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Application of the "in-oil nanoprecipitation" method in the encapsulation of hydrophilic drugs in PLGA nanoparticles

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Abstract

Three hydrophilic model drugs with different characteristics and molecular weights, namely protamine sulphate, diclofenac sodium and N^6-Cyclopentyladenosine (CPA), were nano-encapsulated in poly(D,L-lactide-co-glycolide) (PLGA) using a novel "in-oil nanoprecipitation" method recently developed for the purpose. Although the same settings were used for all three model drugs, the drug loading efficiency was greatly dependent on their chemical-physical characteristics, being considerably higher for protamine (roughly 93%), intermediate for diclofenac (roughly 50%), and very low for CPA (roughly 7%). The resulting particle size and drug release rates were also strictly model-drug dependent. In the attempt to improve the characteristics of the CPA-loaded nanoparticles, the respective effects of adding an excipient (lauric acid) and substituting PLGA with poly(D,L-lactide) polymer (PLA) were investigated by measuring *in vitro* drug release and drug degradation kinetics in human whole blood. The results indicate that the proposed method seems promising for the nanoencapsulation of hydrophilic drugs in hydrophobic polymers, and easily modifiable to suit molecules that are difficult to incorporate into a polymeric matrix.

Keywords: Poly(lactic/glycolic) acid (PLGA, PLA); Nanoprecipitation; Diclofenac Sodium; Protamine; N^6-Cyclopentyladenosine; Lauric acid.

Chemical compounds: Diclofenac sodium (PubChem CID: 5018304); N^6-Cyclopentyladenosine (PubChem CID: 657378); Poly(glycolide-co-lactide) (PubChem CID: 7139).

1. INTRODUCTION

Polymeric nanoparticles are promising drug delivery systems, being able to cross biological barriers and release their contents in a controlled fashion. Furthermore, nanoparticulate systems are able to induce sustained drug release kinetics and more favourable drug accumulation in tumours and the CNS with respect to traditional delivery systems [1-3].

One of the most widely used polymers in nanoparticulate drug delivery systems is poly(lactic-co-glycolic acid) (PLGA), a biodegradable polyester that has been approved for human administration by the FDA. Formulated as biodegradable colloidal particles, PLGA is an excellent carrier, not only for drugs, but also for genes, proteins and various other macromolecules [4-6]. As it is soluble in several organic solvents, PGLA has been prepared by various nanoparticle formulation methods, including nanoprecipitation, a simple technique relying on rapid diffusion of organic solvent into a non-solvent phase, resulting in the precipitation of small colloidal particles [7]. Nanoprecipitation is generally performed using acetone as the water-miscible solvent and water as the non-solvent [8], and the hydrophobic nature of PLGA molecules makes this nanoparticle-loading method very efficacious for lipophilic compounds, including several anticancer and neuroactive drugs [9-15]. However, the PLGA nanoprecipitation method does present significant limitations in terms of encapsulating water soluble molecules, mainly due to the hydrophobicity of the polymer and the rapid partitioning of hydrophilic drugs in the aqueous phase, a phenomenon that is extremely difficult to prevent [16-19]. Therefore, despite the unquestionable advantages of the nanoprecipitation method, including ease of execution, fast processing time and high batch-to-batch reproducibility, its poor applicability to hydrophilic molecules makes further amendments a necessity [20, 21].

Hence, modifications to the standard nanoprecipitation method have been proposed, including: (i) incorporation of salt additives, (ii) pH variation and (iii) alternative solvents.

1 Although these do induce significant improvements in encapsulation efficiency and, consequently,

2 drug loading [17, 22, 23], most of these adaptations were designed for specific drugs with particular

3 chemical-physical features and, therefore, cannot be considered universally applicable to all types

4 of hydrophilic drugs. Indeed, this high specificity is one of the main shortcomings of polymeric

5 nanoparticle methods, as the different encapsulation processes are liable to vary in efficiency,

6 depending on the water solubility, molecular weight (MW) and/or octanol/water partitioning

7 coefficient (Log P value), etc., of the drug in question [24].

8 In order to widen the range of applicability of nanoencapsulation, therefore, and to expand

9 the somewhat scarce literature on the topic, we recently proposed "in-oil nanoprecipitation" (ION),

10 a method based on the use of a mixture of cottonseed oil and Tween-80 as the non-solvent phase.

11 This technique enabled the incorporation of a polar, slightly hydrophilic, anti-ischemic model drug,

12 N^6-Cyclopentyladenosine (CPA), in the hydrophobic polymeric matrix of PLGA nanoparticles [25].

13 To build on these promising results, here we set out to compare them with those obtained by

14 extending the ION encapsulation technique to two further hydrophilic model drugs with very

15 different physical-chemical characteristics (chemical structure, MW, water solubility and Log P

16 values) to CPA, namely diclofenac sodium and protamine sulphate. To determine the influence of

17 the type of drug on the standardized ION method, the main formulation parameters were kept

18 constant, and nanoparticles loaded with each of the model drugs were compared in terms of size,

19 drug content and drug release. As the encapsulation efficiency previously reported for CPA was

20 found to be relatively poor with respect to the other two drugs selected for this study, further

21 formulation and characterization was performed to investigate the kinetics and evaluate the stability

22 of its nanoparticles in fresh human whole blood.

23

24

25

1 **2. MATERIAL AND METHODS**

2 **2.1 Materials**

3   N^6-Cyclopentyladenosine (CPA, MW: 335.36 Da; water solubility: 0.67±0.04 mg/mL; log

4 P: 1.22 [26]. N^6-Cyclohexyladenosine (CHA), diclofenac sodium (MW: 318.13 Da; water solubility

5 50 mg/mL, Log P as free acid: 4.21), protamine sulphate (MW: 7000 D; water solubility:10

6 mg/mL), Tween 80 and lauric acid (LA) were obtained from Sigma Aldrich (St. Louis, MO, USA).

