***In vitro* investigations on dopamine loaded Solid Lipid Nanoparticles**

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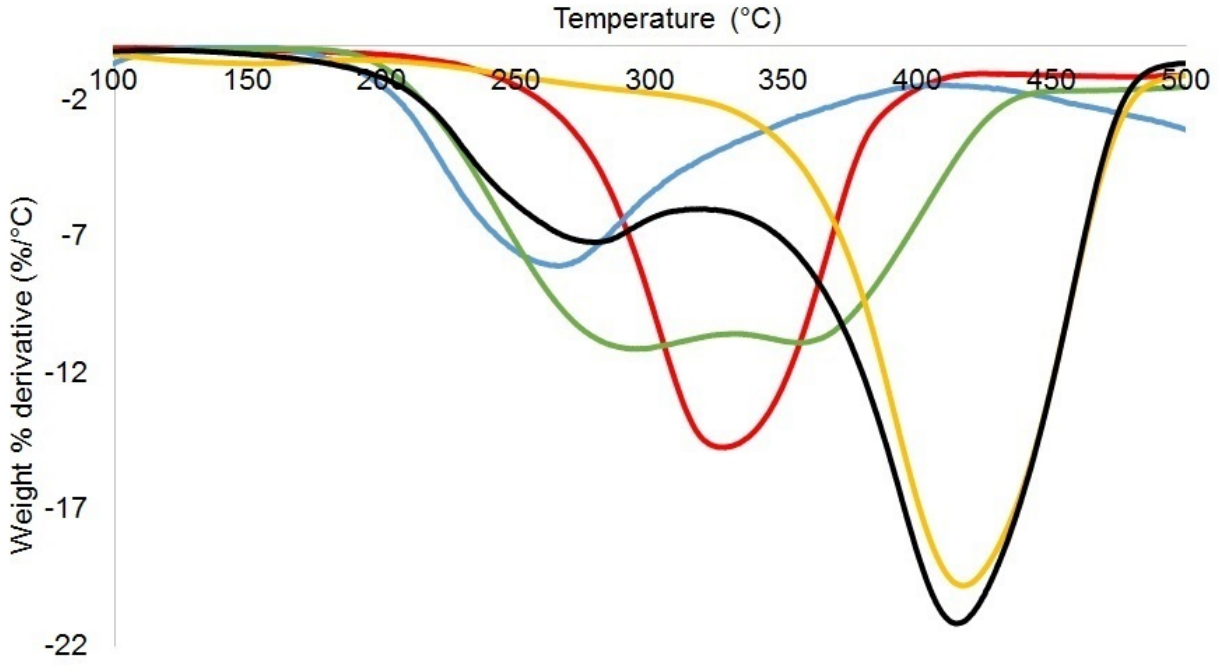
HIGHLIGHTS

1) Dopamine Solid lipid nanoparticles were prepared with the adhesive glycol chitosan
2) Glycol chitosan-Dopamine solid lipid nanoparticles showed 81% of neurotransmitter content
3) Thermal analysis evidenced high stability of lipid carrier and entrapped Dopamine
4) In 48 hours in buffer intact Dopamine was released from solid lipid nanoparticles
5) Overall, ATR, TGA and XPS analyses highlighted the effective-DA entrapment in SLN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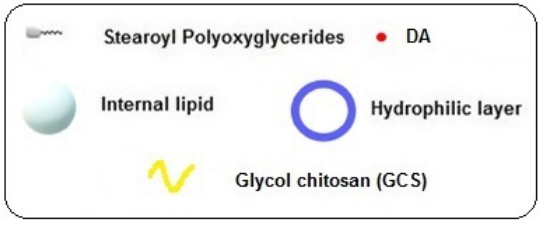
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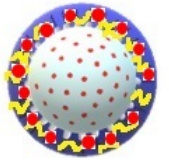
Hydrophilic layer
Glycol chitosan (GCS)



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Abstract

The progressive degeneration of nigrostriatal neurons leads to depletion of the neurotransmitter dopamine (DA) in Parkinson's disease (PD). The hydrophilicity of DA, hindering its cross of the Blood Brain Barrier, makes impossible its therapeutic administration. This work aims at investigating some physicochemical features of novel Solid Lipid Nanoparticles (SLN) intended to enhance DA brain delivery for PD patients by intranasal administration. For this aim, novel SLN were formulated in the presence of Glycol Chitosan (GCS), and it was found that SLN containing GCS and DA were smaller than DA-loaded SLN, endowed with a slightly positive zeta potential

value and, remarkably, incorporated 81% of the initial DA content. The formulated SLN were accurately characterized by Infrared Spectroscopy in Attenuated Total Reflectance mode (FT-IT/ATR) and Thermogravimetric Analysis (TGA) to highlight SLN solid-state properties as a preliminary step forward biological assay. Overall, *in vitro* characterization shows that SLN are promising for DA incorporation and stable from a thermal viewpoint. Further studies are in due course to test their potential for PD treatment.

