



Combining orthogonal measurements to unveil diclofenac encapsulation into polymeric and lipid nanocarriers

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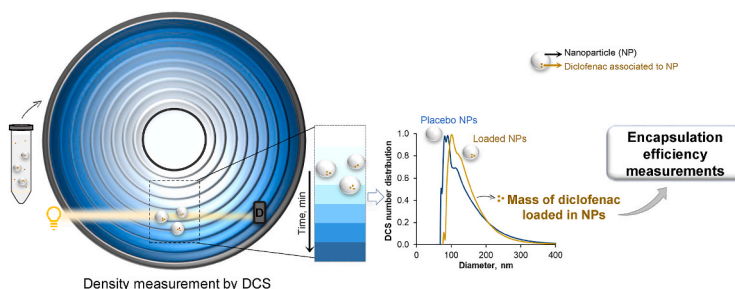
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HIGHLIGHTS

- Reliable encapsulation efficiency (EE) measurements require complementary analysis.
- The measurement of loaded vs. empty nanoparticles mass is proposed for assessing EE.
- Diclofenac-loaded polymeric nanoparticles and lipidic carriers were analysed.
- Batch-to-batch consistency and encapsulation yield monitoring were accomplished.
- Analysis was performed within 15 min, with results aligned with those from HPLC.

GRAPHICAL ABSTRACT



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ABSTRACT

The quantification of the drug associated to nanoparticle carriers, often expressed in terms of encapsulation efficiency, is a regulatory requirement. The establishment of independent methods to evaluate this parameter provides a means for measurement validation, which is critical in providing confidence in the methods and enabling the robust characterization of nanomedicines. Chromatography is traditionally used to measure drug encapsulation into nanoparticles. Here, we describe an additional independent strategy based on analytical centrifugation. The encapsulation of diclofenac into nanocarriers was quantified based on the mass difference between placebo (i.e. unloaded) and loaded nanoparticles. This difference was estimated using particle densities measured by differential centrifugal sedimentation (DCS) and size and concentration values measured by particle tracking analysis (PTA). The proposed strategy was applied to two types of formulations, namely poly(lactic-co-glycolic acid) (PLGA) nanoparticles and nanostructured lipid carriers, which were analysed by DCS operated in sedimentation and flotation modes, respectively. The results were compared to those from high performance liquid chromatography (HPLC) measurements. Additionally, X-ray photoelectron spectroscopy analysis was used to elucidate the surface chemical composition of the placebo and loaded nanoparticles. The proposed approach enables the monitoring of batch-to-batch consistency and the quantification of diclofenac association to PLGA nanoparticles from 0.7 ng to 5 ng of drug per 1 µg of PLGA, with good linear correlation between DCS and HPLC results ($R^2 = 0.975$). Using the same approach, similar quantification in lipid nanocarriers was possible for a loading of diclofenac ≥ 1.1 ng per 1 µg of lipids, with results in agreement with the HPLC method ($R^2 = 0.971$).

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Hence, the strategy proposed here expands the analytical tools available for evaluating nanoparticles encapsulation efficiency, being thus significant for increasing the robustness of drug-delivery nanocarriers characterization.

1. Introduction

Nanotechnology has opened new avenues in the pharmaceutical field, offering a range of strategies to overcome solubility, permeation, stability, and off-target delivery issues for several active ingredients [1]. Over the past 20 years, the promise of medicines with improved therapeutic index has prompted an extensive development (> 70 000 publications between 2002 and 2022 in Web of Science®) of different types of nanocarriers with increasingly sophisticated features [2–4]. Still, their translation into the pharmaceutical market has been slow [5–7], with less than 100 nanoparticle-based products in clinical use so far [1].

The diversity and complexity of nanoscale drug carriers preclude the adoption of a unique set of broadly applicable standard procedures for their characterisation, and poses challenges in the regulatory assessment of their physicochemical properties (such as size, shape, drug encapsulation, and stability), batch-to-batch consistency, and biological fate [7–10]. Considering that these parameters have a direct impact on therapeutic efficacy, quality, and safety of medicines, their thorough characterization is required [11,12]. In this context, it is critical the measurement of the same attribute by independent or orthogonal methods, to validate results and highlight potential procedure-related biases [13,14].

Although a number of approaches to the evaluation of nanoparticles size have been proposed [13,15,16], independent and validated methods for the measurement of the amount of drug associated with nanocarriers [17,18], either inside or bound to nanoparticles [11], are scarce and lack standardisation. There is a clear need to identify methods useful to this purpose. In fact, the amount of drug associated with nanocarriers is a critical quality attribute to evaluate the dose and efficacy of a nanomedicine formulation. Robust, accurate and validated procedures are also needed to evaluate batch-to-batch consistency, batch stability throughout storage, and to screen formulations as well as manufacturing processes during developmental studies. Liquid chromatography coupled to UV–Visible or mass spectrometry detectors is the gold standard to quantify drug encapsulation efficiency into nanocarriers [18]. However, the chromatography method requires the separation of nanoparticles from free drug prior to quantification, which is usually a cumbersome procedure that must be optimised for each nanoformulation [19–21], during which drug leakage may occur [18, 19]. Considering these hindrances, alternative methods for measuring encapsulation efficiency have been proposed, based on analytical ultracentrifugation (UC) [22,23], capillary electrophoresis (CE) [24,25], field-flow fractionation (FFF) [26,27], nuclear magnetic resonance (NMR) [28,29], and small-angle X-ray scattering (SAXS) [30,31]. Nevertheless, some of these solutions rely on expensive equipment that is not available in routine quality control labs and often requires specialized operators or complex data treatment [26–31].

Hence, as a routine procedure, for example in the context of product quality control, the determination of encapsulation efficiency would benefit from faster methods with minimal sample preparation and fit-for-purpose analytical performance. Moreover, methods that have operating principles that are independent from liquid chromatography would provide a route to the validation of results.

Differential centrifugal sedimentation (DCS) is a promising technique to accomplish the abovementioned goals due to its robustness in terms of measurement repeatability, ease of operation and minimal sample preparation requirements [32,33]. DCS is a high-resolution particle separation technique that measures the time required by particles to sediment through a fluid under a centrifugal field. The sedimentation time is a function of the nanoparticle size and density

according to Stokes' law and correction factors to account for deviation from a spherical particle geometry can be applied [34–37]. In the case of spherical particles, if the nanoparticle average diameter is known through an independent measurement, DCS can be employed to measure particle density. With knowledge of the particle volume, the difference in density between the placebo (unloaded) and loaded nanoparticles can be converted to differences in mass [38], permitting drug encapsulation to be measured. Because DCS operates in both sedimentation and flotation modes, nanoparticles with densities both greater than and less than that of the fluid can be analysed [35].