7 Poly(D,L-lactide-co-glycolide) (PLGA 50:50; Resomer RG 504, Mw 60-75 kDa) and Poly(D,L-

8 lactide) (PLA homopolymer; Resomer R203H MW of 16–28 kDa) were obtained from Boehringer-

9 Ingelheim, (Ingelheim am Rhein, Germany). High-performance liquid-chromatography (HPLC)-

10 grade methanol, acetonitrile and water were obtained from Merck (Darmstadt, Germany). The

11 reversed-phase column (Hypersil BDS C-18 5U cartridge column, 150 mm x 4.6 mm i.d.) was

12 obtained from Alltech Italia Srl BV (Milan, Italy). All other reagents and solvents were of analytical

13 grade (Sigma).

14

15 **2.2 Preparation of nanoparticles**

16   Drug (protamine sulphate, diclofenac sodium or CPA, 5 or 10 mg) and polymer (PLGA or

17 PLA, 125 mg) were dissolved in 2.5 mL of acetone (acetone phase) in the presence or not of lauric

18 acid (100 mg). Meanwhile, 2.6 g of Tween 80 was dispersed at room temperature into 40 mL of

19 cottonseed oil (oil phase). The acetone phase was then added drop-wise into the oil phase under

20 mechanical stirring (900 rpm), which was continued until the acetone had completely evaporated

21 (~3 h). The nanoparticles thus formed were separated from the oil phase by vacuum ultra-filtration,

22 using a polycarbonate holder (SM 16510; Sartorius, Gottingen, Germany) equipped with a

23 polypropylene filter (cut off 0.2 µm; Pall Corporation, Ann Arbor, Michigan, USA). The oil-free

1 nanoparticles were recovered and suspended in deionized water by two vortex cycles (30 seconds,

2 Zx3 , VELP Scientifica, Milan, Italy), and, finally, freeze-dried over 24 h (Lyovac GT2; Leybold-

3 Heraeus, Hanau, Germany). Empty nanoparticles were prepared according to the procedure

4 previously described, omitting the presence of the drug.

5

6 **2.3 Particle size measurement and morphological analysis**

7 The particle size, size distribution and polydispersity index were measured via photon

8 correlation spectroscopy (PCS) using a ZetasizerNano ZS (Malvern, Worcs., UK) after

9 reconstitution of the nanoparticles (NP) using Milli-Q water. Each measurement was repeated three

10 times for each sample. The data are the results of the measurements on three batches of each type of

11 nanoparticle, whose shape and morphology were analysed by scanning electron microscope (SEM)

12 (XL-40 Philips, The Netherlands). Before the analysis, freeze-dried nanoparticles were purified

13 from Tween-80 using a vacuum ultrafiltration method, in order to prevent interference [25]. After

14 purification, 10 µL of each nanoparticle suspension was mounted onto metal stubs and dried for 48

15 h. The stubs were then coated with a 10-nm thick layer of gold palladium alloy (Emitech K550

16 Sputter Coater, Emitech Ltd., Ashford, Kent, UK).

17

18 **2.4 HPLC analysis of CPA**

19 HPLC was used to quantify CPA in all samples generated by the experimental procedures

20 [25]. The chromatographic apparatus consisted of a modular system (Model LC-10 AD VD pump

21 and Model SPD-10A VP variable wavelength UV-Vis detector; Shimadzu, Kyoto, Japan) and an

22 injection valve with 20-µL sample loop (Model 7725; Rheodyne, IDEX, CA, USA). The detector

23 was set at 269 nm. Separation was performed at room temperature on a 5 µm Hypersil BDS C-18

1 column (150 mm × 4.6 mm i.d.; Alltech Italia Srl, Milan, Italy), equipped with a guard column

2 packed with the same Hypersil material. The mobile phase consisted of a ternary mixture of

3 acetonitrile, methanol and 10 mM acetate buffer (pH 4) at a ratio of 5/50/45 (v/v/v). The flow rate

4 was 0.8 mL/min, and the retention times of CPA and CHA were 4.5 and 6.4 min, respectively. CHA

5 was employed as an internal standard for blood samples. 10 µL of samples were injected into the

6 HPLC system for CPA quantification.

7 Data acquisition and processing were carried out using Class-VP software (Shimadzu) on a

8 personal computer . The chromatographic precision for CPA and CHA water solutions were

9 evaluated by repeat analysis (n=6) of the same sample, which yielded R.S.D. (relative standard

10 deviation) values of 1.55, 1.69 and 1.65 for 0.335 µg/mL (1 µM) CPA, 3.35 µg/mL (10µM) CPA

11 and 3.49 µg/mL (10 µM) CHA, respectively. A calibration curves of peak areas *versus*

12 concentration of CPA solution was generated on samples ranging from 0.5 to 10 µM; in this range

13 the calibration curve was linear (n = 6; r > 0.999; p< 0.0001). The LOD for CPA solutions was

14 0.0012 µg/mL (3.6 nM - 0.012 ng/injection) with a signal-to-noise ratio of 3:1. The LOQ value was

15 0.004 µg/mL (12.0 nM - 0.040 ng/injection) with a signal-to-noise ratio of 10:1.

16

17 **2.5 Drug loading analysis**

18 Drug loading was determined by dissolving an accurately weighed amount of nanoparticles

19 (about 5 mg) in dichloromethane (500 µL) and adding 5 mL of deionized water to solubilize the

20 drugs. Loading analysis was performed on the supernatant, after evaporation of the dichloromethane

21 phase (2 h), and centrifugation (14,000 x g for 5 min, Beckman Microfuge 18 Centrifuge, München,

22 Germany). To quantify CPA loading, 10 µL of the supernatant was injected into the HPLC system.

23 Preliminary experiments indicated that concentrations of CPA in water solutions were not altered

1   by the presence of a 10% (v/v) of CH_2Cl_2 after vortex and centrifugation processes both in the

2   absence and in the presence of equivalent amounts of unloaded nanoparticles.

3   For protamine-loaded and diclofenac-loaded nanoparticles, drug quantification was

4   performed by UV-vis spectroscopic analysis of the supernatant at 276 nm for diclofenac, and by

5   Bio-Rad DC Protein Assay (Bio-Rad laboratories, Milan, Italy) at 750 nm for protamine. The

6   absorbance of the drug containing solutions was converted to the amount of drug on specially

7   prepared standard calibration curves constructed using the supernatants of the corresponding

8   unloaded nanoparticles (n=6), in order to eliminate any possible interference in the measurements.