Keywords: *Solid lipid nanoparticles, Dopamine, FT-IR/ATR, TGA, in vitro release*

Dopamine hydrochloride (PubChem CID: 65340)
Chitosan (PubChem CID: 129662530)
Acetic acid (PubChem CID: 450349)
Potassium Chloride (PubChem CID: 4873)
Sodium Chloride (PubChem CID: 5234)

HIGHLIGHTS

1) Dopamine Solid lipid nanoparticles were prepared with the adhesive glycol chitosan
2) Glycol chitosan-Dopamine solid lipid nanoparticles showed 81% of neurotransmitter content
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1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder with typical dyskinesia, due to low levels of the striatal neurotransmitter dopamine (DA). Unfortunately, DA depletion could not be treated with exogenous DA, because the latter cannot cross by itself the Blood-Brain Barrier (BBB), due to its hydrophilicity and ionized form at physiological pH. Therefore, currently, the so-called "dopamine (DA) replacement therapy" mainly consists of the administration of DA prodrugs or DA receptor agonists able to cross the BBB. In this context, L-Dopa still represents the gold standard treatment for PD but severe side effects, connected to its long-term treatment, induce several researchers to find new strategies to reduce PD symptomatology. Several papers have investigated the effect of intranasal administration of dopamine on its cerebral content by means of autoradiographic studies with [^{3}H] DA, microdyalisis experiments and DA nasal metabolism/nasal mucosal transport [1,2], their findings suggesting that the systemic absorption of the neurotransmitter by nasal administration can be considered lower enough than that occurring in the brain. As a consequence, also the corresponding systemic side effects should be more limited and, perhaps, less relevant. Based on these findings, it can be concluded that intranasal administration of DA can be employed for "DA replacement strategy therapy" of PD and can constitute an alternative to the current oral L-Dopa therapy.

Concerning novel formulations capable to deliver DA in the brain, we had previously investigated some DA-polymeric nanoparticles [3,4] in an attempt to supply it into the brain in a "masked" manner, capable to hide its unfavorable physicochemical properties. In addition, some DA receptor agonists for PD treatment have already been described for administration via lipid-based colloidal carriers, namely solid lipid nanoparticles (SLN) [5]. However, to the best of our knowledge, the potential of SLN carriers for DA delivery is still unexplored. Indeed, through SLN, the design of a nanoparticulate system, potentially able to release DA in a sustained manner, could be achieved. Thus, the present work aims at formulating novel SLN, loaded by DA, and to characterize them *in vitro*, in view of a potential intranasal administration for PD treatment. Herein, a DA entrapment efficiency around 80% was achieved, when SLN were formulated with the mucoadhesive polysaccharide Glycol Chitosan (GCS). Moreover, to determine DA content in the SLN, an enzymatic digestion of SLN was herein proposed, followed by HPLC analysis. The characterization of the novel SLN also involved Fourier Transform Infra-Red spectroscopy (FT-IR), in Attenuated Total Reflectance mode (ATR), as well as thermogravimetric analysis (TGA).

2. Experimental

2.1. Materials

Gelucire® 50/13 was kindly supplied by Gattefossè (Milan, Italy). Dopamine hydrochloride (DA), Polysorbate 85 (Tween® 85), acetic acid (HAc), Glycol Chitosan (GCS, MW 400 KDa according to the manufacturer) and carboxyl ester hydrolase (E.C. 3.1.1.1, 15 units/mg solid) from porcine liver were provided by Sigma Aldrich (Milan, Italy). Throughout this work, double distilled water was used. All other chemicals were of reagent grade.

2.2. Preparation of DA-SLN

DA-SLN and GCS-DA-SLN were prepared according to the melt-emulsification method [6, 7]. 60 mg of Gelucire® 50/13 were melted at 70 °C and, in a separate vial, 1.37 mL of a diluted HAc solution, 0.01%, w/v containing the surfactant (Tween 85®, 60 mg) were heated at 70 °C. In the HAc solution, 10 mg of DA were poured, prior to the addition of the resulting mixture to the melted lipid at 70 °C. Then, an emulsion was obtained by homogenization at 12300 rpm for 2 min, with an UltraTurrax model T25 apparatus (Janke and Kunkel, Germany). Finally, the nanosuspension was cooled at room temperature, the resulting SLN centrifuged (16,000 × g, 45 min, Eppendorf 5415D) and the obtained pellet was re-suspended in distilled water for further studies. For FT-IR and TGA analysis (see below) freeze-drying of SLN was performed for 48–72 h using a Lio Pascal 5P (Milan, Italy).

To allow SLN modification in the presence of GCS, the aqueous phase containing 1.37 g of a previously formed solution of GCS (5 mg/mL in HAc 0.01, v/v) was employed. Afterwards, the procedure was the same above reported for DA-SLN. Control SLN were either the ones without DA and GCS (namely, SLN) or the ones without DA, but containing GCS (namely, GCS-SLN).

