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In Vivo and *in Vitro* Assessment of Baseline Blood-Brain Barrier Parameters in the Presence of Novel Nanoparticles

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Purpose. Nanoparticles have advantage as CNS drug delivery vehicles given they disguise drug permeation limiting characteristics. Conflicting toxicological data, however, is published with regard to blood-brain barrier integrity and gross mortality.

Methods. To address this issue two novel nanoparticle types: "emulsifying wax/Brij 78"and "Brij 72/Tween 80 nanoparticles were evaluated *in vivo* for effect on cerebral perfusion flow, barrier integrity, and permeability using the *in situ* brain perfusion technique. Additional evaluation was completed *in vitro* using bovine brain microvessel endothelial cells for effect on integrity, permeability, cationic transport interactions, and tight junction protein expression.

Results. In the presence of either nanoparticle formulation, no overall significant differences were observed for cerebral perfusion flow *in vivo*. Furthermore, observed *in vitro* and *in vivo* data showed no statistical changes in barrier integrity, membrane permeability, or facilitated choline transport. Western blot analyses of occludin and claudin-1 confirmed no protein expression changes with incubation of either nanoparticle.

Conclusions. The nanoparticle formulations appear to have no effect on primary BBB parameters in established *in vitro* and *in vivo* blood-brain barrier models.

KEY WORDS: cerebral perfusion flow; drug delivery; integrity; polybutylcyanoacrylate; permeability.

INTRODUCTION

Brain penetration of therapeutic agents is often limited by the blood-brain barrier (BBB). The BBB is comprised of brain capillary endothelial cells connected by tight junctions (*zonulae occludens*) that circumferentially surround the cell margin. These tight junctions may approximate 100 times greater transendothelial electrical resistance than junctions of peripheral capillary endothelium (1). Thus, the BBB demonstrates similar drug permeation restrictions of a continuous cell membrane (i.e., allowing lipid soluble molecules transport across the membrane) whereas compounds that are hydrophilic, protein bound, or of large molecular weight have little to no permeation (2).

Nanoparticles (NPs) may have utility as drug delivery carriers across the BBB. These colloidal particles (size from 1 to 1000 nm) disguise permeation limiting characteristics of therapeutic molecules with the physical nature of the NP. NPs may use numerous combinations of polymers and surfactants for optimized BBB penetration. CNS penetration of NPs, loaded with drugs once impermeable to brain, may provide therapeutic promise (3–4).

Currently NPs manufactured with polybutylcynoacrylate (PBCA) as the polymer and Tween 80 as the surfactant have been studied as drug carriers across the BBB (4). There are conflicting data, however, with regard to *in vivo* toxicity of PBCA-polysorbate 80 NPs. Olivier *et al.*, (5) demonstrated that dalargin adsorbed onto PBCA-polysorbate 80 NPs resulted in death in 3 to 4 of 10 mice (dose = 166 mg/kg). Furthermore, surviving animals had significantly decreased activity, after a short burst of hyperactivity and apparent discomfort. The authors suggest toxicity was mediated by rapid esterase biodegradation of the PBCA polymer to toxic compounds (6–8). Kreuter (4), however, refuted the toxicity, since the CNS effects were a normal response to the opioid dalargin.

Tight junctions are integral to maintaining the physiologic role of the BBB (i.e. limiting CNS entry of toxins). The junctions may be transiently opened by artificially created osmotic pressure as a CNS drug delivery strategy (9). Barrier opening, however, poses significant risk for CNS toxin entry and subsequent damage (10). Alyautdin *et al.*, (11) intravenously injecting (12) PBCA tween 80 NPs, demonstrated an inulin vascular volume increase of 10% after 10 min and 99% after 45 min in the presence of PBCA-polysorbate 80 NPs. Although the author proposed that there was not a major opening of the BBB, it was significant compared to controls.

In vitro publications of PBCA NP safety have the same disparity as the apparent contradictions of *in vivo* reports.

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ABBREVIATIONS: BBB, Blood-brain barrier; NP, Nanoparticle; PA, Cerebrovascular permeability surface-area product; NPE72, Nanoparticle formulation made from Brij72/Tween 80; NPE78, Nanoparticle formulation made from emulsifying wax/Brij78; PBCA-Polysorbate 80, Nanoparticle formulation with polybutylcyanoacrylate polymers overcoated with polysorbate 80.