9   Drug loading was determined as [27]:

10    $$\text{Drug loading} = \frac{mass\ of\ drug\ in\ nanoparticles}{mass\ of\ nanoparticles} \times 100$$

11   Percentage encapsulation efficiency was calculated as [28]:

12    $$EE\ \% = \frac{entrapped\ amount\ of\ drug\ per\ g\ nanoparticles}{theoretical\ amount\ of\ drug\ per\ g\ nanoparticles} \times 100$$

13

14   **2.6 "In vitro" drug release studies**

15   For the release of CPA, an accurately weighed amount of nanoparticles (about 0.6 mg) was

16   added to 15 mL of deionized water and immediately dispersed by sonication. The samples were

17   maintained at 37°C, and stirred mechanically (100 revs/min). Aliquots (200 µL) were withdrawn at

18   fixed time intervals, filtered upon centrifugation at 13,000 ×g, using Microcon filter devices (YM

19   30, Millipore Corporation, Bedford, MA, USA), and injected (10 µL) into the HPLC apparatus for

20   CPA quantification.

1   To quantify the release of protamine and diclofenac, six Eppendorf tubes of each, containing

2   5 mg of nanoparticles resuspended in 1 mL of deionized water, were prepared and pre-warmed to

3   37° C. After being thoroughly sealed, the Eppendorfs were placed in a thermostat bath at 37° C and

4   maintained in vigorous agitation. One Eppendorf of each sample was taken at predetermined time

5   intervals (15, 30, 60, 180, 240 minutes and 24 hours), and the content subjected to centrifugation

6   (14,000 x g for 5 min, Beckman Microfuge 18 Centrifuge, München, Germany); the supernatant

7   was used for the quantitative determination, as described in the previous paragraph.

8   The amounts of nanoparticles and deionized water were chosen in order to ensure sink

9   conditions for release studies on all samples. The release of each sample was evaluated in triplicate,

10   and data are expressed as means ± standard deviation.

11

12   **2.7 Kinetic experiments in human whole blood**

13   Compounds were incubated at 37° C in three mL of heparinized fresh whole blood obtained

14   from healthy volunteers (final CPA concentrations of 10 µM). The concentration of nanospheres

15   was 1 mg/mL. The samples were shaken continuously, and at regular time intervals 100 µL was

16   taken, haemolysed, and extracted twice with 900 µL of ethyl acetate, after the addition of 50 µL of

17   3 M sodium hydroxide, 50 µL of internal standard (40 µM CHA), and 200 µL of dichloromethane.

18   The organic layer was evaporated to dryness by N_2 flow. 100 µL of mobile phase was added, and,

19   after centrifugation 10 µl was injected into the HPLC system for CPA and CHA detection.

20   The accuracy of the method was determined by recovery experiments. In particular, the

21   percentage recoveries of 10 µM CPA and 10 µM CHA were calculated by comparing the peak areas

22   of the compounds extracted from test samples (n = 6) with those obtained by an equivalent

23   concentration of the analytes dissolved in the mobile phase. The average recoveries ± S.D. of CPA

24   and CHA were 63.7 ± 2.5 and 71.3 ± 2.7%, respectively. The CPA concentrations were therefore

9

1 referred to peak area ratios of CPA and 10 µM CHA, employed as an internal standard. The

2 precision of the method based on peak area ratio was represented by an R.S.D. value of 1.5.

3 The calibration curve of peak area ratios *versus* concentration of CPA was generated with blood

4 samples ranging from 0.5 to 10 µM; in this range it was linear (n = 6; r > 0.998; p< 0.0001).

5 The half-life of CPA was calculated from an exponential decay plot of the peak-area ratio

6 between the compound and internal standard, expressed as percentage, *versus* incubation time,

7 using the computer programme GraphPad Prism (GraphPad, San Diego, CA, USA).

8

9 **2.8 Statistical analysis**

10 The statistical significance was assessed by the t test or one-way ANOVA (GraphPad

11 PrismProgram). Differences were considered to be significant at a level of $P < 0.05$.

12

13 **3. RESULTS**
14
15 **3.1 Physical-chemical characterization of drug-loaded nanoparticles**

16 A summary of the characteristics (size, PDI, drug loading and encapsulation efficiency) of

17 the drug-loaded PLGA nanoparticles formulated using the ION method are shown in Table I. As a

18 yield of between 90 and 98% was obtained in each case, through purification under the same

19 conditions by a 0.2 µm filter, the amount of small particles able to pass through the filter can be

20 considered negligible. However, under the same formulation conditions, the resulting nanoparticles

21 were of considerably different sizes (from 190 to 296 nm), depending on the drug in question.

22 Specifically, the particles prepared from protamine had the largest diameter (about 290 nm),

23 regardless of the initial drug amount, while CPA-loaded and diclofenac-loaded nanoparticles were

1   very similar in size (about 190 nm). CPA data was obtained from our previous study [25], and is

2   reported in Table I for comparison.

3   Two samples of each model drug (5 or 10 mg) were formulated, but the size of the

4   recovered nanoparticles appeared to be dependent on the type of drug rather than on the amount

5   employed during the formulation process. Indeed, increasing the amount of drugs in the formulation

6   processes induced no significant variations in PDI values, which appeared to be high (≥ 0.33) for all

7   preparations reported in Table I, despite the monomodal particle size distribution (data not shown).

8   As shown in Figure 1, which reports the unloaded and CPA-5-PLGA samples as representative

9   examples, the particles were not spherical and very irregular in shape.

10   An increase in the initial amount of the drug in the formulation phase did, however, lead to

11   an increase in drug loading, albeit accompanied by a small reduction in the EE% (Table I), as

12   expected from previous reports in the literature [8, 29]. This reduction in EE% appeared to be

13   dependent on the greater loss of drug during the formulation phase since, as already observed, the

14   size of the particles did not increase with the initial amount of drug, and the loading capacity

15   remained constant. Nevertheless, the initial amount of drug being equal, the model drugs were

16   incorporated to very different extents. In fact, while protamine was incorporated with a very high

17   EE (roughly 93%), diclofenac displayed lower EE values (47–55%), and CPA very low indeed

18   (about 7%).

