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A NIR labelled PLGA-PEG/PHEA-*g*-Dy700-*g*-PLA self-traceable nanoplatform for intranasal delivery

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In the present work, near infrared (NIR) fluorescent polymeric nanoparticles were developed as a traceable platform to investigate their path following intranasal administration. Nanoparticles were produced by a nanoprecipitation method using blends of polyethylene glycol–polylactic acid-co-glycolic acid (PLGA-PEG) and an amphiphilic graft copolymer, α,β -poly(*N*-2-hydroxyethyl)-D,L-aspartamide-*g*-Dy700-*g*-polylactide (PHEA-*g*-Dy700-*g*-PLA), in which the NIR fluorophore Dy700 was covalently bound to ensure signal stability. Physicochemical analyses using dynamic light scattering (DLS) and atomic force microscopy (AFM) demonstrated that all nanoparticle formulations exhibited appropriate colloidal properties for intranasal administration (a mean size of <130 nm, a polydispersity index of <0.3, and ζ -potential ranging from –25 mV to –1 mV) and a spherical shape. Quantitative ¹H-NMR confirmed the surface PEGylation of the nanoparticles, supporting their potential mucus-penetrating behaviour. The nanoparticles maintained their physicochemical properties after incubation in simulated biological fluids for up to three hours. *In vitro* studies on olfactory ensheathing cells (OECs) demonstrated cytocompatibility and efficient cellular uptake. Pareto analysis and a desirability function were applied by considering both normalized cellular uptake and a fluorescence signal as response variables, in order to identify the most suitable formulation for fluorescence molecular tomography (FMT) and subsequent *in vivo* evaluation. Following intranasal administration in healthy mice, FMT enabled non-invasive, three-dimensional monitoring of nanoparticle distribution, revealing rapid and preferential accumulation of fluorescence signals within the brain region.

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1. Introduction

Over the past decade, the nose-to-brain (N2B) route of administration has garnered increasing interest in biomedical

research as a strategy for central nervous system (CNS) drug delivery, as evidenced by the growing body of literature on this topic.^{1–3} This route can offer a direct passage of drugs from the nasal cavity to the cerebrospinal fluid (CSF) *via* the trigeminal and olfactory pathways, effectively bypassing the blood–brain barrier (BBB). The olfactory route involves both axonal transport along olfactory neurons to the olfactory bulb and extracellular diffusion *via* perineural and perivascular spaces into the CSF, enabling rapid and direct access to multiple brain regions. The trigeminal route transports molecules along trigeminal nerve branches to the brainstem and, in some cases, directly to the olfactory bulb, supporting both intracellular and extracellular transport, generally at a slower rate.⁴ In addition to these direct routes, indirect systemic transport can contribute to CNS exposure, in fact substances absorbed in the nasal respiratory mucosa may enter the bloodstream and reach the brain *via* the BBB. Traditional nasal devices, such as simple droppers or standard sprays, predominantly deposit formulations in the respiratory mucosa, leading to systemic absorption rather than direct CNS transport. Efficient N2B delivery, however, critically depends on deposition in the olfactory region. Optimizing device selection is

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crucial for achieving efficient and reliable CNS delivery: specialized intranasal devices, such as ViaNaseTM, Precision Olfactory Delivery[®], OptiMistTM and NaltosTM, have been developed to enhance deposition in the olfactory region employing various strategies, including nebulized vortices, gas-propelled aerosols, positive pressure and fine mist sprays.^{5–7} Despite these advantages, challenges such as mucociliary clearance, enzymatic degradation, local pathological conditions and the limited volume that can be administered remain. To address these issues, numerous preclinical studies have demonstrated encouraging results using nanoparticles (NPs) for N2B delivery, which can offer improved retention, controlled release and enhanced N2B transport.^{8–11} Moreover, thanks to their small size, NPs could be phagocytised by olfactory ensheathing cells (OECs), Schwann cell-like glial cells encircling olfactory neurons, and according to recent hypotheses, this may assist their access to the brain *via* retrograde axonal transport along the olfactory nerve pathway.^{12,13}

Despite its potential, clinical translation remains limited due to an incomplete understanding of the transport mechanisms and the complexity of the cranial nervous system.¹⁴ In this context, reliable tracking of nanocarriers is essential for the correct interpretation of biodistribution data.^{15–17} Fluorescent NPs are widely used for this purpose; however, the physical encapsulation of fluorophores may introduce additional experimental complexity, as probe leakage, photobleaching or signal redistribution can potentially occur. As a result, extensive complementary investigations, such as release and stability studies, are often required to verify signal stability and to exclude imaging artifacts, particularly in *in vivo* settings.^{16,18,19}

In previous work, Bonaccorso *et al.* reported the biodistribution of intranasally administered positively charged NPs, specifically chitosan-coated PLGA NPs, using fluorescence molecular tomography (FMT), showing a time-dependent accumulation of the fluorescence signal in specific brain regions following administration.^{13,18} Subsequent studies explored surface modifications, including PEGylation and the use of covalently linked fluorescent probes, to improve mucus penetration and enhance the reliability of imaging data.²⁰ While probes absorbing and emitting light in the visible spectrum are well suited for cellular-level investigations, their spectral properties limit their direct translation to whole-body and tomographic *in vivo* imaging approaches, due to tissue absorption, autofluorescence and scattering.²¹ This consideration motivated the adoption of near-infrared (NIR) fluorophores specifically compatible with FMT for *in vivo* biodistribution studies. In this framework, distinguishing between signals arising from intact NPs and those originating from released cargo remains an important methodological consideration for studies aimed at investigating N2B delivery. In the present work, we report the design and production of polymeric NPs for potential N2B applications based on blends of PLGA-PEG and the α,β -poly(*N*-2-hydroxyethyl)-D,L-aspartamide-*co*-polylactide graft copolymer (PHEA-*g*-Dy700-*g*-PLA). Dy700, a NIR fluorophore suitable for real-time, non-invasive deep tissue imaging, was covalently linked to the polymer backbone to obtain an amphiphilic copolymer with stable fluorescence in the NIR window.^{22–24} PLGA-PEG was selected to provide a hydrophilic surface and enhance brain transport.²⁵ Based on previous evidence

showing that NP size, internal organization and fluorescence output are strongly dependent on polymer composition and dye distribution within the matrix, a systematic modulation of the copolymer ratio was adopted.²⁶ Rather than focusing on a single formulation, a series of NPs with increasing amounts of the fluorescent graft copolymer were investigated to identify compositional windows in which colloidal properties and fluorescent detectability could be simultaneously preserved. To address this, we implemented a stepwise enrichment strategy by gradually increasing the PHEA-*g*-Dy700-*g*-PLA fraction (20, 40, 60, and 80 wt% of the total polymer weight) while correspondingly reducing the PLGA-PEG fraction. Following NP production by nanoprecipitation, the resulting NPs were characterized in terms of their physicochemical properties, morphology, and stability under storage conditions and in simulated nasal fluid.

Biological characterization included cytocompatibility and cellular uptake studies in OECs. This stepwise approach provided a rationale for selecting an optimal formulation suitable for both cellular studies and non-invasive *in vivo* imaging. Finally, a selected formulation was evaluated *in vivo* after intranasal (IN) administration in healthy mice using FMT as a proof-of-concept for non-invasive biodistribution imaging.

2. Materials and methods

2.1 Materials

Poly(ethylene glycol) methyl ether-*block*-poly(L-lactide-*co*-glycolide) (PEG average M_n 5.000; PLGA M_n 55.000), polyoxyethylene sorbitan monooleate (Tween[®] 80), D,L-poly(lactic acid) (PLA acid terminated, M_w 10–18 kDa), and mucin (mucin from porcine stomach type II) were purchased from Sigma Aldrich (Milan, Italy). Ethanol was purchased from Honeywell International Inc. (North Carolina, USA). Analytical-grade acetonitrile was purchased from Romil Ltd (Waterbeach, United Kingdom). A dialysis membrane (molecular weight cut off (MWCO), 3500 Da; diameter, 29 mm; Spectra/Por[®]) was purchased from VWR International PBI Srl (Milan, Italy). Deionized water was used throughout this study.

2.2 Synthesis of the PHEA-*g*-Dy700-*g*-PLA graft copolymer

α,β -Poly(*N*-2-hydroxyethyl)-DL-aspartamide (PHEA) was synthesized and characterized according to a previously reported procedure.^{22,23}

The PHEA-*g*-Dy700-*g*-PLA graft copolymer was synthesized, purified, and characterized according to a two-step procedure.²⁴ The first step was the coupling of the hydroxyl groups of PHEA with the carboxylic acid groups of Dy700 to obtain the PHEA-*g*-Dy700 copolymer. In detail, Dy700 was dissolved in DMF-a (1.1 mg mL^{-1}), and an appropriate amount of CDI was added according to $R_1 = \text{mmol CDI per mmol Dy700} = 4$. The mixture was stirred for 4 hours at 40 °C. Meanwhile, PHEA was dissolved in DMF-a (37.5 mg mL^{-1}), according to $R_2 = \text{mmol Dy700 per mmol of repeating units on PHEA} = 0.003$. DEA, as a catalyst, was added to this solution ($R_3 = \text{mmol DEA per mmol Dy700} = 20$). After activation, the PHEA organic dispersion was added dropwise to the Dy700 solution and left to react for 68 hours at 40 °C. The reaction mixture was then precipitated into diethyl ether,

and the solid precipitate was collected by centrifugation (9800 rpm, 10 min, 4 °C), washed twice with ethanol, vacuum-dried, and redissolved in distilled water. Final purification was performed *via* size-exclusion chromatography (Sephadex G-25, water as an eluent), and the purified fractions were freeze-dried. The PHEA-*g*-Dy700 graft copolymer was obtained with a yield of 72 wt% based on the starting copolymer (Fig. S1).

¹H-NMR (300 MHz, D₂O, 25 °C, TMS) of PHEA-*g*-Dy700: δ 1.06 (m, 15 H Dy700 CH₃CH₂-); δ 2.69 (m, 2 HPHEA -COCHCH₂CONH-); δ 3.23 (t, 2 HPHEA -NHCH₂CH₂O-); δ 3.52 (t, 2 HPHEA -NHCH₂CH₂O-); δ 4.61 (m, 1 HPHEA -NHCH(CO)CH₂-); δ 8.00–8.40 (m, 11 HDy700 H-Ar) (Fig. S2).