2.3. Physicochemical properties and determination of Encapsulation Efficiency

Particle size and polydispersity index (PDI) of SLN were determined by a ZetasizerNanoZS (ZEN 3600, Malvern, UK) apparatus in photon correlation spectroscopy (PCS) mode. Each sample was diluted 1:1 (v:v) with double distilled water. Laser Doppler anemometry (ZetasizerNanoZS, ZEN 3600, Malvern, UK) was used to determine the zeta-potential values after dilution of the sample 1:20 (v:v) in presence of KCl (1 mM, pH 7).

To determine the loading efficiency of DA in the SLN, freeze dried SLN were firstly obtained by lyophilization of previously centrifuged and re-suspended particles. Afterwards, they were cleaved upon enzymatic digestion operated by esterase. Firstly, the enzyme was dissolved at 12 I.U./mL in phosphate buffer (pH 5) and aliquots of freeze dried SLN in the range 1-2 mg were incubated with 1 mL of the enzyme solution for 30 minutes in an agitated (40 rpm/min) water bath set at 37 °C (Julabo, Milan, Italy). Afterwards, samples were centrifuged (16,000 × g, 45 min, Eppendorf 5415D) and the resulting supernatant was analyzed by HPLC for DA content, according to the

HPLC method already described in [8]. The encapsulation efficiency (E.E.%) was calculated as follows:

E.E.% = DA in the supernatant after esterase assay/Total DA *100

where total DA is intended as the starting amount of neurotransmitter used for SLN preparation. Each measurement was performed in triplicate.

2.4. FT-IR/ATR

Pure materials and SLN samples were analyzed with a Spectrum Two PE instrument (PerkinElmer, USA) equipped with the universal ATR accessory (UATR, Single Reflection Diamond/ZnSe), transferring the freeze-dried formulations directly onto the crystal of the horizontal ATR accessory. FT-IR/ATR spectra were acquired from 400 to 4000 cm^{-1}, with a resolution of 4 cm^{-1}. The recorded signals were reported as transmittance percentages.

2.5. TGA

Thermal behavior of SLN samples, as well as their feed materials, was studied heating 5-10 mg of sample in nitrogen-saturated atmosphere, using a PerkinElmer TGA-400 instrument (PerkinElmer Inc., Waltham, MA). The heat range was set between 30 and 600°C at a flow rate of 20°C/ min. The gas flow was set at 20 mL/min. Thermograms (TG) with respective derivative curves (DTG) were recorded and data were analyzed using the software TGA Pyris series.

2.6. In vitro DA release from SLN

For *in vitro* release studies, DA-SLN and DA-GCS-SLN were freshly prepared and each pellet of the particles, originated from centrifuged SLN, was aliquoted in order to provide DA in the range of 1- 1.2 mg. Simulated Nasal Fluid (SNF) was prepared dissolving $CaCl_2 . 2H_2O$ (0.32 mg/mL), KCl (1.29 mg/mL) and NaCl (7.45 mg/mL) in water, providing a final pH value in the range 5-5.5.

In each Eppendorf tube 1.5 mL of SNF was poured and in *vitro* release test started once the abovementioned aliquots of SLN pellet were pipetted at 37 °C-40-rpm/min. At scheduled time-points (0, 2, 5, 17, 24 and 48h), the Eppendorf tube was centrifuged at 16,000 × g for 45 min, (Eppendorf 5415D, Germany), and the DA concentrations were determined in the resulting supernatants by HPLC, as previously described. All release experiments were carried out in triplicate.

2.7. Statistical analysis

Data from physic-chemical characterization properties of SLN formulations were statistically analyzed by one-way analysis of variance (ANOVA) and Bonferroni's post hoc test for multiple comparison at 99.9% confidence level was used throughout data analysis (GraphPad Prism v.4 software). Differences were considered statistically significant when $p < 0.05$.

