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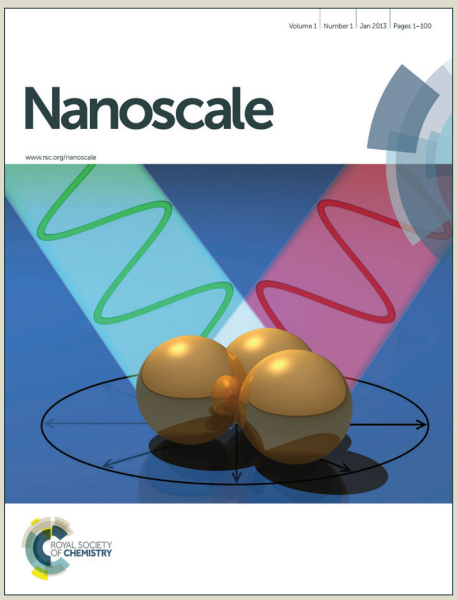

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Galantamine-loaded PLGA nanoparticles, from nano-emulsion templating, as novel advanced drug delivery systems to treat neurodegenerative diseases

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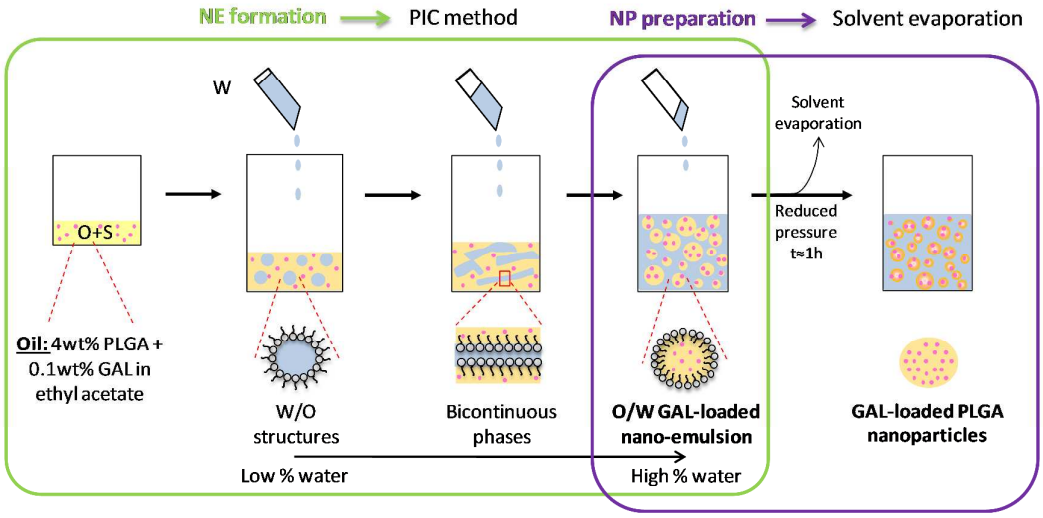
Schematic representation of the methodology used in this work to prepare GAL-loaded PLGA nanoparticles from nano-emulsion templating: nano-emulsification using the PIC low-energy method followed by solvent evaporation. This is the first time that GAL-loaded polymeric nanoparticles have been designed, demonstrating their suitability as novel therapeutic formulations for neurodegenerative diseases.



Abstract

Polymeric nanoparticles could be promising drug delivery systems to treat neurodegenerative diseases. Among the various methods of nanoparticle preparation, nano-emulsion templating was used in the present work, preparing galantamine-loaded nano-emulsions by a low-energy emulsification method followed by solvent evaporation to obtain galantamine-loaded polymeric nanoparticles. This approach was found suitable, since biocompatible, biodegradable and safe nanoparticles, with the appropriate features (hydrodynamic radii around 20nm, negative surface charge and stability higher than 3months) for their intravenous administration were obtained. Encapsulation efficiencies higher than 90wt% were obtained, with a sustained drug release profile, as compared to that from aqueous and micellar solutions. The enzymatic activity of the drug was maintained in 80% after its encapsulation in nanoparticles that were non-cytotoxic at the required therapeutic concentration. Therefore, novel galantamine-loaded polymeric nanoparticles have been

designed for the first time, using the nano-emulsification approach and showing the appropriate features to become advanced drug delivery systems to treat neurodegenerative diseases.

Keywords

Polymeric nanoparticles; nano-emulsion templating; galantamine; neurodegenerative diseases; advanced drug delivery systems

