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Feature Article

Nanoparticles as drug delivery agents specific for CNS: in vivo biodistribution

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Abstract

The pharmacological treatment of neurological disorders is often complicated by the inability of drugs to pass the blood-brain barrier. Recently we discovered that polymeric nanoparticles (NPs) made of poly(D,L-lactide-co-glycolide), surface-decorated with the peptide Gly-L-Phe-D-Thr-Gly-L-Phe-L-Leu-L-Ser(O-β-D-glucose)-$CONH_2$ are able to deliver, after intravenous administration, the model drug loperamide into the central nervous system (CNS). This new drug delivery agent is able to ensure a strong and long-lasting pharmacological effect, far greater than that previously observed with other nanoparticulate carriers. Here we confirmed the effectiveness of this carrier for brain targeting, comparing the effect obtained by the administration of loperamide-loaded NPs with the effect of an intracerebroventricular administration of the drug; moreover, the biodistribution of these NPs showed a localization into the CNS in a quantity about two orders of magnitude greater than that found with the other known NP drug carriers. Thus, a new kind of NPs that target the CNS with very high specificity was discovered.

From the Clinical Editor: This paper discusses a nanoparticle-based technique of targeted drug delivery through the blood-brain barrier. The biodistribution of these novel nanoparticles showed two orders of magnitude greater efficiency compared to other known NP drug carriers.

Key words: Nanoparticles; Brain diseases; Nanotechnology; Biodistribution; CNS; In vivo experiments

Polymeric nanoparticles (NPs) are actively investigated as drug carriers, so as to deliver therapeutic agents to several sites of action and to promote a suitable, selective and specific targeted therapy. Among the most widely studied diseases, the pathologies affecting the brain are difficult to treat because of the presence of the blood-brain barrier (BBB). Even if some pathologies alter the permeability of this barrier, the BBB may restrict the entry of drugs for the pharmacological treatments of neurological and psychiatric disorders.[1,2] The presence of this barrier excludes many compounds that otherwise would be effective in treating CNS diseases from reaching a suitable concentration in the brain tissue and producing the desired therapeutic effect. Thus, drug delivery to the brain is a therapeutic challenge.[3]

A very promising approach for CNS drug delivery was recently discovered. Poly(D,L-lactide-co-glycolide) (PLGA), a polymer approved by the US Food and Drug Administration for human use, was conjugated by means of its carboxylic end with the glycosylated heptapeptide Gly-L-Phe-D-Thr-Gly-L-Phe-L-Leu-L-Ser(O-β-D-glucose)-$CONH_2$.[4] NPs prepared with this peptide-modified polymer were able to cross the BBB in rat brain perfusion technique experiments, without damage of the BBB, reaching the nuclei of cerebral parenchyma.[4] These NPs were also able to reach the brain tissues after tail vein administration, allowing loperamide, an opioid agonist used as a model drug, and the fluorescent dye rhodamine-123 (Rh-123) to cross the BBB.[5]

No conflict of interest was reported by the authors of this paper.

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Rh-123 was selected as a marker for these biodistribution studies, because this fluorescent dye is able to cross the BBB in a negligible amount when administered intravenously (IV).[6-8] The ability of the NPs to act as a drug delivery agent for the CNS was assessed in previous studies dealing with several model drugs as loperamide, because this compound is unable to exert any antinociceptive effect when administered IV, being unable to cross the BBB.[9] Because a pharmacological assay (antinociceptive assay) allows the determination of the drug effect when it reaches the brain, loperamide has been used in many other studies dealing with drug delivery to the CNS.[10-12] Thus, it is possible to compare the pharmacological results obtained following our approach with the literature data obtained by using other kinds of delivery agents.

In particular, it has been observed that loperamide loaded in the NPs prepared with our peptide-modified PLGA is able to exert an antinociceptive activity into rats (hot-plate test) for a period longer by about five times with respect to the same dose of loperamide loaded in other kinds of NPs,[5] although the same maximum effect percentage was reached.

Thus, in the present work we decided to compare the effect exerted by the peptide-modified NPs loaded with loperamide with that obtained administering intracerebroventricularly (ICV) different amounts of this drug.

Moreover, it is well known that the modification of the surface of both PLGA and the closely related poly(D,L-lactide) (PLA) NPs modify considerably the biodistribution of the NPs.[13-16] Even if some of these NPs showed stealth properties, their brain concentration was found close to zero.[14] Very few studies have appeared on the derivatization of polyester NPs (PLGA or PLA) with peptides so as to improve their targeting properties. TAT peptide-decorated NPs have been prepared to improve their in vitro cellular targeting[17]; very recently, PLA NPs derivatized with TAT peptide have been prepared for the CNS delivery of anti–human immunodeficiency virus drugs,[18] and PLGA NPs derivatized with lactoferrin have been shown to cross the BBB[19] in a qualitative way.

Because it had been shown that loperamide embedded into our peptide-modified PLGA NPs is able to exert a marked pharmacological effect,[5] PLGA NPs and the peptide-modified PLGA-NPs were loaded with the dye Rh-123 and injected via tail vein in rats to evaluate their biodistribution into different organs (liver, spleen, heart, lungs, kidneys, and brain) at different times after the administration, in comparison with unmodified PLGA NPs loaded with the dye. The biodistribution results have been correlated with the effect exerted by the loperamide loaded into the NPs.