19

20   **3.2 Drug release**

21   Biphasic drug release profiles were found for all the nanoencapsulated drugs considered,

22   including CPA-5-PLGA and CPA-10-PLGA, as reported in our previous study [25], (Figure 2); in

23   all cases release slowed down after an initial rapid phase (burst effect). The burst releases observed

24   from all batches were compared (Table I), and for each model drug it was observed that the greater

25   the actual loading, the smaller the burst release. Indeed, protamine-loaded samples, characterized by

11

1   a high level of drug loading, showed a relatively low burst effect (10–30%), while CPA-loaded

2   nanoparticles, characterized by very poor loading, showed a relatively high burst (about 40%).

3   The second phase of the observed particle-release patterns evidenced the ability of the

4   nanoparticles to provide controlled release of their encapsulated drugs. As reported in Figure 2, Pro-

5   5 and Diclo-5 samples released about the 60% of drug within 24 hours, showing similar release

6   patterns with respect to the parent samples Pro-10 and Diclo-10, respectively, even though these

7   patterns shifted to reflect the higher burst effect observed. CPA-5-PLGA and CPA-10-PLGA

8   profiles showed similar behaviour in the second release phase.

9

10   **3.3 Adjustments to the formulation of CPA-loaded nanoparticles**

11   Among the model drugs chosen for our study, CPA appeared to be characterized by

12   relatively poor drug loading and greater burst effect with respect to protamine and diclofenac (Table

13   I). However, as the CPA-loading capacity of PLGA nanoparticles via the basic nanoprecipitation

14   method was nearly zero (precisely 0.01 % w/w) [31, 32], even these results were considered

15   promising at the time of their reporting [25]. Hence we continued to study the ION

16   nanoencapsulation of CPA, investigating several modifications to the standard preparation to find a

17   way to improve CPA encapsulation in polymeric nanoparticles and/or the control of its release.

18   The first modification was performed on the polymer matrix, by substituting the PLGA with

19   PLA, which is characterized by a lower hydrophilicity. Nanoparticles obtained by this process

20   displayed a regular, spherical shape (Figure 3a and 3b), and, as reported in Table II, CPA-5-PLA

21   and CPA-10-PLA particles were the same size as those obtained using PLGA, but PDI values were

22   lower ($\leq$0.2). Moreover, using PLA polymer in place of PLGA increased CPA loading when 5 mg

23   of drug were used as the initial amount, doubling the EE value (from 7.1% for CPA-5-PLGA to

24   15.3% for CPA-5-PLA). However, this phenomenon was not observed with 10 mg of CPA, at

which CPA-10-PLGA and CPA-10-PLA EE values were very similar (about 7%). Concerning drug

release (Figure 4), the PLA nanoparticles obtained from 5 mg of CPA (CPA-5-PLA) showed a

slightly larger burst effect (about 48%) with respect to the homologue sample CPA-5-PLGA (about

40%), studied previously [25]. In contrast, the PLA nanoparticles obtained in the presence of 10 mg

of CPA (CPA-10-PLA) produced a very high burst effect (about 87%), revealing the total inability

of this nanoparticulate system to control the release of CPA.

We therefore attempted a second modification of the standard formulation, keeping the

polymer (PLGA) constant, but adding a lipophilic excipient, namely lauric acid (LA), to the

polymeric matrix. The aim was to limit the loss of drug during nanoparticle formation by hindering

drug diffusion, but, although the added excipient induced a small increase in the size of the CPA-

loaded nanoparticles (from 190 nm to about 220 nm, Table II) and no change in shape (data not

shown), their drug loading ability was even lower, the EE% being slightly smaller than those

achieved for the homologue samples formulated without LA. Promisingly, however, the addition of

LA to the PLGA nanoparticles loaded with CPA did reduce the burst effect and slowed the release

in the second phase (Figure 4). Indeed, as reported in Table III, the CPA-5-LA sample produced a

burst effect of about 28%, significantly lower than the value detected for the homologue sample

obtained in the absence of LA (CPA-5-PLGA, burst effect about 40%), a pattern repeated when the

initial drug amount was increased (CPA-10-LA roughly 28%, CPA-10 without LA about 42%).

Furthermore, LA also produced a significantly slower release in the second phase in both cases

(Figure 4), indicating more efficacious incorporation of the drug into the polymeric matrix in the

presence of this excipient.

13

1 **3.4 Degradation in human whole blood**

2   In our previous study we found that the *in vitro* release properties of CPA-10-PLGA

3 nanoparticles allowed for significant drug stabilization in the blood [25]. In light of the promising

4 results mentioned above, in this study we set out to investigate the ability of LA to influence CPA

5 release from PLGA nanoparticles, and to further study the degradation kinetics of CPA (free or

6 encapsulated in PLGA or PLA, and in the presence or absence of LA) in human whole blood. As

7 shown in Figure 5, which also reports previously recorded data on representative sample CPA-10-

8 PLGA [25], in the absence of LA, CPA-5-PLA displayed the ability to reduce the CPA degradation

9 rate, in agreement with its ability to improve control of the release of the drug with respect to CPA-

10 10-PLA. Free CPA was degraded according to a first order kinetic with a half-life of 21.8 ± 2.4

11 min, confirming the values obtained in previous studies on CPA pharmacokinetics [25, 33, 34]**.**

12 The degradation profile was not altered by the presence of unloaded nanoparticles (data not shown),

13 suggesting their good biocompatibility. Following this kinetic pattern, free CPA appeared to be

14 totally degraded in human whole blood after 3 hours, but the degradation of the drug encapsulated

15 in the nanoparticles was significantly reduced. Indeed, after three hours, the degradation of CPA-5-

16 PLGA was about 48%, and CPA-10-PLGA showed a similar degradation pattern (roughly 46% at 3

17 hours), in accordance with their similar CPA release profile in water [25]. Although similar

18 degradation values were also seen at 3 hours in blood when LA was added in the case of CPA-10-

19 LA (42%), a significantly lower degradation rate (P< 0.05) was registered at this time-point for

20 CPA-5-LA (28%).

21   Among the CPA degradation results it is important to remark that after three hours of

22 incubation of the CPA-5-PLA sample in human whole blood, about 40% of the loaded drug was

23 still detectable, indicating the superiority of the ION method in terms of controlling the release of

1 CPA in whole blood, in addition to the greater encapsulation efficiency of the corresponding

2 nanoparticles produced by this technique (roughly 15% with 0.61% drug loading) .

