GREEN TEA EXTRACT LOADED INTO SOLID LIPID NANOPARTICLES

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The aim of this study was to synthesize by high shear homogenization method and to characterize new solid lipid nanoparticles loaded with green tea extract, nanoparticles which show high antioxidant activity. In this respect, cetyl palmitate and glyceryl stearate were used as solid lipids in combination with two nonionic surfactants, Tween20 and Tween80 and an ionic surfactant as lecithin. The obtained solid lipid nanoparticles have been firstly characterized by particle diameter and physical stability using dynamic light scattering method. The particle morphology was examined by transmission electron microscopy. The antioxidant and antimicrobial properties of new developed nano-materials have been also evaluated.

Keywords: solid lipid nanoparticles, green tea extract, high shear homogenization

1. Introduction

In the past few years, many different methods for the preparation of solid lipid nanoparticles have been successfully applied. These methods are high pressure homogenization [1, 2], microemulsion technique [3], ultrasonication [4], solvent injection method [5], solvent diffusion method [6], solvent evaporation [7] and phase inversion [8].

Solid lipid nanoparticles (SLNs) have attracted increasing attention, and are regarded as an alternative carrier system to traditional colloidal systems such as liposomes, emulsions and nanoparticles [9, 10]. The major advantages of these systems are biocompatibility, bioavailability and the fact that there is no problem with multiple routs of administration, such as oral, transdermal and intravenous administration [11, 12]. The literature presents sufficient data which demonstrate that the bioavailability of hydrophilic and lipophilic drugs can be improved when these drugs are encapsulated into SLNs [13].

Recently, due to the high content of flavones, a growing interest in the therapeutic effects of green tea for cancer treatment and other diseases was shown. Flavonoids are natural polyphenols present in vegetables normally consumed by

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human. Many studies reported that green tea can inhibit the proliferation of multiple cancer cell types, including prostate and breast carcinoma cells [14], colon and lung cancer [15], and pancreatic tumor cells [16]. Moreover, it can also reverse multidrug-resistance in cancer cells and enhance the anti-cancer effects of other drugs [17, 18]. Therefore, an oral formulation of green tea is highly desired.

The aim of the present study is to design and characterize green tea extract loaded SLNs (GTE-SLNs) using the high shear homogenization (HSH) technique and to evaluate the antioxidant potential of new SLNs obtained.

2. Materials and metods

2.1. Materials

The composition of GTE used consists in polyphenols (51.8% determined by UV), catechins (39.2%) and caffeine (9.8%) determined by HPLC. The catechin compounds present in GTE are: EGC (9.5%), DL-C (1.92%), EG-GG (15.5%), EC (3.8%), GCG (2.6%), and ECG (5.9%). The other materials used in this study, such as polyethylene glycerol sorbitan monolaurate (Tw20), polyethylene glycol sorbitan monooleate (Tw80) were purchased from Merck (Germany). Synperonic PE/F68 (block copolymer of polyethylene and polypropylene glycol), L-α-Phosphatidylcholine, (Lecithin) and Tris[Hydroxymethyl] aminomethane (Luminol) were purchased from Sigma Aldrich Chemie GmbH (Munich, Germany). n-Hexadecyl Palmitate (CP) 95% was purchased from Acros Organics (USA), while Glycerol Stearate (GS) was supplied by Cognis GmbH. A spectroscopic quality ethanol (Sigma Aldrich) was used as solvent.

2.2. Preparation of SLN

The obtained GTE loaded solid lipid nanoparticles were prepared by a modified high shear homogenization method previously described [19]. Briefly, the HSH method consists in the production of solid lipid nanoparticles (SLNs) by preparing two different surfactant phases, aqueous and lipid obtained separately. The aqueous surfactant phase consisted of Tw20 or Tw80, Synperonic F68, Lecithin block copolymer and double-distilled water and the lipid phase consisted of CP and GS. Both phases were heated at the same temperature of 85 °C for 30 mins. In the lipid phase, two different amounts of GTE were added, 0.1% and 0.17%, respectively. Before mixing the two phases, the aqueous surfactant phase was high speed stirred for two mins at 15000 rpm. Then, a pre-emulsion was produced by high shear stirring and processed by HSH.