Methods

Animals

Male albino Wistar-Hannover rats (Harlan, San Pietro Natisone, Italy), weighing 250 ± 30 g for the biodistribution studies and 260 ± 10 g for the ICV administration, were used for in vivo experiments. The rats were maintained at 25°C for an average period of 15 days before experiments, on standard diet and water ad libitum. The experiments were carried out in accordance with the European Communities Council Directives of 24 November 1986 (86/609/EEC) for experimental animal care and approved by the Ethics Committee of the University of Modena and Reggio Emilia.

Drugs and chemicals

PLGA RG503H was used (Boehringer-Ingelheim, Ingelheim am Rhein, Germany), inherent viscosity (25°C; 0.1% in $CHCl_3$): 0.38 dL/g, ratio of lactic acid to glycolic acid 50:50. PLGA-CONH-Gly-L-Phe-D-Thr-Gly-L-Phe-L-Leu-L-Ser(O-β-D-glucose)-$CONH_2$ (peptide-modified PLGA) was prepared as described in the literature.[4] Loperamide was purchased from Tocris Bioscience (Park Ellisville, Missouri), and Rh-123 was purchased from Sigma-Aldrich (St. Louis, Missouri). All the other chemicals and solvents were obtained from commercial sources and used without further purification. A Milli-Q water system (Millipore, Bedford, Massachusetts), supplied with distilled water, provided high-purity water (18 MΩ) for these experiments.

Preparation of nanoparticles

Rhodamine-123 loaded nanoparticles

NPs were obtained in accordance with the nanoprecipitation procedure.[20] The unmodified PLGA (100 mg) or a mixture of peptide-modified PLGA (75 mg) and unmodified PLGA (25 mg) along with Rh-123 (5.0 mg) were dissolved in acetone (8 mL) to obtain, respectively, Rh-123–loaded unmodified PLGA NPs (Rh-123-B-NP) and Rh-123–loaded peptide-modified PLGA NPs (Rh-123-M-NP). The organic phase was added dropwise into deionized water (25 mL) containing Pluronic F68 (100 mg). After stirring at room temperature for 10 minutes, the organic solvent was removed at 30°C under reduced pressure (20 mm Hg, 15 minutes). The final volume of the suspension was adjusted to 10 mL with deionized water. NPs were then purified by gel-filtration chromatography (Sepharose CL 4B gel; Sigma Aldrich, 160 mL, column 50 × 2 cm), using water as the mobile phase, and freeze-dried (Lyovac GT-2; Leybold-Heraeus, Hanau, Germany) for a 24-hour complete cycle without any cryoprotector.

Loperamide-loaded nanoparticles

NPs loaded with loperamide (Lop-M-NP) were prepared following the same procedure previously reported.[5] Briefly, a mixture of the peptide-modified PLGA (75 mg) and unmodified PLGA (25 mg) was dissolved in acetone (8 mL) along with loperamide (20 mg). The organic phase was added dropwise into deionized water (25 mL) containing Pluronic F68 (100 mg). After stirring at room temperature for 10 minutes, the organic solvent was removed at 30°C under reduced pressure (20 mm Hg, 15 minutes). The final volume of the suspension was adjusted to 10 mL with deionized water. Then, the NPs were purified by gel-filtration

Table 1
Nanoparticles chemico-physical and technological parameters

Preparation	Size (nm) [a]	PDI [a]	Zeta potential (mV) [a]	Rh-123 or loperamide content (mg) [a]
Rh-123-M-NPs	189 (± 42)	0.17 (± 0.02)	–15.5 (± 5.3)	0.34 (± 0.03)
Rh-123-B-NPs	167 (± 54)	0.19 (± 0.01)	–18.2 (± 4.9)	0.35 (± 0.02)
Lop-M-NPs	155 (± 26)	0.13 (± 0.01)	–15.2 (± 5.6)	15.1 (± 0.7)

PDI, polydispersity index.
[a] Values represent means ± SD of $n = 3$ experiments.

chromatography and freeze-dried, as reported above for the preparation of both Rh-123-M-NP and Rh-123-B-NP.

Determination of drug loading

Rhodamine-123 content

Before the purification step of the preparation procedure, an aliquot of Rh-123-M-NP or Rh-123-B-NP suspensions was centrifuged at 90,000 g for 1 hour at 4°C using a freeze-ultracentrifuge (Sorvall RC-28S; Du-Pont Company, Sorval Instruments, Wilmington, Delaware). The concentration of Rh-123 was evaluated analyzing the unbound drug remaining in the solution after the NP preparation by means of a HPLC procedure, using of a reversed-phase column (Varian ChromPack, ChromSep Column SS, 50 × 4.6 mm, C18 Vydac C18, 300-Å pore size, 5-μm particle size; Varian Inc., Palo Alto, California) and fluorescence detector (Fluorescent Detector, Varian 9075) set at an excitation and emission wavelength of 498 nm and 525 nm, respectively. The mobile phase consisted of a mixture of 25 mM phosphate solution and methanol (1:1, vol/vol). The flow rate was 0.5 mL/min, and the temperature was set at 40°C. The retention time (R_t) of Rh-123 was 7.89 minutes.[7] The drug loadings are expressed in milligrams for 100 mg of particles.