Two previous reports suggest a PBCA-polysorbate 80 NP exposure concentration of 10 μg/ml may result in BBMECs tight junction disruption (5,13). Other investigators have shown similar concentrations of PBCA polysorbate 80 coated NPs have no effect on apical to basal movement of impermeable markers (4). This study addresses changes in BBB parameters issues with regard to two novel NP formulations. These preparations were developed with the rationale that polymers and surfactants used in manufacturing should be biocompatible and biodegradeable. Given this premise, we hypothesize that the NP formulations will not demonstrate similar adverse effects at the BBB that are observed *in vivo* with the PBCA-polysorbate 80 NPs.

MATERIALS AND METHODS

Nanoparticles, Radiochemicals, and Antibodies

Emulsifying wax/Brij 78 nanoparticles (NPE78) and Brij 72/Tween 80 nanoparticles (NPE72) were prepared from warm oil-in-water microemulsion templates as described elsewhere (14). Briefly, for the NPE78 formulation 2 mg of emulsifying wax was weighed out into glass vials and melted at 50–55°C. Brij 78 solution (100 mM) was added to the melted oil phase, followed by deionized filtered (0.2 μm) water to obtain a final volume of 1000 μL and surfactant concentration of 3 mM. Microemulsion templates formed solid nanoparticles on cooling to room temperature. NPE72 were engineered using a similar procedure, with 2.3 mM Tween 80 as the surfactant.

The particle size of nanoparticles was measured at 20°C using a Coulter N4 Plus Sub-Micron Particle Sizer (Coulter Corporation, Miami, FL, USA). Nanoparticle suspensions were diluted with filtered water (1:10) prior to particle sizing and size was measured at 90° light scattering for 90 s (n = 3). For sizing of aged NPE72 and NPE78, the NP suspensions were sealed and stored at 4°C for one week. Prior to particle size measurement aliquots of nanoparticles were allowed to equilibrate to room temperature and then were diluted with filtered water to ensure light scattering intensity within the required range of the instrument (5×10^4 to 1×10^6 counts per second).

High specific activity [^{14}C]-thiourea (56.0 mCi/mmol) was obtained from Moravek Biochemicals (Brea, CA, USA). High specific activity [^{3}H]-diazepam (76.0 Ci/mmol), [^{3}H]-choline (79.2 Ci/mmol) and [^{14}C]-sucrose (401.0 mCi/mmol) were obtained from Perkin Elmer Life Sciences (Boston, MA, USA). In each experiment, the [^{3}H]-compound was dried prior to being dissolved in the buffer, to remove volatile tritium contaminants, including [^{3}H]-H_20.

The monoclonal antibodies used were mouse anti-zonulae occuldens (ZO)-1 and rabbit anti-claudin-1 obtained from Zymed Laboratories Inc (San Francisco, CA, USA). The anti- ZO-1 is aimed at the amino acid residues 334-634 of the human recombinant ZO-1 protein. The ZO-1 antibody is specific for ZO-1α^+ and ZO-1α^- isoforms. Claudin-1 recognizes the C-terminus of the human/mouse claudin-1 protein. Anti-mouse IgG and anti-rabbit IgG secondary antibodies were purchased from Sigma (St. Louis, MO, USA)

In Situ Perfusion Procedure

Assessment of *in vivo* effects of NPs was accomplished by using the *in situ* rat brain perfusion technique of Takasato *et al.* (15), with modifications (16–17). Briefly, Male Fischer-344 rats (220–330 g; Charles River Laboratories, (Kingston, NY, USA) n = 3–6) were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneal). A PE-60 catheter filled with heparinized saline (100 U/ml) was placed into the left common carotid artery after ligation of the left external carotid, occipital, and common carotid arteries. The pterygopalatine artery was left open during the experiments (17). Rat body temperature was monitored by rectal probe and maintained at 37°C by a heating pad connected to a feedback device (YSI Indicating Controller, Yellow Springs, Ohio, USA). The catheter was connected to a syringe containing buffered physiologic perfusion fluid (containing [in mM]: NaCl 128, $NaPO_3$ 2.4, $NaHCO_3$ 29.0, KCl 4.2, CaCl 1.5, $MgCl_2$ 0.9, and D-glucose 9) with combinations of 0.15 μCi/ml [^{3}H]-diazepam, 1.0 μCi/ml [^{3}H]-choline, 0.33 μCi/ml [^{14}C]-sucrose, 0.33 μCi/ml [^{14}C]-thiourea, and/or unlabeled NP formulations. Perfusion fluid was filtered and warmed to 37°C and gassed with 95% air and 5% CO_2. Perfusion fluid was titrated to a pH of 7.4 with osmolarity being ~290 mOsm. NPs were prepared for perfusion by diluting the NP stock concentration (2 mg/ml) into physiologic buffer to the desired concentration. The perfusion fluid was infused into the left carotid artery with an infusion pump for 20–60 s at 10 mL/minute (Harvard Apparatus, South Natick, MA, USA) with a total dose 200 μg of NP formulations delivered (unless otherwise specified). Flow was set to maintain a carotid artery pressure of ≈120 mmHg. Rats were decapitated and regional cerebral samples obtained, as described (15), after removal of the arachnoid membrane and meningeal vessels. The brain and perfusion fluid samples were then digested overnight at 50°C in 1 mL of 1M piperidine. Ten milliliters of Fisher Chemical scintillation cocktail (Beckman, Fullerton, CA, USA) was added to each vial and the tracer contents assessed by dual-label liquid scintillation counting. All studies were approved by the Animal Care and Use Committee and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