The second synthetic step was the grafting of PLA onto PHEA-*g*-Dy700. In detail, PLA was dissolved in DMF-a (173.3 mg mL⁻¹) and activated with CDI according to R_4 = mmol CDI per mmol PLA = 2 for 4 hours at 40 °C. In parallel, PHEA-*g*-Dy700 was dissolved in DMF-a (83.3 mg mL⁻¹) according to R_5 = mmol PLA per mmol of UR of PHEA = 0.03, and then added dropwise to the activated PLA dispersion in the presence of DEA as a catalyst (R_6 = mmol DEA per mmol PLA = 5). The reaction was left under continuous stirring for 68 hours at 40 °C.

Afterwards, the mixture was precipitated dropwise into a 15:1 diethyl ether/DCM mixture, and the resulting precipitate was collected by centrifugation (9800 rpm, 10 min, 4 °C), washed repeatedly using the same mixture and dried under vacuum. The PHEA-*g*-Dy700-*g*-PLA graft copolymer was obtained with a yield of 60 wt% based on the starting amount of PHEA-*g*-Dy700.

¹H-NMR (300 MHz, d7-DMF, 25 °C, TMS) of PHEA-*g*-Dy700-*g*-PLA: δ 1.48 and δ 1.96 (2d, 582 HPLA -OCOCH(CH₃)O-); δ 3.0 (m, 2 HPHEA -COCHCH₂CONH-); δ 3.48 (t, 2 HPHEA -NHCH₂CH₂O-); δ 3.71 (t, 2 HPHEA -NH-CH₂CH₂O-); δ 4.3–4.6 and δ 5.3–5.6 (m, 194 HPLA -OCOCH(CH₃)O-), and δ 4.98 (m, 1 HPHEA -NHCH(CO)CH₂-) (Fig. S3).

2.3 Preparation of polymeric nanoparticles (NPs)

Polymeric NPs were prepared using the nanoprecipitation method, as previously described,²⁰ with slight modification.

Briefly, PLGA-PEG and PHEA-*g*-Dy700-*g*-PLA were combined at defined weight ratios (see Table S1), maintaining a total copolymer concentration of 4 mg mL⁻¹ in the organic phase.

Due to its amphiphilic nature, PHEA-*g*-Dy700-*g*-PLA was dissolved separately in an acetonitrile/water mixture (7:3, v/v) (phase S1), while PLGA-PEG was dissolved in acetonitrile (phase S2). The two solutions were then mixed at a 1:1 volume ratio to obtain the final organic phase (phase S).

NPs without PHEA-*g*-Dy700-*g*-PLA (NPs-BL) were prepared using the same procedure, omitting phase S1.

The organic phase (S) was added dropwise under magnetic stirring to the anti-solvent phase (AS), consisting of 3 mL of water/ethanol (1:1, v/v) containing 0.1% (w/v) Tween[®] 80, using a 1:1 volume ratio (S).

Following NP formation, organic solvents were removed under reduced pressure (38–40 °C). The final polymer concentration in the NP aqueous suspensions was approximately 7.06 mg mL⁻¹.

2.4 Purification method

Purification of NPs was carried out with the aim of removing excess surfactant, using dialysis as a mild method of purification.^{27,28} The colloidal suspensions were loaded into dialysis membranes (MWCO 3.500 Da, diameter 29 mm; Spectra/Por[®]) and immersed in 500 mL of distilled water, which was replaced with a frequency of water changes per hour (L per h) of 1 L per h (2 L in 2 h with 4 water changes). The NP suspensions were analysed using DLS as previously described (Section 2.4) in terms of mean size, PDI, and ZP before and after the purification process, to assess any variation due to the dialysis procedure.

2.5 Particle size distribution and zeta potential measurements

The mean size (Z-Ave), polydispersity index (PDI) and ζ potential (ZP) of NP suspensions in deionized water were analysed by dynamic light scattering (DLS) using a Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK). The experiments were carried out at a detection angle of 90° at 25 °C with a 4 mW laser operating at 633 nm as a light source. Each value was measured in triplicate. The results are shown as the mean $\pm$ SD.^{27,28}

2.6 Determination of the surface pegylation

The surface pegylation degree of all the NPs samples and the chain conformation were determined as previously reported.²⁰ Briefly, a PEG calibration curve was established using PEG 5000 Da by analysing the integral of the PEG signal at δ 3.6 ppm in spectra obtained from PEG dispersions in a range of concentrations from 0.3 to 2.5 mg mL⁻¹ ($y = 8325x$, $R_2 = 0.9999$), using RhB (3 mg mL⁻¹) as the internal standard.

Then, ¹H-NMR spectra were recorded on NP dispersions in D₂O at a concentration of 25 mg mL⁻¹ and PEG quantification was done by measuring the integral at 3.6 ppm and comparing it with the calibration curve, with each determination repeated in triplicate.

2.7 Colloidal stability in simulated nasal fluid

Simulated nasal fluid (SNF) was prepared as an aqueous solution containing CaCl₂ (0.58 mg mL⁻¹), KCl (2.98 mg mL⁻¹), and NaCl (8.77 mg mL⁻¹), adjusted to pH 6.64.²⁹ These concentrations refer to the stock SNF solution prior to NP dilution.

NP suspensions (initial polymeric concentration $\sim$ 7.06 mg mL⁻¹) were diluted in SNF at a ratio of 1:3 (v/v, NPs suspension), resulting in a final NP polymeric concentration of approximately 1.76 mg mL⁻¹.

Samples were incubated at 37 ± 0.2 °C. At predetermined time points (1 and 3 h), Z-Ave and PDI were measured by DLS (Section 2.4) at 37.0 °C.

2.8 Interaction with mucin

Mucin (MUC) solutions were prepared by dissolving 0.1 g of mucin in 100 mL of SNF, corresponding to a final concentration of 1 mg mL⁻¹ (0.1% w/v). The mixture was maintained under continuous stirring overnight to ensure complete hydration and homogeneity, following previously reported procedures.

2.8.1 Colloidal stability assessments. NP suspensions (initial polymeric concentration $\sim 7.06 \text{ mg mL}^{-1}$) were diluted in SNF containing MUC (0.1% w/v) at a 1:1 (v/v) ratio, in accordance with a protocol previously standardized by our research group, allowing direct comparison with existing literature data on NP–mucin interactions.²⁹ Samples were incubated at $37 \pm 0.2^\circ\text{C}$, and Z-Ave and PDI were measured by DLS (Section 2.4) at T_0 and 3 h to assess possible changes in the particle size distribution and colloidal stability upon exposure to MUC.

2.8.2 Turbidimetric mucoadhesion assay. NP–MUC mixtures were prepared at a 1:1 (v/v) ratio (NP suspension:MUC solution). MUC solution (1 mL) was mixed with 1 mL of water and measured against SNF as a blank.

Samples were incubated at $37 \pm 0.2^\circ\text{C}$, and absorbance at 650 nm was recorded using UV-vis spectroscopy (UH5300, Hitachi, Europe, Milan, Italy) at 15 min (T_0), 30 min, 1 h, 2 h, 3 h, and 24 h. Variations in absorbance were interpreted as indicative of changes in turbidity associated with potential NP–MUC interactions or complex formation.²⁹

2.8.3 ZP interaction assay. Surface charge modifications upon MUC exposure were evaluated by ZP measurements. MUC solution was first analysed in deionized water after a 1:10 (v/v) dilution. This dilution step was applied to reduce the ionic strength of the medium and to improve the measurement stability and reproducibility of electrophoretic mobility values.

For interaction studies, NP formulations were mixed with MUC solution at a 1:1 (v/v) ratio (NP suspension:MUC solution) and incubated under gentle agitation for 15 min. The resulting dispersions were then diluted 1:10 (v/v) with deionized water prior to ZP analysis.

All samples were incubated at $37 \pm 0.2^\circ\text{C}$. ZP measurements were performed at predefined time points (15 min, 30 min, 1 h, 2 h, 3 h, and 24 h) to evaluate potential time-dependent changes associated with MUC adsorption or complexation phenomena.²⁹

2.9 Atomic force microscopy (AFM) analysis

AFM imaging was performed on adlayers prepared by drop-casting at room temperature on freshly cleaved muscovite mica substrates (Ted Pella, Inc., Redding, CA, USA). Briefly, 10 μL of dialyzed NP suspension, $\sim 7.06 \text{ mg mL}^{-1}$ (4 h/4 L, 8 water changes), was deposited on the mica. After 5 min, the surface was rinsed with 1 mL of Milli-Q water, gently dried under a nitrogen stream and imaged. AFM measurements were carried out using a Cypher instrument (Asylum Research, Oxford Instruments, Santa Barbara, CA, USA) equipped with a scanner providing an XY range of 30 μm (closed loop) or 40 μm (open loop). Imaging was conducted in air, in AC-mode, with silicon tetrahedral tips mounted on 30 μm rectangular cantilevers (AT240TS, Olympus, Oxford Instruments, Santa Barbara, CA, USA). Images covering surface areas between 1 and 10 μm^2 were acquired, and particle sizes were analysed using the free tool in the MFP-3DTM offline section analysis software.

2.10 Storage stability studies

2.10.1 Mean size and ζ potential analysis. NP aqueous suspensions were collected after purification and stored at

4°C . A Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK) was used as previously described (Section 2.4) to measure any variation in the mean size, PDI and ZP of NPs during storage at 4°C after 7, 14, 21, 28, 60, 90 days from the preparation. Each value was measured in triplicate.

2.10.2 Fluorescence measurements. Fluorescence was measured using a Hitachi F-7000 spectrometer with a 1 cm path-length cuvette. Measurements were performed on aqueous nanosuspensions, with the excitation wavelength set at 700 nm and emission collected at 725 nm.^{30,31} NPs were analysed after 7, 14, 21, 28, 60, and 90 days of storage at 4°C .

2.11 Differential scanning calorimetric (DSC) analysis

A Mettler Toledo DSC 1 STARE system equipped with a PolyScience temperature controller (PolyScience Illinois, USA) was used to perform calorimetric analyses. The sensitivity was automatically chosen as the maximum possible through the calorimetric system, and the reference was an empty pan. The calorimetric system was calibrated, in temperature and enthalpy changes, by using indium and by following the procedure of the DSC 1 Mettler TA STARE instrument. Each sample was sealed in a 40 mL aluminium pan, with the pure polymers, physical mixtures of polymers, and freeze-dried NPs, which were subjected to heating and cooling cycles in the temperature range of 15–200 $^\circ\text{C}$ at a scanning rate of 5 $^\circ\text{C min}^{-1}$ (heating) and at a scanning rate of 10 $^\circ\text{C min}^{-1}$ (cooling). The transition temperature and enthalpy changes (ΔH) were calculated from peak areas with a Mettler STARE Evaluation software system (version 18.00) installed on Optiplex3020 DELL.