3

4. DISCUSSION

5   The entrapment of hydrophilic drugs in the hydrophobic polymeric matrix of nanoparticles

6 currently constitutes an important challenge, the low drug polymer affinity and the small size and

7 large surface area of the particles being the main factors contributing to the loss of the drug into the

8 aqueous phase [8, 22]. Indeed, the submicron size of nanoparticulate systems can often induce the

9 loss of either encapsulation efficiency or the ability to control the release of drugs [35]. In this paper

10 the efficacy of an original nanoprecipitation method, the "in-oil nanoprecipitation" technique

11 (ION), in encapsulating water-soluble drugs was evaluated on the model drugs protamine and

12 diclofenac sodium, comparing them with results previously obtained for CPA [25].

13   Protamine is a small cationic protein extracted from salmon sperm that has been FDA-

14 approved for parenteral administration to inhibit the anticoagulant activity of heparin. It consists of

15 32 amino acids, 21 of which are arginine, and since it is a nuclear protein that helps DNA packaging

16 in sperm cells, it is also used as transfection accelerator in the gene delivery [36].

17   Diclofenac sodium, on the other hand, is a potent non-steroidal anti-inflammatory drug

18 (NSAID) that acts as non-selective inhibitor of cyclo-oxygenase (COX). Due to its anti-

19 inflammatory, analgesic and anti-pyretic effects, it has a wide range of clinical applications.

20 Moreover, alongside other COX inhibitors such as diclofenamic acid, it is currently being studied

21 for its application in Alzheimer's disease (AD) [37, 38].

22   The final model drug studied, N^6-cyclopentyladenosine (CPA), is a potent and selective

23 agonist of adenosine A_1 receptors [39], whose activation depresses cardiac and neuronal excitability

24 [40], inducing ischemic tolerance and protection in neuronal and cardiac tissues. However, CPA-

15

1 selective A_1 agonists appear to be poorly adsorbed into the brain [41], and can be quickly degraded

2 *in vivo* or in whole blood [42, 43].

3 Hence, an effective strategy for nanoencapsulating these hydrophilic model compounds in a

4 biodegradable polymeric matrix constitutes a preliminary step towards both increasing the stability

5 of water soluble drugs in physiological fluids and targeting specific body compartments. To this end

6 we show that the ION technique enabled the incorporation of the model drugs in the polymeric

7 matrix of nanoparticles, thanks to their low solubility in oil after the diffusion of acetone in the

8 outer oil phase. As oil is more viscous than water, diffusion of acetone in the oil phase takes place

9 more slowly than the diffusion of acetone in the outer water phase occurring in classic

10 nanoprecipitation [44], and, as a consequence, the nanoparticles obtained when PLGA was

11 employed in their formulation were not perfectly spherical. In contrast, PLA encapsulation resulted

12 in nanoparticles characterized by a more regular shape, presumably due to the lower viscosity of

13 the PLA polymer–acetone solution (60–75 kDa vs. 16–28 kDa). The irregular and asymmetrical

14 shape of PLGA nanoparticles could explain their high polydispersity index, registered by PCS

15 analysis. Indeed, the calculation model used in this type of measurement is based on the equivalent

16 principle, in which each particle is viewed as a sphere, meaning that an irregular shape can have a

17 noticeable influence on the findings [45].

18 As regards the encapsulation efficiency, in PLGA nanoparticles CPA showed loading values

19 one order of magnitude lower that the satisfactory values observed for protamine and diclofenac.

20 Indeed, CPA is classified as a slightly hydrophilic drug (Log P = 1.21 [26]) characterized by a great

21 ability to diffuse out of hydrophobic matrixes during the formation of particulate systems,

22 remaining adsorbed on their external surface [30,33]. This phenomenon could explain the high burst

23 values registered for CPA-loaded nanoparticles with respect to those loaded with the other two

24 model drugs. That being said, all PLGA samples demonstrated the ability to provide controlled

25 release of their encapsulated drugs. As evident from the second phase of particle release patterns

16

1 reported in Figure 2, only roughly 40% of the drug amounts included in the nanoparticulate

2 powders had been released after 24 hours.

3   As ION loading of CPA in PLGA nanoparticles was relatively poor with respect to that of

4 the other model drugs, we extended the investigation, first by adding a lipophilic excipient, lauric

5 acid (LA), during the formulation of PLGA nanoparticles, and then by encapsulating the drug in

6 PLA, which is more hydrophobic with respect to PLGA. We also set out to verify the effects of the

7 ION-generated CPA nanoparticles' encapsulation and controlled release in human whole blood, a

8 model fluid is able to degrade the drug relatively quickly (with a half-life of about 15 min) [42].

9   Although initial loading was slightly reduced in the presence of LA, probably owing to LA

10 acting as a filler in the nanoparticles' polymeric matrix and thereby leaving less space for the drug,

11 encouragingly, the samples obtained in the presence of LA, CPA-5-LA and CPA-10-LA, showed a

12 reduced burst effect with respect to the homologues formulated without LA, followed by a very

13 slow release. This behaviour, perhaps ascribable to the small weight of the drug molecule, appears

14 to be different to that previously observed in the encapsulation of proteins in the presence of

15 additives previously employed as loading enhancers [46, 47].

16   Encapsulation of CPA in PLA rather than PLGA resulted in an increase in drug loading

17 capacity, but, interestingly, this failed to increase further when the initial drug amount was

18 increased, indicating the existence of a drug-loading plateau. Moreover, whereas the CPA-5-PLA

19 sample appeared to provide controlled release of CPA, even though less markedly so with respect to

20 the PLGA homologues, the CPA-10-PLA sample provided no such control. Hence, although

21 increasing the initial amount of CPA in the preparation of PLA nanoparticles did not influence the

22 quantity of drug recovered in the samples, it did cause a total loss of control over drug release. This

23 reflects a similar phenomenon we previously observed in ION-generated PLGA nanoparticles, in

24 which CPA ratios higher than 10 mg per 125 mg of PLGA induced an unremarkable increase in

25 drug loading, but a significantly higher release of the drug [25].