1. Introduction

Neurodegenerative diseases (ND) include a variety of sporadic and/or familiar conditions characterized mostly by a persistent loss of neuronal activity, being Alzheimer's disease (AD) and Parkinson's disease (PD) the most common nowadays. Their prevalence and incidence have experienced an exponential tendency to increase in the last years, mostly due to an aging of the population, which will continue increasing in the next decades; thus resulting in high social and economical costs [1-4]. Their pathogenesis is characterized by an accumulation, aggregation and modification of human proteins, alterations in tissue homeostasis, immunological damages and effects due to viral infections [4]. Current pharmacotherapy for ND consists on a combination of drugs with different targets and mechanisms of action. For example, for the treatment of AD, two classes of drugs have been approved by the FDA: second generation cholinesterase inhibitors, such as galantamine hydrobromide (Reminyl) for the treatment of mild to moderate AD, and memantine (N-methyl-D-aspartate

antagonist) for the treatment of moderate to severe dementia [1,5-6]. Although these therapies have been proved to improve patients' quality of life, they still lack of efficacy. For example, only 25-30% of AD patients responded to these therapies; which was mainly attributed to the presence of the Blood-Brain Barrier (BBB) that limits the entrance of exogenous components to the central nervous system (CNS). In addition, current therapies produce severe side effects, such as functional impairment and medical commorbidity (e.g. gastrointestinal tract adverse effects, headache) [1,7]. Therefore, novel drug delivery systems to enhance the localized therapeutic effects of these drugs and decrease their adverse effects are required.

Polymeric nanoparticles (NP) are a promising alternative as advanced drug delivery systems for ND, since they can: 1) be administered using a systemic route of administration and intended to reach to any human organ because of their nanometric size, 2) protect drugs from enzymatic degradation, 3) solubilize high amounts of lipophilic drugs, 4) produce a controlled and sustained drug release leading to an extended duration of action, 5) target the actives specifically to the BBB, thus increasing the drug pharmacological activity at the CNS and reducing side effects as well as the dosage frequency, improving patients compliance, and 6) they can be prepared using easily scalable and cost effective methodologies [1, 8-11]. Among the diverse methodologies to prepare NPs, nano-emulsion templating from O/W nano-emulsions is advantageous. Nano-emulsions are fine emulsions with droplet sizes typically between 20 – 200nm, showing high kinetic stability and transparent to translucent appearance [12]. Since they are thermodynamically unstable systems, nano-emulsion formation requires an energy input, which can be from

external or internal sources. In the so-called condensation or low-energy emulsification methods, the internal chemical energy of the system is used to trigger emulsification. An interesting low-energy method in view of biomedical applications is the phase inversion composition (PIC) method, which is performed at constant temperature (e.g. room temperature) and mild process conditions, thus being advantageous not only to obtain high encapsulation efficiencies and sustained drug release, but also in terms of safety issues and method development in mild conditions (e.g. 25ºC) [12- 14]. If the oil component of an O/W nano-emulsion is composed of a preformed polymer dissolved in a volatile solvent, by solvent evaporation, polymeric nanoparticles are obtained [12,14-15]. In addition, the preparation of polymeric nano-emulsions by nano-emulsion templating has the advantage of allowing the control of the physico-chemical properties (e.g. size, surface charge), which depends not only on the nano-emulsion components (e.g. surfactant) but also on nano-emulsion composition (e.g. percentage of oil phase, presence of additives) and process variables (e.g. temperature) [12]. Previous studies of our group showed the high versatility of the nano-emulsification approach, being nano-emulsions prepared by the PIC method, to obtain tailored nanoparticles with the required physico-chemical properties [14,16-17], depending on the objective of the study. For example, nanoparticle size can be tuned to the desired sizes by changing diverse parameters of the template nano-emulsion. the addition of electrolytes in the aqueous phase of the nano-emulsion template produced a marked reduction of nano-emulsions and nanoparticle sizes. Small nanometric polymeric nanoparticles of radii lower than 50 nm were obtained using the nano-emulsion templating system PBS 0.16M (W)/ polysorbate 80 (S) / [4wt%

PLGA in ethyl acetate] (O), at high aqueous phase contents (> 85wt%) and O/S ratios corresponding to the central part of the nano-emulsion domain [17], for example. The type of polymer used and the surfactant were other parameters that influenced droplet sizes [14,16-17].

In this context, the objective of the present work was to take advantage of the nano-emulsification strategy to prepare galantamine-loaded polymeric nanoparticles as novel advanced drug delivery systems to treat neurodegenerative diseases. Polymeric nanoparticles were prepared with biocompatible and biodegradable components, using poly-(lactic-co-glycolic acid) (PLGA) as the polymer and polysorbate 80 as the surfactant, since both are FDA approved for parenteral administration [18-23]. Galantamine was chosen as a “gold standard” drug, currently used to treat neurodegenerative diseases. Although galantamine has been approved for the treatment of mild to moderate AD, to our best knowledge, a therapy with advanced nanoparticles carrying this drug to improve its pharmacotherapeutic activity has not been reported yet. Therefore, this study represents an innovative contribution to the field of therapeutic treatments for neurodegenerative diseases.