The melting procedure of the lipid to produce the hot pre-emulsion and the homogenization process can have potential effect on chemical stability of the lipid.

Lyophilization is one of the most employed methods to obtain dehydrated formulations which can be stored and shipped at room temperatures. Trehalose at a concentration of 5 w% was used in the freeze-drying process as cryoprotector. First, the SLNs suspensions were frozen in an aqueous trehalose solution at -25 °C for 24 hours, and then the samples were transferred to the freeze-dryer at -55 °C for 72 hours. After that, the SLNs powders were collected for further measurements. The obtained lyophilized samples exhibit a powdery aspect and light yellow color.

2.3. Particle size and zeta potential measurements

Size distribution and polydispersity index of the SLNs were determined by dynamic light scattering (DLS) method using a Zetasizer Nano ZS, (Malvern Instruments Ltd.). DLS measurements were performed at 90° scattered light and the temperature was 25 °C. All the samples were diluted in deionized water to an adequate scattering intensity prior to the measurement.

The zeta potential was measured using the same Zetasizer. The electrophoretic mobility was converted into zeta potential by using the Helmholtz–Smoluchowski equation. Zeta potential measurements were performed in distilled water with a conductivity adjusted to 50 μS/cm by addition of sodium chloride solution. All the measurements were done in triplicate.

2.4. TEM analysis

Morphology of SLNs was examined using a 300 Kv Tecnai G^2 F30 S-TWIN transmission electron microscope equipped with STEM and with HAADF detector, EDX, EELS. Before measurements, a drop of the SLN dispersion sample was deposited on the holey copper grid and dried at room temperature. Microphotographs were recorded.

2.5. UV-Vis analysis

The UV-VIS absorption spectra of lyophilized GTE-SLNs were recorded at the wavelength range 220-2200 nm using a Jasco double-beam V670 Spectrophotometer. The lyophilized samples were prepared in pellets form in the presence of MgO, which has been also used as reference.

2.6. DSC analysis

Differential scanning calorimetry (DSC) measurements were performed using a DSC 204 F1 apparatus (Netzsch). Approximately 10 mg of lipid bulk material or equivalent SLN dispersion were filled into an aluminum pan and

sealed. It was then heated from 25 °C to 100 °C at a rate of 5 °C/min. The crystallinity of the particles was quantified as so called crystallinity index, what it means that the melting enthalpy of the lipid in the SLN dispersion was expressed as percentage of the melting enthalpy of the bulk lipid [20]. The bulk lipid was considered being fully crystalline, corresponding to the index of 100%. Of course, this index is only a rough measure because the particles can crystallize partially in a different modification, while peak separation is in most cases not possible. The crystallinity index (Ci) was calculated using the following equation:

$$Ci = \frac{\Delta H_{NLC}\,[J/g]}{\Delta H_{bulk}\,[J/g]\,C_{lipid\ phase}} x100\% \quad (1)$$

2.7. Antioxidant activity

The antioxidant activity (AA) of free-SLNs and GTE-SLNs was determined and compared with that of GTE in bulk by the chemiluminescence method (CL) using a Chemiluminometer Turner Design TD 20/20, USA. A cyclic hydrazide (luminol) was used as light amplifying substance. The luminol increases the detection sensitivity of activated oxygen species in the sample. H_2O_2 was used as generator system for free radicals in a tampon TRIS-HCl solution with a pH=8.6. The antioxidant activity of ethanol solutions of green tea and ethanol solutions of lyophilized GTE-SLNs with the same concentration of active compound was calculated using the following equation:

$$AA = \frac{I_0 - I_s}{I_0} x100\% \quad (2)$$

2.8. Antibacterial activity

The antibacterial activity of free-SLN, GTE-SLNs and GTE in bulk were tested against the action of *Escherichia coli* K 12-MG1655 bacteria. The antibacterial activity measurements were performed by agar well diffusion method [21].

The bacterial strains were grown in Luria Bertani Agar (LBA) plates at 37 °C with medium composition: peptone - 10 g/L; yeast extract - 5 g/L, NaCl - 5 g/L and agar - 20 g/L. The stock culture was maintained at 4 °C. All the bacteria containing plates were incubated at 37 °C for 24 h.