1   The degradation profiles of the nanoparticulate samples analysed in whole blood indicate

2   that the drug stability of CPA encapsulated in both PLGA and PLA nanoparticles can be modulated

3   in a manner consistent with the respective release profile patterns of the model drugs.

4   **5. CONCLUSIONS**

5   The ION method enabled the nanoencapsulation in PLGA of different kinds of hydrophilic

6   drugs, but with different extents of drug loading. The results indicate that particle size is influenced

7   mainly by primary and secondary operating parameters, while the final drug load depends strongly

8   on the nature of the drug in question. The type of drug also affected the release pattern, but this may

9   be successfully modulated by the addition of the excipient lauric acid. However, before a simple,

10   universal protocol for nanoprecipitation that provides precise and reproducible control over the key

11   nanoparticle characteristics, enabling its industrial application, can be developed, it is necessary to

12   ensure the method is applicable to a wider range of drugs of different physical-chemical

13   characteristics.

14

15   **ACKNOWLEDGMENTS**

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4

Table I

Size, polydispersity index (PDI), actual loading and encapsulation efficiency values forthe encapsulation of model hydrophilic drugs in PLGA nanoparticles. Data are reported as the mean ± SD of three independent experiments.

Sample	Drug	Particle size (nm) ± SD	PDI	Actual loading (%, w/w)	Encapsulation efficiency (%)	Drug released in initial burst (%)
Unloaded PLGA-Np	-	253± 33	0.442	-	-	
Pro-5	Protamine (5 mg)	296 ± 26	0.391	3.9 ± 0.4	94.3± 1.5	10.5 ± 0.4
Pro-10	Protamine (10 mg)	288 ± 23	0.412	7.4 ± 0.3	92.5± 1.3	30.3 ± 1.3
Diclo-5	Diclofenac (5 mg)	194 ± 30	0.460	2.2 ± 0.2	55± 0.3	21.4 ± 0.9
Diclo-10	Diclofenac (10 mg)	190± 30	0.512	3.8 ± 0.1	47.5± 0.7	36.4 ± 1.5
CPA-5-PLGA[a]	CPA (5 mg)	194 ± 32	0.328	0.27 ± 0.01	7.10 ± 0.02	39.9 ±4.0
CPA-10-PLGA[a]	CPA (10 mg)	193 ± 58	0.430	0.50 ± 0.03	6.90±0.04	42.1 ±3.8

[a]Data reported from [25]

Table II

Size, polydispersity index (PDI), actual CPA loading, encapsulation efficiency, and burst effect values for CPA encapsulation in PLGA and PLA nanoparticles in the presence or absence of lauric acid (LA). Data are reported as the mean ± SD of three independent experiments.

Sample	CPA amount	Polymer	Particle size (nm) ± SD	(PDI)	Actual loading (%, w/w)	Encapsulation efficiency (%)	Drug released in initial burst (%)
Unloaded PLA-Np	-	PLA	220±37	0.298	-	-	-
CPA-5-PLA	5 mg	PLA	190 ±37	0.178	0.61 ± 0.04	15.3±1.3	48.5 ± 3.6
CPA-10-PLA	10 mg	PLA	205 ± 25	0.207	0.58± 0.03	7.3±0.8	86.6 ± 4.0
CPA-5-LA	5 mg	PLGA	206 ± 45	0.405	0.19 ± 0.02	4.8± 0.4	28.0 ± 3.3
CPA -10-LA	10 mg	PLGA	245 ± 25	0.407	0.39± 0.03	5.6± 0.6	28.1 ± 3.5

Figure Legends

Figure 1. Scanning electron microphotograph (SEM) of unloaded PLGA nanoparticles [A] and CPA-loaded nanoparticles formulated via the ION method in the presence of 5 mg of the drug (PLGA-5-CPA) [B] after removing the excess of Tween-80.

Figure 2. Release profiles of protamine (Pro), diclofenac (Diclo) and N^6-cyclopentyladenosine (CPA) from PLGA nanoparticles formulated via the ION method in the presence of 5 mg (Pro-5, Diclo-5 and CPA-5 PLGA) or 10 mg (Pro-10, Diclo-10 and CPA-10-PLGA) of the model drugs. Data are reported as the mean ± SD of three independent experiments.

Figure 3. Scanning electron microphotograph (SEM) of CPA-loaded PLA nanoparticles after removing the excess of Tween-80. The loaded nanoparticles were formulated via the ION method in the presence of 5 mg [A] or 10 mg [B] of CPA.

Figure 4. [A] Release profiles of CPA from PLGA (CPA-5-PLGA, CPA-10-PLGA, CPA-5-LA, CPA-10-LA) and PLA nanoparticles (CPA-5-LA, CPA-10-LA) formulated via the ION method in the presence or absence of lauric acid (LA). [B]: zoom in of plot [A] with expanded X-scale (time) from 0 to 3 hours. Data are reported as the mean ± standard error of three independent experiments.
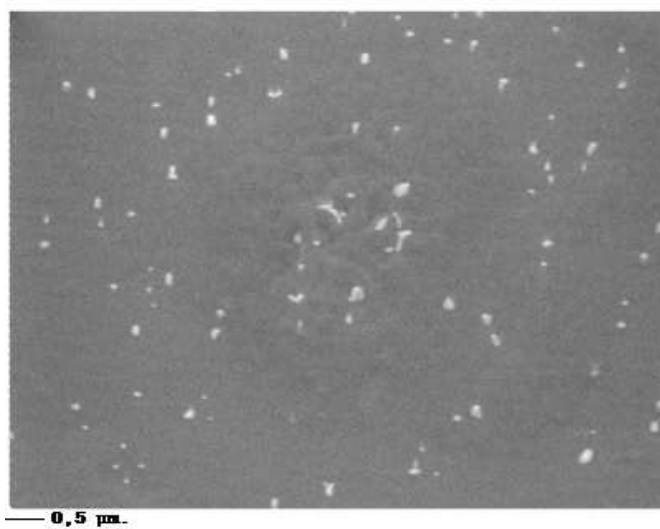
Figure 5. Degradation kinetics of free and nanoencapsulated CPA (ION method) in human whole blood. Data are reported as the mean ± SD of three independent experiments.