2.12 Cell cultures

OECs were isolated from olfactory bulbs of 2 day-old mice pups (mouse strain C57BL6/J) and all experimental procedures in this study were carried out in compliance with the Italian Guidelines for Animal Care (D.L. 116/92; 26/2014) and in accordance with the European Communities Council Directive (2010/63/EU), in order to minimize animal suffering and reduce the number of animals used. Pups were decapitated, and the bulbs were removed and dissected out in cold ($+4^\circ\text{C}$) Leibowitz L-15 medium (Sigma) containing 25 mM HEPES. Subsequently, bulbs were digested in medium essential medium-H (MEM-H, Sigma) containing 0.03% collagenase (Sigma) and 0.25% trypsin (Sigma) for 15 min at 37°C , as described by Pellitteri *et al.*³² Trypsin activity was stopped by using Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% foetal bovine serum (FBS, Sigma). The digested tissue was mechanically dissociated by filtration through 80 μm nylon mesh and centrifugation at $500\times g$ for 10 min. Finally, cells were resuspended in fresh DMEM medium containing 10% FBS, 2 mM L-glutamine (Sigma), penicillin (50 $\mu\text{g mL}^{-1}$, Sigma) and streptomycin (50 U mL^{-1} , Sigma). Cytosine arabinoside (10^{-5} M), an antimitotic agent, was added 24 h after initial plating, in order to reduce the number of dividing fibroblasts. The purity of OECs was verified through immunocytochemical procedures using p75 and S-100 markers; the percentage of S-100/p75 positive OECs was about 80–90% (data not shown).

Purified OECs were plated both in 96 well plates (at a density of 1.5×10^4 in 200 μL of medium) and in 14-mm diameter poly-L-lysine (PLL; 10 g mL^{-1}) coated glass coverslips into 12 well plates (NuncTM MicroWellTM, Thermo Fisher Scientific) (at a density of 4×10^4 in 1000 μL of medium) for cellular viability and cellular uptake, respectively, and cultured in DMEM/FBS at 37 °C.

2.13 Treatment of OECs

NP concentrations were selected based on previously reported experimental conditions for PLGA-PEG-based NP systems in olfactory ensheathing cells (OECs).²⁰ In this study, NPs were evaluated within a concentration range of 0.1–5.0 mg mL^{-1} . Based on our knowhow, three polymeric concentrations were selected for the present study: 0.140 mg mL^{-1} (low), 0.700 mg mL^{-1} (medium), and 1.4 mg mL^{-1} (high). For improved dose comparability across nanomaterial systems, NP exposure was additionally expressed as particle number per cell (PPC), as detailed in the SI. The highest concentration was selected below the threshold reported to induce morphological alterations in OECs. Finally, the treated cells were incubated at 37 °C in a humidified chamber (5% CO_2 –95% air) for 24 hours. Untreated cells were used as a control.

2.14 Cellular viability

The cellular viability of OECs was evaluated by a quantitative colorimetric method, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT).³³ Briefly, MTT was added to each multiwell with a final concentration of 1.0 mg mL^{-1} . After 2 hours of incubation in a CO_2 incubator, the medium was gently removed and acid-isopropanol/SDS (MTT solvent) was added to block the reduction of MTT (pale yellow) to formazan (violet). Then, cells were placed on an orbital shaker for 15 minutes and the absorbance was measured using a multiskan reader at 570 nm.³⁴ The results are expressed as the percentage MTT reduction of control cells.

Cell viability was calculated using the following equation:

$$\text{Cell viability (\%)} = \frac{\text{Int}_{\text{s}}}{\text{Int}_{\text{control}}} \times 100 \quad (1)$$

where Int_{s} is the colorimetric intensity of cells incubated with NPs, and $\text{Int}_{\text{control}}$ is the colorimetric intensity of untreated cells.

2.15 Cellular uptake assay

After 3 and 24 hours of treatment with NP dispersions at three polymeric concentrations (0.14, 0.70, and 1.40 mg mL^{-1}), OECs plated onto 14 mm diameter poly-L-lysine (PLL; 10 g mL^{-1}) coated glass coverslips at a final density of 1×10^4 cells per coverslip and fed with DMEM/FBS medium were used for the uptake study. Coverslips were analysed through a Zeiss fluorescence microscope (Zeiss, Germany), measuring Dy700 fluorescence at excitation/emission wavelengths of 700/725 nm, and images were captured with an Axiovision Imaging System.

2.15.1 Fluorescence quantification. Fluorescence images were analysed using Fiji (ImageJ, NIH).^{35,36} All images were converted to 8-bit prior to analysis, and background subtraction was applied to reduce a non-specific signal.

For each image, background intensity was determined by manually selecting a cell-free region of the image. Individual cells were subsequently selected as regions of interest (ROIs), and fluorescence intensity was quantified on a per-cell basis. Four cells per image were analysed to account for intra-sample variability.

Fluorescence intensity was quantified as corrected total cell fluorescence (CTCF), calculated as previously reported^{37,38} following eqn (2):

$$\text{CTCF} = \text{integrated density} - (\text{area} \times \text{mean background}) \quad (2)$$

To enable comparison between formulations with different fluorescence properties, CTCF values were normalized using min-max scaling to a 0–1 range. Two parameters were considered: (i) fluorescence signal intensity (CTCF) and (ii) normalized uptake, obtained by correcting fluorescence values for the PHEA-*g*-Dy700-*g*-PLA content in each formulation.

A multi-objective optimization approach was applied to evaluate the trade-off between the fluorescence signal and cellular uptake. The analysis was conducted separately for 3 h and 24 h time points to distinguish early internalization from longer intracellular persistence. For each formulation, the mean values obtained from the three tested concentrations were used to avoid selection bias related to a single dose condition. Pareto dominance analysis was used to identify non-dominated formulations, defined as those for which no other formulation simultaneously exhibited a higher signal and uptake.

To select a single optimal formulation, a weighted geometric mean desirability function was applied to normalized values³⁹ (eqn (3)):

$$D = (\text{signal}_{\text{norm}})^{0.6} (\text{uptake}_{\text{norm}})^{0.4} \quad (3)$$

where greater weight was assigned to the fluorescence signal to reflect its primary relevance for imaging applications.

2.16 Animals and housing conditions

Male C57BL6/J mice (6–8 weeks old, body weight 20–26 g; $n = 4$) were purchased from Envigo Srl (Italy) and underwent health evaluation shortly after arrival. Mice were housed in plastic cages under controlled environmental conditions (room temperature: 22 ± 2 °C, room relative humidity: $55 \pm 10\%$, 24-hour cycle of 12 hours light/12 hours dark, 7 am–7 pm); food and water were administered *ad libitum*. All the experiments were performed in mice under controlled general anaesthesia (isoflurane 2.5%) to avoid pain and discomfort. Their care and husbandry were in compliance with national (D.L.vo n. 26/2014) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, Dec 12, 1987; Guide for the Care and Use of Laboratory Animals, US National Research Council, 2011); the protocol was approved by the Institutional Animal Care and Use Committee of the University of Milano-Bicocca.

2.17 Non-invasive monitoring of the fluorescence NP signal following intranasal administration

The path of the fluorescent NPs to the brain was evaluated using a fluorescence molecular tomography system (FMT1500, PerkinElmer). IN administrations were performed on mice kept under anaesthesia during the whole administration and fluorescence detection procedure. IN administration in mice under anaesthesia required inducing a light-to-moderate plane of anaesthesia, positioning the mouse, and delivering micro-droplets slowly to allow inhalation. Inhaled isoflurane (2.5%) has been employed during procedures to keep the animal immobile and reduce sneezing or expulsion of the NP suspension. NP suspension has been typically delivered gradually at the external opening of the naris, allowing the animal to inhale the material naturally without inserting the delivery tip deep into the nasal passage.¹⁸

For each administration, NPs were used at a polymeric concentration of 7.059 mg mL⁻¹ (theoretical estimated NP concentration 6.22×10^{12} particles per mL, see SI S1.1–S1.4), in a volume of 10 μ L per nostril.

After NP administration, anaesthetised mice were placed into the imaging cassette inside the chamber of the instrument. The total amount of fluorophore (expressed in picomoles) in a selected three-dimensional region of interest (ROI) was calculated using TrueQuant software using previously generated standards of the appropriate dye. Mice were analysed 5, 30, 60, 90, 120, and 180 minutes after the administration. All animals were shaved prior to imaging sessions to minimize signal attenuation caused by fur.

Acquisitions were performed with the animals in the prone position for head FMT imaging and in the supine position for whole-body FMT imaging. ROI placement was guided by the signal generated using the imaging instrument, ensuring that the relevant deep signal was consistently captured across animals. Fluorescence intensity was normalised by volume for all ROIs to ensure the comparability of the measurements. During the whole experiment, no changes in animal weight and behaviour were observed.

2.18 Statistical analysis

Statistical analysis was performed using GraphPad Prism. Data are presented as mean $\pm$ standard deviation (SD) from independent experiments. One-way ANOVA followed by Tukey's multiple comparisons test was used for comparisons among NP formulations under a single experimental condition. For datasets involving two factors, two-way ANOVA was applied. When significant effects were found, differences among formulations within each condition were assessed using simple effects analysis.

A *p*-value of <0.05 was considered statistically significant. Detailed statistical information is reported in the figure legends.

3. Results and discussion

The present study aimed to develop and characterize a self-traceable polymeric NP platform suitable for investigating potential N2B transport following IN administration, with particular emphasis on physicochemical stability, biological

behaviour at the olfactory interface and non-invasive *in vivo* biodistribution monitoring.

Based on PLGA-PEG fluorescent polymeric NPs previously described and proposed by our research group, we produced fluorescent NPs through blending between PLGA-PEG and the near infrared (NIR) labelled graft copolymer α,β -poly(*N*-2-hydroxyethyl)- D,L -aspartamide-*co*-Dy700-*g*-polylactide (PHEA-*g*-Dy700-*g*-PLA). PHEA, selected as the polymeric backbone due to its biocompatibility and its high chemical versatility, allows for the grafting of various functional moieties to tailor the copolymer's structural and functional properties for specific applications.²³ Dy700, a fluorescent probe with excitation/emission wavelengths 700/725 nm, was covalently grafted to the PHEA backbone *via* its primary amine groups, ensuring stable fluorescence in the NIR region, while PLA allows the formation of NPs in aqueous media, by conferring hydrophobic behaviour to the resulting copolymer⁴⁰ (Fig. S4 and S9).