3. Results and discussion

3.1. Particle size measurements and TEM analysis

Evaluation of SLNs size distribution was achieved in two optimized systems types Tw20 or Tw80/lecithin/ block copolymer. Fig. 1 illustrates the

particle size distribution of free-SLNs and GTE loaded SLNs samples prepared with Tw20 and Tw80, respectively.

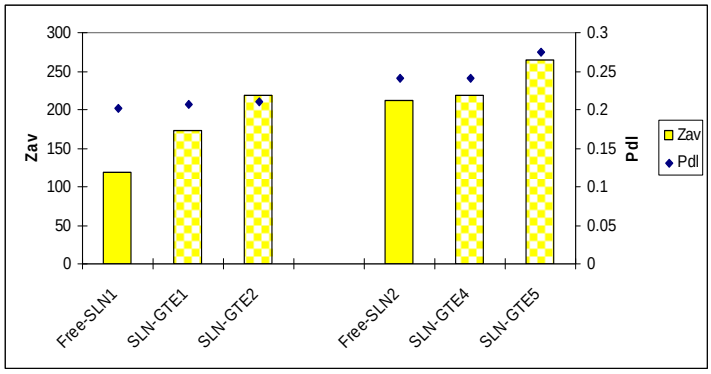

Fig. 1. DLS dimensional analysis of GTE-SLNs systems

The compositions of all free-SLNs and GTE loaded SLNs samples is given in table 1. Concerning the effectiveness of the two types of nonionic surfactants used, one may observe that the lipid matrix prepared with Tw20 exhibits smaller average particle diameter for the free lipid matrix (Zave=119 nm), while for the matrix prepared with Tw80 higher value was obtained: Zave=211 nm.

As a first observation, could be affirmed that the size of all SLNs is increasing with the GTE content. The highest values were obtained when Tw80 was used as surfactant (see Fig. 1). Moreover, the values obtained for Pdl, ranging between 0.2 - 0.27, reveal good degree of homogeneity for all the investigated systems. It can be observed that the Pdl is depending on surfactant used, being almost the same for all SLNs prepared with Tw20, while when Tw80 is used, Pdl is increasing with the increase of green tea content.

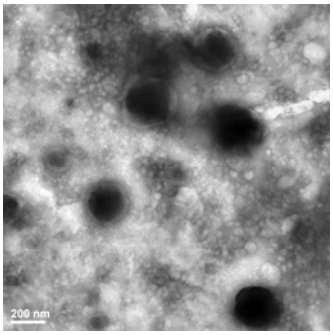

Fig. 2. TEM image of SLN-GTE2

These size evaluations were confirmed by the transmission electron microscopy (TEM). TEM measurement was carried out to get the information of the real size and morphology characteristics of SLNs.

Fig. 2 shows as example one of the TEM images of SLNs. It can be seen that the obtained SLNs are nearly spherical in shape and the size distribution is narrow. From the TEM photograph, the particle size distributes in the range of 100 - 200 nm. It is known that the drug incorporation into lipid matrix always leads to their size increase comparing with the free-SLNs [22]. Corroborating all the information obtained concerning the size of the nanoparticles it can be affirmed that GTE was loaded into SLNs.

3.2. Nanodispersion stability

Table 1

Composition and physico-chemical characterization of free-SLNs and GTE-SLNs

Sample	Composition[a]		ZP (mV)	DSC caracteristics	
	GTE %	Main Surfactant[b]		C.I.	P.t./shoulder °C
free-SLN1	-	Tw20	-44.9±1.07	70.45	51.8
SLN-GTE1	0.1	Tw20	-36.3±1.07	68.15	51.8
SLN-GTE2	0.17	Tw20	-46.6±0.702	60.35	53.6/47.7/44.0
free-SLN2	-	Tw80	-52.3±0.551	-	-
SLN-GTE3	0.1	Tw80	-34.4±2.55	66.76	51.8
SLN-GTE4	0.17	Tw80	-34.1±0.596	62.76	53.4

[a] All SLNs samples were prepared with 10% (w/w) lipid mixtures, in a ratio of CP:GS = (1:1) .