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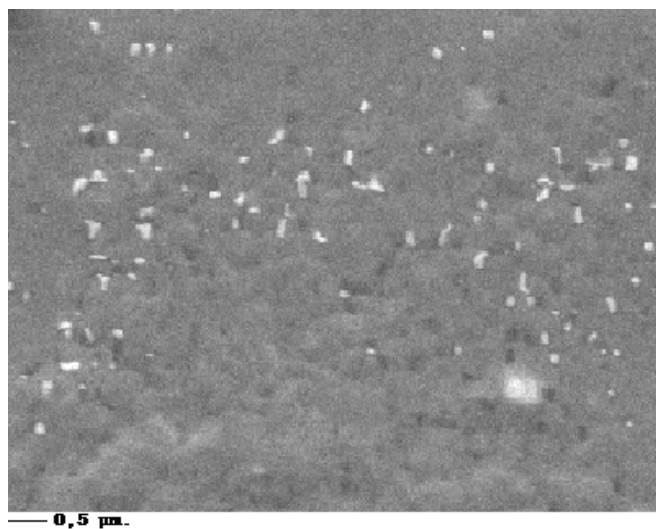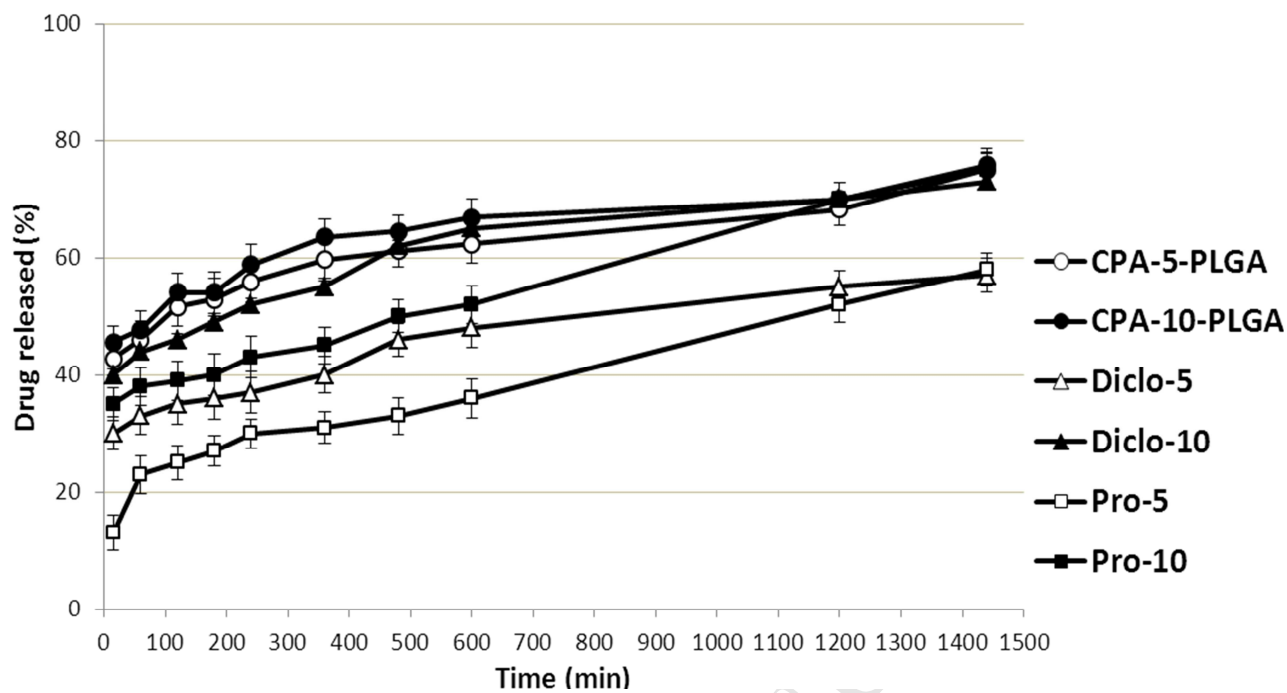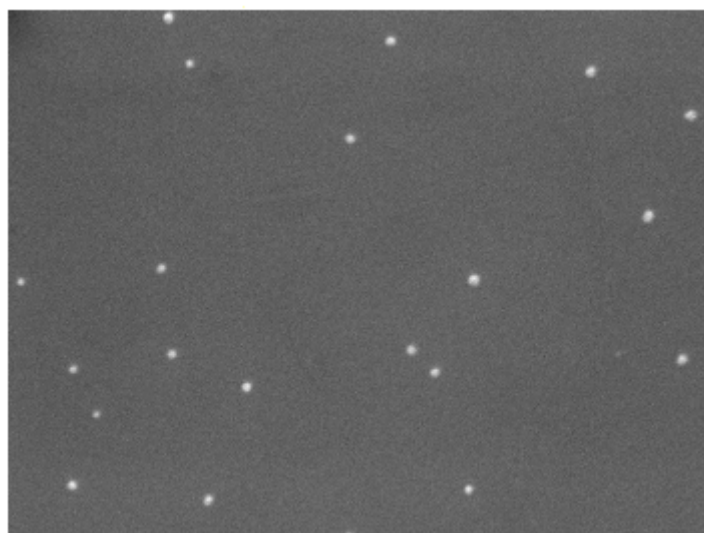

