Long-Circulating PEGylated Polycyanoacrylate Nanoparticles as New Drug Carrier for Brain Delivery

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Purpose. The aim of this study was to evaluate the ability of long-circulating PEGylated cyanoacrylate nanoparticles to diffuse into the brain tissue.

Methods. Biodistribution profiles and brain concentrations of [^{14}C]-radiolabeled PEG-PHDCA, polysorbate 80 or poloxamine 908-coated PHDCA nanoparticles, and uncoated PHDCA nanoparticles were determined by radioactivity counting after intravenous administration in mice and rats. In addition, the integrity of the blood-brain barrier (BBB) after nanoparticles administration was evaluated by *in vivo* quantification of the diffusion of [^{14}C]-sucrose into the brain. The location of fluorescent nanoparticles in the brain was also investigated by epi-fluorescent microscopy.

Results. Based on their long-circulating characteristics, PEGylated PHDCA nanoparticles penetrated into the brain to a larger extent than all the other tested formulations. Particles were localized in the ependymal cells of the choroid plexuses, in the epithelial cells of pia mater and ventricles, and to a lower extent in the capillary endothelial cells of BBB. These phenomena occurred without any modification of BBB permeability whereas polysorbate 80-coated nanoparticles owed, in part, their efficacy to BBB permeabilization induced by the surfactant. Poloxamine 908-coated nanoparticles failed to increase brain concentration probably because of their inability to interact with cells.

Conclusions. This study proposes PEGylated poly (cyanoacrylate) nanoparticles as a new brain delivery system and highlights two requirements to design adequate delivery systems for such a purpose: a) long-circulating properties of the carrier, and b) appropriate surface characteristics to allow interactions with BBB endothelial cells.

KEY WORDS: PEGylated nanoparticles; brain targeting; polysorbate 80; surfactant coating.

INTRODUCTION

The blood-brain barrier (BBB) is a unique membranous barrier that tightly segregates the brain from the circulating blood. This barrier, formed by special endothelial cells sealed with tight junctions and a complete absence of pinocytic activity, restricts the molecular exchange to transcellular transport (1). In fact, only unionized, lipophilic, and low molecular weight molecules can diffuse freely through the endothelial membrane and may passively cross the BBB. Polar molecules and small ions are totally excluded. Other essential compounds such as amino acids, hexoses, neuropeptides, and proteins need specific carriers or transport systems to permeate the brain (2). Consequently, the therapeutic value of many promising drugs is diminished, compromising, importantly, the treatment of neurological and psychiatric disorders.

Traditional approaches to overcome brain drug delivery obstacles include direct intracerebral drug injection or disruption of the BBB by infusion of hyperosmotic solutions (3). Although these procedures can significantly increase drug levels into the brain, all of them are associated with a high risk for the patient. Chemical modifications of drugs as an increase in lipophilicity or the conjugation of the drug with a BBB specific transport vector are interesting alternatives (4). Nevertheless, in the later case, the carrying capacity of these molecular carriers is very limited because these systems generally involve a 1:1 stoichiometry of vector to drug. The carrying capacity of the vector could be greatly expanded by incorporation of the non-transportable drug into particulate systems such as liposomes or polymer nanoparticles. A number of authors have developed several liposome based drug carriers such as antibody-coated liposomes (5) or mannosylated liposomes (6) with the aim to increase the brain uptake by improving the adhesion of liposomes to the endothelial cells of BBB. Even though these approaches may improve the brain uptake, the results of these studies were not convincing enough.

Polymeric nanoparticles have been proposed as an interesting alternative. It is noteworthy that these colloidal systems allow the enhancement of therapeutic efficacy and reduction of toxicity of a large variety of drugs (7). However, their rapid clearance from blood circulation by the tissues of the Mononuclear Phagocyte System (MPS) is the major obstacle to the delivery of drugs to organs or cells other than MPS. Different strategies have been proposed to modify the body distribution of polymeric nanoparticles, most of them being based on the modification of the hydrophobic particle surface by physical adsorption of a hydrophilic polymer. One of the most commonly used polymers for particle hydrophilization is the series of linear or branched copolymers of poly (ethylene oxide) and poly (propylene oxide) (Pluronic/Tetronic™ or Poloxamer/Poloxamine™) (8–10). Another approach includes the synthesis of amphiphilic copolymer in which the hydrophobic block itself is able to form a solid phase (particle core), while the hydrophilic part remains as a surface-exposed "protective cloud." Examples are the block-copolymer of PEG and poly (lactide-glycolide) (PEG PLAGA) (11), or the block-copolymer of PEG and n-hexadecylcyanoacrylate (PEG-PHDCA) (12). With such types of hydrophilic coatings, the natural blood opsonization process of the particles are reduced, hence a relative avoidance of the recognition by macrophages in liver and spleen is conferred, increasing particle blood half-life and therefore, their extravasation to non-RES tissues. Among the different surfactants used to modify particle biodistribution, polysorbate 80-coating was the only one to undergo brain passage of

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the nanoparticles although biodistribution toward other organs was not modified (8). Moreover, central analgesic drugs such as dalargin (13,14), loperamide (15), tubocurarine (16), and MRZ 2/576 (17), a novel N-methyl-D aspartate (NMDA) receptor antagonist, and the antitumor drug doxorubicin (18) have improved their pharmacologic activity or brain concentration when associated with polysorbate 80-coated nanoparticles. The authors have hypothesized that the coated nanoparticles were transported across the BBB after endocytosis by the brain capillary endothelial cells. However, the mechanism underlying such endocytosis remained very obscure, even leading to a certain polemic. Indeed, Olivier *et al.* (19) recently suggested a non-specific permeabilization of the BBB caused by polysorbate 80-coated -poly (butyl cyanoacrylate) nanoparticles.