In Situ Kinetic Analysis

Brain uptake of radiolabeled tracers was determined by calculation of a single time point blood-to-brain transfer coefficient (K_{in}) as previously described by Takasato *et al.* (15), and Smith (16), from the following relationship:

$$K_{in} = [C_{tot} - V_v C_{pf}]/(C_{pf}/T) \quad (1)$$

where: $C_{tot} = C_{br} + C_{vas}$, the sum of the amount of tracer remaining in the perfusate in the blood-brain vessels and the amount of tracer that has penetrated into brain, V_v is brain vascular volume, defined as a ratio of the vascular marker [^{14}C]-sucrose in brain to perfusion fluid concentration, C_{pf} is the perfusion fluid concentration of the radiolabeled tracers, and T is the net perfusion time with the assumption that uptake is linear.

Apparent cerebrovascular permeability surface-area product (PA) was determined using the following relationship:

$$PA = F\ln(1 - k_{in}/F) \quad (2)$$

where F is the cerebral perfusion flow determined from the uptake of [^{3}H]-diazepam (18). Regional perfusion flow was

used for regional PA determination to account for regional flow variations.

Bovine Brain Microvessel Endothelial Cell Transport Method

Bovine microvessel endothelial cells (BBMEC) were isolated as previously published (19–21). Briefly, fresh bovine brain was obtained from a local meat slaughterhouse and placed in ice-cold buffered essential medium. Meninges and large surface vessels were carefully removed and discarded. Cerebral gray matter was aspirated from the cerebral cortex. To release microvessels from the gray matter a 2.5 h dispase (4 ml of 12.5% dispase sol./50 g gray matter) digestion at 37°C was performed. Tissue debris was removed by centrifugation with 13% dextran. Removal of pericytes and astrocytes was accomplished by a 4-h incubation with collagenase/dispase (3 mg each in 3 ml of sol /microvessel g). Finally, a percoll gradient centrifugation removed other cellular contaminants.

Cells were seeded on 12-well Transwell® (Costar, Cambridge, MA, USA) plates (0.4 μm pores) at a density of 50,000 cells/cm^2, grown to confluency and used on days 10–12. Culture media was removed prior to transport experiments and allowed to equilibrate for 10 min in physiologic buffer using an oscillating-table (122 mM NaCl, 3 mM KCl, 25 mM Na_2PO_4, 1.3 mM K_2HPO_4, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM glucose, and 10 mM Hepes; pH~7.4). The basolateral chamber contained 1.5 mL of buffer; apical chamber contained 0.5 mL, to ensure no change in pressure gradient existed. Transport experiments were conducted in the apical to basal direction for 2 h with sampling times of 15, 30, 60, and 120 min using an oscillating-table for circulation of well content. Buffer was maintained at 37°C throughout experimental time frame. Studies were completed in the absence (control) and presence of two NP formulations (200 μg/ml; it was believed that a dose significantly higher than would be presented to the BBB with physiologic concentrations required in-clinical therapy) $n = 6$ in all experiments. Study initiation began when 0.5 mL of transport medium supplemented with test compounds was placed in the apical chamber. Fifty micro liters were sampled from the apical chamber (with replacement) at time zero for exposure concentration. Serial 100 μl sampling of the basolateral chamber (with replacement) occurred at the times listed. At the conclusion, 50 μl were sampled from the apical chamber. Fisher Chemical scintillation cocktail (3 ml) (Beckman, CA, USA) was added to each sample and tracer contents assessed by dual-label liquid scintillation counting.

In Vitro Kinetic Analysis

Flux of radiolabeled compounds through BBMECs were determined by calculation of apparent permeability coefficients (P) as previously described (22) from the following relationship:

$$P = Flux/(A * C_{do})$$

where flux is the slope (calculated by linear regression) of pmoles appearing in the receiver chamber vs. time (minutes); A is the area of the membrane in cm; and C_{do} is the donor concentration at time zero.