[b] A mixture of 1% Lecithin:SynperonicF68 (1:1) was added to the main nonionic surfactant

Different studies have proved that disaccharides are good stabilizers for nanoparticles [23, 24], and one of them, trehalose, has been extensively demonstrated to provide good stabilization of lyophilized SLNs [25, 26]. The stabilizer sugar should have high glass transition temperature (Tg) to maintain the temperature during lyophilization below it.

The lipid nanodispersion stability analysis, based on electrokinetic potential values, revealed that all the obtained free-SLNs and GTE loaded SLNs samples possess a good physical stability. As presented in Tab. 1, the zeta potentials of the SLNs were between -34 and -52 mV [27].

The stability of GTE loaded SLNs obtained is depending on surfactant used being higher when Tw20 was used. At the same time, the thermodynamic stability of GTE-SLNs containing Tw20 is increasing when the quantity of GTE is increasing. While for GTE-SLNs containing Tw80 the values of zeta potential obtained do not depend on the quantity of GTE. Nevertheless, the electrokinetic potential is lower when GTE is loaded onto SLNs, but these values show a good stability of the nanoparticles.

3.3. Electronic spectra

In order to attest the presence of GTE into SLNs and to observe its stability after encapsulation, electronic spectra of free-SLNs, GTE-SLNs have been registered and compared with that of GTE in bulk (Fig. 3).

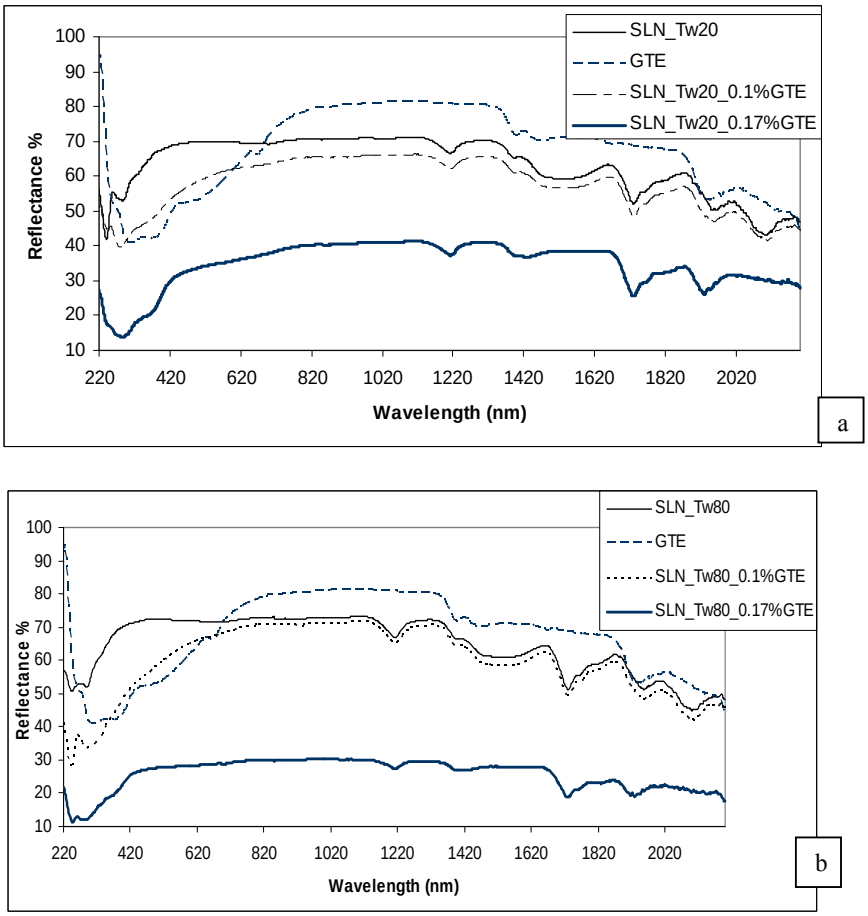

Fig. 3. UV-VIS absorption spectra of SLNs loaded with GTE

Green tea absorbs in the UV-Vis domain, with maximum absorbance at 312-364 nm doublet. The light yellow color of the powder is originating from polyphenolic compounds. UV-Vis reflectance spectra of the new GTE loaded SLNs prepared with the two surfactants Tw20 (Fig. 3a) and Tw80 (Fig. 3b) clearly evidence the presence of GTE into lipid nanoparticles, by means of shoulders in the above mentioned region accompaning the bands characteristic to surfactants in UV region.

