.20 μm filters to remove the entrapped drug and, after centrifugation of the particles, to assess the most suitable method to remove the free drug from the system (data not shown). The two methods were not significantly different, and the very similar values of %EE for each type of formulation suggest that the system is not completely saturated, although it was difficult to test higher drug concentrations due to its solubility problems. Thus, the formulation with 1:5 drug:lipid ratio was chosen as the optimized formulation because of the best properties in term of particle size and PDI values (Table 1).

We choose 1:0.4 lipid:surfactant ratio after evaluation of different lipid:surfactant ratios. In this case an optimized particle size with a narrow size distribution was obtained, while increasing the amount of Brij 78

both the mean particle size and polydispersity index rose (Table A.3). High concentration of surfactant could lead to intrinsic thermodynamic instability of the nanoparticle system causing adsorption of surfactant on the particle surface (Feritas and Muller, 1999; Goppert and Muller, 2005). Likely, at a low concentration surfactant prevents aggregation but, on the contrary, rising the concentration could cause the rearrangement of the surfactant molecules to form loops and tails on the particles surface, leading to the bridging among the SLNs followed by particles aggregation.

Chemical and physical instabilities may be observed when the nanoparticles are stored as aqueous suspension (Abdelwahed et al., 2006). In particular, different problems can occur with SLNs, such as lipid crystallization and/or polymorphic transformations, particle growth as a result of Ostwald ripening processes, aggregation/and hydrolysis reactions (Westesen et al., 1997; Muller et al., 2000). For these reasons, nanoparticles are usually subjected to lyophilization process, but this step may generate various stresses resulting in destabilization of the nanoparticles; for example, freezing may lead to crystallization of surface poly(ethylene glycol) (PEG), reducing its steric stabilization properties and leading to particle fusion (Howard et al., 2012). To minimize these stresses and avoid possible particle aggregation and destabilization during the lyophilization process, suitable cryoprotectant were added. In particular, between two tested cryoprotectants we selected sucrose at two different concentrations during the lyophilization process: 5% w/v and 10% w/v aqueous solution (1:10, 1:20 particles:cryoprotectant ratio) of cytoprotectant. As reported in Figure A.1 (Appendices), both dried blank and drug-loaded SLNs, after the reconstitution with deionized water, presented considerable aggregation phenomena, with a greater extent for drug-loaded nanoparticles than the drug-free ones. Higher concentration sucrose (10% w/v) was selected as most suitable compound to maintain the particles size; indeed, it led to a slight increase of the size in the blank nanoparticles (12.9%), although a more considerable raise of particles size in the drug-loaded nanoparticles was observed. From the reported data it is evident the good ability of sucrose as cryoprotectant for this system.

The TEM image of SLNs-LA-MEM is shown in Figure 2. It can be observed from these microscopic images that the lipid particles were disk shaped.

3.2. Characterization of SLNs-LA-MEM by DSC

The most frequent application of DSC in the study of SLNs dispersions is the investigation of the physical state of the lipid. This is due to the changes of the properties of the matrix lipid materials that can occur during the formation of the colloidal dispersed state. Even when crystalline lipids are used for the preparation of the dispersions, the lipid particles are not necessarily present in the solid state after processing, particularly when the preparation method requires heating. Thus, it is possible to observe supercooling melts, where lipid crystallization may not occur even though the sample is stored at a temperature below the melting point of the lipid. The confirmation of the desired physical state of the matrix lipid is thus of crucial importance for the development of nanoparticles based on solid lipid, as the potential advantages of SLNs are associated to the solid state of the lipid (Bunjes and Unruh, 2007).

The calorimetric curve of stearic acid (SA) is characterized by the presence of a small peak at 57.32 °C, and a main peak centred at 69.40 °C due to the melting of the compound; the melting peak of the surfactant is centred at 43.10 °C. Both the blank and the drug-loaded SLNs presented well-defined peaks for lipid and surfactant, but shifted at lower temperatures (66.56 °C and 41.68 °C, 63.01 °C and 36.35 °C for the blank and drug-loaded particles respectively), and slightly broader compared to the bulk materials signals (Figure 3). The lowered lipid melting peak of the SLNs suggests that the SA matrix has been successfully solidified during the preparation of the particles, thus, as previously reported by Lee et al. (2007), these results confirm that solid lipid nanoparticles were obtained. The shift of the endothermic signal to lower temperatures could be related to the particle size, according to the Gibbs-Thomson equation: for a particle of given size, it is possible to observe a decrease in melting temperature compared to the bulk material. Certainly, as the nanoparticles dispersions variously polydispersed, the melting transition could also be broadened since the fractions of different particle sizes melt at different temperatures (Lee et al; 2007).

3.3. In vitro release and stability studies of SLNs-LA-MEM

The oral route continues to be a challenge, even though it is the most attractive way to administer drugs. After oral administration SLNs may enhance the transport of drugs through the GI tract by different mechanisms including lymphatic transport, drug protection efficiency, and sustained drug release (Garcia-Fuentes et al.; 2005). For this reason, the incorporation of drugs into SLNs opens the perspective of enhanced and/or less variable bioavailability and prolonged plasma levels (Runge, 1998; Demirel et al.,

2001). The advantages of SLNs are also linked to their size within the submicron range; therefore, the preservation of the particles size after oral administration is of crucial importance. The first barrier is constituted by the physicochemical environment of the gastrointestinal tract, thus an evaluation of the nanoparticles stability in this medium is necessary. The GI environment may have influence on the physical stability of SLNs formulations due to ionic strength, pH values, and enzymatic activity which destabilize nanoparticles and potentially lead to aggregation.

In this study the SLNs stability was investigated, after the incubation of the nanoparticles in the simulated gastrointestinal fluids, by observing potential changes of the particles size distribution and polydispersity index (Alhnan et al., 2011). The first media used were simulated gastric fluids at pH 1.2 with and without pepsin. As shown in Figure 4, the particles seemed stable in both environments, because no significant aggregation was detected by dynamic light scattering. After 2 hours of incubation the size and the PDI were maintained. In literature, some studies have demonstrated the influence of the particles surface on the gastrointestinal stability. Particularly, in the presence of a PEG coating the stability is improved (Tobio et al.; 2000). In this study, the presence of the Brij 78 may, in a similar way, protect particles from degradation and enzymatic attack from pepsin.