Western Blot Analysis

After a 2-h exposure to NPE72, NPE78 or no treatment, protein was isolated from confluent BBMEC's seeded on 12-well plates at a density of 50,000 cells/cm^2 on days 10 to 12. Isolation was completed with the Tri-reagant protocol (Sigma, St Louis, MO, USA). Briefly, at the conclusion media was removed and 0.4 mL per 10^2/cm tri-reagant LS was added to each well to lyse cells. Separation of DNA, RNA, and protein was completed with addition of 0.2 mL of chloroform (per ml of tri-reagant). Precipitation of DNA and protein from the interphase and organic phase was accomplished by adding ethanol and isopropanol, respectively. Protein samples were then washed three times by a 0.3 M guanidine hydrochloride/95% ethanol solution and centrifugation 7.5 g for 5 min. Protein was prepared for western blot analysis by pellet dissolution with 1% SDS and sonication at 65°C. Protein was quantified with a BCA Pierce assay kit (Pierce, Rockfors, IL, USA). After a standard curve was established ($r^2 = 0.9847$), 20 μg of protein from each group, and molecular weight markers (Amersham Life Science, Buckinghamshire, UK), were separated using a gradient (4–20%) tris glycine polyacrimde gel (Novex, San Diego, CA, USA) The protein markers and samples were electrophoretically transferred to polyvinylidene fluoride membranes (Amersham Life Sciences, Buckinghamshire, UK). The membrane was incubated overnight in a blocking buffer of 5% non-fat dry milk. After blocking membranes were washed three times with 5% non-fat milk for 20 min. Primary antibodies for ZO-1 and Claudin-1 (1:1,000; 1:500; dilutions respectively) were incubated for 2 h at 23°C. Membranes were washed three times with 5% non-fat milk for 20 min after which the respective secondary antibodies (1:5,000; 1:5,000; dilutions respectively) were incubated for 2 h then washed again. Membranes were developed using ECL plus (PerkinElmer Life Sciences Inc, Boston, MA, USA). Protein bands and molecular markers were visualized on X-ray film.

Statistical Analysis

Data presented are from the frontal cerebral cortex for *in situ* studies unless otherwise specified. Brain PA and permeability coefficients across BBMECs were evaluated by one-way ANOVA analysis followed by a Bonferoni's multiple comparison test. Errors are reported as standard error of the mean unless otherwise indicated. Differences were considered statistically significant at $p < 0.05$. (GraphPad Prism version 2.01 for Windows, GraphPad Software, San Diego,CA, USA). Dual labeled scintillation counting of samples were accomplished with correction for quench, background and efficiency. Western blot analysis was completed using Scion Image for Windows, version 4.02 Beta (Scion Corp., USA).

RESULTS

Effect of NP Types on Cerebral Perfusion Flow

To determine the effect of the NPs on cerebral perfusion flow, we evaluated brain permeation of [^{3}H]-diazepam in the presence or absence of either NP. [^{3}H]-diazepam baseline PA values were determined [$4.05 \pm 0.33 \times 10^{-2}$ ml/s/g (2.43 ± 0.20 ml/min/g)]. Figure 1 shows that in the presence of either NP type no significant ($p > 0.05$) alterations in cerebral perfusion