3.4. DSC analysis

According to the Thomson equation, the melting point decreases while diminishing particle size [28]. Transformation of lipid bulk material into lipid nanoparticles leads to changes of the melting behavior of the lipid accompanied by potential occurrence of lower melting α and β modifications. Generally, the onset temperature and the melting peak of the lipid nanoparticles are approximately with 1 - 3 °C lower compared to the bulk material. Surfactants distributed to the melted lipid phase during the production process can distort the crystallization resulting in a lower melting enthalpy. Fig. 4 shows the corresponding DSC melting curves of loaded SLNs in comparison to the free-SLN and the parameters are given in table 1.

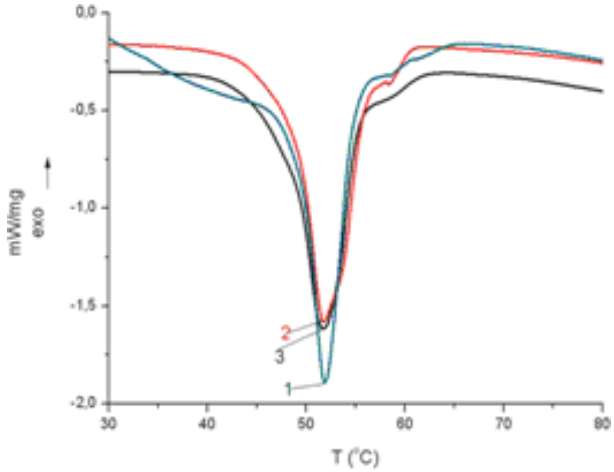

Fig. 4. DSC curves for GTE loaded SLNs, compared with free-SLN: 1 - free-SLN1; 2 – SLN-GTE2; 3 – SLN-GTE4

The GTE loaded SLNs prepared with two kinds of nonionic surfactants show similar endothermic behavior in an appropriate temperature range. This fact indicates that the type of nonionic surfactant used does not lead to significant changes in the lipid network. The melting temperatures of GTE loaded SLNs are shifted with approximately 1-2°C comparing with free-SLN, indicating a slight

increase in the particle size diameter. This observation is in good agreement with DLS analysis.

3.5. Antioxidant activity

The antioxidant properties of the developed SLNs were measured by the chemiluminescence method which is an appropriate technique for measuring the presence of free-oxygen radicals [29]. For comparative purpose the solutions of free-SLNs, GTE-SLNs, and GTE in bulk were exposed to a free radical generator system that releases free, high energy, intermediate radicals.

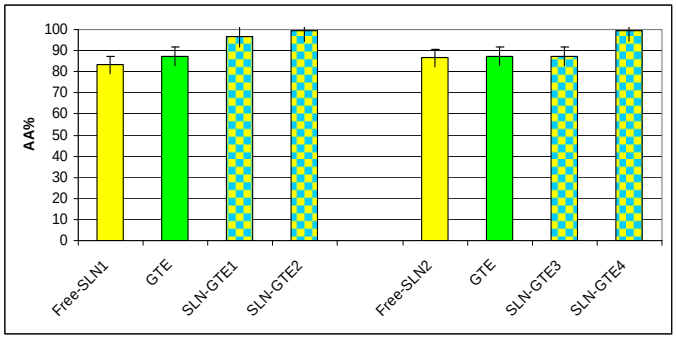

Fig. 5. Antioxidant activity of GTE-SLNs

The antioxidant capacity depends on various factors such as: type of surfactant, type and concentration of encapsulated extract, type of solid lipids. The chosen solid lipids for the synthesis procedure of SLNs were cetyl palmitate and gliceril stearate which lead to free fatty acids formation. It is known that the saturated fatty acids undergo a peroxidation process resulting in peroxy and hydroperoxides free radicals [30].

In case of all GTE loaded SLNs, the AA values are higher than free-SLNs or GTE in bulk as result of a synergistic effect between the complex structural lipid matrix and bioactive GTE encapsulated. The use of the two surfactants Tw20 and Tw80, as well as of two concentrations of bioactive extract (0.1 and 0.17%), does not lead to significant differences. The highest AA was obtained when Tw20 was used as surfactant and a concentration of 0.17% GTE (AA = 99.6%), while the smallest AA was obtained when Tw80 was used and a concentration of 0.1% GTE (AA = 87.3%).