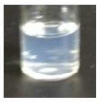
2. Results

2.1. Nano-emulsion and nanoparticle formation and characterization

O/W PLGA nano-emulsions were prepared by the PIC method, using the PBS 0.16M (**W**) / polysorbate 80 (**S**) / [4wt% PLGA + 0.1wt% GAL in ethyl acetate] (**O**) system. Nano-emulsions with 90 wt% electrolyte solution (0.16M) and an O/S weight ratio of 70/30 were chosen to prepare PLGA nanoparticle

dispersions by solvent evaporation. The physico-chemical properties of the templating GAL-loaded nano-emulsion and the corresponding GAL-loaded PLGA nanoparticles are shown in Table 1. The visual appearance of all dispersions is nearly transparent, with a characteristic bluish shine (Table 1), indicating a small nanometric droplet / nanoparticle size, which was confirmed by DLS measurements, resulting in droplet radii of around 33 nm for the GAL-loaded nano-emulsion. The size was reduced after the evaporation of the solvent, as expected [14,16], resulting in nanoparticle radii around 22 nm. The surface charge of all dispersions was negative due to the carboxylic groups of the PLGA polymer.

Table 1: Characterization of the 90wt % electrolyte solution (0.16M), 70/30 O/S ratio GAL-loaded nano-emulsion (NE(GAL)) and the corresponding nanoparticles (NP(GAL)) by means of DLS and electrophoretic mobility determinations.

Sample	Visual appearance	Hydrodynamic radius(nm)	ζ potential (mV)
NE(GAL)		32.73 ± 0.39	-24.15 ± 6.74
NP(GAL)		21.50 ± 0.25	-11.18 ± 0.89

2.2. Galantamine entrapment efficiency, loading and release

The GAL entrapment efficiency, assessed by the indirect filtration/centrifugation method described in Section 2.2.5, was higher than 98wt% (Table 2).

Regarding GAL loading, the values obtained were around 57mg/100mL, and the drug concentration was around 6 mg/g NP (Table 2).

Table 2: Parameters of the entrapment efficiency, drug loading and drug concentration of GAL encapsulation.

Encapsulation efficiency (EE) (%)	98.47 ± 0.43
Drug loading (mg GAL/g PLGA)	56.87 ± 3.48
Drug concentration (mg GAL / g NP)	6.01 ± 0.01

GAL release was studied from nanoparticle dispersions, as described in Section 2.2.6, and for comparison purposes, from aqueous and micellar solutions (with 3% polysorbate 80 surfactant). Figure 1 shows the percentage of GAL released as a function of time. The release from the aqueous and micellar solutions was fast and completed within approximately 24 and 50 hours, respectively. However, for the nanoparticle dispersion, only 80% of the drug was released within 120 hours (5 days). The release from the micellar solution was slower than that from the aqueous solution, indicating a reservoir effect of the surfactant which could be caused by an obstruction of the diffusion due to the presence of micelles. The GAL release from the nanoparticle dispersion was considerably slower than that from the aqueous and micellar GAL solutions.

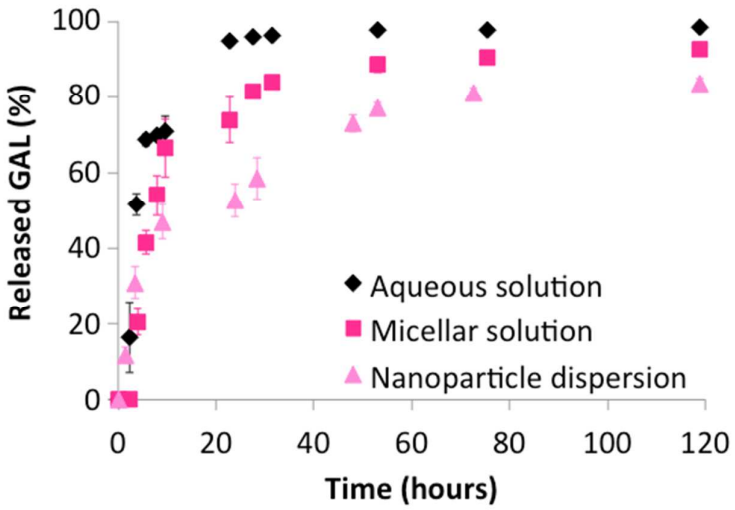


Figure 1: GAL release from a) an aqueous solution; b) a micellar solution with 3 wt. % surfactant and c) a nanoparticle dispersion.

2.3. *In vitro* biocompatibility of nanoparticles

In vitro cytotoxicity of the GAL-loaded nanoparticles (NP(GAL)) was determined using the MTT colorimetric assay [24-25], at 3 mg/mL and 0.3 mg/mL PLGA concentration, in two cell lines. As Figure 2 shows, at 0,3 mg/mL nanoparticle concentration viability was high (around 100%) in all samples (GAL-loaded and unloaded nanoparticles) in both cell lines. Increasing the concentration to 3mg/mL resulted in the same viability values except for GAL-loaded nanoparticles, which produced 50% cytotoxicity on SH-SY5Y cells, although they were non-cytotoxic at this high concentration on HeLa cells.