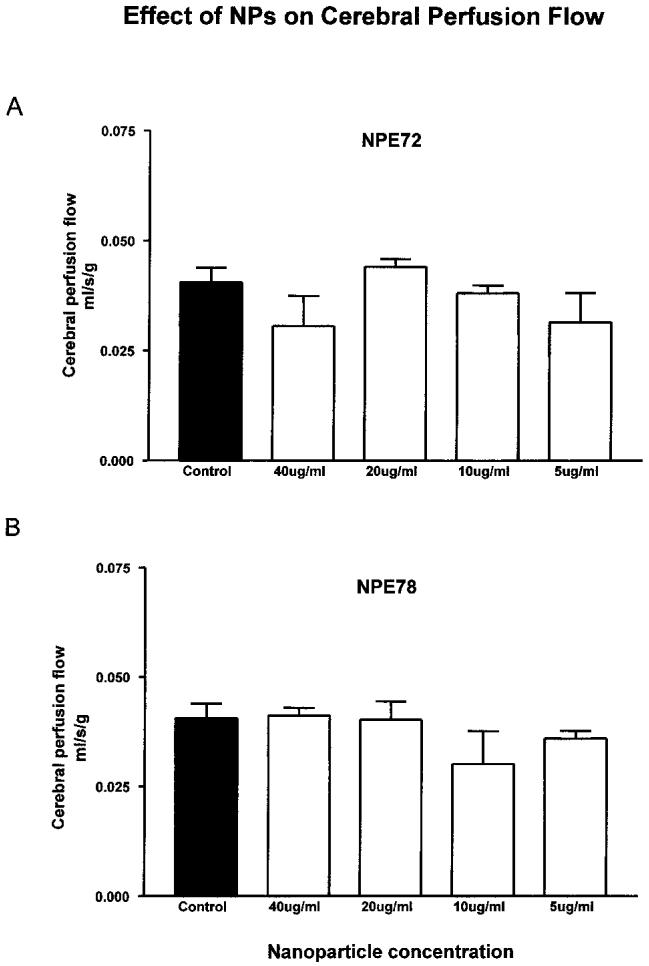

Fig. 1. Calculated frontal cortex [^{3}H]-diazepam (a measurement of cerebral perfusion flow) PA (ml/s/g) in the absence (control) or presence of either NP formulation at concentrations of 5–40 μg/ml (total BBB NP exposure 25–200 μg). (A) represents [^{3}H]-diazepam PA in the presence of NPE72. Figure 1B: represents [^{3}H]-diazepam PA in the presence of NPE78. Data are mean ± SEM (control, n = 6; NP concentrations n = 3–5). No significant differences in PA were observed in the presence of either NP at the concentrations evaluated ($p > 0.05$).

flow were seen at physiologically high NP concentrations (40 μg/ml; total BBB NP exposure 200 μg) [Fig. 1A: NPE72: 3.05 ± 0.68 × 10^{-2} ml/s/g (1.83 ± 0.41 ml/min/g); Fig. 1B: NPE78: 4.10 ± 0.18 × 10^{-2} ml/s/g (2.46 ± 0.11 ml/min/g)]. Furthermore, lesser concentrations had no significant impact on cerebral perfusion flow.

Because there is regional variability of cerebral perfusion flow within the CNS, differences in regional BBB [^{3}H]-diazepam PA were evaluated. Figure 2A shows no overall significant ($p > 0.05$) regional differences in cerebral perfusion flow in the presence of either NP type. A slight statistical reduction of cerebral perfusion flow was seen in the parietal cortex [control: 6.27 ± 0.87 × 10^{-2}ml/s/g (3.76 ± 0.5 ml/min/g)] in the presence of NPE78 [4.39 ± 0.40 × 10^{-2} ml/s/g (2.63 ± 0.24 ml/min/g); $p < 0.05$.

Effect of NP Types on BBB Integrity

To determine if either NP formulation alters the integrity of the BBB *in vivo*, [^{14}C]-sucrose was incorporated in the perfusion fluid in the presence and absence of either NP. Figure 3, shows no significant changes ($p > 0.05$) for frontal cortex vascular volume (ml/g) under control conditions (1.20 ± 0.19 × 10^{-2} ml/g) compared to the presence of 40 μg/ml (total BBB NP exposure 200 μg) of either NP formulation (Fig. 3A: NPE72: 0.84 ± 0.14 × 10^{-2} ml/g; Fig. 3B: NPE78: 1.125 ± 0.001 × 10^{-2} ml/g). Replicated experiments showed subsequent lesser NP concentrations did not alter the vascular space measurements from control. Regional characterization of the effects of the NP's on *in vivo* vascular volume are shown in Fig. 2B. No significant alterations in vascular volume were noted in the presence of either NP formulation with the exception of cerebellum volume (1.38 ± 0.17 × 10^{-2} ml/g) in the presence of NPE72 (0.91 ± 0.11 × 10^{-2} ml/g).

For *in vitro* evaluation of BBB tight junction integrity the authors evaluated apical to basal permeation of [^{14}C]-sucrose across a BBMEC culture in the presence or absence of either (NPE72; NPE78) formulation. Fig. 4 shows the cumulative appearance of sucrose picomoles in the receiver chamber vs. time under control and experimental conditions. For all sampling time points (15, 30, 60, and 120 min) no significant ($p > 0.05$) differences for total [^{14}C]-sucrose in the receiver chamber between experimental groups (NPE72; NPE78) were observed. A permeability coefficient was calculated for [^{14}C]-sucrose as described. Table I illustrates no significant ($p > 0.05$) differences in [^{14}C]-sucrose permeability coefficients observed in the experimental design.

