Supplementary Information

Methods

Synthesis of amino derivative DY405-PLGA conjugate (DY405-PLGA)

The terminal carboxylic groups of the PLGA 503H were conjugated to the DY405 amino derivative by means of an amide bond.

Briefly, the carboxylic groups of the polymer were activated through the formation of N-succinimide ester. With this aim dicyclohexylcarbodiimide (DCC) (2.2 mg, 10.6 µmol) and N-hydroxysuccinimide (NHS) (1.2 mg, 10.6 µmol) were added to a solution of PLGA (100 mg, 8.8 µmol) in anhydrous dioxane (1.5 ml), and the mixture was stirred for 4 hr r.t. Then, the dicyclohexylurea was filtered and the activated polymer (PLGA-NHS) was precipitated with diethyl ether. PLGA-NHS was subsequently solubilized in DMSO (1.5 ml), and triethylamine (0.8 µl, d=0.726g/ml, 5.6 µmol) and DY405-amine (2 mg, 2.8 µmol) were added. The solution was stirred for 7 hrs at r.t. and then poured in anhydrous diethyl ether to precipitate the fluorescent polymer as an oil. The oily layer obtained was then solubilized in CH_2Cl_2 and some drops of a saturated solution of HCl in diethyl ether were added to neutralize the excess of triethylamine. The polymer (DY405-PLGA) was purified two times with a precipitation/dissolution technique using CH_3OH/CH_2Cl_2 and performing the centrifugation of the methanolic solution at 9800 rpm for 10 min to promote the polymer precipitation.

Derivatization yield was calculated by indirect evaluation of the not-conjugated dye present in mother liquors (coming from purification passages of the synthesis and allowed to dry) by means of a spectrophotometric procedure on the basis of the logε (logε 4.47 in water) of the dye at the maximum λ (400 nm). Before the analysis, a calibration curve, linear in the range between 98.0 and 20.7 µg/ml, was set up starting from stock solutions of dye in ethanol. Derivatization yield was about 8%.

Checking the integrity of Blood-Brain Barrier

The Blood-Brain Barrier integrity was assessed by Evans blue (EB) test, after i.p. administration of NPs samples and osmotic opening by hypertonic mannitol solution. Details of procedures are reported in Supplementary Information.

Evans blue (EB) fluorescent dye ($C_{34}H_{24}N_6Na_4O_{14}S_4$; Mol. Wt. 960.8, Sigma-Aldrich) was used as a tracer for assessing BBB integrity. Briefly, 50 µl of a 2% (w/w) saline solution of EB was injected i.p. in mice at fixed times (0, 30 min, 2 hrs, 6 hrs, 24 hrs; two animals for each time) soon after i.p. administration of an exact amount of *Rhod-CRM197-NPs* or *DY675-g7-NPs or DY405-C-NPs* (1 mg of each sample suspended in 1 mL of sterile saline solution).

EB was allowed to circulate for 10 min. Afterwards, the mice were sacrificed and the brains were collected. Arachnoid membrane and meningeal vessels were removed and the organs were submitted to conservative procedures by washing with saline solution and then frozen at -80°C. Each frozen tissue was cut in all its thickness by a cryotome into 5 µm slides. The slides were then fixed in absolute MeOH and stored at 4°C in a black box to preserve the possible loss of fluorescence for a maximum period of 24 hrs before the microscopic examination.

As a positive control for this experiment, it was necessary to check the EB permeability under conditions of altered BBB. For this purpose, an osmotic opening of the BBB was obtained by an intracarotid infusion of a hypertonic mannitol solution [51]. Four mice were anesthetized with chloral hydrate (400 mg/kg/10 ml, i.p.) and a 30 G needle (connected to a polyethylene-10 cannula) was inserted upward into the left common carotid artery. A total of 300 µl of a filtered 25% (w:v) mannitol solution was then steadily injected within 30 s. Then, 10 min later, 50 µl of a 2% (w/w) saline solution of EB was injected i.p.. Two mice were sacrificed 30 min after the mannitol injection and two mice after 2 hrs; their brains was collected and treated as above described. Since EB emits red fluorescence after laser excitation at 470 nm, brain sections from all experiments were analyzed using confocal microscopy to determine the NPs and EB distribution in the brain.

Intracardiac perfusion and tissue preparation for immunohystochemical labeling

For histological evaluation, animals were anesthetized with chloral hydrate (400 mg/kg/10 mL, i.p.). Intracardiac perfusion was performed with 4% (w/w) paraformaldehyde and 0.2% picric acid (w/w) for 10 min (7 ml/min) and the organs (brain, liver, spleen, lung and kidney) were dissected. The brain was postfixed in the same solution (4% paraformaldehyde and 0.2% picric acid) for 12 hrs. Before to be frezze-dried, the organs were cryoprotected in 15% (w/w) sucrose PBS 1x pH 7.4 (diluted from PBS 10x, Sigma Aldrich) for about 12 hrs and then in a 30% (w/w) sucrose in PBS 1x pH 7.4 for 1 day. The organs were frozen using dry ice, and coronal 50 µm thick sections series were cut at a cryotome (Cryotome CM3000, Leica Instruments), washed three times in cold PBS pH 7.4 and stored at -20°C in a glycerol-PBS pH 7.4 solution.