After demonstrating the stability of SLNs in the stomach, intestinal stability was also studied. Figure 5 shows the stability of the particles after incubation in the intestinal simulated fluids at pH 6.8, in the presence or absence of pancreatin. As reported, in both media the particles were subjected to aggregation as well as increase in particle size and PDI. This phenomenon seems to be related to the pH increase of the environment more than to the presence of the enzyme, even if in the latter case the determination of the particle size by dynamic light scattering was particularly compromised by the presence of very large aggregates. Although the question concerning the instability of the particles in the intestine is not fully understood, it is possible that the alkaline pH assists the formation of hydrophobic interactions between the particles and the codrug released in the media, and the Brij 78 coating is not sufficient to protect particles from these interactions. As one of the objectives of the present work was the GI absorption of intact particles and their possibility to reach the brain, the evaluation of the release profile in the simulated gastrointestinal fluids was required. The *in vitro* release studies were conducted in sink conditions in simple SGF and SIF without enzymes as media, at pH 1.2 and 6.8, respectively. Solubility of LA-MEM in

simulated gastric and intestinal fluids at room temperature was <10 μg/mL; to provide sink conditions, two different concentrations of Cremophor ELP (1% v/v, 0.1% v/v) were tested. Even if 1% (v/v) Cremophor ELP led to a greater solubility increase in the fluids (about 100 μg/mL), it could not be used because of its ability to form micelles in the media. The dynamic light scattering of the system (SLNs, SGF or SIF with 1% v/v of Cremophor) showed a peak of the nanoparticles and a peak related to the presence of micelles at lower values. This did not happen with 0.1% (v/v) of Cremophor in which the solubility was about 60 μg/mL, allowing it to be selected as final concentration in the media. The presence of common non-ionic surfactant in the release media could also better mimic *in vivo* conditions because it takes into account the large number of surface-active molecules in the body (Wischle and Schwendeman, 2008).

The codrug release from SLNs in the SGF was shown in Figures 6-7. The results indicated that in both media, a small burst of release in the first hour was present, followed by a more sustained trend. In particular, in gastric and intestinal simulated fluids after 2 and 4 hours, respectively, approximately only 20% of the compound was released. Thus, it is reasonable to assume that the particles may be absorbed before a considerable amount of drug is released in the gut, improving its bioavailability. After the lyophilization of LA-MEM loaded SLNs, a one month stability study was conducted to evaluate the particles size stability after storage at 4 ± 2 °C; comparisons were made with SLNs stored as aqueous suspensions. As reported in Figure A.2 (Appendices) LA-MEM loaded SLNs showed a significant increase in particles size in the case of the aqueous suspensions; on the contrary, lyophilized SLNs showed greater consistency in their particles size measurement. Regarding the PDI values, they all remained < 0.3, generally considered the acceptable limit of monodispersity. The entrapment efficiency decreased in the suspension after one month storage from 88% to 62%, while overall it remained unchanged after storage for the lyophilized SLNs. The lyophilization process seemed to be effective in terms of nanoparticles long-term stability.

3.4. Citotoxicity studies of SLNs-LA-MEM

To quantify cell death in response to different SLNs formulations, MTT and LDH assays were performed. The cultured PHWB and N2a NB cells exposed to 10 μM of SLNs, SLNs-LA, SLNs-MEM, and SLNs-LA-MEM did not show any significant changes in cell viability during 24 hours, as determined by MTT and

LDH assays (data not shown). Likewise, the results of both cytotoxicity assays showed that 100 μM of SLNs, SLNs-LA, and SLNs-LA-MEM did not cause any significant changes in cell viability, but the application with 100 μM of SLNs-MEM caused a slight toxicity ($p > 0.05$, $p < 0.1$) compared to the untreated group. Moreover, when the PHWB and N2a NB cells were exposed to 50 μM H_2O_2, MTT absorbance values were respectively 56.63 ± 4.25% and 47.62 ± 5.32% of that of untreated controls, indicating that H_2O_2 caused cell death. H_2O_2-induced cell death was also clearly evidenced by about four- and five-folds increases in the LDH activities of positive controls of the cultures (Figures 8-9). The applications with 10 μM of SLNs, SLNs-MEM, and SLNs-LA-MEM did not lead to any alterations in TAC and TOS levels compared to control value, while 10 μM concentrations of SLNs-LA and SLNs-LA-MEM supported a slight antioxidant activity ($p > 0.05$) in both cell types without changing TOS levels (data not shown). A concentration of 100 μM significantly ($p < 0.05$) increased TAC levels in both PHWB and N2a NB cells. Furthermore, TAC and TOS levels were similar in 100 μM SLNs-LA-MEM treated and untreated cultures, while SLNs-MEM treatments altered the oxidative status. In addition, these alterations were more prominent in N2a NB cells than PHWB cells. Our results also illustrated that H_2O_2 exposure for 24 hours resulted in significant decreases in TAC levels and elevations of TOS levels, compared to those of the control groups. Figures 10-11 show the *in vitro* effects of different formulations of SLNs at 100 μM on TAC and TOS levels, respectively.

It has been reported that the benefit-risk balance deriving from the use of NPs for the treatment of CNS diseases should be carefully evaluated for each type of new engineered NP intended for brain-specific drug delivery (Re et al., 2012). In this study the results of our cytotoxicity assays (MTT and LDH) showed that even 100 μM of SLNs alone did not cause any significant changes in cell viability and oxidative status. In parallel to our findings, recent reports indicated that SLNs could be used as safe and efficient delivery systems for brain since they had no toxicity (Blasi et al.; 2013). On the contrary, in the present investigation the application of 100 μM of SLNs-MEM induced a slight citotoxicity leading to a reduction of cell viability likely related to oxidative alterations in N2a NB and PHWB cells. Similar to these findings, MEM applications at 100 μM led to notable decreases of cell viability in both N2a NB and MDCK cell lines, as determined by MTT assay during 24 h (Coleman et al.; 2013). Again, the determined TAC and TOS levels were similar in 100 μM SLNs-LA-MEM treated and untreated cultures while SLNs-MEM treatments

altered the oxidative status. In fact, antioxidant-loaded SLN systems - containing resveratrol, quercetin, idebenone, and ferulic acid, respectively - were found to be effective in decreasing ROS accumulation, thus exerting antioxidant activity in both *in vitro* and *in vivo* experimental models (Dhawan et al.; 2011). For the first time, cytotoxicity and oxidative damage analyses revealed that SLNs-LA-MEM could be a suitable DDS for an *in vivo* examination for the brain delivery of LA-MEM codrug which could be able to mitigate the oxidative damage and increase the antioxidant capacity in many neurodegenerative disorders, particularly in AD.

4. Conclusion

The new nanoparticulate delivery system loaded with LA-MEM was successfully prepared and characterized in terms of physicochemical properties, entrapment efficiency, and release profile in simulated gastrointestinal fluids. The optimized formulation (1000 rpm, 1:5 drug:lipid ratio and 0.03% of surfactant) had an average intensity diameter of 170 nm, a PDI of 0.072, a zeta potential of -33.8 mV, and an entrapment efficiency of 88%, in distilled water. Also the data obtained from our preliminary *in vitro* biological tests clearly revealed that SLNs-LA-MEM could be screened as a suitable DDS for the brain, from a toxicological point of view. Our preliminary results suggest the suitability of these nanoparticulate devices as carrier systems for LA-MEM codrug for further *in vivo* studies.