To date, DCS has been successfully applied to describe particle size distribution in multimodal (i.e. more than one size population) [15] and aggregated/agglomerated samples [39–41], to measure particle density [35], to monitor nanoparticle shell thickness and coating [33,42], and for *in vitro* dosimetry studies [43]. The use of DCS to analyse nanoparticle drug loading has seldom been exploited despite the promising findings reported by Mable et al. [44] on the quantification of silica nanoparticles (ca. 18 nm) encapsulation into copolymer vesicles. To the best of our knowledge, DCS has not been applied so far to analyse the loading of small molecules (< 1 nm) into nanocarriers.

In this work, DCS measurements were applied to measure the amount of diclofenac associated with polymeric and lipid nanocarriers. Diclofenac is a potent anti-inflammatory drug, with high permeability and poor solubility in water (class II of the Biopharmaceutics Classification System) [45]. Diclofenac is widely used for the management of inflammatory conditions but causes pronounced adverse effects such as gastrointestinal problems and has a short half-life [46,47]. Thus, the vehiculation of diclofenac into a delivery system could offer a way to provide an improved pharmacokinetic and safety profile for long-term uses [47]. Nanoparticles loaded with different amounts of diclofenac were synthesized and characterized by ultrafiltration-high performance liquid chromatography (HPLC), dynamic light scattering (DLS), DCS and particle tracking analysis (PTA). The latter two methods provided information on the density, size, and concentration of the particles providing a means to measure drug encapsulation efficiency. The DCS results were compared with those obtained from HPLC, providing a validated strategy to measure the drug encapsulation efficiency of these nanocarriers.

2. Materials and methods

2.1. Chemicals

Dipotassium hydrogen phosphate, potassium dihydrogen phosphate, polyvinyl alcohol (PVA) (molecular weight 13 000–23,000, 87–89% hydrolysed), and diclofenac sodium salt were acquired from Sigma-Aldrich (St Louis, MO, USA). Witepsol® E85 and Miglyol® 812 used for the preparation of nanostructured lipid carriers (NLCs) were acquired from Cremer Oleo (Hamburg, Germany). Poly-D,L-lactide-co-glycolide (PLGA) (50:50 PURASORB® PDLG 5004A) was kindly provided by Purac Biomaterials (Gorinchem, The Netherlands). Acetone (analytical grade) was purchased from Acros Organics (Geel, Belgium). Methanol (HPLC grade) used for HPLC analysis was acquired from Rathburn Chemicals (Peeblesshire, United Kingdom). All the abovementioned chemicals were used as supplied, with no further purification.

Phosphate buffer (pH 7.0; 0.1 M), containing 0.06 M dipotassium hydrogen phosphate and 0.04 M potassium dihydrogen phosphate, was used for the preparation of the chromatographic mobile phase. A phosphate buffer (pH 6.0; 0.1 M), containing 0.01 M dipotassium

hydrogen phosphate and 0.09 M potassium dihydrogen phosphate, was used during the production of F2 nanoparticles formulation. A diluted solution (20 mM) of this buffer was employed during the preparation of F3 formulation and sucrose solutions, and also to dilute the nanoformulations prior to analysis whenever needed.

2.2. Nanoparticle preparation and purification

PLGA nanoparticles were prepared using the single emulsion-solvent evaporation technique [48,49]. Nanoparticle formulations F1 and F2 were prepared by dissolving 40 mg of PLGA in 3 mL of acetone. After polymer solubilization, diclofenac powder (3 mg) was added to the organic phase (loaded nanoparticles). Then, the organic phase was slowly added to 5.0 mL of a 1% (w/v) PVA solution prepared in water (F1) or phosphate buffer (pH 6.0, 0.1 M) (F2). The mixture was subjected to probe sonication (CPX 130, Cole-Parmer Instruments, Vernon Hills, IL, USA) for 1 min (70% amplitude) and 0.1% (w/v) PVA solution was added up to a final volume of 20 mL. The resulting nanoemulsion was maintained under magnetic stirring (300 rpm) for 8 h at room temperature to enable acetone evaporation. F3 was prepared by dissolving 20 mg of PLGA in 1 mL of acetone, with subsequent addition of diclofenac (2 mg) after polymer solubilization. The organic phase was slowly added to a final volume of 20 mL of 1% (w/v) PVA prepared in phosphate buffer (pH 6.0, 20 mM), and maintained under magnetic stirring (300 rpm) for 8 h at room temperature for acetone removal. F1, F2 and F3 placebo nanoparticles were prepared using the same procedures, without adding diclofenac to the organic phase.

Nanostructured lipid carriers (NLCs) were prepared by hot ultra-sonication method [50]. Briefly, 150 mg of Witepsol® E85 (solid lipid) and 45 mg of Miglyol® 812 (liquid lipid) were melted in a water bath at 65 °C. Diclofenac powder (2, 4 and 6 mg) was then added to the lipid phase and 7 mL of pre-warmed (65 °C) PVA solution (6.7 mg mL⁻¹) were added. The resulting emulsion was subject to probe-sonication for 5 min (55% amplitude), followed by cooling to room temperature. These nanoparticles, prepared with increasing quantities of diclofenac (2, 4 and 6 mg) were designated as “loaded A, B and C” NLCs for simplicity. Placebo NLCs were prepared using the same procedure without addition of diclofenac to the lipid phase.

The loaded nanoparticles were separated from free diclofenac in the suspension by ultrafiltration using Amicon® Ultra centrifugal filters (molecular weight cut-off 50 kDa) of regenerated cellulose (Merck, Darmstadt, Germany). PLGA nanoparticles were diluted 12 times in phosphate buffer (pH 6.0, 20 mM), placed into the centrifugal filters and centrifuged for 15 min at 2100×g (centrifuge 5430, Eppendorf, Hamburg, Germany). Hence, through the application of a centrifugal force, the free diclofenac molecules were expected to pass through the filter pores from the upper to the lower compartment (ultrafiltrate), while nanoparticles were expected to be retained in the upper compartment because they are larger than the pore cut-off. To mitigate potential interactions of the free drug with the filter, washing steps using two aliquots of 1.8 mL of phosphate buffer were applied to the retentate followed by centrifugation (2100×g, total 20 min) to ensure the removal of free diclofenac potentially bound to filter membrane at the end of the first 15 min of centrifugation. Then, the portion of sample retained in the upper compartment and the ultrafiltrate were collected, weighed and analysed (the first by DCS, PTA and HPLC, the second by HPLC only).

Similarly, lipid nanoparticles were diluted 36 times in phosphate buffer (pH 6.0, 20 mM), placed into centrifugal filters and centrifuged for 5 min (2100×g). After centrifugation, the portion remaining in the upper compartment and the ultrafiltrate were collected and weighted. After the collection of these portions, two aliquots of 1.8 mL of phosphate buffer were added to the filter device followed by centrifugation (2100×g, total 20 min), to ensure the removal of free diclofenac potentially bound to filter membrane. This approach was selected due to the reported instability of lipid particles during long centrifugation processes [21].