To obtain NPs, we employed the nanoprecipitation technique, due to the well-known reproducibility and scalability in laboratory settings.⁴¹ Several NPs were formulated by varying the matrix composition, in terms of the PHEA-*g*-Dy700-*g*-PLA weight ratio wt% on the total polymer weight.^{22,24} The covalent incorporation of the fluorescent dye into the NP matrix offers several advantages: it prevents dye leakage, ensures long-term fluorescence stability, and enables reliable intracellular tracking *via* fluorescence microscopy.³¹ Furthermore, this strategy allows for non-invasive, real-time imaging and quantification of NP biodistribution in live animals using FMT, providing valuable insights into the temporal and spatial dynamics of NPs following intranasal administration.

3.1 Synthesis of the PHEA-*g*-Dy700-*g*-PLA graft copolymer

The fluorescent copolymer was synthesized by covalently grafting on the PHEA backbone, the fluorescent NIR dye Dy700 and the PLA, respectively, at 0.2 mol% and 4.3 mol%, by following the procedures already described (Fig. 1).^{20,24} Stability studies have already demonstrated that the NIR dye is stably linked to the PHEA backbone, with the hydrolysis less than 0.2% after 24 hours.²⁴

3.2 Production and characterization of NPs: effects of the polymer weight ratio on mean size, polydispersity index, zeta potential, and surface pegylation

NPs were produced by nanoprecipitation using blends of PLGA-PEG and PHEA-*g*-Dy700-*g*-PLA at increasing weight ratios, allowing systematic modulation of composition and surface properties. Formulations containing 0 wt%, 20 wt%, 40 wt%, 60 wt%, and 80 wt% of PHEA-*g*-Dy700-*g*-PLA relative to the total copolymer weight were designated as NPs-BL, NP1-20, NP1-40, NP1-60, and NP1-80, respectively.

To improve colloidal stability, all formulations were prepared in the presence of polysorbate 80 (Tween[®] 80), a surfactant playing a crucial role in reducing interfacial tension and preventing aggregation during the preparation process.^{42,43}

DLS analysis showed that all formulations exhibited average hydrodynamic diameters ranging from 80 to 109 nm and low

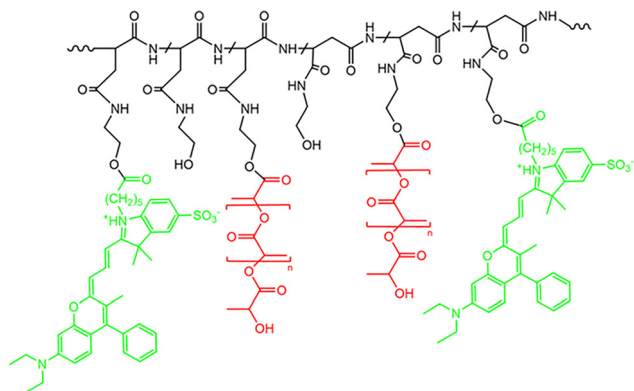


Fig. 1 The chemical structure of the PHEA-g-Dy700-g-PLA graft copolymer ($n = 194$).

PDI values (0.180–0.199), indicating a narrow size distribution (Fig. S5).

Dialysis, used as a mild purification step, was used to remove excess surfactant. Two-way ANOVA showed no significant intra-formulation differences between pre- and post-dialysis samples for any of the analysed parameters (Fig. 2A). However, post-dialysis inter-formulation analysis by one-way ANOVA followed by Tukey's test highlighted significant differences only for the ZP of NP1-80 compared with the other formulations. ZP measurements revealed dependence on formulation composition. Before dialysis, a progressive shift toward less negative values was observed with increasing PHEA-g-DY700-g-PLA content, culminating in a positive ZP for NP1-80 (+6.68 mV). This behaviour indicates an increasing contribution of protonatable amine groups associated with the PHEA-DY700 component to the effective interfacial electrostatic properties.

After dialysis, all formulations exhibited more negative ZP values, while NP1-80 shifted to approximately neutral values (−0.93 mV) (Fig. 2B). This decrease in ZP following purification is consistent with previously reported effects of purification processes on the surface properties of NPs, where a reduction in ZP is attributed to the removal of surface-associated masking molecules (e.g., residual surfactants or weakly adsorbed species).^{44,45} In agreement with this interpretation, dialysis may contribute to the removal of weakly associated components from the NP surface, leading to a more accurate reflection of the intrinsic surface electrostatic properties of the polymeric matrix.

In PEGylated NP systems, PEG chains form a highly hydrated interfacial layer that increases steric stabilization and shifts the hydrodynamic shear plane away from the NP surface. Consequently, PEG can effectively attenuate the measured ZP by masking the contribution of underlying charged functional groups.^{46,47}

Accordingly, the progressive increase in PHEA-g-DY700-g-PLA content leads to a higher surface contribution of protonatable amine groups, which progressively counterbalances PEG-mediated shielding effects.

To further characterize the surface properties of NPs, the extent of PEG exposure was quantified using a previously reported method.^{20,48}

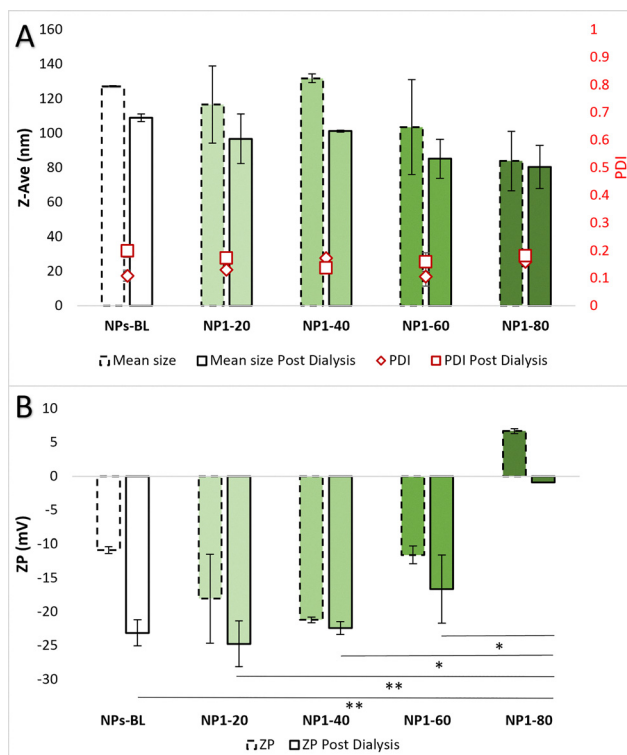


Fig. 2 (A) Z-Ave, PDI and (B) ZP of NPs-BL, NP1-20, NP1-40, NP1-60 and NP1-80 samples, before (dashed lines) and after the dialysis purification process (solid lines). Statistical analysis was performed using two-way ANOVA to evaluate intra-formulation differences before and after dialysis, followed by one-way ANOVA with Tukey's multiple comparison test for inter-formulation comparisons on post-dialysis values. No significant differences were observed before vs. after dialysis within each formulation. Significant differences were detected only for NP1-80 in terms of ZP compared to the other formulations (* $p < 0.05$, ** $p < 0.01$).

The analysis revealed that samples NP1-20, NP1-40, and NP1-60 exhibited comparable levels of PEG exposed on the surface, with values of 3.9 wt%, 3.7 wt%, and 4.1 wt% of the total sample weight, respectively. In contrast, NP1-80 showed a significantly lower pegylation level, approximately 1.9 wt%.

Despite these differences, all the samples have a high-density surface pegylation with PEG chains adopting a straighter (brush-like) structure, which is generally associated with reduced non-specific interactions and enhanced stability in biological fluids.⁴⁹

This aspect is also confirmed by the characterization data obtained after lyophilization, which showed the absence of aggregation of the particles as well as a non-significant variation in the ZP (data not shown). Stability studies under refrigerated storage conditions (4 °C) confirmed that Z-Ave and PDI remained substantially unchanged within the time frame relevant for the *in vitro* experiment and sample handling (Fig. S6 and S7), whereas significant variations in ZP were observed after 28 days of storage (Fig. S6). These results indicate that the formulations maintain acceptable colloidal properties during short- to medium-term handling, while also highlighting limitations in long-term storage. In this context, lyophilization may represent a valuable strategy to improve storage stability and support future translational development.⁵⁰

3.3 AFM characterization

AFM was used as a complementary qualitative technique to assess NP morphology. AFM images revealed that all formulations exhibited a predominantly spherical shape (Fig. 3), with no major structural differences among samples, although slight surface variations were observed at higher PHEA-*g*-Dy700-*g*-PLA content. The particle dimensions observed by AFM (approximately 45–67 nm; Table S2) were consistently smaller than those measured by DLS, as expected due to the different measurement principles. While DLS determines the hydrodynamic diameter, including the solvation layer surrounding the NPs, AFM measures particles in the dried state after sample deposition. Therefore, dehydration and collapse of the hydration shell during sample preparation likely account for the reduced dimensions observed by AFM.⁵¹

3.4 Colloidal stability and mucin interaction in simulated nasal conditions

Stability studies conducted in SNF demonstrated that all NP formulations preserved their colloidal integrity over a 3-hour incubation period at physiological temperature (Fig. 4A). This time frame is compatible with the *in vivo* potential brain distribution after IN administration.⁵² Neither Z-Ave and PDI exhibited aggregation, indicating resistance to salt-induced aggregation and compatibility with the nasal environment. To further evaluate NP interactions with the mucus barrier, additional studies were performed in a MUC-containing SNF medium (1:1 ratio) (Fig. 4B). Overall, the results consistently demonstrated a high degree of colloidal robustness across all formulations. No significant changes in Z-Ave or PDI were observed over time, indicating

the absence of aggregation phenomena or formation of complexes with MUC protein. In agreement with these findings, absorbance measurements did not reveal the formation of NP–MUC complexes, further supporting the lack of detectable associative interactions (Fig. 4C). Moreover, ZP values showed time-dependent fluctuations without a consistent monotonic trend across formulations (Fig. 4D). A comparable degree of variability was also observed for MUC alone, suggesting that these fluctuations reflect the intrinsic variability of ZP measurements in mucin-containing dispersions over time, rather than a specific, reproducible NP–MUC interaction. Overall, ZP values remained within a narrow range (0 to –10 mV) across all timepoints and formulations, without evidence of a progressive or directional shift indicative of MUC adsorption.