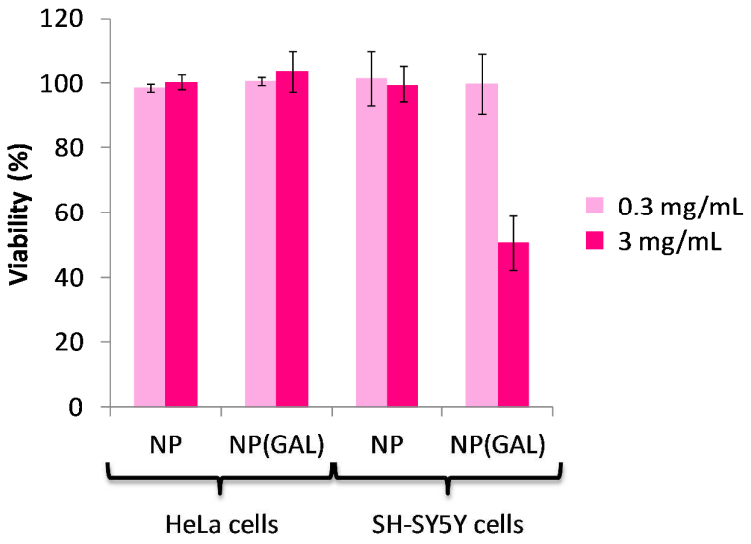


Figure 2: Viability (in %) of HeLa cells and SH-SY5Y cells after their incubation with NP(GAL) at increasing concentrations, by means of the MTT assay. Non-loaded nanoparticles viability is also included for comparative purposes.

2.4. Inhibition of the acethylcholinesterase

The acetylcholinesterase activity was studied for the NP(GAL), using GAL aqueous and micellar solutions, as positive controls and a PBS solution and non-loaded nanoparticle dispersion as negative controls. The results are presented in Figure 3. The inhibition of the acetylcholinesterase is plotted as a normalized percentage between the maximum and the minimum inhibition. NP(GAL) achieved an inhibition of around 80wt % of acetylcholinesterase, as compared with both positive controls; thus indicating that after its encapsulation into the nanoparticles, GAL maintains its activity.

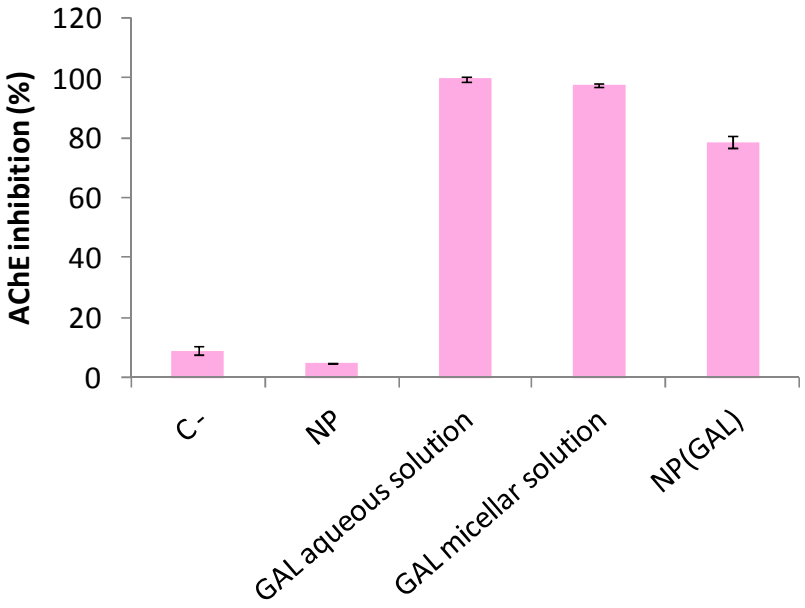


Figure 3: Acetylcholinesterase (AChE) inhibition (in %) for the tested samples with a same GAL concentration.

3. Discussion

The current study demonstrates for the first time the possibility to develop GAL-loaded PLGA nanoparticles with interesting properties to be used as advanced drug delivery systems for the symptomatic treatment of AD. These nanoparticles were prepared by nano-emulsion templating, a very versatile technology to obtain polymeric nanoparticles for diverse biomedical applications [12-14]. To our best knowledge, this is the first study that reports the encapsulation of GAL in polymeric nanoparticles, specifically, in PLGA nanoparticles.

As described in the results section, the designed polymeric nanoparticles have radii around 22 nm (Table 1) and negative surface charge. These nanoparticles

were previously prepared without any loading [17], with sizes and surface charges very similar to those obtained in the present study. Therefore, the incorporation of GAL did not influence the physico-chemical properties of template nano-emulsions (size, surface charge and stability), which determine the properties of PLGA nanoparticles. It is noteworthy that nanoparticle sizes achieved in the present work are very small as compared with previous studies [9,11,26-28], which was attributed to the nano-emulsification approach of nanoparticle preparation. These small nanometric sizes could be advantageous when considering *in vivo* future therapies, not only for the decrease on the immune detection and further nanoparticle elimination from the blood circulation, but also for the crossing of the Blood-Brain Barrier (BBB), required for the local treatment of neurodegenerative diseases, advantageous in terms of enhanced therapeutic effect and reduced adverse effects [9-10,26,29]. In addition, although they are not highly surface charged, as required for the electrostatic stabilization [1, 30]; the presence of the surfactant enables their steric stabilization, making these nanoparticles stable for, at least, 3 months, as reported in our previous study [17].