Loperamide content

Loperamide content in NPs was determined as previously described[5] by means of a high-performance liquid chromatography (HPLC) method,[21] analyzing the unbound drug remaining in the solution after the NP preparation. Aliquots were injected into a C18 column (Vydac C18, 4.6 × 250 mm, C18, 300-Å pore size, 5-μm particle size; Varian, Inc.) and analyzed by an ultraviolet detector (UV-1575; Jasco Europe, Cremella, Italy) (λ = 220 nm, Rt 22.0 minutes). Flow rate was 1.0 mL/min, mobile phase acetonitrile–sodium phosphate solution (pH 2.3, 20 mM)–diethylamine (40:60:0.8, vol/vol/vol). The drug loadings are expressed in milligrams for 100 mg of particles.

In vitro release studies

Rhodamine-123 in vitro release

An exact amount of Rh-123-B-NP and Rh-123-M-NP (about 50 mg) was suspended in pH 7.4 phosphate buffer solution (1 mL) and placed into an acetate-cellulose membrane dialysis tube (molecular weight cutoff = 12,000–14,000 distribution; Spectrum Laboratory Inc., Miami, Florida). The dialysis tube was placed in 25 mL phosphate buffer solution

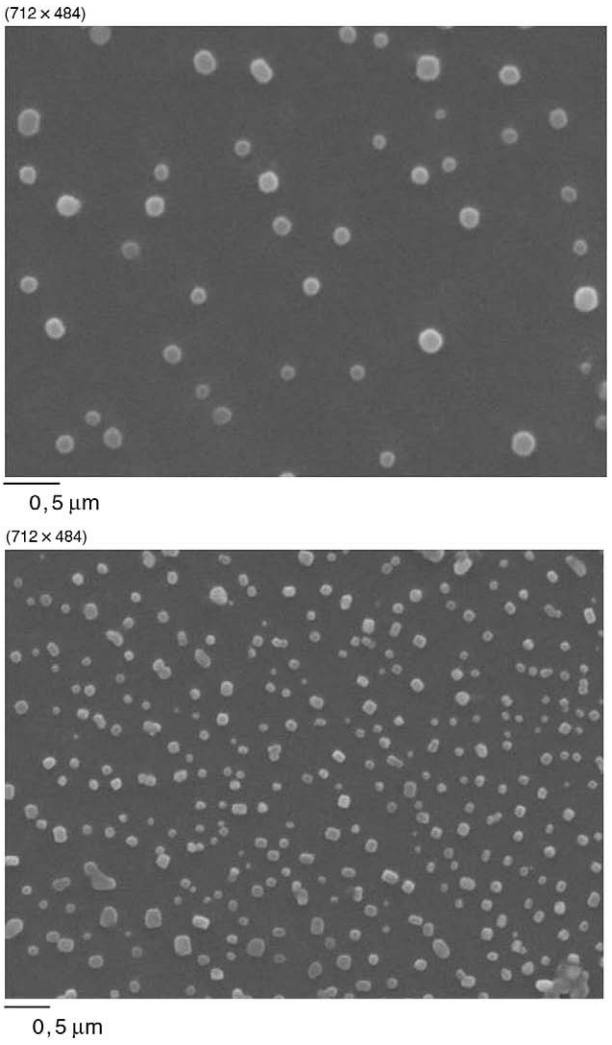

Figure 1. **(A)** SEM image of peptide-modified NPs (Rh-123-M-NP) (magnification 10,000×). **(B)** SEM image of unmodified NPs (Rh-123-B-NP) (magnification 8500×).

and gently shaken with a magnetic stirrer at 100 rpm in a water bath at 37 ± 0.1 °C. Samples (0.2 mL) were taken at fixed intervals from the medium outside the dialysis tube, and the medium was immediately supplemented with the same amount of buffer. Rh-123 concentration was determined by HPLC analysis (see above, "Rhodamine-123 content"). The results are expressed as mean of at least three tests.

Loperamide in vitro release

The loperamide release from Lop-M-NP was determined by using the same procedure described above under "Rhodamine-123 in vitro release". Loperamide concentration was determined by HPLC analysis (see above, "Loperamide content"). The results are expressed as mean of at least three tests.

Microscopic analysis

A scanning electron microscope (SEM) (XL-40; Philips, Eindhoven, The Netherlands) operating at 8 kV was used to