With this data in mind we evaluated if a prolonged permanence of particles in blood automatically leads to an increase in nanoparticle concentration in the brain, and if this is the case, is the integrity of the BBB preserved. We have compared the body distribution of the following different types of long-circulating and conventional nanoparticles: PEGylated nanoparticles made by a new amphiphilic block-copolymer (PEG-PHDCA), poloxamine 908-coated, polysorbate 80-coated, and uncoated PHDCA nanoparticles. The integrity of the BBB after nanoparticle administration was evaluated by quantifying the diffusion of [^{14}C]-sucrose across BBB *in vivo* to observe modifications of the BBB permeability caused by either polymeric nanoparticles or their associated surfactants. In addition, the location of nanoparticles in the brain was also investigated using epi-fluorescent microscopy.

MATERIALS AND METHODS

The poly [methoxy poly (ethylene glycol) cyanoacrylate-co-hexadecyl cyanoacrylate] (PEG-PHDCA 1:4) copolymer was synthesized by condensation of methoxy poly (ethylene glycol) cyanoacetate (MePEG, 2000 Da of molecular weight) with n-hexadecyl cyanoacetate (HDCA) in ethanol, in presence of formalin, and pirrolidine as described elsewhere (20). HDCA was also condensed by a similar reaction to obtain the poly (hexadecyl cyanoacrylate) (PHDCA) used as a control. For body and brain distribution studies, radiolabeled PEGylated PHDCA ([^{14}C]-PEG-PHDCA 1:4) and non-PEGylated PHDCA ([^{14}C]-PHDCA) polymers were synthesized by a procedure reported previously (12). Specific radioactivities were 1.5 μCi/mg and 5.8 μCi/Mg for [^{14}C]-PEG-PHDCA 1:4 and [^{14}C]-PHDCA, respectively.

Poloxamer 188 (Lutrol® F68) and Poloxamine 908 (Synperonic® T908) were a gift from ICI (Middlesborough, England). Polysorbate 80, (Montanox® 80), was a gift from SEPPIC (Castres, France). [^{14}C]-sucrose (492 mCi/mmol) was obtained from ICN (Orsay, France). All other reagents were of analytical grade.

Animals

This work was done in accordance with the Principles of Laboratory Animal Care (NIH Publication #86-23, revised 1985). Male OF1 mice (20–25 g) were obtained from IFFA Credo Breeding Laboratories (St Germain sur l'Arbresle, France) and male rats Dark Agouti DA/Rj (200–230 g) were obtained from R. Janvier Breeding Centre (Le Genest St-Isle, France). Animals were housed in plastic cages, were given food and water *ad libitum* and were maintained in temperature- and humidity-controlled rooms.

Preparation of Nanoparticles

PEGylated and non-PEGylated PHDCA nanoparticles were elaborated by the nanoprecipitation technique (21). Briefly, the copolymer PEG-PHDCA 1:4 or the polymer PHDCA (75 mg) was dissolved in 5 ml of acetone. The polymer solution was then added, under magnetic stirring, to 10 ml of an aqueous solution of poloxamer 188 (0.25% w/w). Particle precipitation occurred immediately. After solvent evaporation under vacuum, an aqueous suspension of nanoparticles was obtained. Nanoparticle suspensions were ultracentrifuged (120.000 × g, 1 h 30 min at 4°C) and then redispersed in the appropriated volume of an aqueous solution of poloxamer 188 (0.25% w/w) to improve their stability. It was previously shown that such a small amount of poloxamer 188 has no influence on the biodistribution of nanoparticles (8,22). After resuspension, nanoparticles were filtered (prefilters Millex® AP20, 1.2 μm Millipore) and stored at 4°C.

Nanoparticle size was measured at 20°C by Quasi-Elastic Light Scattering (QELS) using a Nanosizer Coulter® N4MD (Coulter Electronics, Inc., FL, USA). The physico-chemical characterization of PEGylated and non-PEGylated PHDCA nanoparticles was previously described in detail (23). For poloxamine 908 and polysorbate 80-coated PHDCA nanoparticles, PHDCA nanoparticles were ultracentrifuged (120,000 × g, 1 h 30 min, 4°C) and resuspended in an aqueous solution containing 1% of poloxamine 908 or 1% of polysorbate 80. Incubation occurred under gentle stirring at room temperature overnight.

Radiolabeled nanoparticles were prepared by the same procedure but using an appropriated isotopic dilution of [^{14}C]-PEG-HDCA 1:4 or [^{14}C]-PHDCA with the respective non-radiolabeled polymers in order to obtain the desired specific activity, as shown in Table I.

Body Distribution of ^{14}C Labelled Nanoparticle Suspensions

For mice body distribution studies, OF1 mice, body weight between 20–25 g, were used. 500 μl of [^{14}C]-radiolabeled nanoparticle formulations were injected intravenously into the tail vein (dose 60 mg/kg, 95 μCi/kg). At time points 1 and 4 h after the injection, animals were anaesthetized with ketamine and the blood was collected into heparined tubes by cardiac puncture. The animals were dissected and each tested organ (spleen, liver, lungs, kidneys, muscle, and brain) was removed. The whole organs were weighed immediately after removal. The brain was dissected into three parts, cerebellum, right hemisphere, and left hemisphere, and each part was exactly weighed into scintillation vials and dissolved in tissue solubilizer Soluene® 350, (Packard BioScience B.V. Groningen, The Netherlands). The vials were stored at 50°C until total tissue solubilization. 200 μl of 30% hydrogen peroxide were then added followed by 10 ml of scintillation cocktail (Hionic Fluor®, Packard BioScience B.V. Groningen, The Netherlands). The samples were stored for 2 days in darkness and the radioactivity was counted in a