Importantly, these results should be interpreted considering the surface characterization reported in Section 3.2, which demonstrated a consistently high PEG surface content across formulations (3.7–4.1 wt% for NP1-20, NP1-40 and NP1-60), with only NP1-80 exhibiting a reduced PEGylation level (~1.9 wt%). Despite these differences, all formulations are likely to present a dense PEG coating, which is expected to adopt a brush-like conformation. This structural organization is known to be critical for the establishment of antifouling interfaces, as it promotes steric stabilization and a strongly hydrated surface layer that limits non-specific interactions with biological macromolecules.⁵³

Accordingly, the absence of measurable interactions with MUC can be rationalized by the presence of a sufficiently dense PEG brush, which prevents MUC interaction with the polymer corona. This interpretation is fully consistent with the mechanistic framework proposed by Huckaby and Lai, who discussed

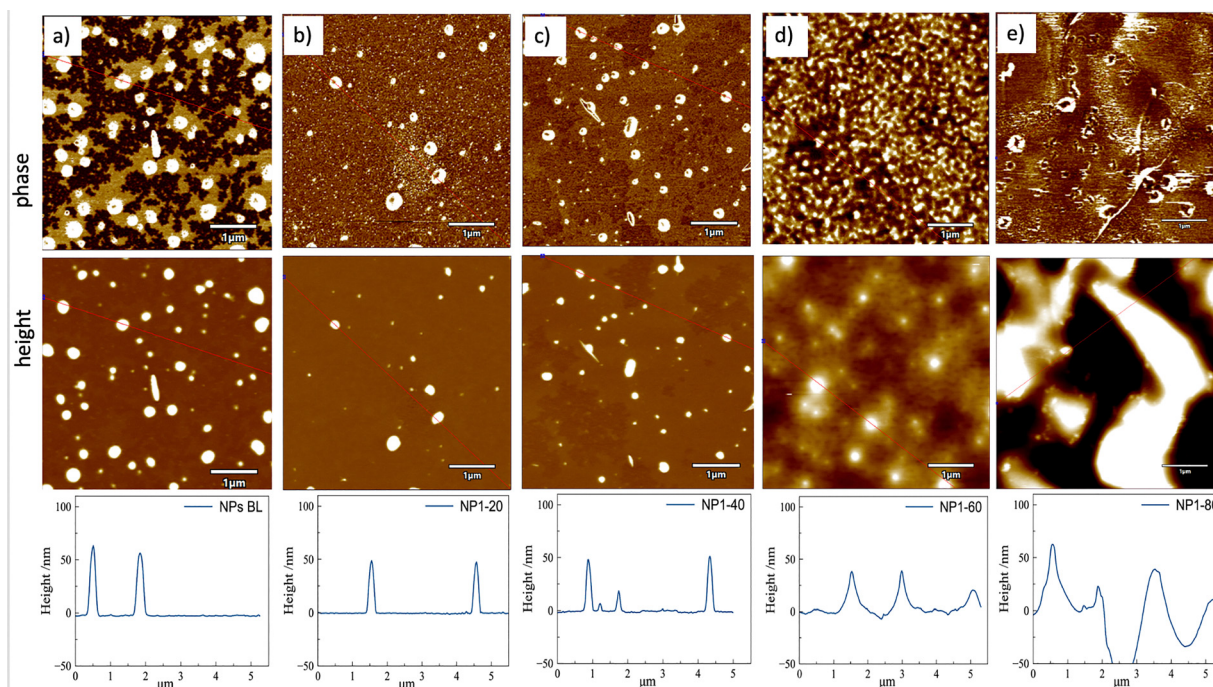


Fig. 3 From top to bottom: AFM phase images, height images (*z* scale = 40 nm), and section profiles of (a) NP1–BL and samples (b) NP1–20, (c) NP1–40, (d) NP1–60, and (e) NP1–80. The scale bar is 1 μ m.

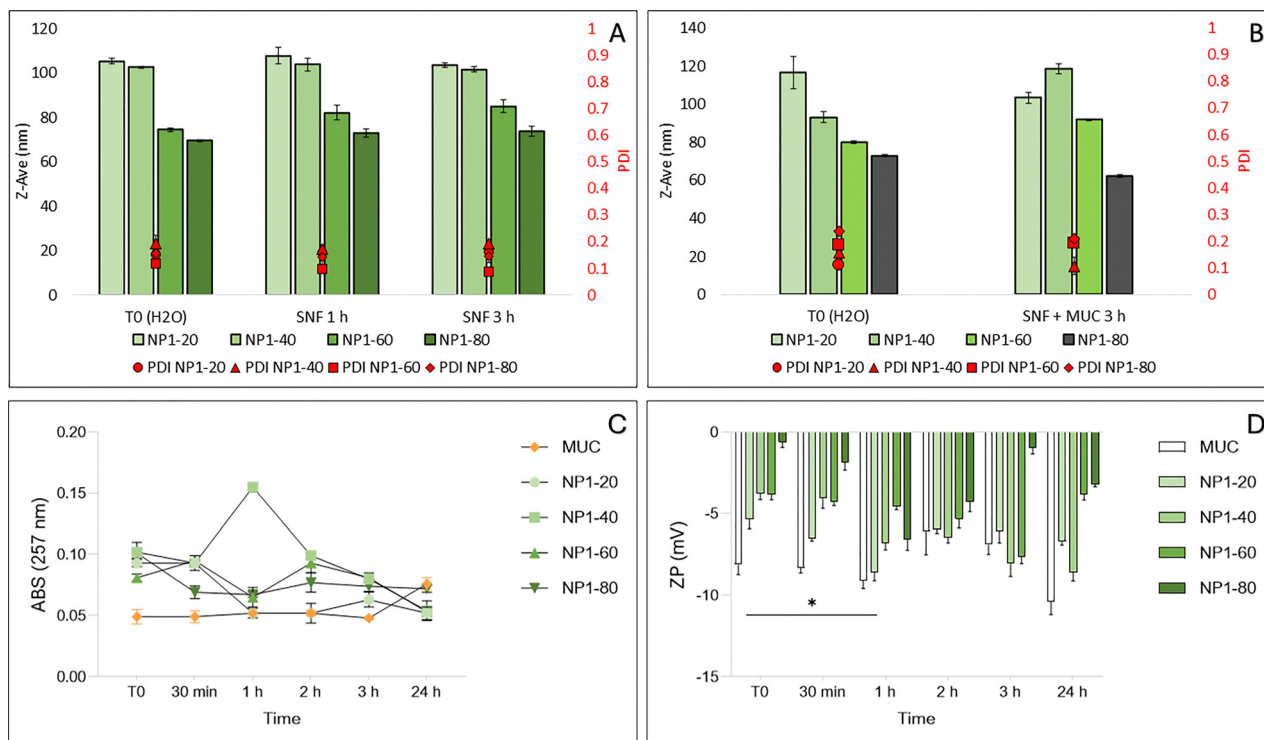


Fig. 4 (A) Time-dependent Z-Ave (primary axis) and PDI (secondary axis) of NPs in SNF (1 : 3 dilution) at 0, 1, and 3 h. (B) Time-dependent Z-Ave (primary axis) and PDI (secondary axis) of NPs in SNF + mucin medium (1 : 1 dilution) at 0 and 3 h. (C) Mucin adhesion assay measured as absorbance at 650 nm over 24 h, indicating time-dependent interaction of NPs with mucin. (D) Zeta potential measurements of nanoparticle formulations, over 24 h, indicating time-dependent interaction of nanoparticles with mucin. Statistical significance is reported within each formulation (* $p < 0.05$), while differences without asterisks were considered not statistically significant (ns).

that mucus-penetrating or mucoinert behaviour is not solely dictated by PEGylation *per se*, but rather by the combined effect of PEG chain density and the conformational regime, which together govern the extent of hydrophobic interactions, hydrogen bonding, and interpenetration with the MUC mesh.⁵⁴

3.5 Differential scanning calorimetric (DSC) analysis

To investigate the thermal properties of the obtained samples, DSC analysis was carried out. Fig. 5(a) shows the thermograms of the individual copolymers and their physical mixtures, corresponding to the compositions of each NP sample. In particular, the thermogram of PLGA-PEG displayed a broad endothermic transition at approximately 36 °C, consistent with previous reports.⁵⁵ As reported in the literature, the PLGA T_g is 50 °C,⁵⁶ and the incorporation of PEG into the PLGA matrix significantly lowers the peak of the endothermic transition, due to the plasticizing effect of PEG chains,⁵⁷ which enhance the segmental mobility of the polymer.⁵⁸ Moreover, the thermal event at 58 °C in the thermogram of PHEA-g-Dy700-g-PLA can be attributed to the relaxation of the PLA amorphous domains, as already reported.^{59,60}

All physical mixtures exhibited a single endothermic transition, reflecting the predominance of one of the copolymers used in each blend.⁶¹ Specifically, the endothermic transitions of the 20 wt% and 40 wt% physical mixtures were closer to that of PLGA-PEG (35 °C and 38 °C, respectively), while those of the

60 wt% and 80 wt% mixtures were more similar to PHEA-g-Dy700-g-PLA (60 °C and 59 °C, respectively).

Fig. 5(b) reports the thermograms of all the NP samples. Notably, NP1-40 and NP1-60 exhibited two distinct endothermic transitions, at 40 °C and 58 °C, likely resulting from the overlapping transitions of the blended copolymers. Endothermic peaks at 98 °C are due to water desorption.⁶² In the case of samples NP1-20 and NP1-80, the thermograms showed a single endothermic peak at 43 °C and 47 °C, respectively. This behaviour can be attributed to a greater molecular homogenization occurring at the compositional extremes, where one copolymer becomes dominant and is able to impose its organization on the minor component, resulting in a single cooperative thermal event rather than two separated transitions.