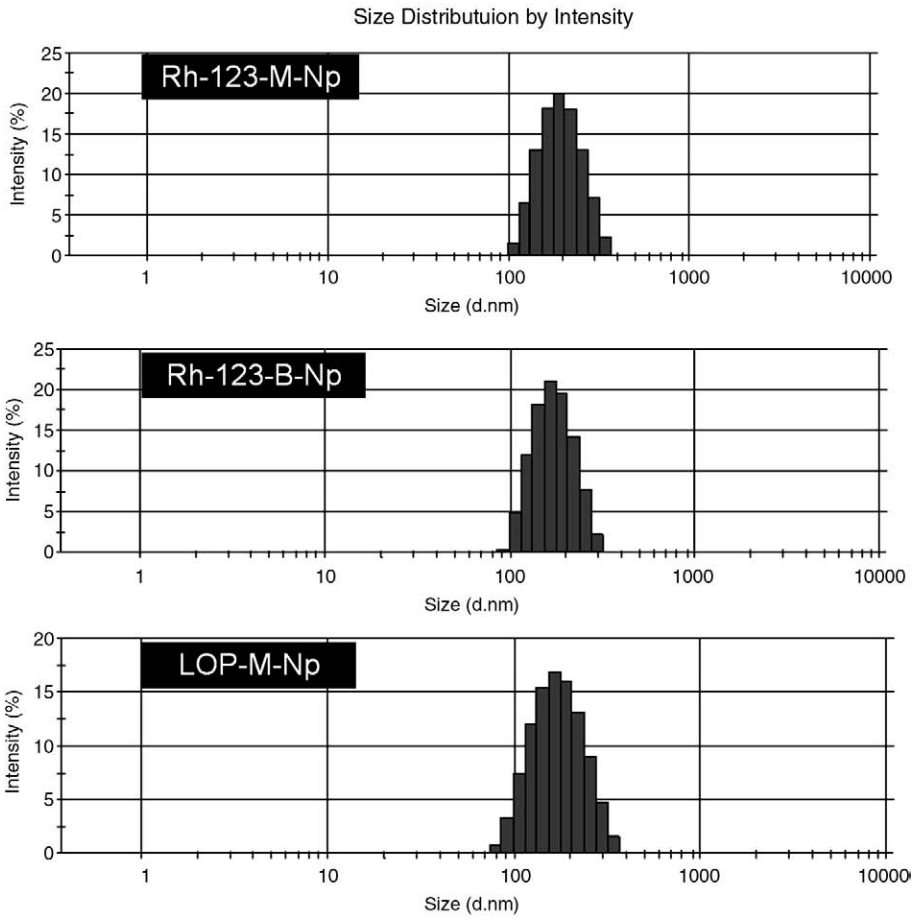

Figure 2. PCS analysis: histogram of size distribution by intensity of a representative sample of Rh-123-loaded peptide-modified NPs (Rh-123-M-NP), Rh-123–loaded unmodified NPs (Rh-123-B-NP), and loperamide-loaded peptide-modified NPs (Lop-M-NP).

evaluate the morphology of NPs. The NPs were resuspended in distilled water after several water washing steps, and a drop of the suspension was placed onto a SEM sample holder and dried under a laminar flow hood (Asal-air laminar flow hood model 700; Asal S.r.l., Cernusco, Milan, Italy) and then completely dried under vacuum (10^{-2} mm Hg). The dried samples were coated under argon atmosphere with a 10-nm gold palladium thickness (Emitech K550 Super Coated; Emitech Ltd, South Stour Avenue Ashford, Kent, United Kingdom) to increase electrical conductivity. The NPs were then processed for the evaluation of their morphology and shape by analyzing images at different magnifications (7500× to 17,500×).

Particle size and zeta potential measurements

NPs were analyzed for particle size (in distilled water) and zeta potential (in simulated-plasma fluid composed of NaCl 128 mM, NaH_2PO_4 2.4 mM, $NaHCO_3$ 29.0 mM, KCl 4.2 mM, $CaCl_2$ 1.5 mM, $MgCl_2$ 0.9 mM, D-glucose 9 mM, generally used for in vivo studies to produce an isotonic and iso-osmolar aqueous solution, pH 7.4 ± 0.1) by photon correlation spectroscopy (PCS) and laser Doppler anemometry using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, United Kingdom; laser 4 mW He-Ne, 633 nm, laser attenuator automatic, transmission 100% to 0.0003%, detector avalanche photodiode, Q.E. > 50% at 633 nm, t = 25°C). The results were normalized with respect to a polystyrene standard solution.

Biodistribution studies

Animal procedure

An exact amount of both Rh-123-M-NP and Rh-123-B-NP dispersed in saline buffer was injected via tail vein to male albino rats (three animals for each time and for each preparation). In particular, each animal was treated with NPs containing 68 μg/kg of Rh-123. Then, the animals were killed by an overdose of barbiturate at fixed times (0.25 hour, 0.50 hour, 1.0 hour, 1.5 hour, 4.0 hours, 5.0 hours, and 24.0 hours); the organs (brain, kidneys, liver, heart, lungs, and spleen) and a sample (1 mL) of blood were removed and washed with clean buffer saline and immediately iced (–20°C) until the procedure for the sample preparation.

Sample preparation

Plasma: according to the literature,[7] an ice-cold aliquot of 180 μL methanol-water (1:1 vol/vol) and 150 μL Tris-

HCl buffer (0.05 M, pH 7.4) was added to 20 μL of plasma and vortexed for 10 seconds. To extract Rh-123, 5 mL of ethylacetate/*n*-butanol (9:1, vol/vol) was added and the mixture vortexed for 1 minute and then centrifuged (4°C, 4000 rpm, 20 minutes). Then the upper layer (4 mL) was removed and evaporated to dryness under reduced pressure (10 mm Hg) at 37°C and stored at −20°C until the HPLC analysis.

Tissues

Whole organs were weighed, and an aliquot (five times the weight of the tissue) of ice-cold Tris-HCl buffer (0.05 M, pH 7.4) was added. The organs were then homogenized on ice (Ultraturrax25, IKA9; Janke & Kunkel, Staufen, Germany). Organ homogenates (100- to 500-μL aliquots) were extracted with 5 mL of the ethylacetate/*n*-butanol solution (9:1, vol/vol) and vortexed for 1 minute. After centrifugation (4°C, 4000 rpm, 20 minutes) the supernatant was decanted and evaporated to dryness under reduced pressure (10 mm Hg). The samples were stored at −20°C until the HPLC analysis.