Table I. Nanoparticle Formulations, Physico-Chemical Characteristics, and *In Vivo* Tests Performed

Formulations	Composition	Particle size (nm)	Nanoparticle concentration (mg/ml)	Specific activity (μCi/mg)	Test
NP [PEG-PHDCA]	–[^{14}C]-(PEG-PHDCA)	137 ± 21	7.6	1.56	mice
	–[^{14}C]-(PEG-PHDCA) + PEG-PHDCA (isotopic dilution 1:5.9)		30	0.22	rats
NP [PHDCA]	–[^{14}C]-PHDCA + PHDCA (isotopic dilution 1:2.3)	164 ± 52	7.6	1.94	mice
	–[^{14}C]-PHDCA + PHDCA (isotopic dilution 1:8.3)		30	0.62	rats
NP [PHDCA]-P80	–[^{14}C]-PHDCA + PHDCA + Polysorbate 80 1% (isotopic dilution 1:2.3)	159 ± 25	7.6	1.94	mice
	–[^{14}C]-PHDCA + PHDCA + Polysorbate 80 1% (isotopic dilution 1:8.3)		30	0.61	rats
N [PHDCA]-polox 908	–[^{14}C]-PHDCA + PHDCA + Poloxamine 908 1% (isotopic dilution 1:2.3)	147 ± 30	7.6	1.94	mice
	–[^{14}C]-PHDCA + PHDCA + Poloxamine 908 1% (isotopic dilution 1:8.3)		30	0.62	rats

scintillation counter (Beckman LS 6000 LL, Beckman Instruments, Fullerton, CA, USA).

For rat body distribution studies, DA/Rj rats, body weight between 200–230 g, were used. 500 μl of [^{14}C]-nanoparticle formulations were injected intravenously into the penis vein (dose 60 mg/kg, 13 μCi/kg). One hour after intravenous injection, the animals were anaesthetized with urethane 20% and the blood was collected into heparined tubes by cardiac puncture. Organs were removed and treated as previously indicated. Brain was dissected in the following structures: olfactory bulb, cerebellum, colliculi, frontal cortex, occipital cortex, hippocampus, striatum, hypothalamus, and thalamus. Each sample was exactly weighed and prepared for counting of radioactivity as previously described. In some cases, a dose of 30 mg/kg of PEGylated PHDCA nanoparticles or polysorbate 80-coated PHDCA nanoparticles was also injected.

The radioactivity content of the tissues and organs was calculated as percentage of injected dose/gram of tissue. For calculating the distribution of radioactivity, muscle and blood volume were assumed to be 45% and 7.5% of the total body weight, respectively.

Brain parenchyma radioactivity (C_{brain}) was calculated by subtracting intravascular radioactivity ($C_{intravascular}$) from net brain regional radioactivity ($C_{net\ brain}$). Intravascular radioactivity was equal to whole-blood radioactivity ($C_{whole\ blood}$) × regional blood volume. Regional blood volumes in mice and rats were calculated by measuring the radioactivity in different brain regions ($brain_{2\ min}$), (identical to those of biodistribution studies) at 2 min after injection of PHDCA nanoparticles.

In addition, the corrections regarding the total brain radioactivity concentrations were applied equally for the different tested formulations.

Thus, $C_{brain} = C_{net\ brain} - C_{intravascular}$, where: C_{brain} = brain parenchyma radioactivity (dpm/g), $C_{net\ brain}$ = net brain regional radioactivity (dpm/g), $C_{intravascular}$ (dpm/g) = $C_{whole\ blood}$ (dpm/ml) × regional blood volume, and regional blood volume = $brain_{2\ min}$ (dpm/g)/$whole\ blood_{2\ min}$ (dpm/ml).

Quantification of the Passage of [^{14}C]-Sucrose Across the BBB Modification of the BBB Permeability by Nanoparticles and Surfactants

The alteration of the cerebrovascular permeability because of the administration of nanoparticles or surfactants was measured by quantifying the passage of [^{14}C]-sucrose into the brain. Sucrose is a slightly permeated molecule across the normal BBB, and it is not metabolized by the rat (24). 500 μl of nanoparticle suspensions (dose 60 mg/kg) or surfactant solutions (1%) containing 5 μCi of [^{14}C]-sucrose were injected intravenously into rats (adult male rats weighing 200–230 g) via the penis vein. One hour after injection, the animals were anaesthetized with urethane 20%. Transcardiac perfusion (150 ml 0.9% NaCl at 100 mm Hg pressure) was done to clear the intravascular compartment of any residual circulating [^{14}C]-sucrose. Afterward, the brains were removed and the following brain regions were dissected: olfactory bulb, cerebellum, colliculi, frontal cortex, occipital cortex, hippocampus, striatum, hypothalamus, and thalamus. Each region was exactly weighed and prepared for counting of radioactivity as previously described. NaCl 0.9% isotonic solution and sucrose 5% were also injected as control solutions.