Effect of NP Ostwald Ripening on Cerebral Perfusion Flow and BBB Integrity *in Vivo*

To assess effects of Ostwald ripening on NPs and the subsequent effects on cerebral perfusion flow and BBB integrity parameters, the NP's were allowed to age for 1 week at 4°C. [size at day 0 (nm): NPE72 98 ± 1.8; NPE78 58 ± 0.8] and at day 7 [size at day 7 (nm): NPE72 206 ± 6.8; NPE78 95 ± 1.1]. NP formulations were then added to the perfusion fluid in the presence of [^{14}C]-sucrose and [^{3}H]-diazepam. Figure 5A reveals no significant ($p > 0.05$) alterations in cerebral perfusion flow in the absence [4.05 ± 0.33 × 10^{-2} ml/s/g (2.43 ± 0.2 ml/min/g)] and in the presence of 40 μg/ml (total BBB NP exposure 200 μg) of either aged NP formulation [NPE72: 2.75 ± 0.45 × 10^{-2} ml/s/g (1.65 ± 0.27 ml/min/g); NPE78: 3.42 ± 0.18 × 10^{-2} ml/s/g (2.05 ± 0.11 ml/min/g)]. Figure 5B illustrates no significant changes ($p > 0.05$) for frontal cortex vascular volume (ml/g) under control (1.20 ± 0.19 × 10^{-2}ml/g) or in the presence of 40 μg/ml of either aged NP formulation (NPE72: 1.31 ± 0.14 × 10^{-2} ml/g; NPE78: 1.24 ± 0.077 × 10^{-2} ml/g).

Effect of NP Formulations on BBB Permeation and Facilitated Choline Transport

To ascertain possible effects of NPs on passive permeation at the BBB *in vivo*, [^{14}C]-thiourea was incorporated into the perfusion fluid. Figure 6A demonstrates the passive permeation of [^{14}C]-thiourea remains unchanged *in vivo* ($p > 0.05$) from control (2.31 ± 0.18 × 10^{-4} ml/s/g) compared to the presence of either NP formulation (13.33 μg/ml) (NP72: 2.42 ± 0.64 × 10^{-4} ml/s/g; NP78: 2.06 ± 0.59 × 10^{-4} ml/s/g). Furthermore, Table I shows control permeability coefficient is