Intracerebroventricular administration

Stainless-steel guide cannulas (23 gauge) (Plastics One, Roanoke, Virginia) were stereotaxically implanted in the right lateral ventricle, to a depth of 0.5 mm above the ventricle (measured in millimeters from the bregma: AP = −0.8; L = 1.4; V = 3.25)[22] under ketamine plus xylazine anesthesia (115 + 2 mg/kg, intraperitoneally) and fixed to the skull with screws and dental acrylic. A removable plug, which extended 0.5 mm below the tip of the guide cannulas, was kept in place except during the drug injections. At the end of the experiment, after decapitation under ethyl ether anesthesia and dissection of the brain, the correct placement of the cannulas was verified. This was done by injecting 2 μL of toluidine blue dye through an internal cannula used for the drug (or saline) injection (that extended 0.5 mm below the tip of the implanted guide cannula). Data obtained from improperly implanted animals were discarded. Loperamide was dissolved in a solution of 30% propylene glycol and 70% distilled water. A volume of 5–10 μL was injected ICV corresponding to doses of 5, 10, 20, 40, and 80 μg of loperamide. At the same time, as control, a solution of 30% propylene glycol and 70% of distilled water was injected.

Nociceptive testing

After loperamide solution ICV administration the nociceptive threshold was measured by the hot-plate test, based on the method of Eddy and Leimbach.[23,24] Briefly, the animals were placed on a surface (23 × 23 cm) maintained at 52 ± 0.2 °C and surrounded by a Plexiglas wall 20 cm high. The apparatus was equipped with a timer and a thermocouple for maintaining a constant temperature. Licking of the forepaws or lifting of one of the hindpaws from the surface was used as the end point for the determination of response latencies. Failure to respond within 60 seconds resulted in the termination of the test (cutoff). Comparison among animals was made by means of an analgesia index (see "Statistics" below), which expresses the change in hot-plate latency in terms of the differences between baseline latency and the latency itself. The latency was assessed every 30 minutes between 0 and 120 minutes after drug administrations. The data are expressed as mean of three determinations.

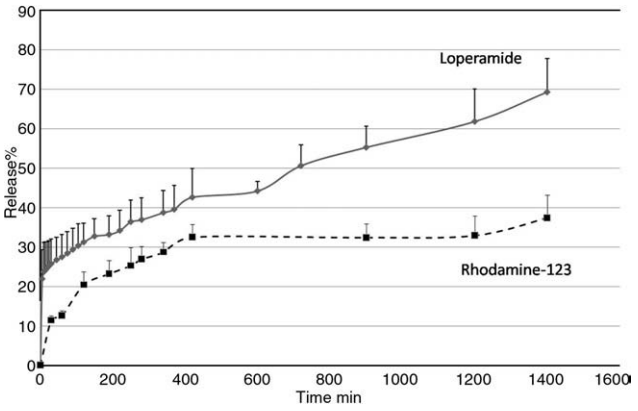

Figure 3. In vitro release of loperamide (◊, *gray line*) and Rh-123 (▪, *broken black line*) from peptidide-modified NPs performed in pH 7.4 buffered solution. Each value is the mean of at least three experiments.

Statistics

The hot-plate response latency was recorded and converted to percentage of the maximal possible effect (MPE) using Eq. (1) and are presented as mean ± standard error (SEM)

$$\text{MPE} = [(\text{latency after drug} - \text{baseline latency}) / (\text{cutoff time}(60\ \text{sec}) - \text{baseline latency})] \times 100 \quad (1)$$

For each time point, data were submitted to analysis of variance (ANOVA). When ANOVA revealed significant difference among groups, means were compared by Student-Newman-Keuls tests for multiple comparisons.

Results

Morphology, size, and zeta potential characterization

The diameter (D90) of the Rh-123-B-NP and of the peptide Rh-123-M-NP was 160–180 nm (Table 1). Both the samples appeared spherical and well formed (Figure 1). The Lop-M-NP showed similar shape and morphology, with comparable size (155 nm), surface charge (zeta potential −15 mV), and narrow polydispersity index value (0.13) (Table 1). Figure 2 shows the histograms referred to the PCS analysis of Rh-123-M-NP, Rh-123-B-NP, and Lop-M-NP.

Because loperamide loaded into peptide-modified NPs (Lop-M-NP) showed D90 size values and surface charge