Acknowledgments

This work was supported by MIUR 2013 60% Grant.

Disclosure

The author reports no conflicts of interest in this work.

References

Abdelwahed, W., Degobert, G., Stainmesse, S., Fessi, H., 2006. Freeze-drying of nanoparticles: formulation, process and storage considerations. Adv. Drug Deliv. Rev. 58, 1688-1713.

Al Haj, N.A., Abdullah, R., Ibrahim, S., Bustamam, A. 2008. Tamoxifen drug loading solid lipid nanoparticles prepared by hot high pressure homogenization techniques. Am. J. Pharmacol. Toxicol. 3, 219-224.

Alhnan, M.A., Murdan, S., Basit, A.W., 2011. Encapsulation of poorly soluble basic drugs into enteric microparticles: A novel approach to enhance their oral bioavailability. Int. J. Pharm. 416, 55-60.

Anand, R., Gill, K.D., Mahdi, A.A., 2014.Therapeutics of Alzheimer's disease: past, present and future. Neuropharmacol. 76, 27-50.

Blasi, P., Schoubben, A., Traina, G., Manfroni, G., Barberini, L., Alberti, P.F., Cirotto, C., Ricci, M., 2013. Lipid nanoparticles for brain targeting III. long-term stability and in vivo toxicity. Int. J. Pharm. 454, 316-323.

Bunjes, H., Unruh, T., 2007. Characterization of lipid nanoparticles by differential scanning calorimetry, X-ray and neutron scattering. Adv. Drug Deliv. Rev. 59, 379-402.

Cacciatore, I., Baldassarre, L., Fornasari, E., Cornacchia, C., Di Stefano, A., Sozio, P., Cerasa, L.S., Fontana, A., Fulle, S., Di Filippo, E.S., La Rovere, R.M.L., Pinnen, F., 2012. (R)-α-Lipoyl-Glycyl-L-Prolyl-L-Glutamyl dimethyl ester codrug as a multifunctional agent with potential neuroprotective activities. Chem. Med. Chem. 7, 2021-2029.

Cacciatore, I., Cornacchia, C., Fornasari, E., Baldassarre, L., Pinnen, F., Sozio, P., Di Stefano, A., Marinelli, L., Dean, A., Fulle, S., Di Filippo, E.S., La Rovere, R.M.L., Patruno, A., Ferrone, A., Di Marco, V., 2013. A glutathione derivative with chelating and in vitro neuroprotective activities: Synthesis, physicochemical properties, and biological evaluation. Chem. Med. Chem. 8, 1818-1829.

Cavalli, R., Caputo, O., Carlotti, M.E., Trotta, M., Scarnecchia, C., Gasco, M.R., 1997. Sterilization and freeze-drying of drug-free and drug-loaded solid lipid nanoparticles. Int. J. Pharm. 148, 47-54.

Coleman, N., Ates-Alagoz, Z., Gaye, B., Farbaniec, M., Sun, S., Adejare, A., 2013. Toxicity studies on novel N-substituted bicyclo-heptan-2-amines at NMDA receptors. Pharmaceuticals 6, 536-545.

Demirel, M., Yazan, Y., Müller, R.H., Bozan, B., 2001. Formulation and in vitro-in vivo evaluation of piribedil solid lipid nanoparticles. J. Microencapsul. 18, 359-371.

Dhawan, S., Kapil, R., Singh, B., 2011. Formulation development and systematic optimization of solid lipid nanoparticles of quercetin for improved brain delivery. J. Pharm. Pharmacol. 63, 342-351.

Di Stefano, A., Sozio, P., Iannitelli, A., Cerasa, L.S., 2009. New drug delivery strategies for improved Parkinson's disease therapy. Exp. Opin. Drug Deliv. 6, 389-404.

Di Stefano, A., Iannitelli, A., Laserra, S., Sozio, P., 2011. Drug delivery strategies for Alzheimer's disease treatment. Expert. Opin. Drug Deliv. 8, 581–603.

Erel, O., 2004. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clin. Biochem. 37, 277-285.

Erel, O., 2005. A new automated colorimetric method for measuring total oxidant status. Clin. Biochem. 38, 1103-1111.

Esposito, E., Fantin, M., Marti, M., Drechsler, M., Paccamiccio, L., Mariani, P., Sivieri, E., Lain, F., Menegatti, E., Morari, M., Cortesi, R., 2008. Solid lipid nanoparticles as delivery systems for bromocriptine. Pharm. Res. 25, 1521-1530.

Evans, H.J., O'Riordan, M.L., 1975. Human peripheral blood lymphocytes for the analysis of chromosome aberrations in mutagen tests. Mut. Res. 31, 135-148.

Feritas, V., Müller, R., 1999. Correlation between long –term stability of solid lipid nanoparticles (SLN) and cristallinity of the lipid phase. Eur. J. Pharm. Biopharm. 47, 125-132.

Garcia-Fuentes, M., Prego, C., Torres, D., Alonso, M.J., 2005. A comparative study of the potential of solid triglyceride nanostructures coated with chitosan or poly(ethylene glycol) as carriers for oral calcitonin delivery. Eur. J. Pharm. Sci. 25, 133-43.

Goppert, T., Muller, R., 2005. Protein adsorption patterns on poloxamer and poloxamine-stabilized solid lipid nanoparticles (SLN). Eur. J. Pharm. Biopharm. 60, 361-372.

Howard, M.D., Lu, X., Jay, M., Dziubla, T.D., 2012. Optimization of the lyophylization process for long-term stability of solid-lipid nanoparticles. Drug Dev. Ind. Pharm. 38, 1270-1279.

Kanwar, J.R., Mahidhara. G., Kanwar, R.K., 2009. Recent advances in nanoneurology for drug delivery to the Brain. Curr. Nanosci. 5, 441-448.

Lee, M.K., Lim, S.J., Kim, C.K., 2007. Preparation, characterization and in vitro cytotoxicity of paclitaxel-loaded sterically stabilized solid lipid nanoparticles. Biomaterials 28, 2137-2146.

Luo, Y., Chen, D., Ren, L., Zhao, X., Qin, J., 2006. Solid lipid nanoparticles for enhancing vinpocetine's oral bioavailability. J. Control. Release 114, 53-59.

Müller, R.H., Mäder, K., Gohla, S., 2000. Solid lipid nanoparticles (SLN) for controlled drug delivery-a review of the state of the art. Eur. J. Pharm. Biopharm. 50, 161-177.

Nassimi, M., Schleh, C., Lauenstein, H.D., Hussein, R., Hoymann, H.G., Koch, W., Pohlmann, G., Krug, N., Sewald, K., Rittinghausen, S., Braun, A., Müller-Goymann, C., 2010. A toxicological evaluation of inhaled solid lipid nanoparticles used as a potential drug delivery system for the lung. Eur. J. Pharm. Biopharm. 75, 107-116.