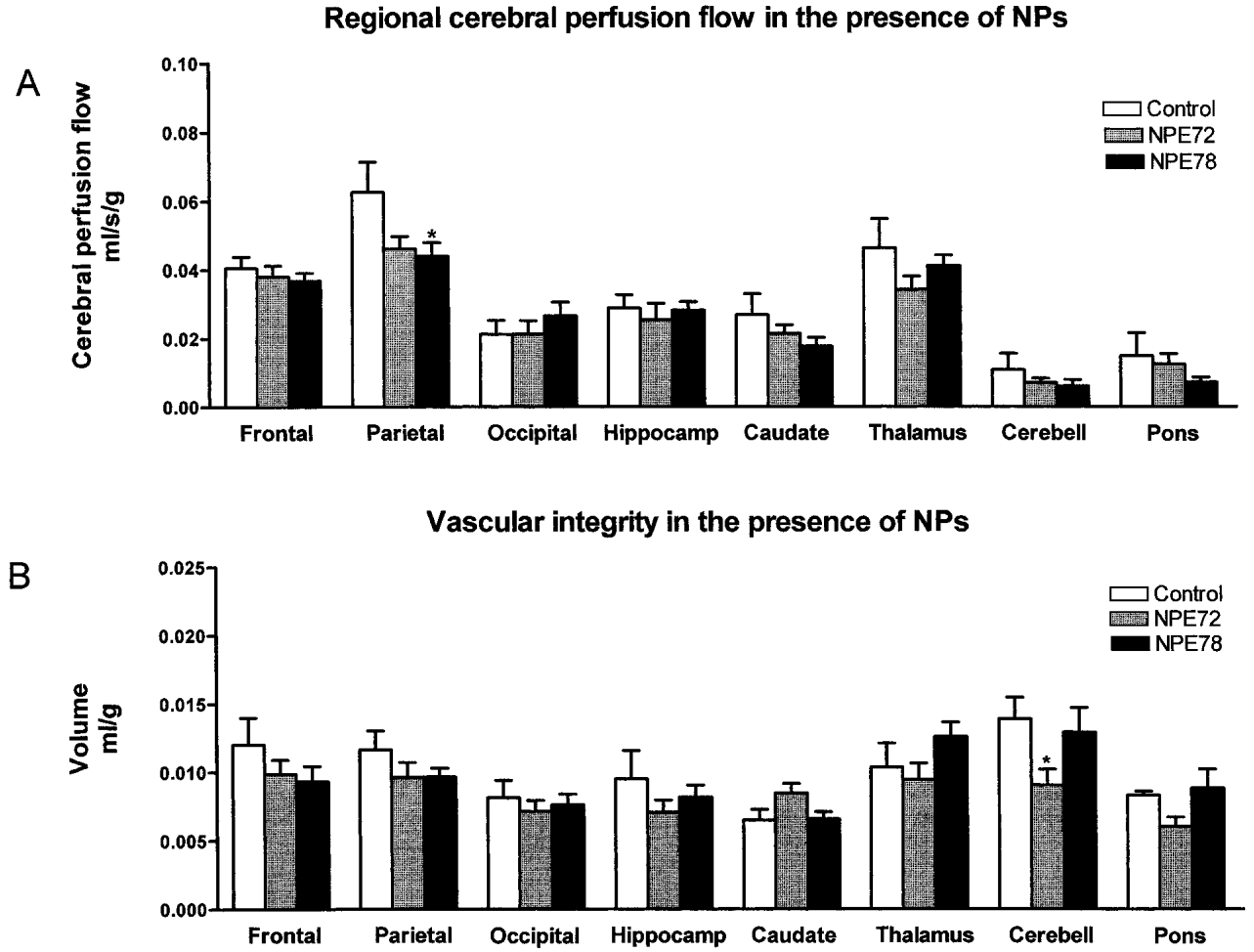

Fig. 2. Evaluation of regional brain [^{3}H]-diazepam PA (ml/s/g) (A) and frontal cortex vascular volume (measured by [^{14}C]-sucrose) (ml/g) (B) in the absence (control) and presence (40 μg/ml; total BBB NP exposure 200 μg) of either NP formulation (control, n = 6; NP experimental groups n = 3–4). Data are mean ± SEM. An asterisk (*) indicates a significant difference ($p < 0.05$). Frontal = frontal cortex, Parietal = parietal cortex, Occipital = Occipital cortex, Hippocamp = Hippocampus, Caudate = Caudate/putamen region, Thalamus = thalamus/hypothalamus region, and Cerebell = Cerebellum, Pons = Pons/medulla region.

not significantly altered in the presence of either NP formulation *in vitro*.

Characterization of [^{3}H]-choline transport in the presence of the NPs was also performed to evaluate the effects of these NPs on cationic BBB transport. Figure 6B illustrates the *in vivo* baseline PA of brain choline ($1.24 \pm 0.02 \times 10^{-3}$ ml/s/g) and in the presence of either NP formulation (13.33 μg/ml). Facilitated transport of choline remains unchanged ($p > 0.05$) (NPE72: $1.50 \pm 0.16 \times 10^{-3}$ ml/s/g; NPE78: $1.35 \pm 0.04 \times 10^{-3}$ ml/s/g) during the 60-s perfusion. Similar lack of effect was seen *in vitro* (Table I).

Effect of NPs on BBMECs Tight Junction Protein Expression

To determine if NP exposure resulted in changes of tight junction protein expression, western blot analyses of Z0-1 and claudin-1 was completed. Figure 7 illustrates protein expression of ZO-1 and claudin-1 in control (lanes 1 and 2) and after exposure to NPE72 (lanes 3 and 4) and NPE78 (lanes 5 and 6). No significant differences were noted between percent control and experimental groups using imaging software (data not shown).

DISCUSSION

The results of these studies demonstrate that two novel NP formulations lack adverse effects *in vivo* and *in vitro* at the BBB. Parameters evaluated include: cerebral perfusion flow, integrity, permeability, and the facilitated transport of choline.

Cerebral perfusion flow has not previously been studied with regard to NP administration. Yet there are data suggesting microspheres can be used to decrease cerebral blood flow to the point of significant impedance in the cortical, striatal, and hippocampal regions with subsequent infarction and necrosis (23). Although NPs are an order of magnitude smaller than microspheres, there is concern NPs may aggregate *in vivo* producing larger particles. Therefore, cerebral perfusion flow was determined in the presence of each NP formulation at doses high enough to potentially result in occlusion. Evaluation of flow was accomplished by calculation of [^{3}H]-