It is also worth noting the limitations of working with a single solvent as the oil component in terms of nano-emulsification and achievement of enough drug encapsulation, as pointed out in previous bibliography [31]. However, in the present work, this limitation has been overcome using ethyl acetate as the only solvent in the oil component of the template nano-emulsions and the PIC emulsification method, an easily scalable methodology to be used in the pharmaceutical industrial production [13].

Another important achievement of this work deals with GAL loading and release. A very high encapsulation efficiency (higher than 98%) was obtained, as described in results section (Table 2). In addition, the total GAL added to the sample remained intact after nanoparticle preparation, being the vast majority encapsulated inside nanoparticles, as described in results section (Table 2). It is worth noting that GAL was only quantified in the filtrated fraction, since traces of polysorbate 80 in the original and non-filtrated nanoparticles interfered with the detection wavelength, not enabling GAL quantification. However, to confirm that this indirect filtration – centrifugation methodology is useful to quantify encapsulated compounds, a mass balance was performed with another compound, a fluorescent dye that can be detected at a wavelength where polysorbate 80 does not interfere. The mass balance enabled to confirm that 94% of the initial encapsulated compound was found after the filtration. Therefore, degradation and / or adsorption of the encapsulated compound during filtration can be ruled out (see Table S1, SI). The high entrapment efficiency was attributed to the nano-emulsification approach to prepare nanoparticles, as previously described for other drugs [16] together with the low topological polar surface area of galantamine [32], thus preferring the location in the nanoparticle core rather than in the nanoparticle surface. In the nano-emulsion template strategy presented here, the drug is added to the oil component of the nano-emulsion, prior to nano-emulsion formation. Therefore, the entire drug is encapsulated in the oil droplets if its solubility in the aqueous phase is low and high in the oil component. Although some drug molecules may diffuse with the solvent during the evaporation process, most of them remain imbibed in the nanoparticle polymeric matrix. Since no previous studies on GAL

encapsulation in polymeric nanoparticles exist, GAL encapsulation efficiency was compared with encapsulation efficiencies of other drugs in PLGA nanoparticles [21]. GAL encapsulation efficiency reported in the present work was higher, due to the above-mentioned reasons (low GAL water solubility and emulsion templating). GAL encapsulation was previously tested in nanoliposomes, but encapsulation efficiencies [33] were lower than those reported here. In addition, compared with tacrine encapsulation efficiency, another model anticolinergic drug, from chitosan nanoparticles coated with 1wt% polysorbate 80 after their formation, encapsulation efficiencies achieved here were also markedly higher (99% in the present paper vs 15% for tacrine in previous studies [34]).

Although GAL encapsulation efficiency was high, GAL loadings were not enough to achieve therapeutic concentrations (8 – 32 mg GAL/day depending on the severity of the disease of each patient) [2,35-37] neither in mice models nor in clinical assays, using the intravenous route of administration, due to the requirement of high volumes of administration. For this reason, a concentration step would be required before *in vivo* administration of nanoparticles. The concentration step, followed by nanoparticle purification to achieve the physiological conditions (pH = 7.4 and 300mOsm/kg), as described in our previous publication [16], did not modify nanoparticles physico-chemical properties. Therefore, these nanoparticles could be easily concentrated to achieve therapeutic concentrations.

Concerning GAL release, as indicated in the Results (discussion of Figure 1), the release from the nanoparticle dispersion was sustained as compared with the micellar and aqueous solutions. After 24 hours, for example, practically

100% of the GAL had been released from the aqueous solution, 75% from the micellar solution and only 60% from nanoparticle dispersions. The sustained release from nanoparticle dispersions had been previously reported in preceding studies using the same kind of nanoparticles to encapsulate other drugs, loperamide and dexamethasone [16-17]. However, a difference was observed for GAL, the initial release suffers a burst that was not observed for loperamide or dexamethasone. This fact could be related to the size of the nanoparticles. While NP(GAL) have hydrodynamic radii of around 22nm, loperamide and dexamethasone-loaded nanoparticles have bigger hydrodynamic radii, around 100nm and 130nm, respectively. These results are in good agreement with previous studies, which described a reduction of the initial burst release on larger nanoparticles [38].

To study more deeply GAL release, the experimental release data were fitted to Fickian diffusion theoretical curves, in order to determine the apparent diffusion coefficients [39] (Figure S2, SI). The results are presented in Table 3. The release from the aqueous solution is about half an order of magnitude faster than that from the micellar solution and more than an order of magnitude faster than from the nanoparticle dispersion, thus indicating a sustained release, namely by nanoparticles. As discussed before, since previous studies of GAL encapsulation in polymeric nanoparticles do not exist, these apparent diffusion coefficients were compared with the release of tacrine from chitosan nanoparticles coated with 1wt% polysorbate 80. This release followed also a Fickian diffusion pattern, but tacrine was markedly faster released from chitosan nanoparticles than GAL from designed nanoparticles [1]. Nevertheless, compared to the release of dexamethasone from the same type of

nanoparticles [39], GAL release is nearly two orders of magnitude faster, as are the release from the micellar and the aqueous solutions. This can be attributed to the higher solubility of GAL to an aqueous receptor solution as compared to that of dexamethasone (Table S3, SI). However, the fact that GAL release was sustained as compared to the aqueous solution makes nanoparticles appropriated to be used as advanced drug delivery systems for a novel promising pharmacological approach; not only to control the pharmacokinetics of this drug, but also to decrease the number of drug administrations required for the patients, since the effects of the drug would last for a prolonged period of time, thus increasing patients compliance [40].