Parihar, M.S., Hemnani, T., 2004. Alzheimer's disease pathogenesis and therapeutic interventions. J. Clin. Neurosci. 11, 456-467.

Patel, M., Souto, E.B., Singh, K.K., 2013. Advances in brain drug targeting and delivery: limitations and challenges of solid lipid nanoparticles. Expert Opin. Drug Deliv. 10, 889-905.

Re, F., Gregori, M., Masserini, M., 2012. Nanotechnology for neurodegenerative disorders. Maturitas 73, 45-51.

Runge, S.A., 1998. FesteLipidnanopartikel (SLN®) als kolloidaler Arzneistoffträger für die orale Applikation von Ciclosporin A, PhD thesis, Freie Universität Berlin.

Schwarz, C., Mehnert, W., 1997. Freeze-drying of drug free and drug-loaded solid lipid nanoparticles (SLN). Int. J. Pharm. 157, 171-179.

Shahgaldian, P., Da Silva, E., Coleman, A.W., Rather, B., Zaworotko, M.J., 2003. Para-acyl-calix-arene based solid lipid nanoparticles (SLNs): a detailed study of preparation and stability parameters. Int. J. Pharm. 253, 23-38.

Silva, A.H., Filippin-Monteiro, F.B., Mattei, B., Zanetti-Ramos, B.G., Creczynski-Pasa, T.B., 2012. In vitro biocompatibility of solid lipid nanoparticles. Sci. Total Environ. 432, 382-388.

Sjöström, B., Kaplun, A., Talmon, Y., Cabane, B., 1995. Structures of nanoparticles prepared from oil-in-water emulsions. Pharma Res. 12, 39-48.

Sozio, P., D'Aurizio, E., Iannitelli, A., Cataldi, A., Zara, S., Cantalamessa, F., Nasuti, C., Di Stefano, A., 2010. Ibuprofen and lipoic acid diamides as potential codrugs with neuroprotective activity. Arch. Pharm. 343, 133-142.

Sozio, P., Marinelli, L., Cacciatore, I., Fontana, A., Türkez, H., Giorgioni, G., Ambrosini, D., Barbato, F., Grumetto, L., Pacella, S., Cataldi, A., Di Stefano, A., 2013a. New flurbiprofen derivatives: synthesis, membrane affinity and evaluation of in vitro effect on β-amyloid levels. Molecules 18, 10747-10767.

Sozio, P., Cerasa, L.S., Laserra, S., Cacciatore, I., Cornacchia, C., Di Filippo, E.S., Fulle, S., Fontana, A., Di Crescenzo, A., Grilli, M., Marchi, M., Di Stefano, A., 2013b. Memantine-sulfur containing antioxidant conjugates as potential prodrugs to improve the treatment of Alzheimer's disease. Eur. J. Pharm. Sci. 49, 187-198.

Tobìo, M., Sanchez, A., Soriano, I., Evora, C., Vila-Jato, J.L., Alonso, M.J., 2000. The role of PEG on the stability in digestive fluids and in vivo fate of PEG -PLA nanoparticles following oral administration. Colloid Surf. B: Biointerfaces 18, 315-323.

Üner, M., Yener, G., 2007. Importance of solid lipid nanoparticles (SLN) in various administration routes and future perspectives. Inter. J. Nanomed. 2, 289-300.

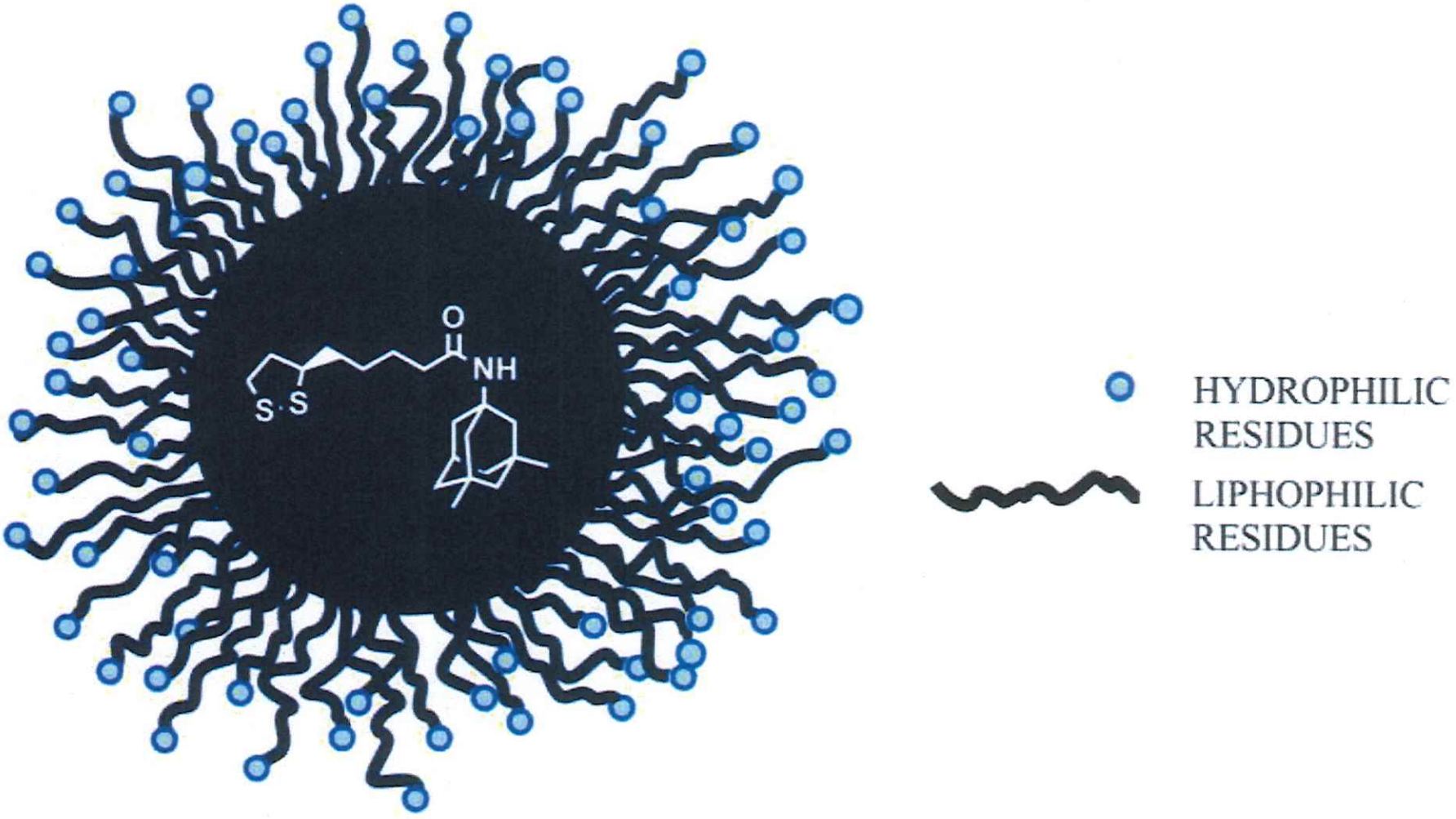
Verger, M.L.L., Fluckiger, L., Kim, Y.I., Hoffman, M., Maincent, P., 1998. Preparation and characterization of nanoparticles containing an antihypertensive agent. Eur. J. Pharm. Biopharm. 46, 137-143.