3.6 Biological characterization

Olfactory sensory neurons (OSNs) located in the olfactory epithelium connect directly to the brain *via* their axons, which project to the olfactory bulb through perforations in the cribriform plate.⁶³ These axons are ensheathed by OECs, a specialized type of glial cell that plays a crucial role in OSN survival and regeneration, guiding repair processes following damage or during normal cellular turnover.^{64,65} Several studies have highlighted the importance of OECs in supporting olfactory axons.^{66–70} Based on this evidence, OECs were selected as the

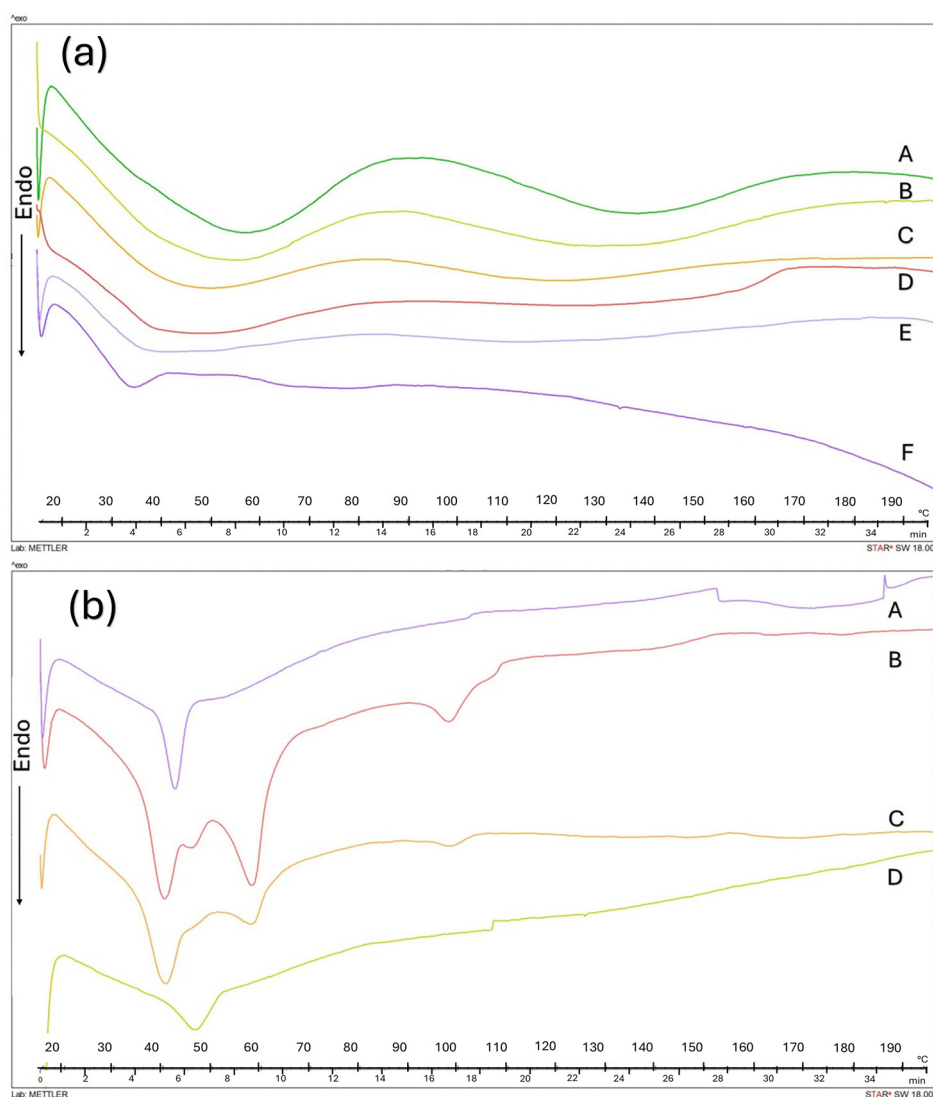


Fig. 5 (a) Heating DSC thermograms of physical mixtures of PHEA-*g*-Dy700-*g*-PLA (A), PHEA-*g*-Dy700-*g*-PLA 80% w/w to PLGA-PEG (B), PHEA-*g*-Dy700-*g*-PLA 60% w/w to PLGA-PEG (C), PHEA-*g*-Dy700-*g*-PLA 40% w/w to PLGA-PEG (D), PHEA-*g*-Dy700-*g*-PLA 20% w/w to PLGA-PEG (E), and PLGA-PEG (F); (b) heating DSC thermograms of NP1-20 (A), NP1-40 (B), NP1-60 (C) and NP1-80 (D). All thermograms were normalized for heating rates (heat flow endo down).

in vitro model for evaluating both NP biocompatibility and uptake in the context of N2B delivery.

3.6.1 Cytotoxicity studies. The MTT assay demonstrated that all NPs maintained cell viability above 80% at the tested concentrations, indicating acceptable cytocompatibility according to ISO guidelines (Fig. 6).⁷¹ Morphological observations revealed that formulations with higher PHEA-*g*-Dy700-*g*-PLA content (NP1-60 and NP1-80) induced concentration-dependent alterations in cell morphology and an apparent reduction in cell number at the highest tested concentration (1.4 mg mL^{-1}) (Fig. S8). Notably, these effects were concentration-dependent and observed only at the highest tested concentration (1.4 mg mL^{-1}), while lower concentrations (0.14 and 0.70 mg mL^{-1}) did not show comparable alterations, supporting the suitability of NP1-80 for subsequent *in vivo* evaluation.

3.6.2 NP uptake by OECs. The choice of OECs for NP uptake studies was based on their key role within the olfactory

pathway involved in N2B transport. OECs surround olfactory axons along their route from the olfactory epithelium to the olfactory bulb and actively contribute to the maintenance and functional support of olfactory neurons.⁷² Importantly, OECs also play a relevant immunological role within the olfactory system, being involved in the production of inflammatory mediators and protective factors in response to external stimuli or mucosal damage. Previous studies suggested that OEC-mediated immune responses may influence drug distribution and internalization following intranasal administration.^{73,74} Considering their anatomical localization and their potential impact on NP uptake and trafficking, OECs represent a biologically relevant *in vitro* model for the preliminary evaluation of intranasal N2B biopharmaceuticals.⁷⁵

The physicochemical properties of NPs, such as surface charge, shape, size, composition, and surface decoration with ligands, play a crucial role in determining their cellular

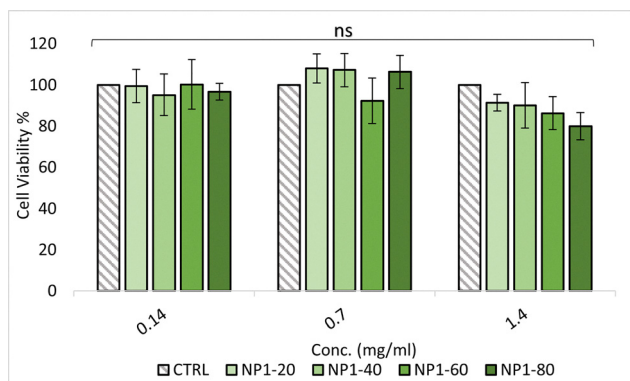


Fig. 6 MTT assay on NP treated OECs at different NP concentrations (0.14, 0.70, and 1.40 mg mL⁻¹), and the incubation time is 24 h. Data are expressed as a percentage of cell viability normalized to untreated control cells (CTRL = 100%). Since CTRL values were used for normalization, no standard deviation is displayed for this group. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparison test, and ns means not significant statistically.

internalization.^{76,77} Before reaching the target cell membrane, NPs interact with the surrounding biological environment, where phenomena such as agglomeration or aggregation may occur, potentially influencing their interaction with cells and their *in vivo* fate.⁷⁸ The cellular uptake of the NP formulations was evaluated at 3 h and 24 h by fluorescence microscopy (Fig. 7) and analysed through quantification of corrected total cell fluorescence (CTCF).

At 3 h, all formulations showed significantly higher fluorescence intensity compared to the untreated control, confirming early internalization. In the non-normalized analysis, a clear concentration-dependent trend was observed (low < medium < high), with NP1-80 displaying the highest absolute CTCF values, particularly at medium and high concentrations (Fig. 8A and B). At first glance, these results could suggest the superior uptake of formulations enriched in PHEA-DY700-PLA. However, because these systems intrinsically contain a larger amount of the DY700-labelled polymer, the raw fluorescence signal alone may overestimate the true efficiency of cellular internalization.

For this reason, CTCF values were normalized to the fluorescent polymer content. Interestingly, after normalization, this trend was substantially reversed: after 3 hours of treatment, the CTCF of NP1-20 was significantly higher than that of NP1-40 and NP1-60 at medium concentrations, and even higher than that of NP1-80 at the highest concentration (Fig. 8C). This behaviour may be influenced by the different PLGA-PEG contents among the formulations. NP1-20 contains a higher proportion of the PEGylated polymer, whereas NP1-40, NP1-60, and NP1-80 are progressively enriched in the fluorescent copolymer.

PEGylation is classically associated with reduced nonspecific interactions and prolonged circulation due to the formation of a hydrated steric barrier on the NP surface.⁷⁹ This effect is strongly influenced by PEG density, chain length, and surface conformation, with brush-like PEG arrangements generally providing more effective steric stabilization and reduced early protein adsorption.⁸⁰ However, increasing evidence suggests

that under biologically relevant conditions, particularly in the presence of serum proteins, PEG does not necessarily reduce cellular uptake. Instead, PEG surface density can strongly influence the composition of the protein corona, which ultimately defines the biological identity and cellular recognition of NPs.⁸¹ Since our uptake studies were performed in FBS-containing medium, a similar mechanism may contribute to the higher internalization observed for NP1-20.

Recent studies on PLGA-PEG NPs demonstrated that a higher PEG content may lead to unexpected increases in macrophage uptake despite reduced total protein adsorption, highlighting that the qualitative composition of the protein corona is more relevant than the total amount of adsorbed proteins.⁸¹ In particular, the balance between opsonins and dysopsonins appears to be a critical determinant of cellular internalization.⁸² Similarly, PEGylated particles were shown to be preferentially internalized by primary human phagocytes under plasma-containing conditions, whereas this effect was not observed in standard culture media, further supporting the role of biological fluids and cell type in modulating PEG-mediated uptake.⁸³

In parallel, PEGylation has also been shown to affect the specific endocytic pathways involved in NP internalization. PEGylated lipid NPs were reported to display a stronger preference for caveolae-mediated and clathrin-mediated endocytosis compared to non-PEGylated systems, likely as a consequence of altered surface charge and membrane interactions.⁸⁴ This aspect may be particularly relevant for OECs, which have been reported to possess membrane lipid rafts enriched in caveolin-1 and flotillin-1, indicating the presence of caveolae-associated membrane microdomains potentially involved in endocytic processes.⁸⁵

The higher PLGA-PEG content could favour both the formation of a protein corona that promotes NP interaction with OECs and a preferential engagement of caveolae-associated uptake pathways during the early stages of exposure.

Interestingly, NP1-80 showed higher uptake at the intermediate concentration compared to the highest dose. Two-way ANOVA revealed a significant effect of both the NP formulation and the concentration on cellular uptake at 3 h ($p < 0.0001$ for all factors). The concentration accounted for the largest proportion of variance (52.82%), followed by formulation (18.99%), indicating that both the dose and the NP composition significantly influence internalization. Importantly, a significant interaction between the two factors (15.45%, $p < 0.0001$) was observed, demonstrating that the effect of concentration on uptake depends on the specific NP formulation. In the case of NP1-80, the increase in uptake at the intermediate concentration, followed by a relative decrease at the highest dose, may reflect the combined influence of multiple factors acting simultaneously in a concentration-dependent manner. These may include the near-neutral ZP of NP1-80, which can reduce electrostatic repulsion with the negatively charged cell membrane, as well as possible changes in the NP dispersion state and protein corona organization in FBS-containing medium.