Location of Fluorescent-Labelled Nanoparticles into the Brain

PEG-PHDCA nanoparticles were fluorescently labelled using a core of fluorescent polystyrene nanoparticles (Fluorobrite™ nanoparticles) and then coated with PEG-PHDCA copolymer. 30 mg of Fluorobrite™ nanoparticles were incubated with 18 mg of PEG-PHDCA in 1 ml of water overnight. 200 μl of fluorescent PEG-PHDCA-coated nanoparticles were injected into the penis vein (dose 60 mg/kg) of rats. At 24 h after the injection, the animals were anaesthetized with urethane 20% and the blood was cleared from the circulation by transcardiac perfusion (150 ml 0.9% NaCl at 100 mm Hg pressure). The brains were removed and fixed in a PBS solution of 4% paraformaldehide for 12 h. After that, samples

Table II. Percentage of the Injected Dose of [^{14}C]-PEGylated PHDCA Nanoparticles, Polysorbate 80-Coated [^{14}C]-PHDCA Nanoparticles, Poloxamine 908-coated [^{14}C]-PHDCA Nanoparticles and Uncoated [^{14}C]-PHDCA Nanoparticles in Different Tissues at 1 and 4 h After Intravenous Administration into Mice

	NP [PHDCA]		NP [PEG-PHDCA]		NP [PHDCA]—Polox 908		NP [PHDCA]—P 80	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
% dose, 1 h								
Blood	11.470	4.362	29.452	2.077	43.100	8.853	6.352	2.345
Liver	52.163	7.377	9.931	2.248	13.462	3.359	42.244	8.176
Spleen	8.236	1.202	2.109	0.306	8.638	0.836	10.377	3.092
Lungs	0.619	0.197	0.845	0.176	0.696	0.161	0.403	0.144
Kidneys	1.354	0.363	1.782	0.176	0.232	0.190	1.338	0.267
Muscle	5.896	1.929	6.727	1.760	6.385	0.478	7.051	4.210
% dose, 4 h								
Blood	3.211	1.103	28.319	1.886	30.853	2.162	1.368	0.300
Liver	40.734	11.116	13.457	3.914	26.698	4.633	65.997	13.874
Spleen	8.126	1.154	1.850	0.247	10.556	2.157	7.520	1.173
Lungs	0.457	0.130	0.617	0.126	0.717	0.110	0.347	0.022
Kidneys	1.057	0.166	1.757	0.199	1.680	0.091	0.941	0.502
Muscle	5.764	1.271	6.530	1.337	5.699	1.167	3.433	0.593

Note. Injected Dose: 60 mg/kg. Mean and SD, n = 4.

were placed in 20% sucrose for 24 h. Following this, the brains were embedded in OCT compound and frozen at –60°C in isopentane. Frozen sections of 5 μm thickness were then prepared with a cryotome Cryostat (DIS, France). The samples were viewed with an epi-fluorescence microscope (Olympus, Japan).

RESULTS

Biodistribution Studies

Table II and Table III show the concentration of nanoparticles in blood and selected organs of mice and rats, respectively, as the percentage of the injected dose. The results showed, as expected, that PEGylation of the PHDCA nanoparticles or their coating by poloxamine 908 decreased dramatically their uptake by the organs of the MPS, especially the liver and the spleen, and, concomitantly, kept them in the blood for a longer period of time. In fact, 1 h after intravenous injection into mice (Table II), about 30–40% of the administered dose of PEGylated nanoparticles or poloxamine 908-coated particles remained in the blood whereas only 11% of uncoated nanoparticles were located in this compartment. Four hours after injection the liver uptake of the poloxamine 908-coated nanoparticles was significantly increased in comparison with PEGylated nanoparticles. Similar conclusions could be drawn from the results obtained in rats (Table III), however the recovered doses in blood were two times those in mice. In this sense, it is interesting to indicate that the biodistribution of PEGylated nanoparticles in rats was different from mice. Indeed, 1 h after injection in rats, 35% of PEGylated nanoparticles was accumulated in the liver, which was not very different from the percentage obtained with uncoated nanoparticles (46%).

On other hand, polysorbate 80 coating modified significantly the biodistribution of PHDCA nanoparticles in rats, but not in mice, reducing the liver uptake to 15% and keeping the particles in blood circulation (68% against 23% with PHDCA nanoparticles). The results in the other tissues (lungs, kidneys, and muscle) fell in line with those in blood and were complementary to the liver and spleen levels.

Brain Distribution Studies

The brain concentrations of nanoparticles achieved with the different formulations were calculated as brain parenchyma concentration and expressed as percentage of the injected dose/gram of tissue. The values of regional blood vol-

Table III. Percentage of the Injected Dose of [^{14}C]-PEGylated PHDCA Nanoparticles, Polysorbate 80-Coated [^{14}C]-PHDCA Nanoparticles, Poloxamine 908-coated [^{14}C]-PHDCA Nanoparticles and Uncoated [^{14}C]-PHDCA Nanoparticles in Different Tissues at 1 h After Intravenous Administration into Rats

	NP [PHDCA]		NP [PEG-PHDCA]		NP [PHDCA]—Polox 908		NP [PHDCA]—P 80	
% dose	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
Blood	23.057	8.976	49.974	6.083	84.500	3.167	68.505	4.758
Liver	46.852	5.157	35.591	6.098	5.148	0.640	15.800	4.619
Spleen	4.462	0.325	1.098	0.373	2.266	0.733	3.872	0.697
Lungs	0.675	0.141	1.705	0.714	1.211	0.140	1.275	0.197
Kidneys	0.515	0.209	0.827	0.298	1.095	0.177	0.758	0.123
Muscle	2.279	0.440	4.204	1.359	4.077	0.459	4.879	1.683

Note. Injected Dose: 60 mg/kg. Mean and SD, n = 4.