3. Results and discussion

3.1. Characterization of DA containing SLN

The main physicochemical properties of DA-loaded SLN prepared by the melt-emulsification method with or without GCS are shown in Table 1. Firstly, being DA susceptible of spontaneous autoxidation when pH value of an aqueous solution is above 7, [8], a diluted solution of HAc (pH 3) [3,9] was adopted as aqueous phase during emulsion. GCS was selected to study its potential modulation role onto the final particle properties for the following reasons. Firstly, GCS has been already seen to be endowed with a mucoadhesive effect and this activity was also expected to take place in the nasal mucosa. Secondly, its P-glycoprotein efflux pump inhibition activity [10] could lead to a penetration enhancer mechanism at the level of the nasal mucosa. Finally, unlike parent chitosan, GCS is highly soluble in PBS, hence its precipitation at pH values higher than 6.5 is prevented. As shown in Table 1, the controls SLN and GCS-SLN, prepared without DA, were similar in particle size to the corresponding ones embedding DA (170 *vs* 171 nm, respectively). On the other hand, unloaded GCS-SLN displayed higher mean diameter as compared to GCS-DA-SLN (265 nm *vs* 147 nm, respectively, $p \leq 0.001$). It is not clear why such decrease in size for GCS-DA-SLN occurs but a similar situation was already observed in the case of DA-loaded chitosan-based nanoparticles [3]. As in the case previously examined, it can be hypothesized that, in the presence of neurotransmitter, a conformational reorganization of the GCS occurs, leading to GCS-DA-SLN shrinkage. However, it should be noted that the size of GCS-DA-SLN ≤ 200 nm is suitable for their transport to the brain via the olfactory and trigeminal nerve pathways [11]. Furthermore, for SLN and DA-SLN, zeta potential values were close to neutrality (*i.e.,* -4.9 mV and -2.0 mV respectively, see Table 1). Moreover, the presence of GCS in the formulation always led to slightly positive zeta potential values (*i.e.,* +8.5 mV and +5.2 mV), as expected for the presence of a positively charged polycation, i.e. GCS, in acidic medium. These zeta potential values near to neutrality could suggest physical instability over time of SLN but results from preliminary studies indicate that the particle size is maintained at least for one week. The detailed investigation in this regard will be reported in a forthcoming paper (manuscript in preparation). As reported in Table 1, the most promising result consists in the high DA E.E.%, that reached 81% in GCS-DA-SLN. Conversely, without the polycation GCS, the DA E.E.% was limited to 19%, as resulted from enzymatic digestion. According to manufacturer, Gelucire® 50/13 is composed of PEG-esters (stearoyl polyoxyl-32 glycerides), a small glyceride fraction and free PEG chains. The cleavage of ester linkages, enzymatically performed on lyophilized SLN, allowed us to calculate the DA amount effectively entrapped into each formulation. Available literature measures drug content in Gelucire® 50/13-based SLN subtracting the amount of drug released in the supernatant after centrifugation from the

total drug used for particle formulation. To the best of our knowledge, this is the first study in which esterase assay is performed onto Gelucire® 50/13-embedding SLN, leading to high DA E.E.%, not previously found in colloidal carriers for potential applications in PD (e.g. liposomes [8, 12], polymeric NPs based on polysaccharides [13,14]). To account for the marked increase in E.E.% observed for GCS-DA-SLN, it is necessary to consider the core-shell structure suggested for the Gelucire® 50/13-based SLN. In particular, they should be constituted by a hydrophilic shell of polyoxyethylene chains together with an internal lipid core comprising the stearoyl moieties [6]. In this model, the neurotransmitter could be adsorbed on the particle surface or entrapped in the hydrophilic shell as well as in the lipidic core as DA nanoemulsion, being Gelucire® 50/13 able to self-emulsify on contact with aqueous media. However, in the case of DA-SLN, leakage of the neurotransmitter adsorbed on the particle surface or entrapped in the hydrophilic shell could occur during sample manipulations lowering the corresponding E.E.%. Instead, as for GCS-DA-SLN, the observed marked increase in E.E.% may be due to the formation of a network structure resulting from hydrogen bonding and polar interactions involving the polyoxyethylene chains, GCS and the functional groups of DA. Besides the leakage of the neurotransmitter adsorbed on the particle surface, this network could hamper leakage of DA entrapped in the hydrophilic shell. Furthermore, the melt-emulsification method, in combination with the acidic environment adopted, led to DA protection during encapsulation in the SLN, as demonstrated by HPLC analysis, which did not reveal DA metabolites derived from autoxidation [8].

[Insert Table **1**]

3.2. FT-IR (ATR) characterization

To gain insight into the solid-state composition of SLN, FTIR and TGA analyses were performed on lyophilized particles. FT-IR is widely employed in literature to ascertain interactions between chemical compounds of drug formulations, or modifications of the encapsulated active molecules [15]. SLN FT-IR spectra, in ATR mode, are shown in Fig. 1. For all systems, the spectra appeared similar, except for the phenol O-H stretching at about 3270 cm^{-1} in the DA-loaded SLN samples, with and without GCS, as well as in the wavenumber range reported in the inset of Fig.1. A significant prominence of the absorption features relevant to the lipid matrix made of Gelucire® 50/13 was observed. However, an effective drug entrapment in the vehicles was obtained, especially when GCS was used in the formulation. Indeed, in DA-GCS-SLN system, both DA and GCS were clearly present, as demonstrated by the contributions in the spectra reported in the inset, in which the peak at 1560 cm^{-1} was ascribable to the N-H bending of protonated amine groups belonging to GCS (absent in neutral GCS, [16]). Moreover, the absorption at 1522 cm^{-1} can be referred to DA aromatic C=C stretching [17]. Finally, the absence of keto-carbonyl peaks in both formulations (i.e.,

DA-SLN and DA-GCS-SLN) at about 1740 cm^{-1}, typical of quinones [18], supported the hypothesis that DA was in 5,6-dihydroxyindole form, rather than in indole-5,6-quinone.