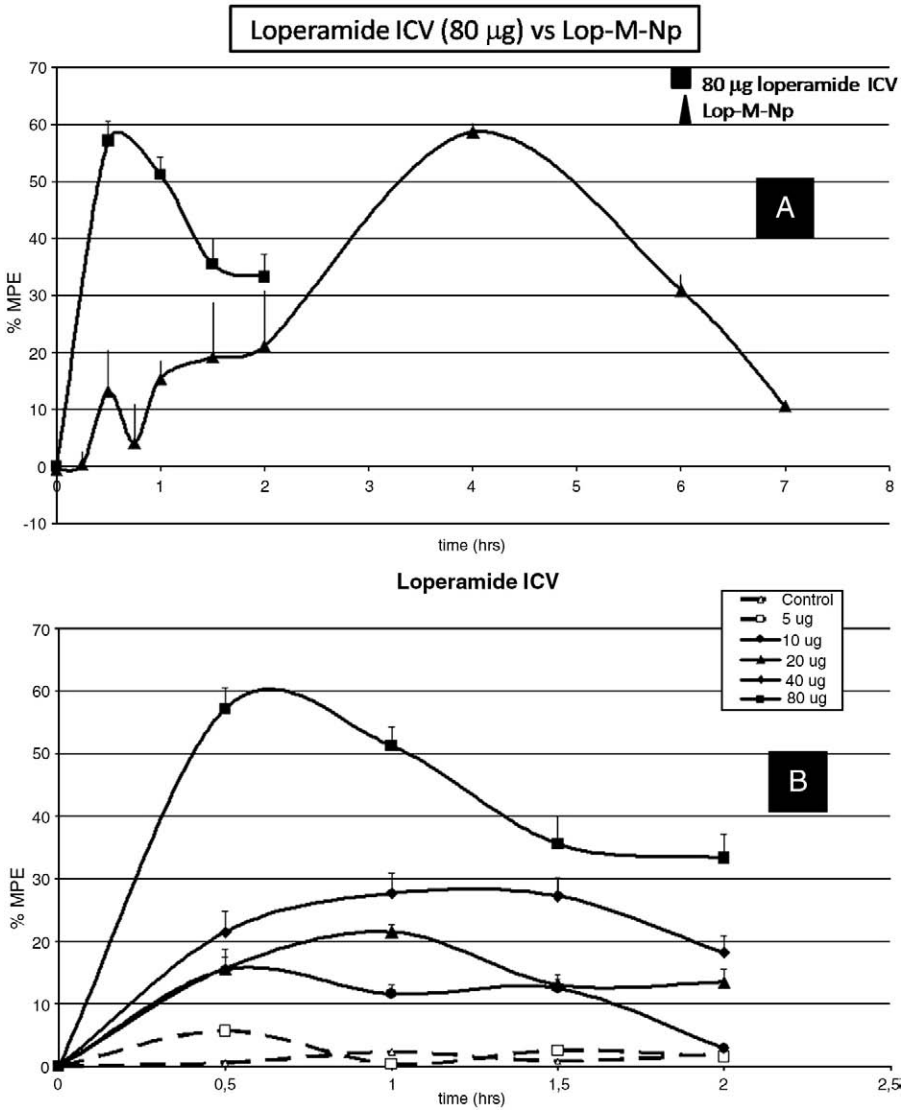

Figure 4. **(A)** Antinociceptive effect exerted by the ICV administration of loperamide (*square*) in comparison with the effect exerted by loperamide loaded in the peptide-modified NPs (Lop-M-NP) (*triangle*). **(B)** Antinociceptive effect exerted by loperamide ICV administration at different dosages. Data are presented as mean of at least three experiments ± SEM (*bars*) and were analyzed by means of ANOVA followed by Student-Neuman-Keuls tests for multiple comparisons. At each time point every group is significantly (at least $P < .05$) different from the other groups; some exceptions were recognized, namely: at 1 hour, control group vs 5 μg and 40 μg vs 20 μg; at 1.5 hours 20 μg vs 10 μg and 0 μg vs 5 μg; at 2 hours, control vs 5 μg, control vs 10 μg, 5 μg vs 10 μg, and 20 μg vs 40 μg. MPE, maximum possible effect.

very similar to those of both Rh-123–loaded NPs, we decided to use Rh-123-M-NP and Rh-123-B-NP to perform biodistribution studies.

Loperamide and rhodamine-123 in vitro release

To assess the release profile of loperamide and Rh-123 from peptide-modified PLGA NP, in vitro studies on Lop-M-NP and Rh-123-M-NP were performed. As shown in Figure 3, loperamide is released from Lop-M-NP by a biphasic profile, with an initial burst effect in which loperamide release reached 30% of the drug entrapped over 180 minutes. This burst effect may be related to the release of loperamide embedded near the nanosphere surface.[25-30] After this first phase, a constant slow loperamide release up to 70% of the total loaded drug amount was observed over 1440 minutes, showing a typical controlled drug release.

Rh-123 was released much more slowly than loperamide. After 2 hours about 15% and after 24 hours only 35% of Rh-123 was released from Rh-123-M-NP (Figure 3). Moreover, we selected Rh-123 as the marker of the NPs for the biodistribution studies, because only a negligible amount of this dye crosses the BBB when administered IV[6-8] and because its release from the NPs is very slow. Besides, it has been shown that there are no differences in the Rh-123 release if considering Rh-123-B-NP and Rh-123-M-NP.[4]