Table IV. Regional Blood Volumes in Brain Tissues

RATS	Blood Volume %
Olfactory bulbs	3.29 ± 0.35
Cerebellum	3.28 ± 0.10
Colliculi	2.15 ± 0.09
Frontal cortex	2.20 ± 0.20
Parietal cortex	2.06 ± 0.27
Striatum	1.90 ± 0.28
Hippocampus	1.93 ± 0.16
Hypothalamus	2.88 ± 0.28
Thalamus	2.48 ± 0.34
MICE	
Cerebellum	2.37 ± 0.24
Left hemisphere	1.79 ± 0.14
Right hemisphere	1.89 ± 0.18

Note. Values are means ± SD, n = 4. Blood volume is defined as the [^{14}C]-PHDCA nanoparticle space, (dpm/g brain)/(dpm/ml whole blood), immediately after iv nanoparticle injection.

umes used to correct brain radioactivities (Table IV) were in perfect accordance with those published previously (24,25). In rats, ten different brain structures were analysed to know any possible preference of particle uptake, which has never been done in previous studies. By contrast, and due to their small size, mice brains were only divided into three regions, the two hemispheres and the cerebellum.

In Fig. 1a the concentrations in brain achieved in mice 1 h after nanoparticle injection are shown. PEGylated nanoparticles were observed to be the most effective carrier to improve nanoparticle brain concentrations. A three-fold increase of radioactivity was obtained in mice with these PEGylated nanoparticles in comparison to uncoated PHDCA particles. In rats, similar observations were made (Fig. 1b and 1c). The concentrations of PEGylated nanoparticles were, in most of the brain structures, significantly higher than those of uncoated PHDCA nanoparticles especially in the deeper regions of the brain (striatum, hippocampus, hypothalamus, and thalamus). Surprisingly, polysorbate 80 coating had not improved the concentration of PHDCA particles in the brain

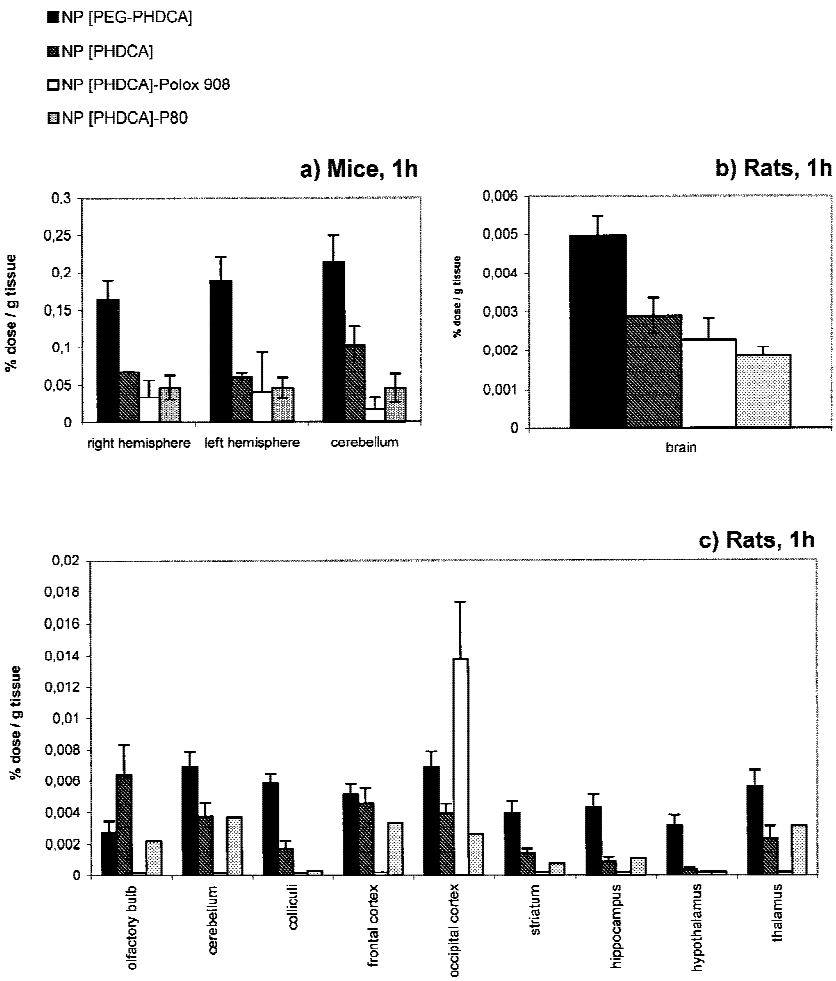

Fig. 1. Concentration of radioactivity (% dose/g tissue) in total brain and brain tissues, after intravenous administration of 60 mg/kg of: [^{14}C]-PEGylated PHDCA nanoparticles, poloxamine 908-coated [^{14}C]-PHDCA nanoparticles, polysorbate 80-coated [^{14}C]-PHDCA nanoparticles, and uncoated [^{14}C]-PHDCA nanoparticles: a) mice, at 1 h post-injection. b and c) rats, at 1 h post-injection. Values are means and SE, $n = 4$.

and the same conclusion could be drawn for poloxamine 908 coating. For this later formulation, it should be pointed out that for two of the four tested animals a low level of radioactivity was found in some brain structures. In fact, measurable concentrations were only obtained in the occipital cortex.

To understand these results, a smaller dose of polysorbate 80-coated PHDCA nanoparticles and also PEGylated nanoparticles (dose 30 mg/kg) were injected in rats. Results in Fig. 2 show that with this lower nanoparticle dose, polysorbate 80 coating significantly increased the amount of particles found in the brain. However, no dose effect was found with PEGylated particles.

Quantification of the Passage of [^{14}C]-Sucrose into the Brain

To investigate eventual modifications on the BBB permeability due to nanoparticles and/or surfactants, additional experiments were done using [^{14}C]-sucrose. This molecule is excluded from the BBB in normal conditions and is not metabolized in rats. Results in Fig. 3a show that none of the nanoparticle suspensions modified the BBB low passage of sucrose. No differences were found between nanoparticles and controls (NaCl 0.9% or sucrose 5% solution treated rats) (Fig. 3b). By contrast, polysorbate 80 solution (1%) increased notably the concentration of sucrose in all the brain structures. However, this effect was not observed when the same dose of polysorbate 80 was injected as adsorbed onto PHDCA nanoparticles (60 mg/kg).