3.6. Antibacterial activity

The antibacterial activity of the microorganisms with free-SLNs, SLNs-GTE and GTE in bulk was determined by measuring the size of inhibition zone

(IZ, mm) as a clear, distinct zone of inhibition surrounding agar wells. The reported results are average values of three experiments and are given with the standard deviation (SDs).

We have tested the unloaded and loaded green tea extract SLNs for their potential antibacterial activity against *Escherichia coli* bacteria which is accepted as an indicator of food products contaminations. All tested SLNs resist to this bacteria. Moreover, some of the GTE-SLNs are highly effective against bacteria development.

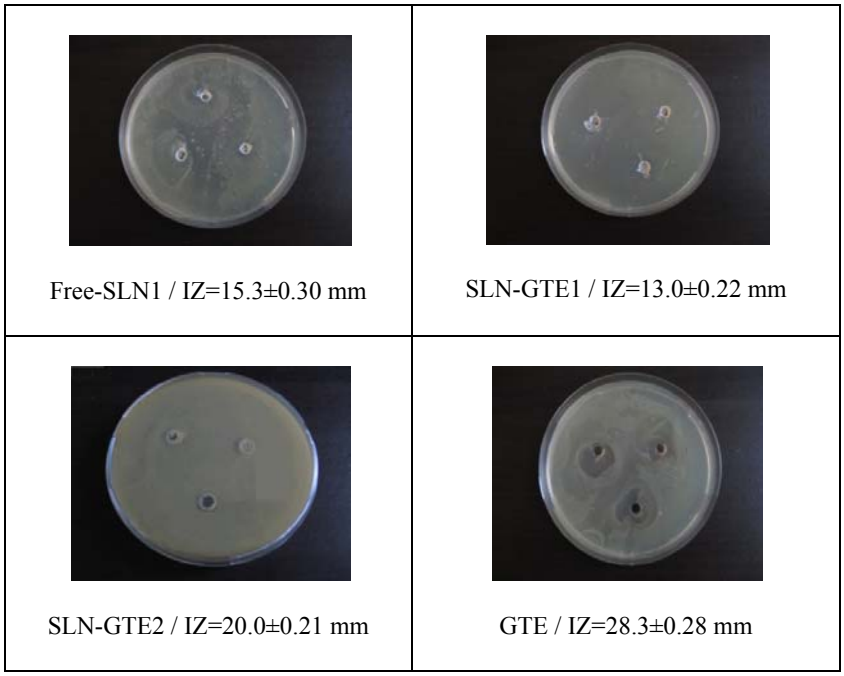

Fig. 6. Antibacterial activity of SLNs-GTE against *Escherichia coli* K 12-MG1655

The best antibacterial activity against the tested bacteria was observed for the sample SLN-GTE2 prepared with Tw20 and containing 0.17% GTE for which an inhibition zone of 20.00 mm was observed. The smallest effect was registered in SLN-GTE4 prepared with Tw80 and a concentration of 0.17% GTE. These facts are in good agreement with the results obtained from DLS and antioxidant activity. The bioactive extract green tea shows a good antibacterial activity with inhibition zone of 28.30 as compared to 11.17 mm obtained for the ethanol solution used as a reference.

4. Conclusions

Green tea loaded solid lipid nanoparticles (SLNs) with high antioxidant and significant antibacterial activities were successfully produced using the high shear homogenization technique. By the present method, the SLNs with small sizes were successfully produced, most of the nanoparticles being smaller than 200 nm and having narrow diameter distribution. The obtained SLNs show a good physical stability, with zeta potential values ranging from -34.1 mV to -52.3 mV. Differential scanning calorimetry shows that the melting temperatures of GTE-SLNs are shifted by 1-2°C comparing with free-SLN, which indicates that the GTE was loaded into SLNs. The evaluation of *in vitro* antioxidant properties has shown that all the obtained GTE-SLNs have high antioxidant activity, for both types of surfactants. Moreover, some of the tested samples were highly efficient against *E. coli* bacteria.

Acknowledgement

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R E F E R E N C E S

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