Table 3: Apparent diffusion coefficients of GAL from nanoparticle dispersions and for an aqueous and a micellar solution.

	Diffusion coefficient (m^2/s)
Aqueous solution	$1.05 \cdot 10^{-8}$
Micellar solution	$4.22 \cdot 10^{-9}$
Nanoparticle dispersion	$1.92 \cdot 10^{-9}$

Nanoparticle cytotoxicity is a key factor to take into account when designing them for biomedical applications. In this work, the cytotoxicity was tested in two cell lines: HeLa cells, as a versatile model cell line, widely used in nanomedicine tests, and SH-SY5Y cells, as a model of neuroblastoma cells, representing a cellular type corresponding to the target tissue, the central nervous system. The cytotoxicity of GAL-loaded nanoparticles is low at the concentrations tested, except for SH-SY5Y cells at the highest concentration (3mg/mL). The cytotoxic effects described for GAL-loaded nanoparticles at

3mg/mL would not be a drawback for nanoparticles *in vivo* administration, for two main reasons. On the one hand, they only produced toxicity in the neuroblastoma cell line (SH-SY5Y cells). On the other hand, although a concentration step is required for the *in vivo* administration, as justified above, when intravenously administered, nanoparticles would found themselves in the bloodstream, resulting in an immediate decrease on nanoparticles concentration. Therefore, as diluted nanoparticles (Figure 2), they would not be toxic when intravenously administered.

As described in our previous study [39], these nanoparticles do not need a purification step after their preparation, since biocompatible components have been used in their preparation, including surfactant, polysorbate 80. This is advantageous, as direct administration of GAL-loaded nanoparticle dispersions avoids time consuming purification steps and derived aggregation problems. In addition, in a previous work, we demonstrated that these PLGA nanoparticles did not produce damaging effects on the blood: they did not produce hemolysis, neither marked protein adsorption, neither a high activation of the immune response [41].

It is noteworthy that the pharmacological activity of GAL is maintained up to 80% when encapsulated in nanoparticles. The loss of a 20wt % of activity could be due to the obstruction that nanoparticles represent for the drug to be in close contact with the enzyme. In addition, this decrease on the pharmacological activity of GAL is in good agreement with the sustained GAL release: at early times, only a very small fraction of GAL has been released. Therefore, these GAL-loaded nanoparticles could be advantageous among current therapies for various reasons. Taking into account that these nanoparticles are intended for

the local drug delivery specifically in their target organs, and considering the central nervous system, the same therapeutic effects could be obtained than those with the current drug preparations, at the same equivalent doses, which produce systemic effects. Due to the sustained drug release, the pharmacological activity of GAL-loaded NP would be prolonged, thus maintaining the anticholinergic activity in the central nervous system.

It is also remarkable to point out the desired localized drug action of GAL. Although in this work a study on nanoparticle biodistribution was out of the scope, we previously demonstrated the capability of PLGA nanoparticles to cross the BBB and produce analgesic central effects because of the permeabilizing effect of the polysorbate 80 (which is part of the formulation, not added afterwards as in most reported studies), increased when nanoparticles were functionalized with an specific targeting moiety (unpublished results). Therefore, it could be hypothesized the nanoparticles behavior *in vivo.* Thanks to their small nanometric size, their low negative charge and the presence of polysorbate 80 as an active targeting of GAL-loaded nanoparticles to the BBB, a BBB crossing is expected. Consequently, GAL-loaded nanoparticles designed in here are expected to produce a central inhibition of the acetylcholinesterase, enhancing therapeutic effects and reducing side effects [1]. In addition, due to the sustained drug release, as discussed above, a longer therapeutic effect is hypothesized when intravenously injected *in vivo*, thus reducing the dosage frequency and enhancing patients' quality of life. Therefore, these GAL-loaded designed nanoparticles are expected to represent an improved pharmacological therapy in a near future.