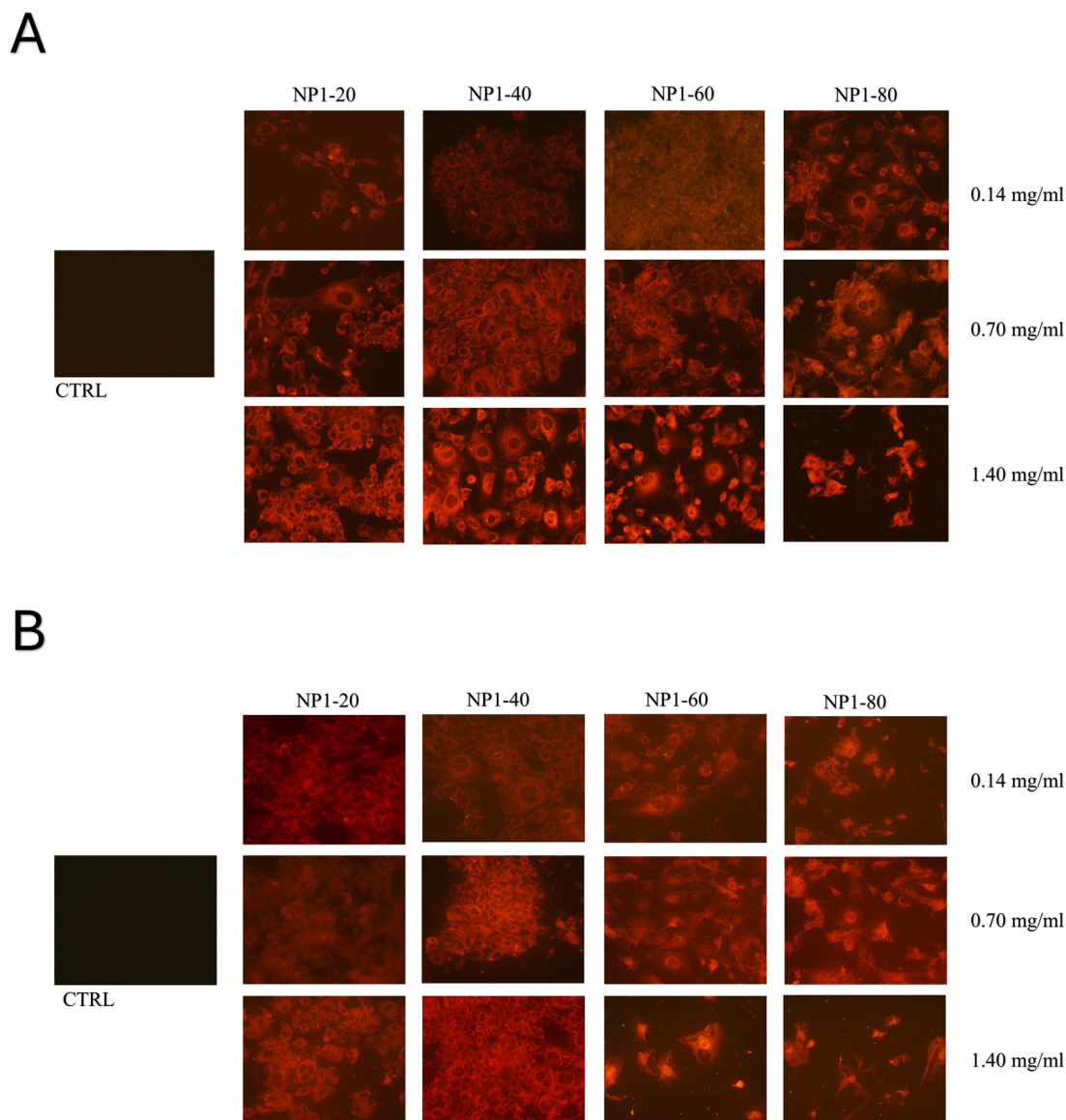


Fig. 7 Images through a fluorescence microscope of OECs incubated for 3 h (A) and 24 h (B) with NP1-20, NP1-40, NP1-60, and NP1-80 at different polymeric concentrations of 0.14 mg mL⁻¹, 0.70 mg mL⁻¹, and 1.40 mg mL⁻¹.

The Pareto analysis integrating fluorescence intensity and normalized uptake further clarified this balance. At 3 h, the formulations were distributed across distinct performance regions, with NP1-20 and NP1-80 showing higher uptake efficiency, while NP1-60 and NP1-80 provided stronger fluorescence output (Fig. 9). At 24 h, the uptake pattern observed at early time points was no longer maintained, and a clear concentration-dependent trend was not evident across formulations. In addition, increased variability was observed among replicates, suggesting a higher heterogeneity in NP-cell interactions over time (Fig. 9).

Importantly, the desirability-based multi-parameter optimization identified NP1-80 as the optimal formulation. Although NP1-20 showed superior uptake efficiency after normalization likely due to the beneficial effect of a higher PEG content, NP1-80 provided the best overall compromise between fluorescence

intensity, normalized uptake, and positioning within the Pareto-optimal region, making it the most suitable candidate for subsequent imaging studies.

3.7 *In vivo* fluorescence molecular tomography (FTM) imaging

The *in vivo* localization assessment by means of FMT, thanks to its safety and ease of use, is a promising approach to investigate the localization of small molecule imaging agents or larger therapeutic biomolecules, *i.e.* NPs, into organs and tissues.^{86,87}

Based on the physicochemical and biological characterization data, the NP1-80 sample was selected for the *in vivo* imaging studies, taking advantage of the high fluorescence conferred to the NPs by the Dy700 probe.

The *in vivo* imaging technique was used to track the fluorescence signal kinetics of NP1-80 following IN administration,

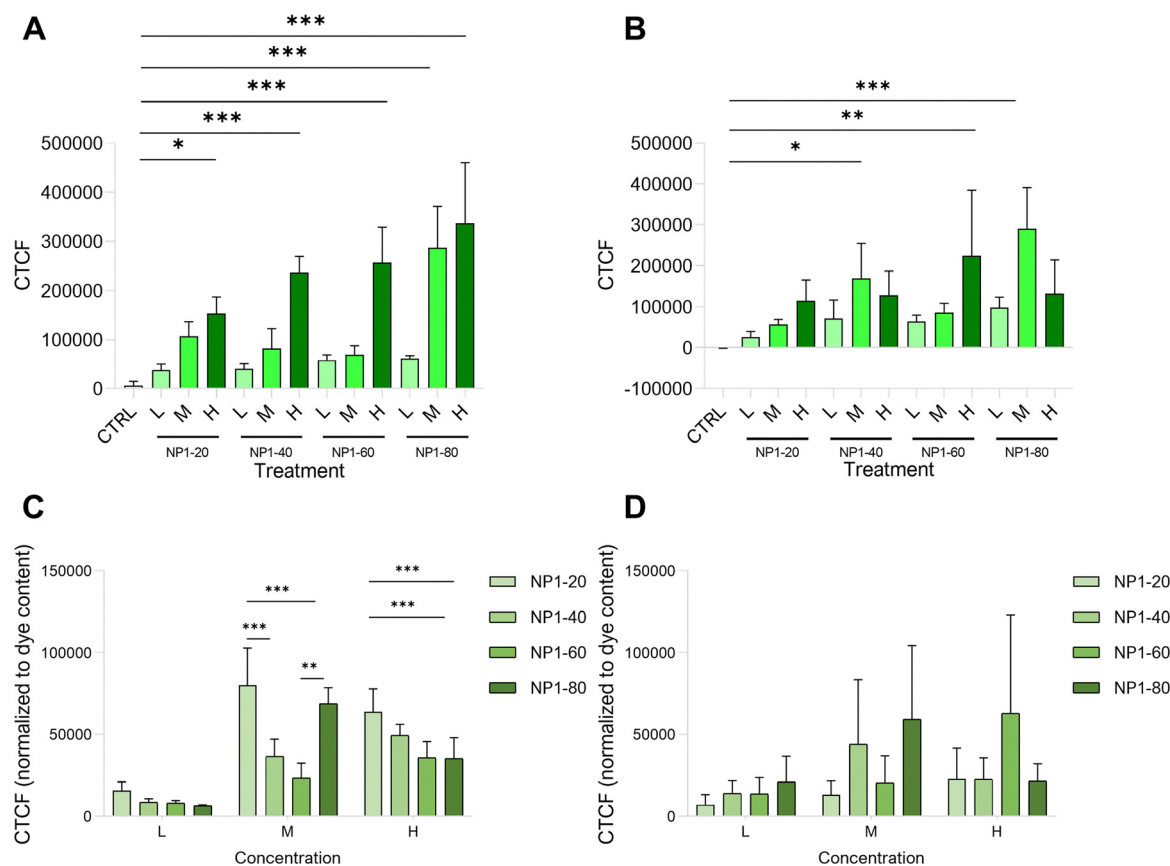


Fig. 8 Quantification of cellular uptake expressed as corrected total cell fluorescence (CTCF) at 3 h (A) and 24 h (B), and corresponding CTCF values normalized to the PHEA-*g*-Dy700-*g*-PLA content of each formulation at 3 h (C) and 24 h (D), for CTRL and NP1-20, NP1-40, NP1-60, and NP1-80 formulations. L, M, and H indicate low (0.14 mg mL^{-1}), medium (0.72 mg mL^{-1}), and high (1.4 mg mL^{-1}) NP concentrations used for cell treatment, respectively. Statistical significance is reported for each formulation compared with the control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$), while differences without asterisks were considered not statistically significant (ns).

monitoring the NPs' path to the brain over a time course ranging from 5 minutes to 3 hours post administration (Fig. 10). The amount of fluorescence in the brain was calculated by drawing a ROI corresponding to the whole brain volume of the mouse (Fig. 10A).