Westesen, K., Bunjes, H., Koch, M.H.J., 1997. Physicochemical characterization of lipid nanoparticles and evaluation of their drug loading capacity and sustained release potential. J. Control. Release 48, 223-236.

Wischle, C., Schwendeman, S.P., 2008. Principles of encapsulating hydrophobic drugs in PLA/PLGA microparticles. Int. J. Pharm. 364, 298-327.

Zhang, J.Q., Liu, J., Li, X.L., Jasti, B.R., 2007. Preparation and characterization of solid lipid nanoparticles containing silibinin. Drug Deliv. 14, 381-387.

*Graphical Abstract (for review)



Supplementary Material

Click here to download Supplementary Material: Appendices.docx

Tables and Figures

Solid Lipid Nanoparticles Loaded With Lipoyl-Memantine Codrug: Preparation and Characterization

Sara Laserra,[1] Abdul Basit,[2] Piera Sozio,[1] Lisa Marinelli,[1] Erika Fornasari,[1] Ivana Cacciatore,[1] Michele Ciulla, [1] Hasan Türkez,[3] Fatime Geyikoglu,[4] Antonio Di Stefano[1*]

[1]Department of Pharmacy, "G. d'Annunzio" University of Chieti-Pescara, Via dei Vestini 31, 66100 Chieti, Italy; [2]Department of Pharmaceutics, UCL School of Pharmacy, University College London, WC1N 1AX, London, UK; [3]Department of Molecular Biology and Genetics, Erzurum Technical University, Erzurum 25240, Turkey; [4]Ataturk University, Faculty of Science, Biology Department, 25240, Erzurum, Turkey.

Correspondence: Prof. Antonio Di Stefano

Department of Pharmacy, "G. d'Annunzio" University of Chieti-Pescara, Via dei Vestini 31, 66100 Chieti, Italy

Tel +39 0871 3554708

Fax +39 0871 3554706

Table 1 Drug Loading Capacity (DLC) and Entrapment Efficiency (EE) of several ratios of LA-MEM loaded SLN.

LA-MEM loaded SLN Codrug:Lipid Ratio	Size (nm)	PdI	ZP (mV)	Recovery (%)	EE (%)	DLC (%)
Blank	160.0 ± 1.2	0.099 ± 0.01	-27.4 ± 0.8	95.50 ± 1.3	-	-
1:2.5	195.6 ± 1.6	0.090 ± 0.01	-33.90 ± 1.05	95 ± 2.8	92 ± 2.8	-
1:5	169.9 ± 4.3	0.072 ± 0.01	-33.8 ± 0.9	96.0 ± 1.7	88 ± 1.8	12.5 ± 1.3
1:10	183.05 ± 1.9	0.120 ± 0.01	-32.9 ± 2.4	97.5 ± 0.7	90 ± 1	-
1:20	173.5 ± 1.4	0.084 ± 0.01	-35 ± 2.1	97.0 ± 0	90 ± 0.7	-

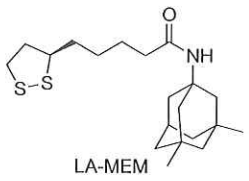


Fig. 1. Chemical structure of LA-MEM codrug.

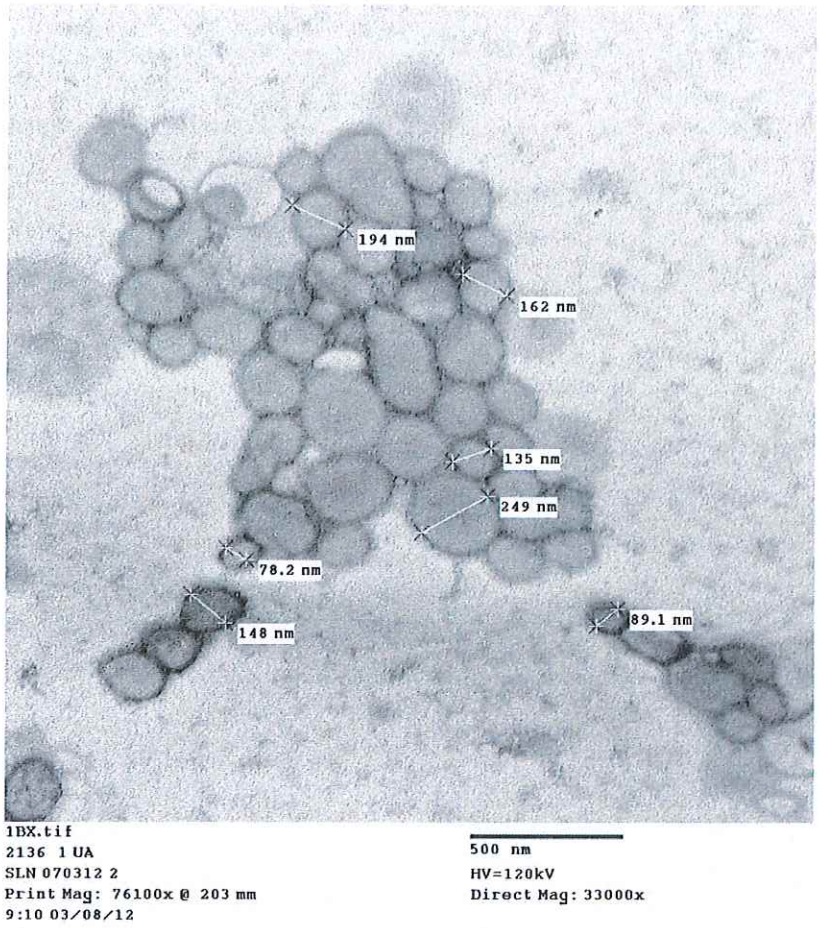


Fig. 2. TEM image of LA-MEM loaded SLN.