2.3. Nanoparticle characterization

2.3.1. X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS) analysis was performed for placebo and loaded PLGA nanoparticles (F1-batch 1) and NLCs (loaded NLCs A batch), previously purified from free drug by ultrafiltration. Samples were prepared according to two different protocols to measure both the chemical composition of the nanoparticle surface and their bulk (core + surface) composition. In the first case, 20 µL of nanoemulsion were deposited by drop-casting onto a 1 cm × 1 cm square of clean silicon wafer and dried in a vacuum desiccator. This procedure was repeated twice to achieve a uniform and thick deposit, thus minimizing the contribution of the substrate to the observed spectrum. For the bulk composition, 1:1 (v/v) and 1:2 (v/v) nanoemulsion:ethanol solutions were prepared to disrupt PLGA nanoparticles and NLCs, respectively. These solutions were then deposited by drop-casting (8 × 5 µL) onto a 1 cm × 1 cm square of clean silicon wafer and dried in a vacuum desiccator.

Samples were analysed in a Kratos Axis Ultra XPS instrument, using a monochromated Al Kα X-ray source (photon energy 1486.6 eV) operated at 15 kV, with a 5 mA emission current. The instrument was intensity-calibrated using the NPL (National Physical Laboratory, UK) intensity calibration software [51], and energy-calibrated according to ISO 15472 [52]. Survey spectra were obtained from the samples at a pass energy of 160 eV with a dwell time of 300 ms and a step size of 1 eV. Higher resolution spectra for the carbon 1s peaks were acquired at a pass energy of 20 eV (dwell time of 300 ms, step size of 0.1 eV). All spectra were collected at normal emission, using the hybrid lens mode and slot aperture, with a charge neutralisation balance of 4.1 V. Reference samples of PVA, PLGA, Witepsol, Miglyol and diclofenac were analysed under the same conditions. Data was analysed using CasaXPS software, version 2.3.22. Quantification was performed using NPL average-matrix relative-sensitivity factors (AMRSFs) [53] and a Tougaard background subtraction, and the energy scale was referenced to the hydrocarbon C 1s peak, at 285.0 eV.

Carbon 1s spectra for PLGA nanoparticles were fitted with 3 sets of components, representing PVA, PVA acetate (PVAc, the polymer used was partially hydrolysed with 7%–13% acetate groups remaining), and PLGA, which were each separately constrained in terms of the full width at half maximum (FWHM), relative position, and relative area based on the known chemistry of these materials and the obtained reference spectra. Similarly, carbon 1s spectra for NLCs were fitted for PVA, PVAc, Witepsol and Miglyol.

Using the known stoichiometry of each of these components, the atomic concentrations of each compound were measured. From the intensity ratio of the PVAc and the PVA components, the level of hydrolysis of PVA was also measured.

The relative uncertainties in measured atomic concentrations are approximately 15% for most elements and may be up to ~20% for elements present below 5 at%, increasing to ~100% for elements close to the XPS detection limits [54].

2.3.2. DLS and PTA

Diameter and ζ-potential of PLGA nanoparticles and NLCs were measured by DLS and electrophoretic light scattering using a Zetasizer Nano ZS (Malvern Panalytical Ltd., Malvern, UK) equipped with a maximum 4 mW He-Ne Laser emitting at 633 nm. Samples were diluted in 0.22 µm filtered phosphate buffer (pH 6.0, 20 mM) prior to analysis and placed in disposable folded capillary cells (DST1070, Malvern Panalytical). Measurements were performed at 25 °C, with a 173° measurement angle and after an instrument equilibration time of 300 s. Particle diameter was expressed as the Z-average values, resulting from the cumulant analysis of the DLS correlation curve. Results are expressed as average values and standard deviations based on five measurement repeats. Nanoparticle diameter and concentration were measured using PTA (LM10, Malvern Panalytical, UK) equipped with a 640 nm laser.

Samples were diluted in 0.22 μm filtered phosphate buffer (pH 6.0, 20 mM) prior to analysis. A minimum of 3 videos with a duration of 60 s were recorded for placebo and loaded nanoparticles. Videos were processed using NTA3.2 software. For each sample, results are expressed as the mean values of the mode or mean from the videos along with respective standard deviations.

2.3.3. DCS

DCS measurements were performed with a CPS disc centrifuge (Model DC24000, CPS Instruments, Prairieville, LA, USA) in sedimentation and flotation modes (Fig. S1). In sedimentation mode, the sample was injected at the centre of the spinning disc and settled through a liquid of lower density towards the edge of the disc where a light beam and a photodetector were placed on opposite sides of the disc. Optical extinction at the photodetector position was used to monitor particle sedimentation over time (Fig. S1, supporting information section 2). Poly(vinyl chloride) particles with nominal 264 nm diameter and 1.385 g cm^{-3} density (CPS Instruments) were used as calibrants [55]. In flotation mode, a disc containing 4 capillaries connecting a central injection rim to the disc edge was used, enabling delivery of the particles at the disc edge (Fig. S1b). From here, the particles floated towards the centre of the disc through a fluid of higher density. Polybutadiene particles with nominal 510 nm diameter and 0.91 g cm^{-3} density were used as calibrants. Detector position was determined for both configurations to allow accurate calculation of the average density of the gradient between the points of injection and detection of the particles. In both configurations, a sucrose gradient was built with increasing radial density from 2% to 8% (w/w) for sedimentation and from 18% to 26% (w/w) for flotation measurements, respectively. The gradient was built from nine injections of 1.6 mL for a total of 14.4 mL of gradient volume. The resulting average gradient densities were 1.012 g cm^{-3} and 1.096 g cm^{-3} for sedimentation and flotation experiments, respectively. A thin layer of dodecane (0.5 mL) was added at the top of the gradient to prevent evaporation, and 30 min of thermal equilibration were undertaken before starting the experiments. For measurements in flotation mode, the samples were diluted in 30% (w/w) sucrose solution. After each sample analysis an injection of sucrose solution was performed to remove any particle residue in the rim and capillaries prior to the next run. All experiments were performed at 24 000 rpm using an injection volume of 100 μL .