Location of Fluorescent PEG-PHDCA Coated Polystyrene Nanoparticles

Microscopic observations of horizontal sections of brain have shown that the fluorescent particles are localized mainly in the pia mater, in the outside layers of the brain cortex, and to a greater extent in the ventricles and choroid plexuses (Fig. 4). The particles were mainly found isolated in the cuboidal epithelial cells derived from the pia mater of the brain and in the ependymal cells that line the ventricles in which the villous-like choroid projects. A minor quantity of particles was found in the white matter of the brain and in the cerebellum.

DISCUSSION

The biodisponibility studies have clearly shown that the amphiphilic copolymer PEG-PHDCA is a good approach to

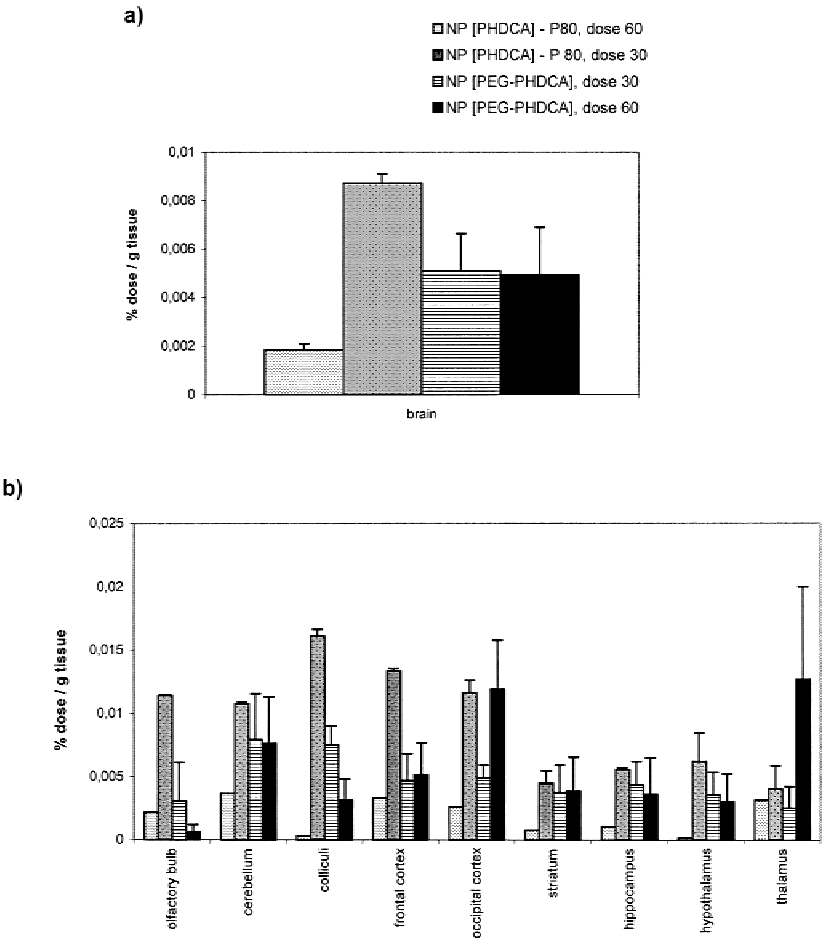

Fig. 2. Concentration of radioactivity (% dose/g tissue) after intravenous administration of 30 mg/kg or 60 mg/kg of [^{14}C]-PEGgylated PHDCA nanoparticles and polysorbate 80-coated [^{14}C]-PHDCA nanoparticles in rats at 1 h post-injection. a) brain, b) brain structures. Values are means and SE, $n = 4$.