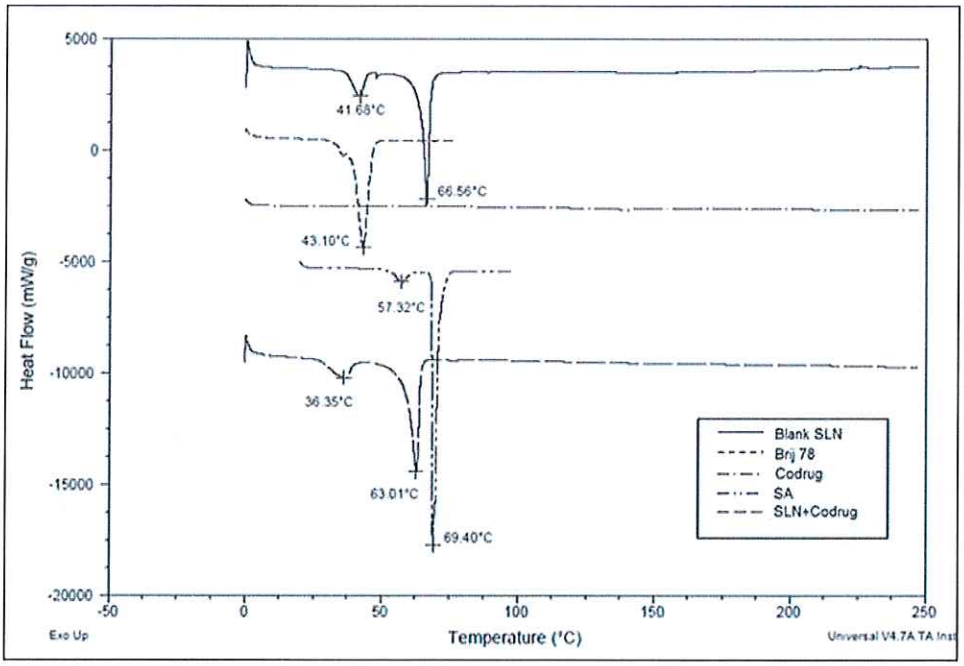


Fig. 3. Differential scanning calorimetry scans of LA-MEM loaded SLN.

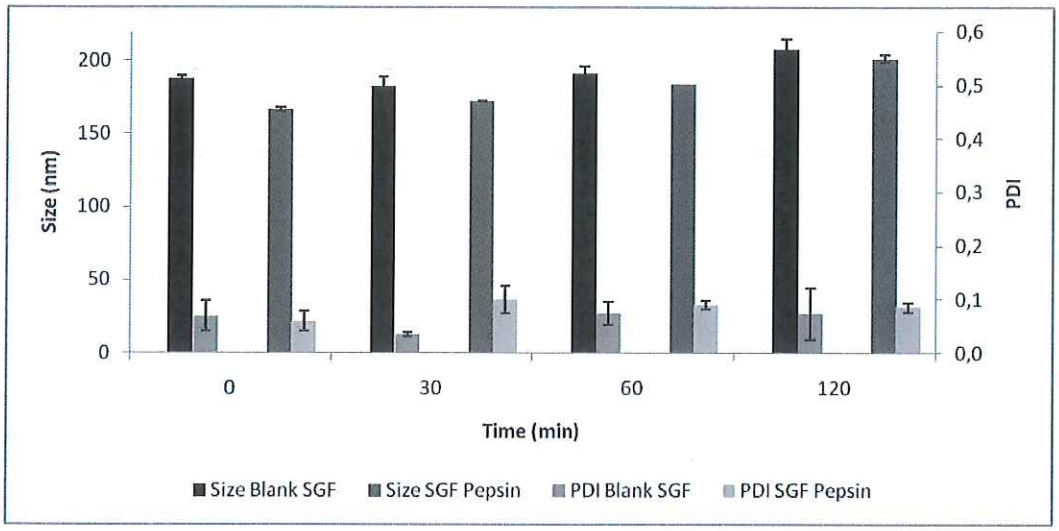


Fig. 4. Size and PDI of SLNs-LA-MEM after incubation in SGF (pH 1.2) at 37 °C, 100 rpm.

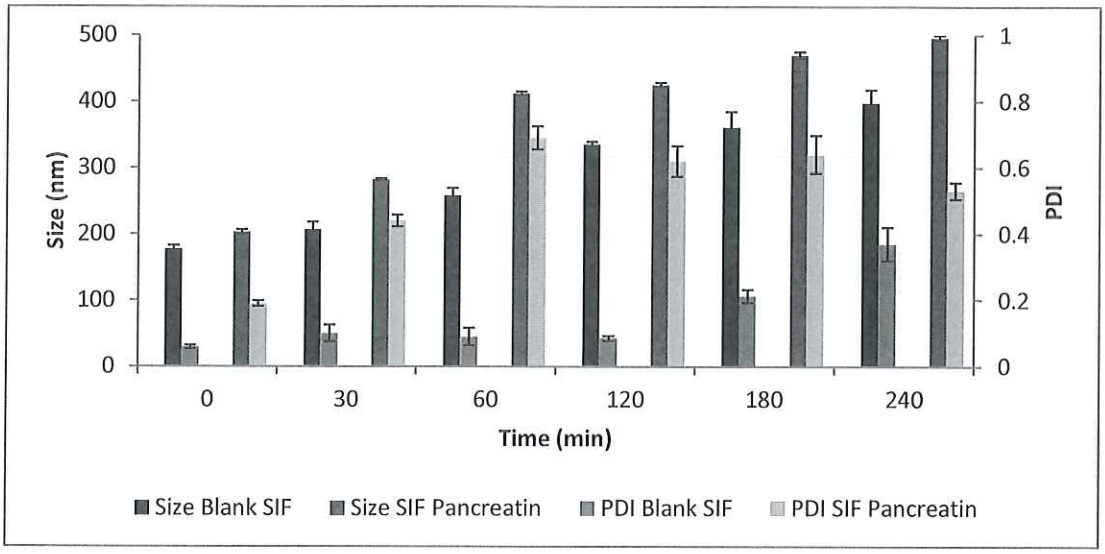


Fig. 5. Size and PDI of SLNs-LA-MEM after incubation in SIF (pH 6.8) at 37 °C,100 rpm.

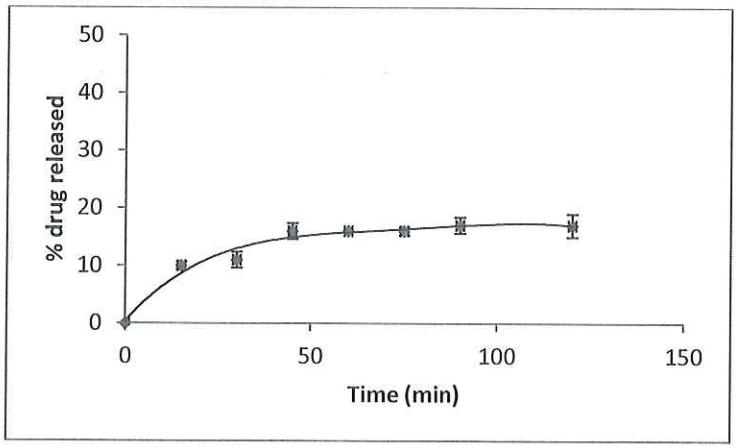


Fig. 6. *In vitro* release of LA-MEM from SLNs after incubation in SGF (pH1.2) and 0.1% (v/v) Cremophor ELP, at 37 °C, 100 rpm.