4. Experimental

4.1. Materials

Poly (lactic-co-glycolic acid), Resomer 752H in the following abbreviated as PLGA (polystyrene equivalent molecular weight PSE - MW ~ 10,000 g/mol, as determined by Gel Permeation Chromathography) was purchased from Evonik. The lactic acid to glycolic acid ratio of the PLGA used was 75/25 and the end groups were free carboxylic acids. Ethyl acetate used as the organic volatile solvent was purchased from Merck. Polysorbate 80 is a nonionic surfactant kindly provided by Croda. It is a yellow liquid at room temperature and has an HLB value of 15 [42]. Galantamine hydrobromide (GAL) (Figure 4) is an antiapoptotic drug, with an enzymatic activity as inhibitor of the acetylcholinesterase, as well as allosteric inhibitor of the nicotinic receptors [2,6]; which was chosen as a “gold standard” for the symptomatic treatment of neurodegenerative diseases, such as AD and PD (MW = 368.27 g/mol) [2,6]. Sodium dihydrogenphosphate hydrate ($NaH_2PO_4 \cdot H_2O$), Disodium monohydrogenphosphatedihydrate ($Na_2HPO_4 \cdot 2H_2O$), Sodium hydroxide (NaOH) and Ortophosphoric acid (H_3PO_4) that were used to prepare the aqueous electrolyte solution (PBS 0.16M), were purchased from Sigma-Aldrich. Water (W) was MilliQ filtered (resistivity of 18.2 MΩ•cm; surface tension = 72.8 nN/m).

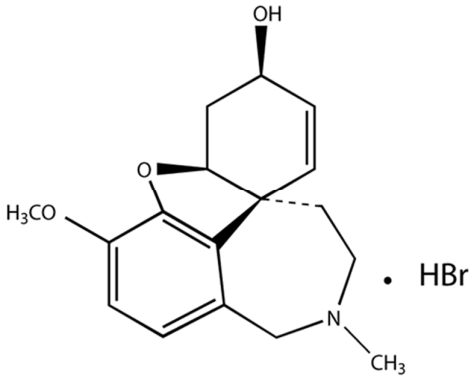


Figure 4: Chemical structure of GAL.

4.2. Methods

4.2.1. Nano-emulsion and nanoparticle preparation

Nano-emulsions with 90wt% W and 70/30 oil/surfactant (O/S) ratio were prepared by the phase inversion composition method (PIC), at 25ºC, as previously described [13-14]. The aqueous phase was PBS 0.16M, the surfactant, polysorbate 80, and the oil phase consisted of 4wt % PLGA and 0.1wt% GAL in ethyl acetate. Briefly, the phase inversion composition (PIC) method consist on: Firstly, preparation of the oil components (PLGA and GAL dissolved in ethyl acetate), secondly, the oil components are mixed with the surfactant, and finally the aqueous component is stepwise added to this mixture, under continuous stirring, resulting in a change on the spontaneous surfactant curvature and the formation of O/W nano-emulsions. The formation of polymeric nanoparticles was achieved by solvent evaporation, under controlled conditions: reduced pressure and 25ºC, during 1 hour [14].

4.2.2. Droplet and nanoparticle size characterization

The mean droplet size and size distribution of nano-emulsions and nanoparticle dispersions were determined by dynamic light scattering (DLS) using a spectrometer (LS Instruments, 3D cross correlation multiple-scattering) equipped with a He-Ne laser (632.8 nm) with variable intensity. Measurements were carried out at a scattering angle of 90º, and at a constant temperature of 25ºC in triplicates, without diluting the samples. Data, by intensity distribution were treated by cumulant analysis, from the particle sizes obtained [43]. The characterization was performed by the Nanostructured Liquid Characterization Unit of the Spanish National Research Council (CSIC) and the Biomedical Networking Center (CIBER-BBN), located at IQAC-CSIC.

4.2.3. ζ potential determination

The surface charge of nano-emulsions and nanoparticles was assessed by Electrophoretic mobility measurements at 25ºC with Zetasizer NanoZS instrument (Malvern Co. Ltd., UK), equipped with a He-Ne red light laser (λ = 633 nm). This instrument measures the velocity of the particles in the electric field (electrophoretic mobility) using the laser Doppler electrophoresis method [44]. The zeta potential (ζ) was calculated from the electrophoretic mobility applying the Smoluchowski equation (Equation (1)):

$$\mu = \frac{\zeta \cdot \varepsilon_r \cdot \epsilon_0}{\eta} \qquad (1)$$

where ε_r represents the relative dielectric constant of water, ε_0 is the vacuum permittivity and η is the viscosity of the liquid. As this equation is only valid if the conductivity is low, samples were diluted 1:20 with distilled water.

4.2.4. Galantamine analysis

GAL concentration was analyzed by fluorescence measurements (Cary Eclipse Fluorescence Spectrophotometer, Varian), since this drug has a maximum emission peak at wavelength around 310 nm when excited at 280 nm (emission measured between 280 – 400 nm, at 500 V fluorescent laser intensity, 25ºC, diluting the samples with milliQ water when the signal was saturated; detection limit under these conditions = 0.5 μg/mL) [45]. Calibration curves were plotted with solution of a known GAL concentration, to correlate each fluorescent signal to GAL concentration. At least three measurements of each sample were performed and GAL concentration was found from the calibration curve.