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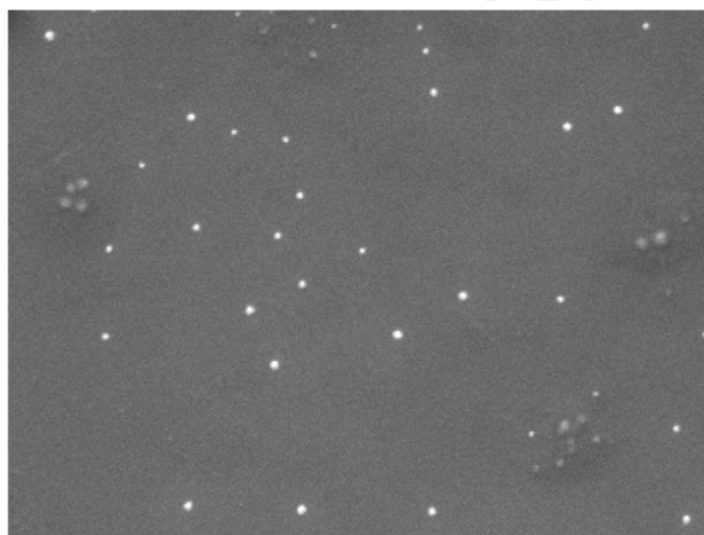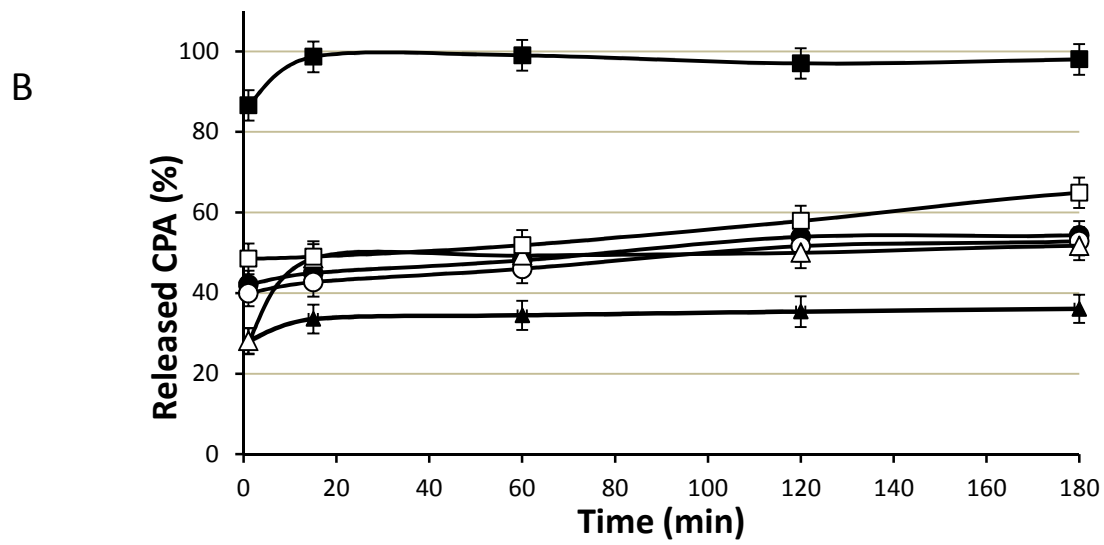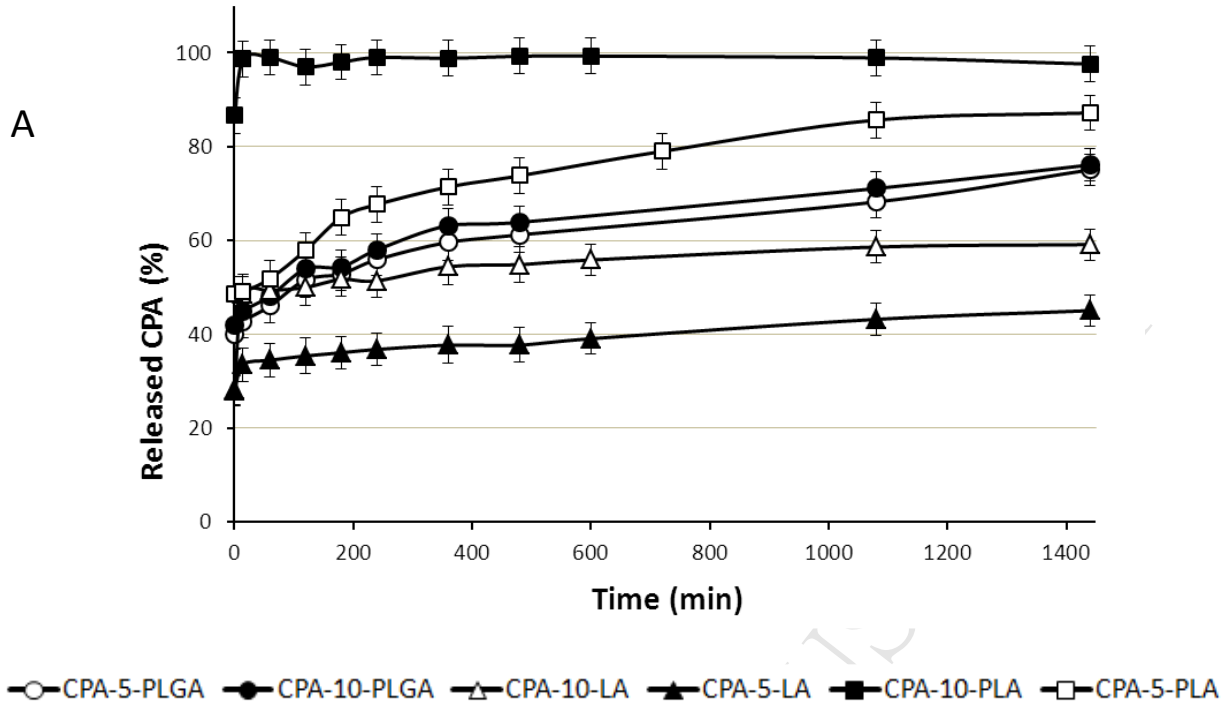
Figure 1

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Figure 2

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Figure 3

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○— CPA-5-PLGA   ●— CPA-10-PLGA   △— CPA-10-LA   ▲— CPA-5-LA   ■— CPA-10-PLA   □— CPA-5-PLA

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Figure 4

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Figure 5