[Insert Figure **1**]

3.3. Thermogravimetric analysis

Additional thermogravimetric analyses (TGA) have been performed to determine the thermo-stability of SLN, as already reported in literature for other delivery systems targeting brain diseases [18, 19]. TG of the feed materials, recorded in inert nitrogen-saturated atmosphere from 30 to 600°C, are reported in Fig. 2A, while the relevant DTG are shown in Fig. 2B. The analysis revealed the following main degradation steps*: i)* for DA, the main pyrolytic event occurred at a T_{peak} = 327°C, with a residue at 600°C of 29%; *ii)* for GCS, the first and more important thermal degradation occurred at 268°C, with a high water/volatiles content (17%) and a residue at 600°C of 16%; *iii)* Gelucire ® 50/13 presented two thermal events of equal entity, at 291 and 355°C, with zero residue at 600° C; *iv)* Tween® 85 presented a decomposition at 416°C, with a water/volatiles content of 3% and a residue at 600°C equal to 4%.

As far as SLN formulations are concerned, the TG and the DTG are reported in Figures 2C and D. Even if an unequivocal attribution of the different thermal events could not be made, due to the complexity of the SLN systems and the overlapping of thermal events, some important speculations can be argued.

Firstly, TGA highlighted the thermal stability of DA-loaded SLN. As shown in Fig. 2D, a mass loss between 25 °C and 130 °C was observed only in GCS-containing SLN, due to the polysaccharide water content. Indeed, the TG curve relevant to DA-GCS-SLN presented a peculiar profile of water surface elimination between 30 and 80°C, with thermal stability between 100 and 160°C. From this result, it could be concluded that DA in the SLN formulation preserved its high stability against temperature. In all the SLN systems, the T_{onset} was between 137 e 164°C, and the moisture content was less than 2%, acceptable in the pharmaceutical industry [20]. The highest residue at 600°C was recorded for DA-GCS-SLN (residue of 11%), likely due to the simultaneous presence of DA and GCS (with respect to DA-SLN, which showed a residue of 1.6%).

[Insert Figure **2**]

3.4. In vitro release of DA from SLN

In vitro release of DA from DA-SLN and DA-GCS-SLN was studied in SNF, without enzymes, in view of a potential intranasal administration of SLN. The resulting DA delivery profiles are reported in Fig. 3. As shown, in 48h, less than 10% of DA was delivered in SNF from DA-SLN and DA-GCS-SLN. However, DA-SLN showed a burst effect within two hours followed by a slower release which reached the highest amount after18 h and then decreased to 4% of DA delivered after 48 h (Fig. 3). Instead, DA-GCS-SLN released the neurotransmitter continuously in a sustained manner leading to about 9 % of DA delivered after 48 h (Fig. 3). Such release profiles could suggest that, as for the DA-SLN, the neurotransmitter is localized on the surface of the particles which is responsible of the prompt burst effect within 2 h. The following slower release can be due to the DA localized in the hydrophilic shell or in the core of the particles. Conversely, for DA-GCS-SLN, the neurotransmitter should be localized either in the hydrophilic shell or in the core of the particles. Furthermore, up to 48h, intact DA was detected in SNF through HPLC analyses for both SLN. On the other hand, after 48h, for both DA-SLN and DA-GCS-SLN, a pink color was optically detected, likely due to autoxidation processes, starting after this time point and probably ascribable to the surface-allocated neurotrasmitter. It could be hypothesized that DA is delivered from SLN systems and then, according to a subsequent reaction, it could be oxidized after exposure to SNF for a long time. Overall, the amounts of neurotransmitter *in vitro* delivered by SLN, in absence of enzymes, are quite similar to the ones we already reported for uncoated DA-loaded liposomes [8]. This finding seems to elucidate that, using lipophilic carriers (i.e. liposomes and SLN), DA release was significantly low, irrespectively of the release medium because of the retention of the neurotransmitter in the carriers themselves. Conversely, when hydrophilic delivery systems are exploited (i.e. chitosan (CS) and chitosan derivatives-based nanoparticles [13]), they promptly release the active DA, leading to 30% of delivered active substance in the receiving medium in the frame time of 1-3 hours, typically with a burst effect. Overall, a sustained DA delivery to the brain is of great interest in clinical treatments, instead of an irregular release of the neurotransmitter, as occurs during frequent and prolonged administration of L-DOPA. Additionally, in the current *in vitro* study, the slow DA release allows the delivery of the neurotransmitter for a long frame time (*i.e.*, 48h) as compared with the 6h release that resulted when the same test was performed with chitosan NPs in PBS [13]. Moreover, DA-GCS-SLN protected DA from autoxidation, while chitosan NPs were unable to prevent this phenomenon, as demonstrated by the darker color of samples collected up to 6h.