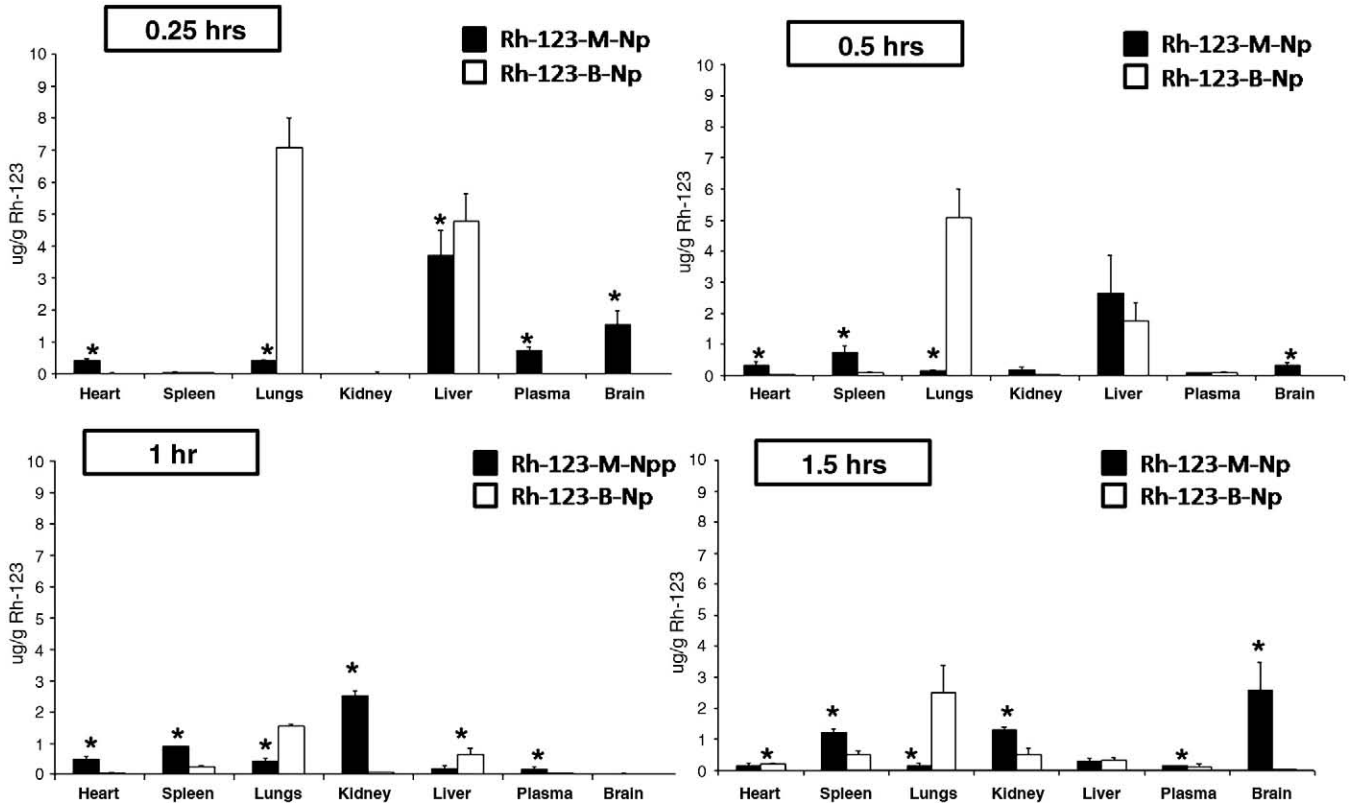

Figure 5. Biodistribution results of Rh-123–loaded peptide-modified NPs (Rh-123-M-NP) (*black*) in comparison with Rh-123–loaded unmodified NPs (Rh-123-B-NP) (*white*). Each value is the mean of at least three experiments. *$P < .05$ (Student's test) Rh-123-M-NP vs Rh-123-B-NP.

Antinociceptive assays

The comparison between the antinociceptive effect of loperamide administered ICV with loperamide delivered to the brain by means of Lop-M-NP[5] confirmed that a great amount of the administered drug is delivered into brain by means of the NPs. After ICV administration 60% of the maximum effect was reached at the dose of 80 μg per animal (Figure 4). The same effect intensity was obtained after the administration of 2.7 mg/kg of loperamide loaded in the Lop-M-NP[5] (~675 μg of loperamide per animal) (Figure 4). Comparing the maximum effect of the loperamide solution ICV injected with that recorded after IV administration of Lop-M-NP, it can be inferred that at least 13% of the injected dose of loperamide loaded into Lop-M-NP is released in the brain from the NPs after 4 hours. There is an obvious time discrepancy in recording the maximum analgesic effect obtained by the ICV administration of loperamide solution and the IV administration of Lop-M-NP (respectively after 0.50 hours and 4 hours). This difference is related to the control of the drug release by the NPs.

When the drug was delivered by means of unmodified NPs no antinociceptive activity was observed.[5]

Biodistribution studies

A biodistribution study of Rh-123 after the administration of Rh-123-M-NP was performed in comparison with the dye biodistribution after the administration of Rh-123-B-NP (Figure 5). Rh-123 was selected because both Rh-123-M-NP and Rh-123-B-NP characteristics (size and zeta potential) were very similar to Lop-M-NP. Moreover, Rh-123 release is very slow as shown in Figure 4 and in the literature.[5] Therefore, the presence of Rh-123 in the tissues can be considered representative of the NP presence, at least until the first 1.5 hours after the NP administration.

Thus, it could be assumed that the fluorescence measured after the first 1.5 hours from the administration of the NPs would be due to the Rh-123 associated to the NPs. Therefore, the NP biodistribution was determined until 1.5 hours, with the exception of the brain, in which the free Rh-123 cannot enter. Therefore, the presence of the dye in the brain can be practically attributed only to the dye associated to the NPs.