4.2.5. Determination of the entrapment efficiency and drug loading

The efficiency of GAL entrapment and drug loading in the nanoparticle dispersion was determined by centrifuging drug-loaded nanoparticles using centrifugal filter units with a molecular weight cut-off of 3,000 Daltons (Amicon Ultra-15 Centrifugal Filter Unit with Ultracell-3 Membrane, Millipore, Billerica, Massachusetts, USA) and analyzing the filtrate by fluorescence. The centrifugation was carried out at 2,300 g during 75 minutes at 25ºC. The drug

entrapment efficiency and drug loading were calculated by using Equations (2) and (3), respectively.

$$Entrapment\ \ efficiency = \frac{initial\ \ drug\ \ amount\ \ -\ free\ drug}{initial\ \ drug\ \ amount} \times 100 \qquad (2)$$

$$Drug\ \ loading = \frac{amount\ \ of\ \ drug\ \ in\ nanopartic\ les}{100mL\ \ of\ \ nanopartic\ les} \times 100 \qquad (3)$$

4.2.6. Galantamine release experiments

Release experiments were performed *in vitro* by the dialysis bag method (e.g. 28] using porous cellulose ester bags (Spectra/Por® Float-A-Lyzer® G2, Spectral Medical Industries, Inc., California, USA, MCWO of 3.5 - 5 kDa). Release of GAL from an aqueous solution, a micellar solution and from nanoparticle dispersions was studied using about 2 g of sample filled in Float-A-Lyzers and submerged in 50 mL of PBS 0.16M (receptor solution), under continuous stirring. The diffusion cells consisted of cylindrical thermojacketed glass vessels connected to a water bath at 25ºC and covered, to avoid loss of the receptor solution due to evaporation [46]. The assays were performed for about five days. Periodically, 125 μL aliquots of the sample were withdrawn to quantify the amount of delivered drug. The volume removed was replaced with the same volume of PBS to keep the volume constant and to ensure sink conditions throughout the experiment [39]. Drug quantification was performed by fluorescence as described in section 2.2.4. For apparent diffusion coefficient determination, experimental results were fitted to theoretical curves based on Fick's law of diffusion [16,39,46-47].

4.2.7. In vitro cytotoxicity assessment

Two cell lines, HeLa and SH-SY5Y, were used for *in vitro* experiments. Cell cultures were grown as described following: HeLa cells, a cervical cancer cell culture widely used as a model cell line, were grown in 96-well plates at an initial density of 6,000 cells per well in 200μL of growth medium for 24 hours. The growth medium consists on Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 wt. % FBS (fetal bovine serum), penicillin and streptomycin. SH-SY5Y cells, a model neuroblastoma cell line selected as a model neural cell line, were grown as explained for HeLa cells, with the exception of the growth medium, that consisted on Dulbecco's modified Eagle's medium (DMEM) supplemented with 15 wt. % FBS (fetal bovine serum).

The metabolic activity of the cells was measured using the MTT test (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); to determine the cytotoxic effect of nanoparticles [24]. Polymeric nanoparticles were incubated at different concentrations. After 4 hours of incubation at 37ºC, the medium containing the incubated samples was removed and replaced for growth medium, which was further incubated for around 18 hours. After this time, 25 μL of MTT at a concentration of 5mg/mL in PBS were added directly in each well and cells were incubated 2 additional hours, at 37ºC. Then, 100 μL of DMSO were added. After 15 minutes agitation, absorbance was measured at 560 nm wavelength on microplate ELISA reader (SpectraMax M5 luminometer, Molecular devices). Results were expressed as a relative percentage to control cells [24-25].

4.2.8. Determination of the acetylcholinesterase activity

The protocol followed for the acetylcholinesterase (AChE) activity measurement is a modification of the Ellman's protocol [48]. In brief, AChE was prepared at 20mM (5U/mL) in the indicated phosphate buffer: 8mM K_2HPO_4, 2.3mM NaH_2PO_4, 0.15M NaCl, 0.05% Tween20, at pH 7.6. Samples to test were diluted in the same buffer at the required concentrations. 50μL of the AChE and 50μL of the samples were added at a well of a 96-well plate and incubated for 30 minutes at room temperature (25ºC). Then, 100μL of the substrate were added to each well and incubated for 3 minutes. The substrate consist on 0.1M Na_2HPO_4, 0.5mM 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB), 0.6mM acetylcholine iodide (ATCI), in milliQ water, at pH 7.5. Finally, absorbances were read at 405 nm [49]. Each sample was studied, at least, three times. Results are given as a mean normalized value +/- the standard deviation.

5. Conclusions

This work represents a novelty concerning basic research in the nanomedical field, required for the development of future pharmacological therapies: the encapsulation of GAL in PLGA nanoparticles, which has not been described previously neither in PLGA nor in other polymeric nanoparticles. The advantage of the nano-emulsion templating technology used to prepare the GAL-loaded PLGA nanoparticles over other preparation methods lies in the versatility of the PIC emulsification method to produce the template nano-emulsions using biocompatible and biodegradable components with the desired features. Using this approach, non-cytotoxic GAL-loaded nanoparticles have been obtained with

high encapsulation efficiencies and sustained drug release, maintaining GAL pharmacological activity, thus enabling long-term therapeutic effects. This is the first time that GAL-loaded nanoparticles have been designed with the appropriate features to become promising advanced drug delivery systems against neurodegenerative diseases.

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