After IN administration, the fluorescence associated with the NP1-80 sample was mainly limited to the brain and no detectable signal was measured in the body. The signal in the brain peaked at 5 minutes in the olfactory bulb and accessory olfactory bulb ($\approx 639 \text{ pmol}$) and gradually distributed to the cortical region associated with the olfactory pathway (the region located on the ventral surface of the frontal lobe and the medial temporal lobe), suggesting rapid initial accumulation followed by clearance. At 30 and 60 minutes, brain retention remained relatively high, indicating short-term stability. In contrast, the total body signal was lower overall but showed a slight increase between 30 and 120 minutes, possibly reflecting systemic redistribution or delayed clearance (Fig. 10B). The whole mouse head in our FMT scan to show the fluorescence signal change over time between the nose and the brain. The signal drops in the nose and olfactory bulbs, but rises in the front and sides of the brain before dropping at

180 minutes. The whole-body view shows signal mostly in the lungs and heart, with no liver or spleen buildup. The body-to-brain ratio remained below 0.2 for most time points (Fig. 10C), supporting a preferential accumulation of the NPs in the brain during the early post-administration phase. The rapid brain fluorescence observed *in vivo* as early as 5 minutes after intranasal administration is compatible with a fast, potential N2B transport, rather than with the slower transneuronal axonal trafficking typically requiring several hours. This interpretation is supported by our *in vitro* data, which show that NP1-80 exhibited strong cellular uptake and efficient internalization despite its lower PEG content, suggesting that NP transport is not determined by PEG density alone, but rather by the combined effect of surface properties, fluorescence performance, and NP-cell interactions. In the presence of serum proteins, the biological identity of NPs is strongly influenced by protein corona formation, which may alter cellular recognition independently of the nominal PEG amount. PEG-based NPs have previously been reported to reach the olfactory bulb within minutes, most likely through the olfactory epithelial pathway. In particular, fluorescent PEG-PLA NPs were detected across multiple olfactory bulb

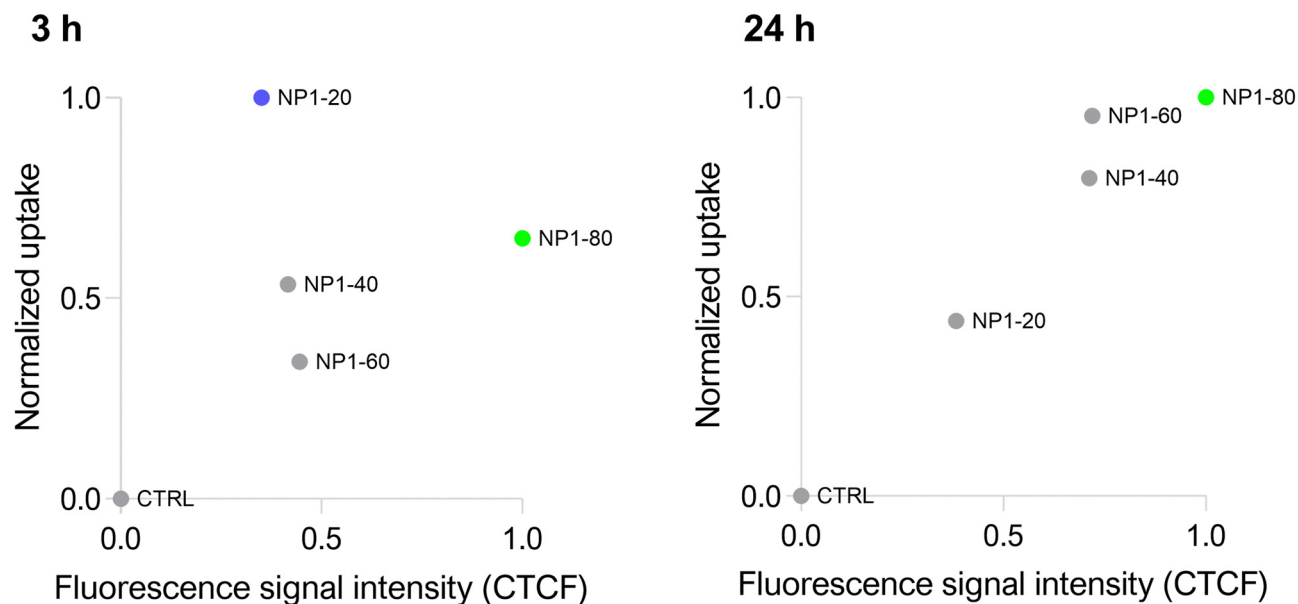


Fig. 9 Correlation between the fluorescence signal intensity (CTCF) and normalized cellular uptake at 3 h and 24 h. Data points represent individual formulations. Pareto-optimal formulations are highlighted in blue, dominated solutions are shown in grey, and the optimal formulation (NP1-80), selected through desirability-based multi-parameter analysis, is highlighted in green.

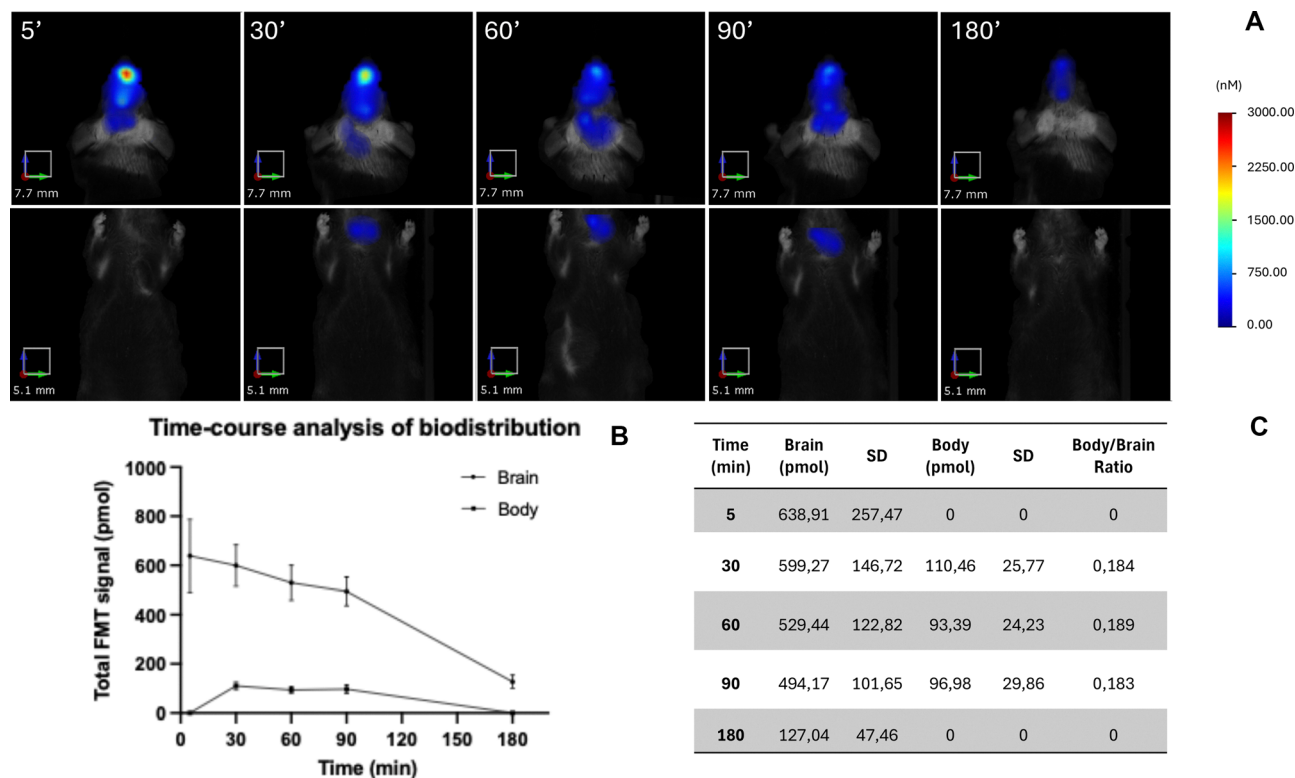


Fig. 10 Images through fluorescence molecular tomography of mice administered intranasally with fluorescent NPs at different time points (A), the time course analysis of the signal (expressed in picomoles) (B) and the body–brain ratio (C).

layers at 5 minutes post-administration, suggesting their transfer through extracellular/paracellular routes facilitated by conduits formed by OECs.⁸⁸ These results suggest that the NP1-80

NP sample exhibits favourable brain-targeting retention after IN administration, although further studies are needed to confirm specificity and evaluate long-term retention.

4. Conclusions

In this study, we developed and systematically characterized NIR-fluorescent NPs based on PLGA-PEG and PHEA-*g*-Dy700-*g*-PLA copolymers for potential N2B delivery. A stepwise fluorophore enrichment strategy allowed the identification of formulations providing sufficient fluorescence signals for both cellular and *in vivo* imaging, without compromising the physicochemical integrity, colloidal stability or cell viability of NPs at low and medium concentrations. *In vitro* studies on OECs confirmed efficient NP internalization for all formulations, with uptake behaviour strongly dependent on the NP composition. After normalization to the fluorescent polymer content, formulations with a higher PLGA-PEG content showed enhanced early cellular uptake, while NP1-80 achieved the best overall balance between the fluorescence signal and cellular internalization. *In vivo* FMT imaging supported this approach, showing rapid and preferential accumulation of NP-associated fluorescence in the head region following intranasal administration, consistent with a potential nose-to-brain transport pathway. Notably, time-course analysis revealed preferential accumulation of the fluorescence signal in the brain ROI relative to the systemic compartment, supporting the potential of the proposed intranasal strategy to promote brain-targeted delivery while limiting systemic exposure. Future studies will extend these investigations to therapeutic drug delivery and evaluate long term biodistribution and clearance profiles, further bridging the gap toward clinical translation of N2B nanomedicines.

Author contributions

Conceptualization: T. Musumeci, E. F. Craparo, and G. Sancini; writing – original draft: S. Salamone, G. Terribile, S. Manin, M. Cabibbo, S. E. Drago, and R. C. Pellitteri; writing – review & editing and visualization: T. Musumeci, E. F. Craparo, G. Sancini, G. Cavallaro, and R. Pignatello; investigation and methodology: S. Salamone, G. Terribile, S. Manin, M. Cabibbo, S. E. Drago, and R. C. Pellitteri (R. C. Pellitteri for *in vitro* studies on OECs financed by Samothrace); data curation: S. Salamone, G. Terribile, S. Manin, M. Cabibbo, S. E. Drago, C. Satriano, S. Manin, and R. Pellitteri; funding acquisition: R. Pignatello and G. Cavallaro; project administration: T. Musumeci, E. F. Craparo, G. Sancini, G. Cavallaro, and R. Pignatello.

Conflicts of interest

There are no conflicts to declare.

Data availability

All data supporting this article are available within the paper and its supplementary information (SI).

Supplementary information (SI) provides additional characterization and supporting data, including NMR and SEC analyses of the polymeric materials, spectroscopic and release studies, nanoparticle size distribution and stability data, microscopic images of treated cells, and fluorescence emission spectra. It also includes

the composition and AFM-based size distribution of the nanoparticle formulations, together with detailed theoretical calculations of nanoparticle number concentration and particles-per-cell (PPC) values for the MTT and cellular uptake studies. See DOI: <https://doi.org/10.1039/d6tb00260a>.

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