[Insert Figure **3**]

4. Conclusions

Solid lipid nanoparticles administering the neurotransmitter DA have been investigated *in vitro* in presence and absence of the polysaccharide GCS. FT-IR (ATR), TGA analyses, as well as physicochemical characterization, including *in vitro* release assays, were the main tools herein explored for a better knowledge of these novel solid lipid nanosystems. The enzymatic degradation operated by esterases allowed the calculation of E.E.%, which resulted of 81% for SLN containing GCS. Overall, ATR and TGA analyses highlighted the high stability against temperature and the effective-DA entrapment (especially when GCS was employed) in the SLN formulations, respectively. Further investigations are currently ongoing, in order to assess the *in vivo* performances of these vehicles for nose-to-brain delivery approach applied to PD treatment.

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Conflict of interest

The authors declare that they have no conflict of interest.

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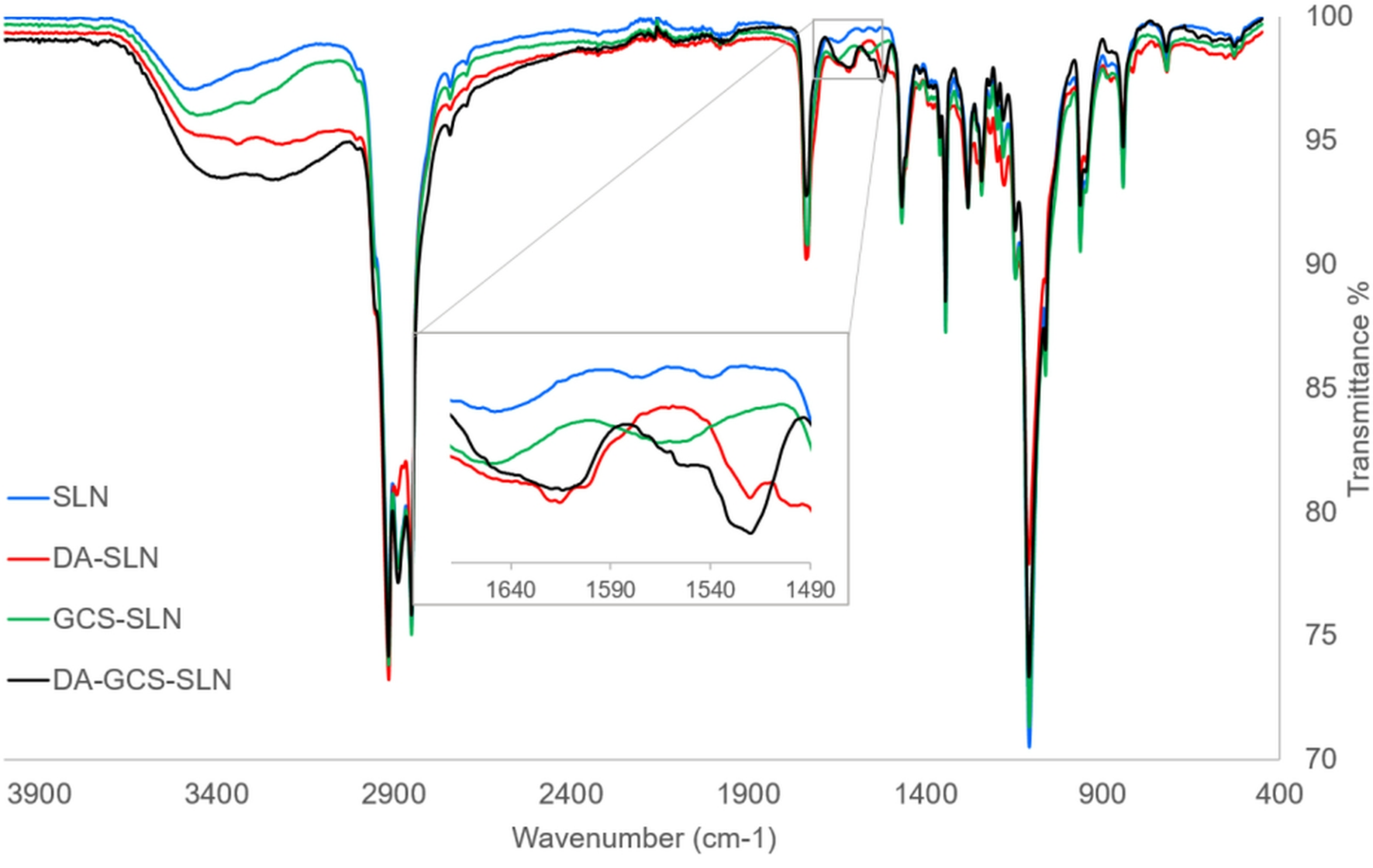
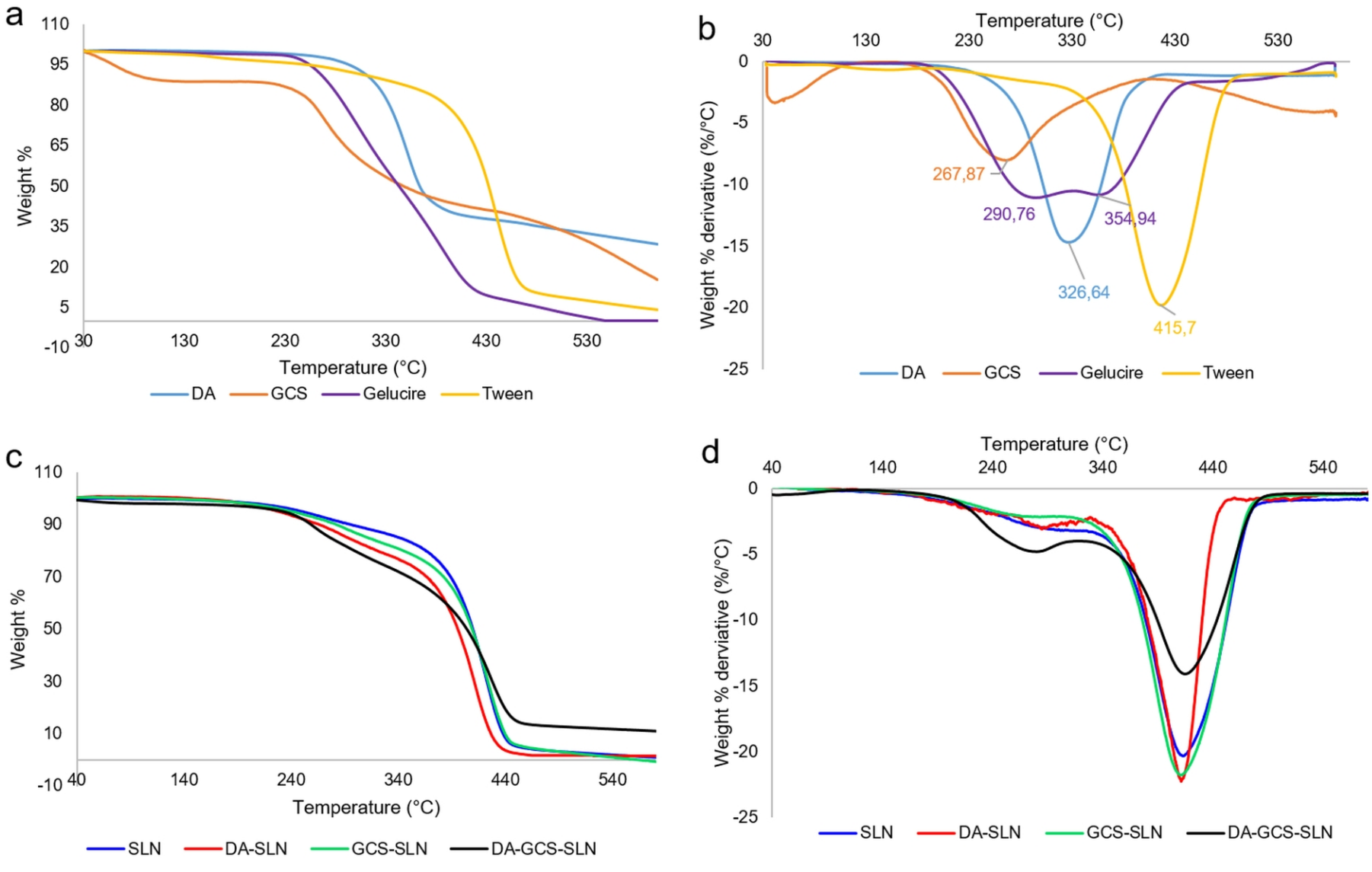
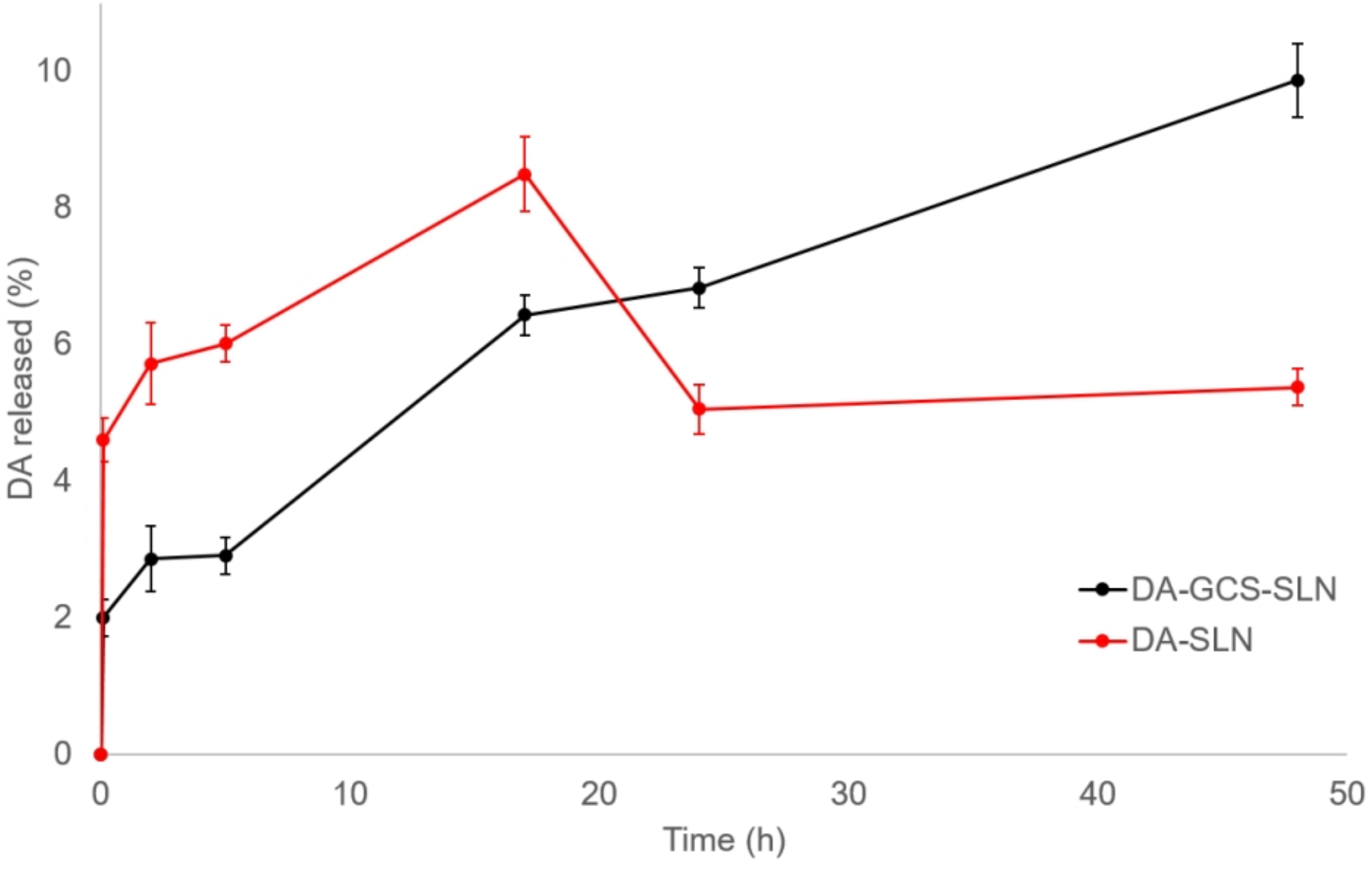