The measurements were performed using an initial estimate of the particle density (ρ_p) based on particle composition. The measured light extinction-based size distributions for placebo and loaded nanoparticles were converted to volume-based and number-based size distributions according to Mie's theory [56] and by modelling the particles as perfect spheres (based on information gathered from previous works [49,50,57]) of refractive index 1.46 and with no absorption. The modal diameters of the DCS number-based size distributions (D_{DCS}) were used along with the modal diameters of the number-based size distributions measured by PTA (D_{PTA}) and the average density of the gradient (ρ_f) to provide an accurate estimate of the particle densities (ρ_p^*) according to Equation (1). Additional information on the DCS method is provided in supporting information (section 1).

$$\rho_p^* = \rho_f + \left(\frac{D_{\text{DCS}}}{D_{\text{PTA}}} \right)^2 (\rho_p - \rho_f) \quad \text{Eq.1}$$

The mass of placebo and loaded nanoparticles in 1 mL of colloidal formulation (M_p) was determined by multiplying the density values measured for each nanoparticle batch by the total volume occupied by the particles in 1 mL (Fig. 1). The latter was estimated by multiplying the average volume occupied by one particle and the particle concentration (C_p) per mL of formulation as measured by PTA (Equation (2)). Here, the number averaged mean diameter (D'_{PTA}) measured by PTA was used to account for the tail at large sizes of the particle size distributions.

$$M_p = \rho_p^* \frac{\pi}{6} (D'_{\text{PTA}})^3 C_p \quad \text{Eq.2}$$

Then, the mass of diclofenac associated with nanoparticles in 1 mL of formulation (M_d) was determined by subtracting the mass of placebo nanoparticles from the mass of loaded nanoparticles (Equation (3)).

$$M_d = M_p^{\text{loaded NPs}} - M_p^{\text{placebo NPs}} \quad \text{Eq.3}$$

The overall strategy based on DCS and PTA measurements to determine the mass of diclofenac loaded into the nanoparticles will hereafter be designated by DCS-PTA for simplicity. Encapsulation efficiency (EE) values (%) are expressed as the total mass of diclofenac associated with nanoparticles, in this case found by DCS-PTA, divided by the amount of diclofenac added to prepare the batch.

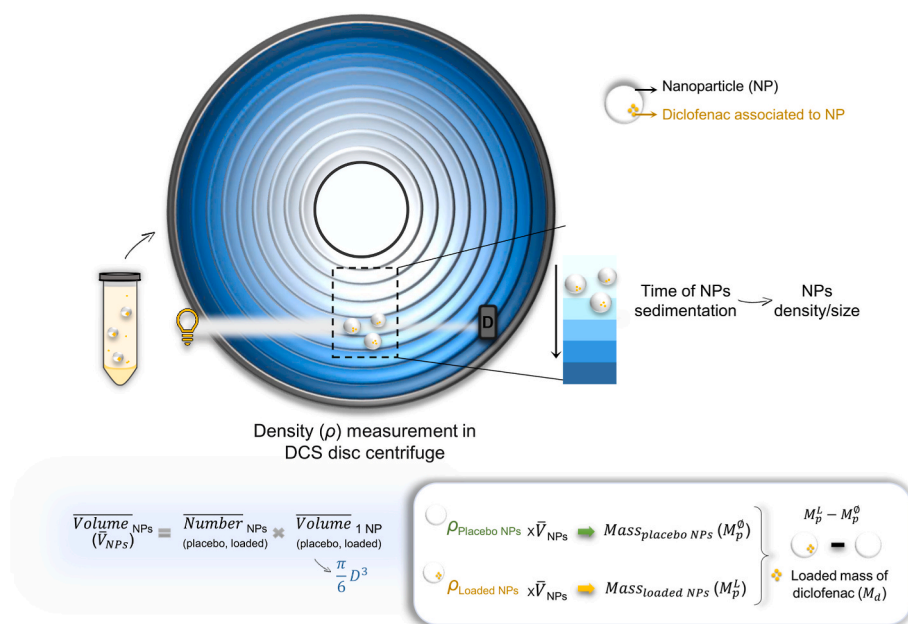


Fig. 1. Schematic representation of the strategy followed for the quantification of diclofenac-associated with nanoparticles (M_d) by the DCS-PTA approach.

2.3.4. High-performance liquid chromatography

HPLC was used to quantify the mass of diclofenac in the sample. Three types of measurements were made: the total diclofenac present in the nanoformulations, the amount associated with nanoparticles, and free diclofenac in the liquid phase.

Chromatographic analysis was performed on a ThermoFisher™ Dionex™ Ultimate™ 3000 HPLC System (ThermoFisher Scientific, Bremen, Germany). Chromatographic separation was accomplished within 5 min using a reserved-phase monolithic column (Chromolith® RP-18e, 100 mm × 4.6 mm i.d., Merck, Darmstadt, Germany) and methanol-phosphate buffer (pH 7.0, 20 mM) (61:39, v/v) as mobile phase. A detection wavelength of 279 nm and a flow rate of 1 mL min⁻¹ were used (further details on supporting information - section 1). Samples and standards were prepared in mobile phase and immediately analysed (200 µL of injection volume).

The mass of diclofenac present in the upper compartment and in the ultrafiltrate of Amicon® filters was calculated from the concentration of diclofenac measured by HPLC and the sample volume in each compartment assessed by weighing. Total diclofenac (i.e. free and loaded forms) was assessed by direct analysis of the formulation (methodological details can be found in the supporting information – section 1).

EE in this case was calculated as the mass of diclofenac associated with nanoparticles as measured by HPLC relative to the amount of diclofenac added to prepare the batch.

Drug loading (DL) [12] was defined as the amount of drug associated with nanoparticles relative to the total mass of nanomedicine material (i.e. drug and carrier). For the mass of the carrier, it was assumed that all the mass of polymer or lipids used to prepare the batches formed nanoparticles.

2.3.5. Data analysis

The size, concentration, density and mass of the nanoparticles and the amount of diclofenac associated to nanoparticles were presented as the mean with standard deviation based on measurement repeats. Paired *t*-test was performed to compare the nanoparticle size, concentration, density and mass between placebo and loaded nanoparticles. The *t*-test values ($|t_{\text{calc}}|$) were calculated and compared to tabulated values (t_{tab}) ($p = 0.05$) using Excel software version 2208 (Microsoft, Redmond, WA, USA).

3. Results and discussion

3.1. Characterization of nanoparticle size, charge, and chemical composition

3.1.1. PLGA nanoparticles

The polymeric nanoparticles under analysis presented diameters within the range suitable for passive delivery to inflammation sites (50 nm–200 nm, Table 1) [58]. For all the tested formulations, placebo

nanoparticles had larger diameters (171 nm–203 nm) than those prepared in the presence of diclofenac (140 nm–177 nm, $p = 0.05$) accordingly to DLS size measurements (Table 1). For all formulations, narrow distributions were obtained, with polydispersity index (PI) values comprised within 0.08 and 0.21.

The larger diameters found by DLS for placebo relative to loaded nanoparticles were consistent with the mean diameters measured by PTA for all the formulations (Table 1, Fig. S2), although significant differences between placebo and loaded nanoparticles were only found by PTA for both batches of formulation F1 ($p = 0.05$). For these batches, PTA analysis also revealed differences in particle number concentration, with loaded nanoformulations exhibiting higher particle concentrations with respect to placebo batches ($p = 0.05$, Table 1). This is consistent with theoretical nanoparticle concentration estimates [59], considering the size (Table 1) and density (Table 2) values found for these particles. It appears that for the loaded particles the presence of diclofenac is likely to affect the interactions taking place during particle assembly, with consequent formation of a higher number of smaller particles with respect to the placebo. Strong hydrophobic interactions between hydrophobic molecules (as diclofenac) and PLGA during nanoparticle

Table 2

Nanoparticle (NP) density as measured by DCS for polymeric NPs, estimated mass of NPs-associated diclofenac for 1 mL of colloidal formulations (M_d) and the corresponding encapsulation efficiency (EE) and drug loading values.