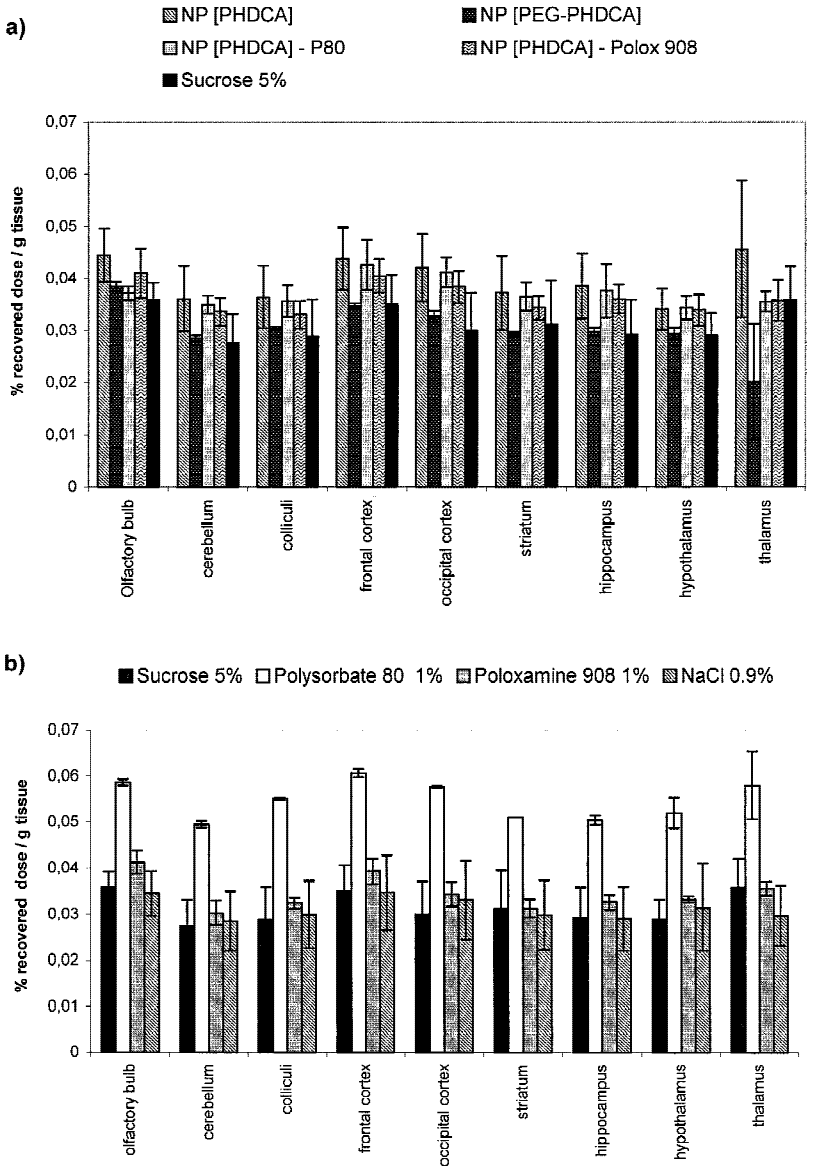

Fig. 3. Concentration of [^{14}C]-sucrose (% dose/g tissue) in different brain structures at 1 h after intravenous administration into rats of 60 mg/kg of. a) nanoparticle suspensions. b) control and surfactant solutions. Values are means and SE, $n = 4$.

obtain stable long-circulating nanoparticles. In fact, in comparison with poloxamine 908-coated nanoparticles, PEGylated nanoparticles have been revealed to be more efficient in avoiding MPS. The explanation could be related with the loss of the protective hydrophilic poloxamine 908 coating after *in vivo* administration as it was recently described by Neal *et al.* (26). For PEGylated nanoparticles, the PEG chains that are covalently attached to the hydrophobic core of the particles produce a stable hydrophilic coating that could explain the constant levels of PEGylated particles observed in blood during the first four hours after intravenous injection. On the other hand, it is interesting to note that the "stealth" effect of the PEG "cloud" was more effective in the case of mice as an animal model than in rats as an animal model. These differences could be attributed to the distinct mechanisms of blood clearance in both animal species. Indeed, previous studies carried out with liposomes demonstrated that the hepatic uptake of liposomes in rats is opsonin-dependent while opsonin-independent or dysopsonin-dependent uptake governs in mice (27). In addition, the cells and the pathways involved in spleen capture of surfactant coated nanoparticles were also different in both species (28).

Concerning brain delivery, PEGylated nanoparticles were the most effective carrier to improve nanoparticle brain concentration. The concentrations of PEGylated nanoparticles were, in most of the different brain structures, significantly higher than those of uncoated PHDCA nanoparticles, especially in the deeper regions of the brain (striatum, hippocampus, hypothalamus, and thalamus). In addition, this increased brain penetration was achieved without modification of the BBB permeability. In fact, no toxic effect arising from the nanoparticles or from their degradation products has been detected toward the permeability of the BBB. By contrast, polysorbate 80 solution notably increased the concentration