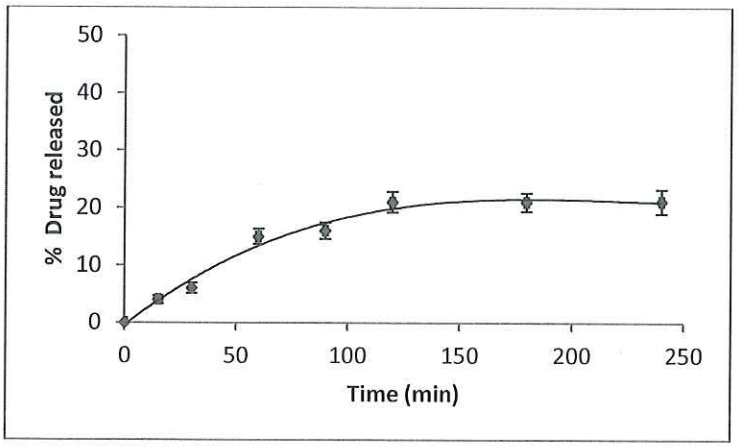


Fig. 7. *In vitro* release of LA-MEM from SLNs after incubation in SIF (pH 6.8) and 0.1% (v/v).

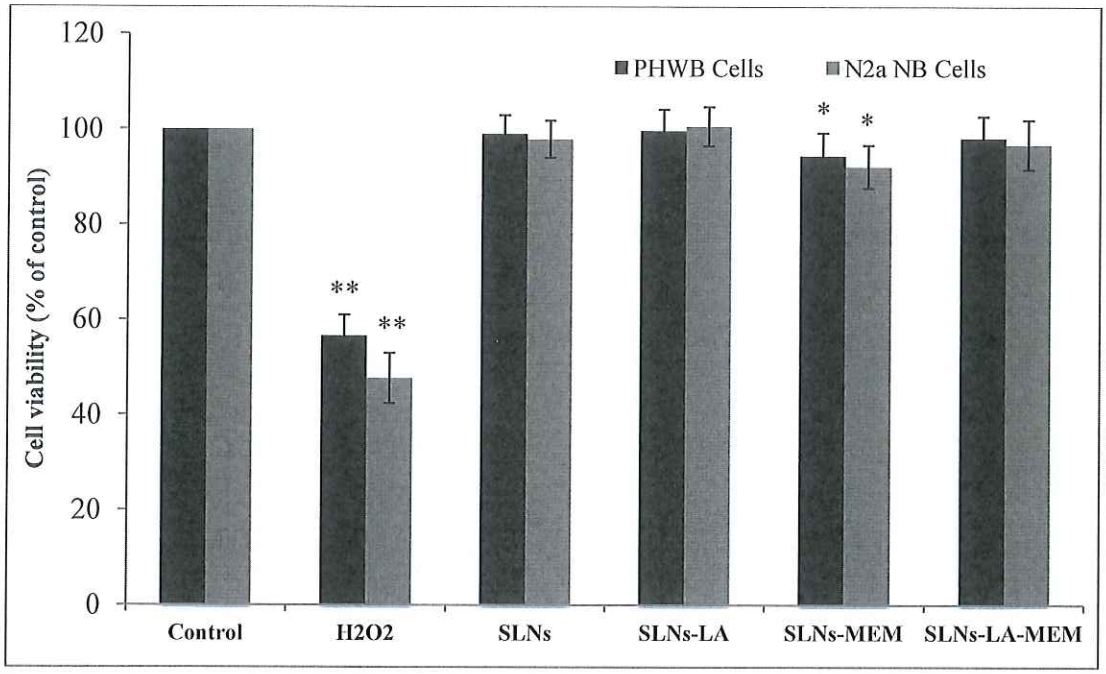


Fig. 8. Viability of PHWB and N2a NB cells after 24 h exposure to (100 μM) different SLNs formulations. The results were presented as percentage of the control group (n = 3). Data are expressed as mean ± SD. ** $p < 0.05$, * $p < 0.1$.

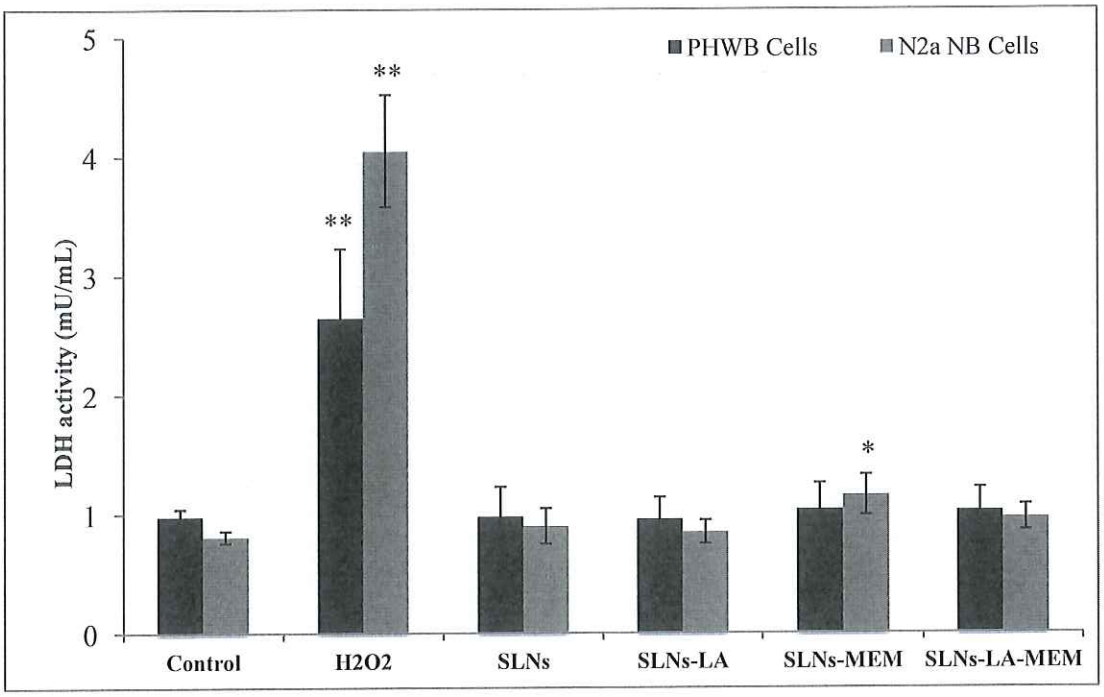


Fig. 9. Extracellular levels of LDH in cultured PHWB and N2a NB cells maintained in the presence of different SLNs formulations (100 μM).