Formulation	Type	Density, g cm ^{-3a}	M_d , µg ^{a,b}	EE ^a , %	Drug loading ^a , %
F1, batch 1	Placebo	1.05 ± 0.01			
	Loaded	1.08 ± 0.01 ^c	13 ± 2	7.5 ± 0.8	0.53 ± 0.06
F1, batch 2	Placebo	1.039 ± 0.007			
	Loaded	1.060 ± 0.007 ^c	5.7 ± 0.5	3.4 ± 0.3	0.23 ± 0.02
F2, batch 1	Placebo	1.035 ± 0.005			
	Loaded	1.043 ± 0.006	2.0 ± 0.4	1.2 ± 0.3	0.08 ± 0.02
F2, batch 2	Placebo	1.036 ± 0.004			
	Loaded	1.043 ± 0.002 ^c	2.0 ± 0.2	1.1 ± 0.1	0.08 ± 0.01
F3, batch 1	Placebo	1.096 ± 0.011			
	Loaded	1.098 ± 0.013	0.5 ± 0.1	0.5 ± 0.1	0.05 ± 0.01

^a Corresponds to mean ± standard deviation of all independent measurements performed for a batch.

^b Determined for each replicate (independent measurements). Results are mean ± standard deviation of all measurements. Similar RSD values were obtained between batches for M_d values.

^c Student's *t*-test showed values to be significantly different ($p = 0.05$) between placebo and loaded nanoparticles of the same batch.

Table 1

Mean values for Z-average, PI, mean and mode diameters and number concentration for the polymeric nanoparticles under study as measured by DLS and PTA with associated standard deviations.

Formulation	Type	DLS		PTA		
		Z-average, nm	PI	Mean diameter, nm	Modal diameter, nm	Number concentration, 10 ¹¹ mL ⁻¹
F1, batch 1	Placebo	180 ± 3	0.11 ± 0.01	200 ± 20	176 ± 20	1.1 ± 0.2
	Loaded	140 ± 2	0.17 ± 0.02	157 ± 10	131 ± 6	2.2 ± 0.2
F1, batch 2	Placebo	171 ± 1	0.12 ± 0.01	168 ± 3	150 ± 10	1.3 ± 0.2
	Loaded	144 ± 2	0.21 ± 0.01	128 ± 10	112 ± 7	2.4 ± 0.4
F2, batch 1	Placebo	197 ± 2	0.08 ± 0.04	160 ± 10	150 ± 10	1.1 ± 0.3
	Loaded	166 ± 1	0.13 ± 0.01	160 ± 10	147 ± 6	1.1 ± 0.3
F2, batch 2	Placebo	189 ± 2	0.09 ± 0.02	170 ± 20	150 ± 10	1.1 ± 0.2
	Loaded	160 ± 1	0.11 ± 0.02	155 ± 8	140 ± 10	1.4 ± 0.4
F3	Placebo	203 ± 2	0.09 ± 0.01	171 ± 8	150 ± 2	1.3 ± 0.2
	Loaded	177 ± 2	0.09 ± 0.03	153 ± 6	132 ± 5	1.8 ± 0.3

assembly have been described, resulting in the preferential partition of these molecules into PLGA nanoparticles' core.

Surface ζ -potential values close to zero at pH $\approx$ 6.5 were found for both placebo and loaded nanoparticles. This result may suggest that the negative charge of the acid-terminated PLGA polymer used for the nanoparticle preparation is being shielded by other component. For example, a neutral surface charge was obtained for PLGA nanoparticles (made of the same polymer used herein) after their coating by polyethylene glycol resulted in a close to neutral charge, in comparison with the slightly negative charge (-7 to -5 mV) measured for uncoated nanoparticles [57]. Likewise, the ability of the surfactant PVA (used during nanoparticle preparation to prevent PLGA coalescence [60]) to coat the surface of PLGA nanoparticles promoting neutral surface charge has been described before [61–63]. This is also consistent with PVA sterically stabilising the particles, as suggested by the absence of extensive sample agglomeration despite the close to neutral surface charge (Fig. S2).

Indeed, XPS analysis shows that although PLGA is the major component of the nanoparticles' surface (within the ~ 10 nm information depth of XPS), coating of the nanoparticles under study by the PVA/PVAc is likely because $\approx 33\%$ and 38% (homogeneous equivalent atomic concentration) of the placebo and loaded nanoparticles' surfaces, respectively, are PVA/PVAc (Figs. S3–4, Table S1), corresponding to an average PVA/PVAc coating thicknesses of (0.6 ± 0.2) nm and (0.8 ± 0.2) nm, respectively [64]. The presence of a PVA/PVAc layer at the surface of PLGA nanoparticles was also described by other authors [65]. Hydrocarbon is a common adventitious contaminant in XPS analysis: nevertheless, no peak corresponding to contamination was required to achieve a suitable fit for the carbon 1s spectra from the nanoparticles. Indeed, in the presence of some adventitious hydrocarbon, the proportion of PVA observed would be lower. Likewise, the relative intensities of the constrained peaks indicate that any carbon contamination present is likely negligible.

No significant difference in the surface chemical composition of the two systems (i.e. placebo and loaded) was found by XPS. Therefore, the size differences between placebo and diclofenac-loaded nanoparticles for both batches of formulation F1 are unlikely to result from differences in their surface (i.e. ~ 10 nm superficial layer) composition. Dissimilarities between the core composition of placebo and loaded nanoparticles could possibly justify these results. To test this hypothesis, we repeated the XPS analysis on the same particles after incubation with 50% (v/v) ethanol, that is expected to destabilize the structure of the particles, thus allowing the analysis of a mixture of core and surface materials. Contrary from previous analysis on the surface of the particles, the results show in this case different PLGA:PVA ratios for placebo and loaded nanoparticles (Fig. S4, Table S1). This result suggests that there are differences in the chemical composition of the two cores of the two types of particles. These differences could be justified by the establishment of different PLGA-acetone and PLGA-PVA interactions during nanoparticles assembly process in the presence and absence of diclofenac, as discussed with further extension on Supporting Information (section 2.1) [48,60,66].

The differences in size found between placebo and loaded nanoparticles, along with differences in concentration and chemistry, informed the decision to measure the nanoparticles mass (M_p), using the size and the concentration values found across placebo and loaded nanoparticles along with the density measurements for each (Eq. (2)) for determining diclofenac association to nanoparticles by the DCS-PTA strategy.