SLN
DA-SLN
GCS-SLN
DA-GCS-SLN
100
95
90
85
80
75
70
Transmittance %
3900
3400
2900
2400
1900
1400
900
400
Wavenumber (cm-1)
1640
1590
1540
1490


a
Weight %
Temperature (°C)
DA
GCS
Gelucire
Tween
b
Temperature (°C)
Weight % derivative (%/°C)
267,87
290,76
326,64
354,94
415,7
DA
GCS
Gelucire
Tween
c
Weight %
Temperature (°C)
SLN
DA-SLN
GCS-SLN
DA-GCS-SLN
d
Temperature (°C)
Weight % derviative (%/°C)
SLN
DA-SLN
GCS-SLN
DA-GCS-SLN


DA released (%)
10
8
6
4
2
0
0
10
20
30
40
50
Time (h)
DA-GCS-SLN
DA-SLN

In vitro investigations on dopamine loaded Solid Lipid Nanoparticles

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Table 1. Particle size, polydispersity index (PDI), zeta potential measurements, encapsulation efficiency of different formulations prepared. $^{**}p \leq 0.001$ for size and zeta is considered significantly different compared with size of GCS-SLN.

Formulation	Size (nm)	PDI	Zeta Potential (mV)	Encapsulation Efficiency (E.E.%)
SLN	170 ±31	0.43-0.47	-4.9±1.7	-
DA-SLN	171±6	0.25-0.27	-2.0.±0.7	19±3
GCS-SLN	265±5	0.46-0.53	+8.5±0.6	-
DA-GCS-SLN	147±24**	0.44-0.58	+5.2±1.7	81±2

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Bari, 28 February 2020

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Giuseppe Trapani: formal analysis;

Sante Di Gioia: data curation;

Laura Dazzi: data curation;

Elvira De Giglio: conceptualization and project administration; writing - review & editing;

Adriana Trapani: supervision; writing - review & editing.

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Captions to Figures

Figure 1. FT-IR (ATR) spectra of SLN formulations. In the inset, the wavenumber range evidencing the main differences occurring in GCS and/or DA presence in the relevant formulations.

Figure 2. TG (a and c) and DTG (b and d) thermograms performed in N_2 between 30 and 600°C of a) Dopamine, Glycol Chitosan, Gelucire ® 50/13 and Tween 85 and c) SLN formulations with or without DA and/or GCS.

Figure 3. *In vitro* release profiles of DA from DA-SLN (red) and GCS-DA-SLN (black) in SNF. Data are shown as mean ± SD (n=3, for each type of SLN).