Both Rh-123-M-NP and Rh-123-B-NP were administered (via rat tail vein) at the same NP dose at which Lop-M-NP were administered, because it was noted that blood clearance and mononuclear phagocyte system uptake of the PLGA NP depends on the dose, perhaps as a result of a saturation of the mononuclear phagocyte system.[31]

As can be seen in Figure 5, both kinds of NPs were removed quickly from the plasma, but when 0.25 and 0.50 hours had elapsed after the administration, marked differences in biodistribution between Rh-123-B-NP and Rh-123-M-NP were recognized. Considering Rh-123-B-NP, they were found mainly in lungs and liver; on the contrary, Rh-

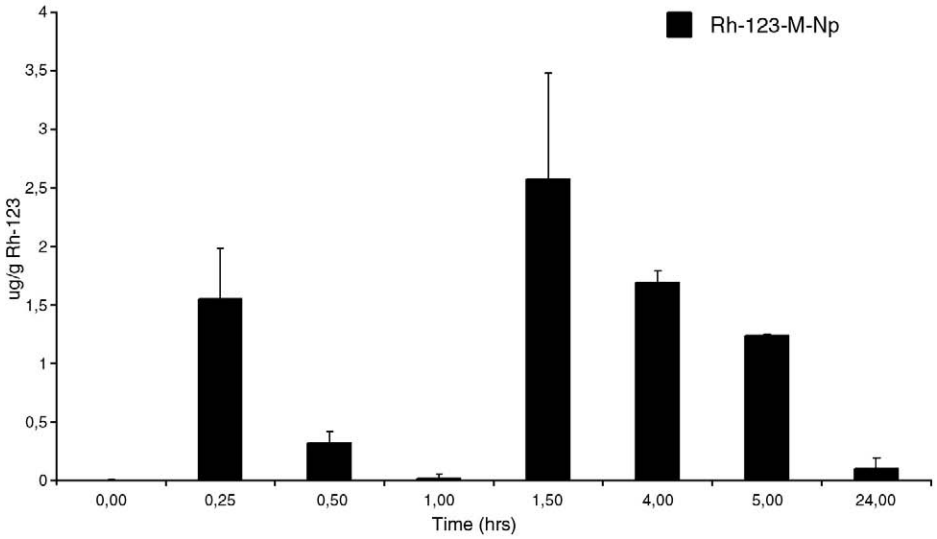

Figure 6. Brain amounts of Rh-123–loaded peptide-modified NPs (Rh-123-M-NP). Each value is the mean of at least three experiments. Bars represent SD.

123-M-NP were found mainly in liver and with a very low level in lungs. Moreover, Rh-123-M-NP were found also in the brain (~about 9% of injected dose per gram of tissue at 0.25 hours after the administration). After 0.25 hours the brain level of the Rh-123 started to decrease, but after 1.5 hours a great amount of Rh-123 (~15% of injected dose per gram of tissue) was found again. The reason for this biphasic behavior is presently unknown. Then, the NP brain levels were monitored at 4, 5, and 24 hours; a gradual decrease in NP amount within the brain was observed (Figure 6).

This result is remarkable, because the other kinds of NPs that have been studied thus far to target the CNS reached a brain level that is of the order of 0.1% to 0.2% of injected dose.[32-38] The results obtained by quantitative brain biodistribution of Rh-123-M-NPs (15% of the injected dose) are comparable with the results obtained by antinociceptive assays with loperamide loaded into Lop-M-NPs (at least 13% of the injected dose inferred by ICV studies). The difference between the time at which the maximum antinociceptive effect was reached after the administration of Lop-M-NP (4 hours) and the time at which the maximum concentration of the dye was observed in the brain after the administration of Rh-123-M-NP (1.5 hours) could be due to the different release rate of the two model molecules from NPs. In fact, as previously explained, considering the first 2 hours, Rh-123 in vitro release from NPs was very slow; thus, it could be hypothesized that only the dye embedded into the Rh-123-M-NPs was recorded in the brain, allowing the detection of Rh-123 to be considered as proof of NP biodistribution.

Discussion

The data regarding the pharmacological effect obtained by means of the ICV administration of loperamide in comparison with the antinociceptive data previously obtained[5] clearly show that a great quantity of loperamide is delivered to the brain by the peptide Gly-L-Phe-D-Thr-Gly-L-Phe-L-Leu-L-Ser(O-β-D-glucose)-$CONH_2$-modified NPs. The NP biodistribution data strongly support these results, in that the amount of peptide-modified PLGA NPs reaching the brain is about 100 times the amount of the other known NPs that target the brain.[32-38] The unmodified NPs are not able to deliver loperamide to the CNS, because the biodistribution studies showed that the unmodified PLGA NPs are unable to cross the BBB.[31] At present the reason of the target properties of the peptide-modified NPs is unknown. In fact, there are no differences between unmodified and peptide-modified NPs with respect to the characteristics that influence the NP behavior such as their size, shape, zeta-potential values, along with the amount of loaded Rh-123. The only difference could be recognized in the presence of the peptide on the NP surface, as pointed out by electron spectroscopy for chemical analysis.[4] This fact, associated with the absence of a pharmacological effect exerted by the NPs modified with a peptide composed of the same amino acids, but with a different sequence,[5] suggests a quite specific interaction of the peptide with some body component(s), such as a specific receptor present on brain vessels or with blood components. In vitro studies are in progress to address this hypothesis.

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