3.1.2. Nanostructured lipid carriers

The diclofenac-loaded nanostructured lipid carriers (NLCs) prepared with different amounts of drug (2 mg–6 mg) exhibited no marked differences in particle mean diameters and concentration values in relation to placebos (Table S2), along with close to neutral surface charge. Likewise, XPS analysis (Fig. S5) suggested no marked differences in the

surface composition of placebo and diclofenac loaded NLCs. Considering the molecular components of the NLCs (PVA, PVAc and the fatty acids Wittepsol and Miglyol) and their carbon and oxygen composition, the XPS results suggest that NLCs' surface was mainly composed of the lipids (with C:O ratio $> 5:1$), while the surfactant PVA (with a C:O ratio of 2:1) was located more inwards away from the surface. As far as the detection of diclofenac is concerned, neither nitrogen nor chlorine were observed by XPS in the survey scans (Fig. S5). Considering that diclofenac consists of approximately 5% nitrogen by number of atoms (excluding hydrogen, which is not analysed by XPS), and the detection limit of this element in these samples by XPS is less than 0.3% (considering XPS detection limits [54]), this puts an estimate for the upper limit on the volume fraction of diclofenac present at approximately 5–6%. Also, no differences in the composition of placebo and loaded NLCs were observed by XPS when NLCs were disrupted with ethanol and analysed.

3.2. Determination of nanoparticle encapsulation efficiency

3.2.1. PLGA nanoparticles

To evaluate the feasibility of assessing EE values using the density values determined by DCS, the mass of diclofenac associated with different batches of PLGA nanoparticles was estimated by the DCS-PTA strategy and by HPLC-UV analysis, and the results compared. Results are shown in Tables 2 and 3 respectively. For the DCS-PTA strategy, particle mass was measured according to equation (2), using the particle density values estimated (Equation (1)) from DCS and PTA number-based distributions (Fig. 2) along with the mean values of the particle diameter and concentration measured by PTA across the loaded and placebo batches. In the case of formulation F1-batch 1, higher particle densities were found for loaded with respect to placebo nanoparticles (Table 2, Fig. S6). Calculating the differences in placebo and loaded nanoparticle masses according to equation (3), M_d results in (13 ± 2) μg for the loaded batch. The same analysis performed on a second batch (F1-batch 2) revealed placebo particles with similar densities to those from batch 1 but loaded particles exhibiting lower densities with respect to batch 1, resulting in a lower amount of loaded diclofenac for the same volume of formulation (Table 2). These results correlated with the HPLC-UV measurements (Table 3). Indeed, for these two batches, the diclofenac mass ratio (batch 1/batch 2) was found to be 2.3 by the DCS-PTA method and 2.5 by the HPLC-UV analysis (Table 3). Hence, the DCS-PTA approach for the measurement of EE was effective in identifying drug loading inconsistencies between two batches prepared according to the same procedure. Moreover, awareness of such inconsistencies could be raised upon observation of the measured DCS and PTA number-based size distributions (Fig. 2A and B), which exhibited different profiles.

This approach was extended to the analysis of the formulations F2 and F3. These were tailored to carry lower diclofenac loading with respect to F1. The analysis of F2 formulation revealed consistency between placebo and loaded nanoparticles for batches 1 and 2 (Table 2).

Table 3

Estimated values of M_d , total diclofenac^a, encapsulation efficiency (EE) and drug loading measured for the polymeric nanoparticles under analysis by HPLC.

Formulation	M_d , μg	Total diclofenac ^a , μg	EE, %	Drug loading, %
F1, batch 1	13.28 ± 0.01	147.4 ± 0.3	7.4 ± 0.3	0.531 ± 0.001
F1, batch 2	5.25 ± 0.04	169.7 ± 0.2	3.1 ± 0.1	0.216 ± 0.002
F2, batch 1	2.62 ± 0.04	172 ± 4	1.45 ± 0.06	0.104 ± 0.002
F2, batch 2	2.84 ± 0.01	161.4 ± 0.1	1.67 ± 0.01	0.117 ± 0.001
F3	0.57 ± 0.01	101.3 ± 0.1	0.55 ± 0.03	0.054 ± 0.001

^a Total diclofenac (associated to nanoparticles and free) found in 1 mL of formulation by HPLC analysis.

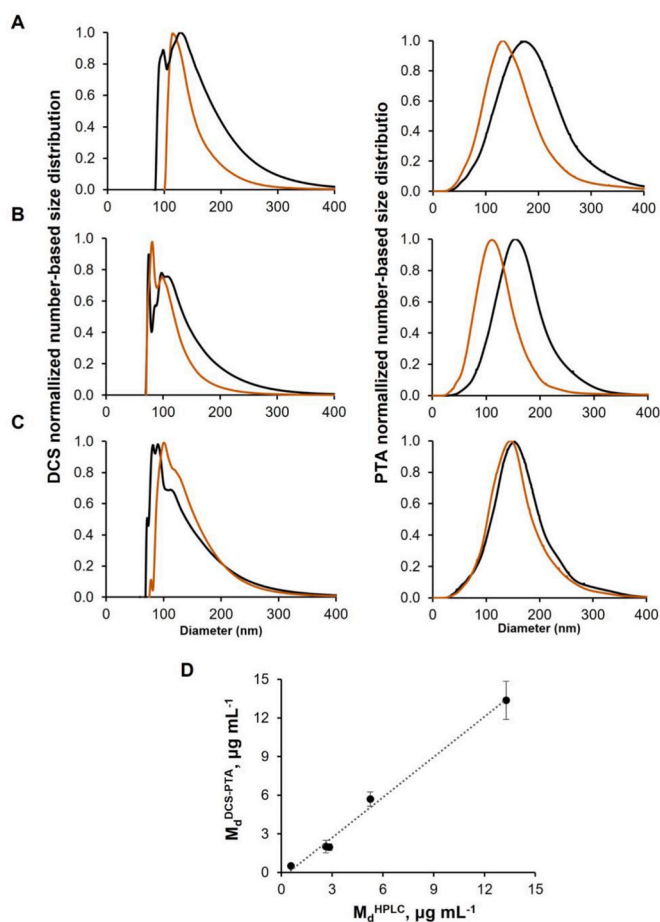


Fig. 2. Average DCS (A–C left panel) and PTA (A–C right panel) number-based size distributions for placebo (black line) and loaded (orange line) nanoparticles from batches F1#1, F1#2 and F2#1 (A, B and C, respectively). Loaded diclofenac mass (M_d , $\mu\text{g mL}^{-1}$) found by DCS-PTA vs. HPLC (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Lower loading efficiencies were obtained with respect to F1, with M_d values of $(1.2 \pm 0.3) \mu\text{g}$ and $(1.1 \pm 0.1) \mu\text{g}$ for batches 1 and 2, respectively. Thus, the results provided by the DCS-PTA approach suggested a consistent preparation between F2 batches, which is reflected in the reproducible number-based size-distributions that were measured (Fig. 2 and Fig. S2). Furthermore, the lower diclofenac encapsulation yield for F2 in relation to F1 batches was confirmed by HPLC-UV analysis (Table 3).

For the F3 nanoparticle formulation, the M_d value was $(0.5 \pm 0.1) \mu\text{g}$, which was also consistent with HPLC results (Table 3).