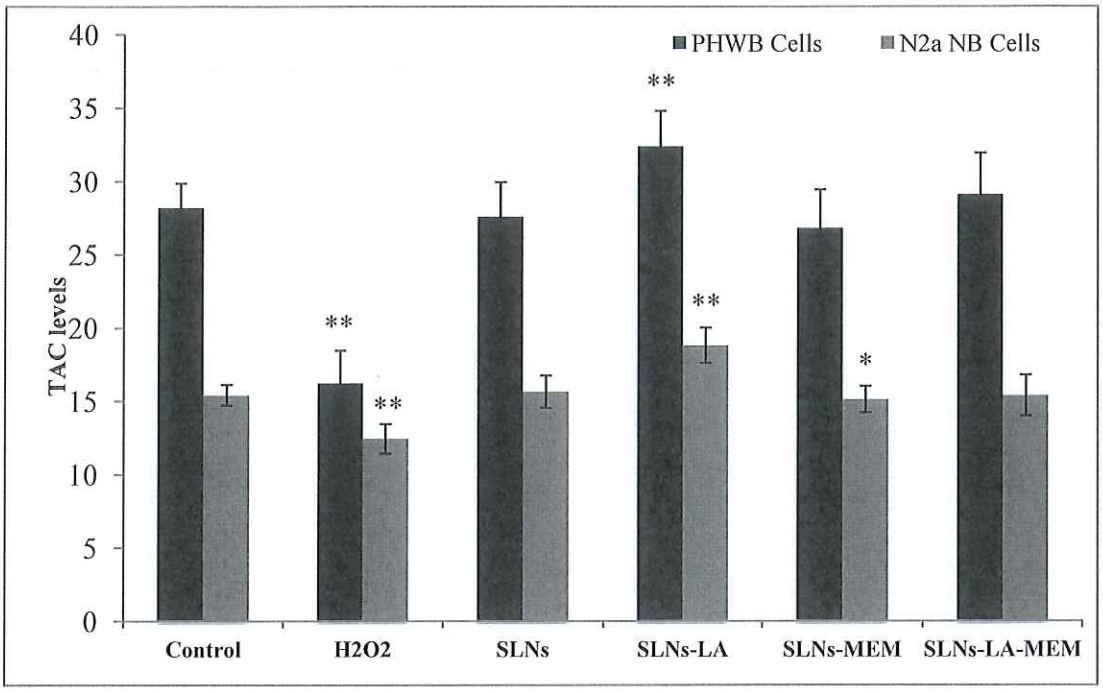


Fig. 10. *In vitro* levels of TAC (as mmol Trolox Equiv./L) in cultured PHWB and N2a NB cells maintained in the presence of different SLNs formulations (100 μM).

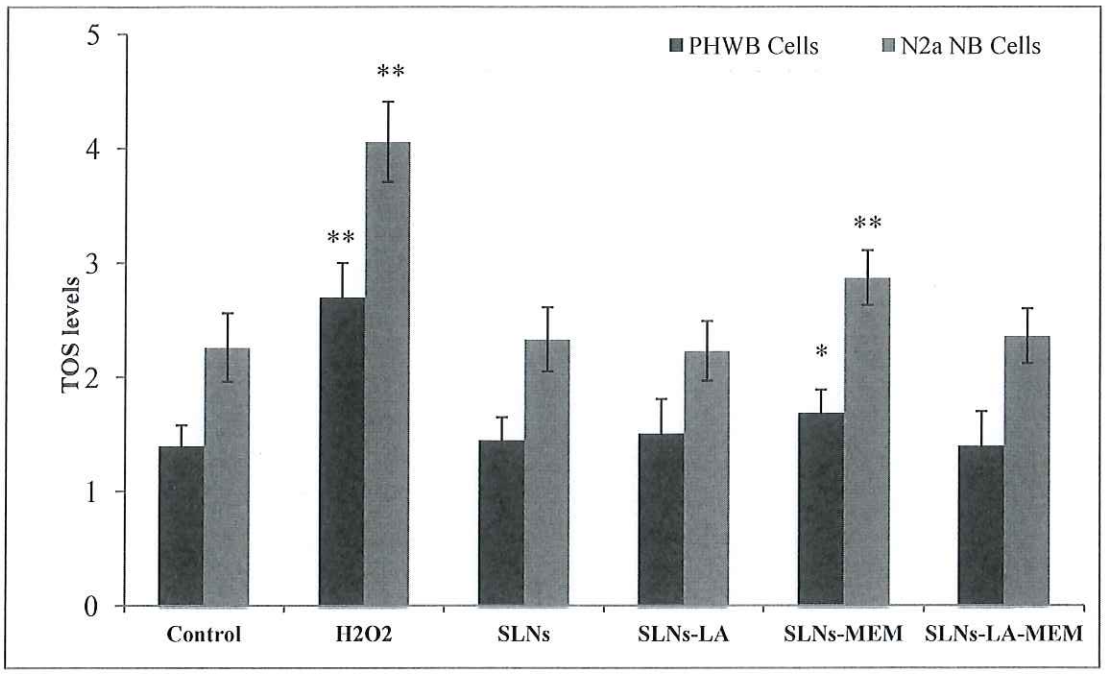


Fig. 11. *In vitro* levels of TOS (as mmol H_2O_2 Equiv./L) in cultured PHWB and N2a NB cells maintained in the presence of different SLNs formulations (100 μM).

IJP AUTHOR CHECKLIST

Dear Author,

It frequently happens that on receipt of an article for publication, we find that certain elements of the manuscript, or related information, is missing. This is regrettable of course since it means there will be a delay in processing the article while we obtain the missing details.

In order to avoid such delays in the publication of your article, if accepted, could you please run through the list of items below and make sure you have completed the items.

Overall Manuscript Details

- Is this the final revised version? X
- Are all text pages present? X
- Are the corresponding author's postal address, telephone and fax numbers complete on the manuscript? X
- **Have you provided the corresponding author's e-mail address?** X
- **Manuscript type – please check one of the following:**
  - Full-length article X
  - Review article ☐
  - Rapid Communication ☐
  - Note ☐
  - Letter to the Editor ☐
  - Other ☐
- **Manuscript section – paper to be published in:**
  - Pharmaceutical Nanotechnology section X
  - Personalised Medicine section ☐

Manuscript elements

- Short summary/abstract enclosed? X
- 3-6 Keywords enclosed? X
- Complete reference list enclosed? X
- Is the reference list in the correct journal style? X
- Are all references cited in the text present in the reference list? X
- Are all **original** figures cited in the text enclosed? X
  - Electronic artwork format? ----------
- Are figure legends supplied? X
- Are all figures numbered and orientation provided? X
- Are any figures to be printed in colour? NO
  - If yes, please list which figures here:----------
- If applicable, are you prepared to pay for reproduction in colour? NO
- Are all tables cited in the text supplied? X

General

- Can you accept pdf proofs sent via e-mail? X