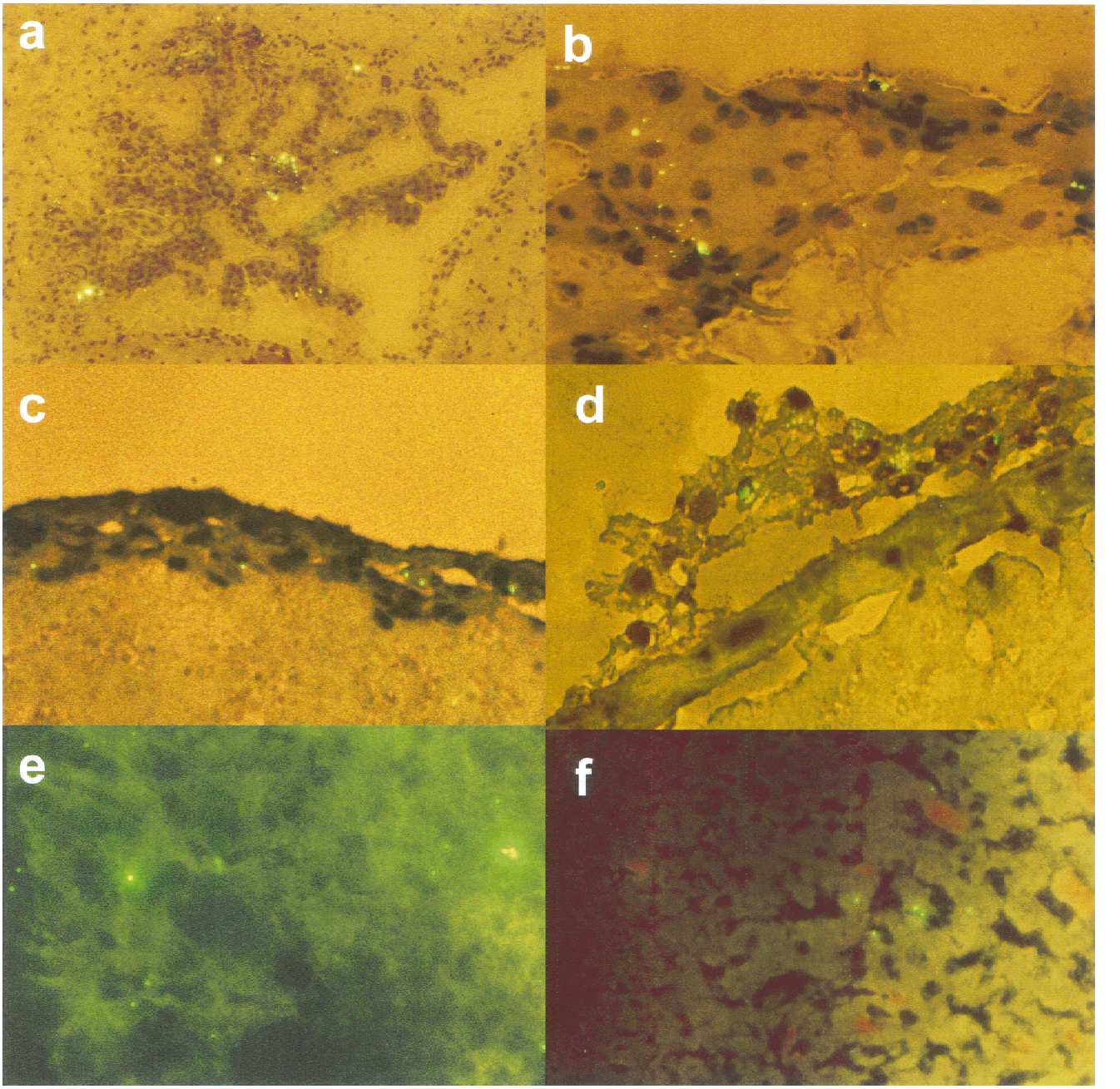

Fig. 4. Fluorescent Fluorobrite™ nanoparticles coated with PEG-PHDCA localized in brain 24 h after intravenous injection. a) choroid plexus, magnification ×380. b) choroid plexus, magnification ×1000. c) and d) pia mater, magnification ×1000. e) and f) white matter, magnification ×1000.

of sucrose in all the brain structures, suggesting a permeabilization effect of this surfactant over the BBB. Surprisingly, this effect was not observed when the same dose of polysorbate 80 was injected as adsorbed onto PHDCA nanoparticles. This result may bo explained by the differences in free polysorbate 80 concentration achieved in blood. Indeed, in the case of polysorbate 80-coated nanoparticles, an important part of the administered surfactant is retained in the nanoparticle surface. This hypothesis was confirmed by the results obtained after administration of a smaller dose of PHDCA nanoparticles (30 mg/kg) but maintaining the amount of polysorbate 80 as previously (20 mg/kg). In this case, it is suspected that the concentration of the free surfactant in the blood is augmented due to the diminished number of particles to be covered. Results clearly demonstrated that the amount of particles found in brain were significantly higher when the ratio polysorbate 80/polymer was increased. It seems, therefore, that polysorbate 80 had a dramatic effect on BBB permeability, which is not the case with PEG coating.

The microscopic examination of the brain after injection of fluorescent nanoparticles showed that the particles were able to penetrate into the brain cells. The nanoparticles were mainly found in the ependymal cells of the choroid plexuses and in the epithelial cell of the pial surface. The retention of the particles by the choroid plexuses is the logical consequence of the function of this tissue as a producer of cere-

brospinal fluid (CFS). The pia mater location could be related to the described extracellular pathways found at the pial surface/subarachnoid space that allow passage between the blood and the CNS interstitial fluid/CFS. These extracellular pathways may allow quasi-equilibrium between plasma and the fluid of the CNS for otherwise impermeable proteins such as albumin (29). In addition, nanoparticles were also found in the white matter, which strongly support the hypothesis that these particles could penetrate into the endothelial cells of BBB as previously reported (13).

Therefore, the higher brain concentrations of particles achieved with PEGylated nanoparticles could be understood because of their higher half-life in blood. However, this conclusion does not support the results obtained with poloxamine 908-coated nanoparticles. This system showed indeed the smallest brain concentration even though poloxamine 908 coating produced an important long-circulating effect. The great capacity of the steric barrier of poloxamine 908 to interfere opsonization, avoiding also the cell membrane recognition step (30), could explain the present observations. Therefore, a compromise between long-circulation characteristics and cell/particle interactions must be strongly considered to design an adequate drug carrier to cross the BBB. This seems to be the case with PEGylated cyanoacrylate system. In addition, it is also interesting to note that the long-circulating characteristics of PEGylated PHDCA nanoparticles could increase the AUC and the transport gradients of particles and/or adsorbed drugs without any induced changes in BBB permeability.

CONCLUSIONS

This study presents a new particulate carrier for brain delivery. Based on their long-circulating characteristics, PEGylated PHDCA nanoparticles made by a PEGylated amphiphilic copolymer penetrate into the brain to a larger extent than all the other tested nanoparticle formulations, without inducing any modification of the BBB permeability. On the contrary, polysorbate 80-coated nanoparticles, described until now as the only particulate carrier capable of brain diffusion, were found to induce permeabilization of the BBB, likely due to toxic effects of polysorbate 80. Another tested long-circulating carrier, poloxamine 908-coated nanoparticles, failed to increase brain concentration probably due to their incapacity to interact with the endothelial cells of the BBB. These results define two important requirements to take into account in the design of adequate brain delivery systems, long-circulating properties of the carrier and appropriate surface characteristic to permit interactions with endothelial cells. In addition, for the first time, biodistribution of nanoparticles in the different tissues of the brain (cerebellum, colliculi, frontal and occipital cortex, striatum, hippocampus, hypothalamus, thalamus, and olfactory bulb) has been investigated. Finally, this article also presents evidence concerning the influence of the animal species on the biodistribution profile of surfactant coated polymer nanoparticles, which has obvious implications in drug carrier design.

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