Mass calculations based on the number-based size distributions measured by the DCS and PTA allowed to identify nanoparticle preparations with different drug loading efficiencies (F1-batch 1 > F1-batch 2 > F2 batch 1 > F2 batch 2 > F3) and to assess batch-to-batch consistency. The results were in agreement with the measurements performed by HPLC-UV, as shown in Fig. 2D. In fact, the fit of the two datasets provided a linear correlation given by $M_d^{DCS-PTA} = (1.05 \pm 0.05) \times M_d^{HPLC} - (0.56 \pm 0.35)$ with a $R^2 = 0.975$, where the superscript specifies the method (Fig. 2D). It should be noted that although the mean measured density values for loaded nanoparticles were higher than the mean measured density values obtained for placebo nanoparticles for all tested formulations (Table 2), significant differences ($p = 0.05$) were only found when diclofenac loading was $\geq 0.75 \text{ ng per } \mu\text{g}$ of PLGA.

To validate the proposed correlation model, the mass of diclofenac associated with nanoparticles per unit volume of formulation was back-

calculated using the values obtained by DCS-PTA approach [67,68]. Relative deviations of $\pm 12\%$ from the nominal mass values were obtained for the F1 formulation batches, which contained $> 2 \text{ ng}$ of diclofenac loaded per $1 \mu\text{g}$ of PLGA (Table 3). The relative deviation increased with decreasing particle loading. For batches 1 and 2 of the F2 formulation relative deviations up to $\pm 15\%$ were found. Higher deviations than the limits accepted by bioanalytical method guidelines ($\pm 20\%$) for the lower limit of quantification were obtained for the F3 formulations (with loadings $< 0.5 \text{ ng of drug } \mu\text{g}^{-1}$ of PLGA). Therefore, considering the deviations obtained, the proposed correlation model revealed suitability for nanoparticles with $\geq 0.7 \text{ ng}$ of loaded compound per μg of polymer [61,68]. This represents the lower limit of quantification for the method.

3.2.2. Application to lipid nanocarriers

The proposed approach was applied to a different type of formulation composed by lipid nanoparticles (nanostructured lipid carriers, NLCs). For these nanoparticles, DCS measurements were performed using a set up in which the particles float in a medium of higher density towards the center of the disc (Fig. S1b). Representative number-based size distributions measured by DCS and PTA are shown in Fig. 3A and B respectively.

The measured density values of NLCs loaded with 2, 4 and 6 mg (NLCs A, B and C) of diclofenac were higher but not significantly different than those measured for placebo nanoparticles for all tested formulations (Table 4, $p = 0.05$; Fig. S7).

We observed a trend where the density differences increased with increasing diclofenac added during nanoparticle assembly process, resulting in M_d values of approximately $14 \mu\text{g}$, $33 \mu\text{g}$ and $48 \mu\text{g}$ for batches #A, #B and #C respectively (Table 4). The EE values determined by the DCS-PTA strategy were consistent with those measured by HPLC analysis, and a linear correlation between the results of the two methods was found (Fig. 3C) with a slope of (0.81 ± 0.07) and a R^2 value of 0.970.

When M_d was back calculated using the values measured by the DCS-PTA strategy, and compared to the value measured by HPLC, relative deviations below 15% were found for amounts of loaded drug $\geq 1.1 \text{ ng}$ per μg of lipids (NLC formulations B and C). For lower drug loadings, higher deviations ($> 20\%$) were observed. Thus, we recommend that the DCS-PTA method is applied to NLCs with drug loading values above 0.11%, which is above the threshold set for the analysis of PLGA nanoparticles under a sedimentation setup.

We showed that NLC density measurements in flotation mode combined with number concentration measurements enabled the observation of differences in the amount of diclofenac associated with the nanoparticles (NLC A < B < C) and obtained good quantitative agreement with the values measured by HPLC. This is a valuable output for quality control operations for lipid nanoparticles, once options for the purification of these nanocarriers prior to HPLC quantification are limited (i.e. ultracentrifugation is challenging for low density nanocarriers [20,69]; long centrifugation during ultrafiltration procedures for complete separations of nanoparticles from free drug often compromises the integrity of these nanoparticles [21]).

Moreover, the slope obtained for $M_d^{DCS-PTA}$ vs. M_d^{HPLC} for the NLCs was close to the values found for the polymeric nanoparticles, despite the different bulk and surface composition of these particles, and the different analysis modes used on DCS (flotation vs. sedimentation). In fact, when the $M_d^{DCS-PTA}$ values determined for all the polymeric and lipidic particle batches were plotted against the M_d^{HPLC} , a linear correlation with a slope of (0.90 ± 0.03) and a R^2 value of 0.986 was obtained (Fig. 4). The same plotting eliminating the highest M_d value (NLC C), thus corresponding to M_d ranging from $\approx 0.5 \mu\text{g}$ to $33 \mu\text{g}$, resulted in a slope of (1.04 ± 0.03) and in a R^2 value of 0.990 (Fig. 4). This result may suggest that organic nanoparticles from different batches and presenting different compositions but with close density values (relative deviations $< 15\%$ for the density values measured across all the polymeric and lipid

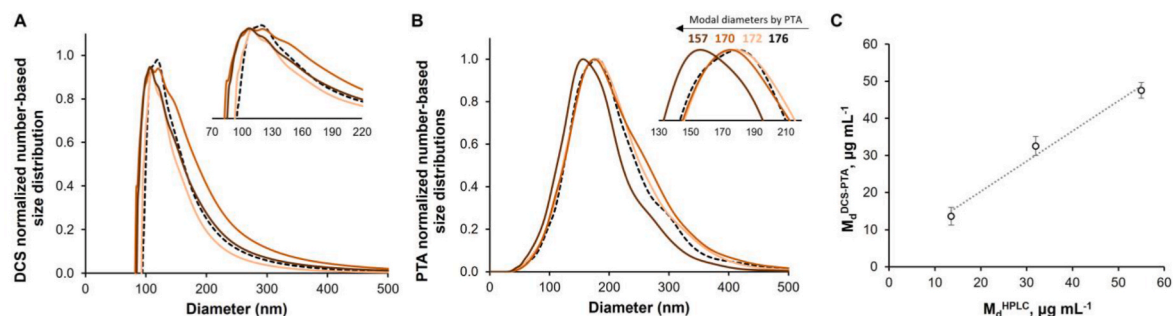


Fig. 3. Representative DCS (A) and PTA (B) number-based size distributions for placebo (black dashed line) and loaded (orange solid lines, darkening representing increased quantities of diclofenac NLCs). Loaded diclofenac mass (M_d , µg) mL⁻¹ nanoformulation found by DCS-PTA vs. HPLC (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 4

Measured density values by DCS, and the estimated M_d and drug loading (DL) values obtained by the DCS-PTA and HPLC-UV approaches for the NLCs under analysis.