Immunohystochemical labeling

The slices of the organs were processed for multiple immuno-fluorescence histochemistry according to the following protocol. After five washes with PBS 1x pH 7.4 for 10 min, blocking was performed for 1 hr at r.t. in a PBS 1x pH 7.4 solution containing 0.1% (w/w) Triton X100 and 1% (w/w) bovine serum albumin (BSA). Incubation with primary antibodies diluted in 0.3% (w/w) Triton X100, 1% (w/w) normal serum (NS) and PBS 1x pH 7.4 was performed overnight at 4°C. After three washes in PBS 1x pH 7.4/0.1% Triton X100, incubation with goat anti mouse Alexa488 (dilution 1:200 from stock solution) or goat anti rabbit Alexa488 (dilution 1:200 from stock solution) secondary antibody in 0.2% (w/w) Triton X100, 1% (w/w) NS and PBS 1x pH 7.4 was carried out for 90 min at r.t. After washing three times with PBS 1x pH 7.4 for 10 min, tissues' sections were placed on gelatinised glass slides, dried and, after incubation with DAPI, mounted for confocal microscopy analysis.

In order to observe cellular structures, the slides were stained for 10 min with 50 µl of a DAPI solution (4´-6-diamidino-2-phenylindole; 125 ng/ml; LabVision Corporation, Fremont, CA, USA), which forms fluorescent complexes with natural dsDNA [50].

To assess NPs localization, confocal microscopy (Confocal Microscope Leica) was used and set in consideration to the confocal experiments on the different type of fluorescent polymer; fluorescent spots, due to the fluorescent dyes, were considered as the visible markers of the dye linked to the NPs, while blue fluorescent spots demonstrated the intercalation of DAPI in the nuclear DNA.

Nociceptive studies and measurements

The nociceptive measurements were performed by hot plate test, based on the method of Eddy and Leimbach [53,54] and reported in our previous papers [38,55,56],. The animals were placed on a surface (23x23 cm) maintained at 55 ± 0.5°C and surrounded by a plexiglass 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 sec resulted in the termination of the test (cut-off). Comparison amongst animals was made by means of an analgesia index (Eq. 1) which expresses the change in hot-plate latency in terms of the differences between base-line latency and the latency itself. The latency was assessed at fixed intervals between 0 and 168 hr after the administrations.

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

MPE = (latency after drug - control latency)/ [cut off time (60 sec) - control latency] (Eq. 1)

For each time point, data were submitted to ANOVA. When ANOVA revealed significant difference amongst groups, means were compared by Student-Newman-Keuls tests for multiple comparisons.

Chemico-phsyical, morphology and surface analysis of NPs

Particle size and ζ potential measurements

Each batch of NPs was analyzed (in distilled water) for particle size and ζ potential by photon correlation spectroscopy (PCS) and laser Doppler anemometry using a Zetasizer Nano ZS (Malvern, UK; Laser 4 mW He–Ne, 633 nm, Laser attenuator Automatic, transmission 100% to 0.0003%, Detector Avalanche photodiode, Q.E>50% at 633nm, t=25 °C). The results were normalized with respect to a polystyrene standard solution.

NPs morphology: Scanning Electron Microscopy

The morphological observations of NPs were performed with a scanning electron microscope (SEM) (XL-40; Philips, Eindhoven, The Netherlands) operating at 8 kV. A drop of a NP suspension in deionazed water (0.05 mg/ml) was placed onto a SEM sample holder and 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, Ashford, 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 (13,000× to 16,000×).

Surface analysis of NPs

Electron Spectroscopy for Chemical Analysis (ESCA) study was used to show the presence of N atoms on the surface of engineering NPs (namely *DY675-g7-NPs* and *Rhod-CRM197-NPs*). ESCA analysis was performed on a 04-153 X-ray source analysis system (Physical Electronics, Chanhassen, MN, USA) and an EA11 hemispherical electron analyzer (Leybold Optics, Alzenau, Germany), using $MgK_{\alpha 1,2}$ radiation. The spectra were recorded in fixed retardation ratio (FAT) mode with 190 eV pass energy. The pressure in the sample analysis chamber was ca. 10^{-9} mbar. Data acquisition and processing were performed with the AugerScan 2 (RBD Instruments, Bend, OR, USA)