Batch	Formulation	DCS			HPLC	
		Density, g mL ⁻¹	M_d^a , µg	%DL	M_d^a , µg	%DL
#A	Placebo	1.072 ± 0.001	–	–	–	–
	Loaded	1.073 ± 0.001	14 ± 2	0.05 ± 0.01	13.6 ± 0.1	0.051 ± 0.001
#B	Placebo	1.068 ± 0.006	–	–	–	–
	Loaded	1.069 ± 0.006	33 ± 2	0.118 ± 0.01	32 ± 1	0.116 ± 0.002
#C	Placebo	1.064 ± 0.005	–	–	–	–
	Loaded	1.066 ± 0.001	48 ± 2	0.178 ± 0.01	55.0 ± 0.1	0.206 ± 0.001

^a Determined for each replicate (independent measurements). Results are mean ± standard deviation of all measurements.

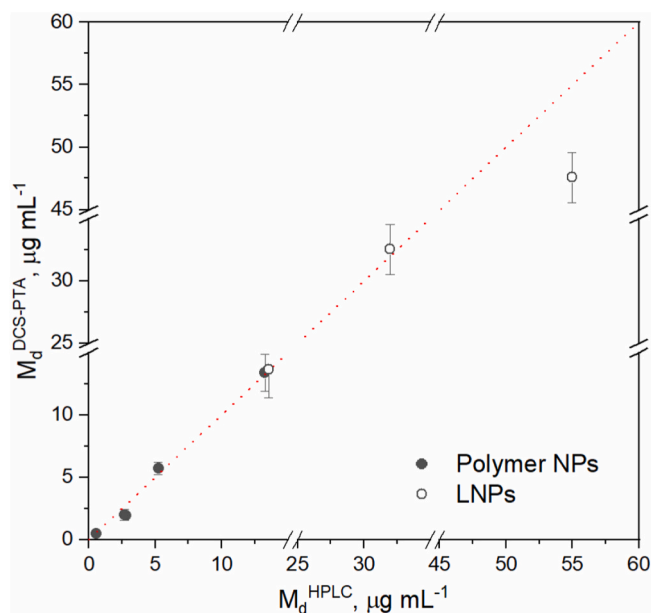


Fig. 4. Loaded diclofenac mass (M_d , µg) mL⁻¹ nanoformulation found by DCS-PTA vs. HPLC for all the polymeric nanoparticles (●) and NLCs (○) under study in this work.

nanoparticles batches), and with M_d values comprised within 0.5 µg–33 µg may be fitted within the same model. Such would provide a universal strategy for drug loading measurement in organic nanocarriers. Nevertheless, additional work is required to further explore the potential of these strategy for nanoparticles with higher drug loading values and for application to nanoparticles with diverse compositions.

3.2.3. Comparison with existing independent and/or orthogonal methods to HPLC for the evaluation of drug encapsulation

In the proposed approach, diclofenac encapsulation into polymeric and lipid nanoparticles was assessed by measuring the differences in density and concentration between loaded and placebo nanoparticles. This approach allowed identification of nanoformulations loaded with different amounts of diclofenac, for amounts of loaded diclofenac ≥ 0.7 ng per 1 µg of PLGA for PLGA nanoparticles, and ≥ 1.1 ng per 1 µg of lipids for NLCs. The amounts of drug loaded per µg of nanocarrier material quantified by the proposed approach are lower than those tested so far by ultracentrifugation, NMR and circular dichroism based methods with reported measurements ranging from 21 to 4385 ng per µg of nanoparticles material [22–25,28,70,71]. Likewise, the proposed approach enabled determinations of drug loaded per µg of nanocarrier material slightly lower than those accomplished by asymmetric flow field-flow fractionation systems coupled to multiple detectors (0.87 ng µg⁻¹ PLGA) [27]. Higher sensitivity has only been reported for SAXS, which can measure interaction between the drug and the nanoparticle at molecular level [30,31]. Moreover, the linear correlation of the results given by the DCS-PTA approach with HPLC-UV method determinations, this latter being considered the gold standard for drug quantification, shows a clear route to method validation for quality control purposes. Density measurements produced relative standard deviation (RSD) values < 3%, while estimated values of nanoparticle mass shown RSD values < 5%. Still, these values represent the experimental variability, and do not correspond to the full uncertainty budget of the DCS-PTA approach, which should also account for the variability of size and concentration measurements performed by PTA, resulting in a larger uncertainty in the final result. Nevertheless, despite the large uncertainty associated with EE measurements by the DCS-PTA approach, the systematic errors in many of the measurements are expected to be cancelled, justifying the use of experimental standard deviation values for error measurement.

The proposed strategy requires minimal sample preparation, has a straightforward operation, with streamlined detection and does not require expensive equipment and experienced personnel in comparison to NMR or SAXS [28–31]. Moreover, it is relatively fast: the density measurements were performed within 15 min.

Finally, calibration curves with drug standard solutions are not required because the measurement is not based on the optical properties of the encapsulated drug. This is an advantage compared to other methods, such as HPLC, capillary electrophoresis and circular dichroism

[24,25,70,71], where the determination of amounts of loaded drug is based on comparison of analytical signals obtained from standards containing the target drug. Since DCS measurements are based on changes in nanoparticles sedimentation time, which in turns depends on their mass, resulting from the loading of substances within the particle carrier, its application for analysing PLGA nanoparticles and NLCs loaded with other small molecules or larger entities (e.g. metallic nanoparticles [44] or proteins [42]) is feasible.

4. Conclusions

In this work, the feasibility of density and concentration measurements to evaluate the amount of drug associated to nanoparticles was demonstrated. Measurements of nanoparticle mass were performed by DCS in sedimentation or flotation modes using nanoparticle diameter and concentration measured by the independent method of PTA. The proposed approach was applied to different batches of diclofenac loaded PLGA polymeric nanoparticles and lipid nanocarriers. A linear correlation and reasonable quantitative agreement between the amount of diclofenac loaded into the nanoparticles determined by the DCS-PTA strategy and HPLC was found.

Density measurements by DCS may be used as an independent approach to HPLC quantification to evaluate drug encapsulation efficiency. Since no standardized methods have been made available so far to assess this parameter, the set of independent procedures for data comparison and validation provides a strategy for the accurate and thorough characterization of nanomedicines and to their smoother translation into clinic application.

Moreover, the application of density measurements by DCS during routine quality control procedures, such as batch-to-batch consistency monitoring, may constitute a simpler and faster approach for batch release with respect to current practice. Therefore, DCS combined with PTA represents a promising expansion of the analytical toolset available to characterize nanoparticles.

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CRediT authorship contribution statement

Sara S. Marques: Investigation, Formal analysis, Data curation, Writing – original draft. **David J.H. Cant:** Investigation, Formal analysis, Data curation, Writing – review & editing. **Caterina Minelli:** Methodology, Resources, Formal analysis, Data curation, Supervision, Project administration, Writing – review & editing, Funding acquisition. **Marcela A. Segundo:** Formal analysis, Supervision, Data curation, Project administration, Writing – review & editing, Funding acquisition.

Declaration of competing interest

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aca.2023.341234>.

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