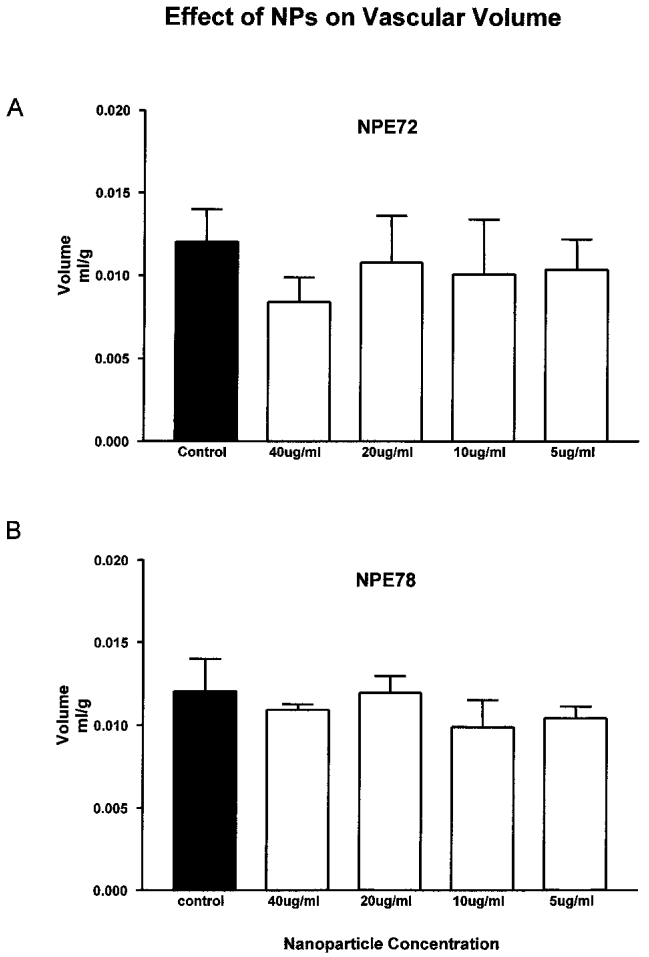

Fig. 3. Analysis of frontal cortex vascular volume (measured by [^{14}C]-sucrose) (ml/g) in the absence (control) and presence of either NP formulation at concentrations of 5–40 μg/ml (total BBB NP exposure 25–200 μg). (A) represents [^{14}C]-sucrose volume in the presence of NPE72. (B) represents [^{14}C]-sucrose volume in the presence of NPE78. Data are mean ± SEM (control, n = 6; NP concentrations n = 3–5). No significant differences were found for vascular volume in the presence (compared to control) of either NP at the concentrations evaluated ($p > 0.05$).

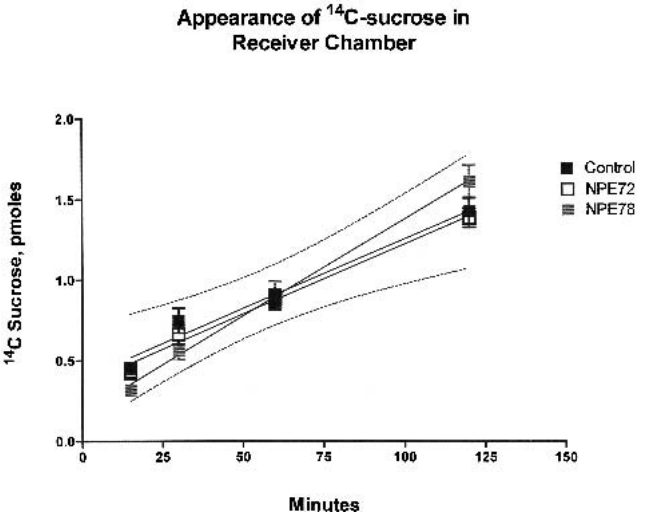

Fig. 4. Cumulative appearance of [^{14}C]-sucrose (picomoles) in the receiver chamber vs. time. Data obtained in the absence (control) and presence of either NP formulation (200 μg/ml). Data are mean ± SEM; n = 6 Slopes are linear regression lines of the accumulation and the 95%confidence interval of control slope is shown. No significant differences were found for any sampling time point vs. control in the presence of either NP at any sampling time ($p > 0.05$).

diazepam PA (ml/s/g) and control values calculated agree with previous results published (15,18) (marker for cerebral flow given an extraction of nearly 100%) (15–16). Figures 1 and 2A, demonstrate that cerebral perfusion flow is not significantly altered in the presence of either NP formulation at doses ranging from 5 μg/ml to 40 μg/ml with the exception of the parietal cortex in the presence of NPE78, where a slight 30% reduction is present.

The mechanism of NP brain entry has not yet been fully elucidated. One hypothesized route is simple paracellular movement after NP induced BBB tight junctional opening (4). This is consistent with reports demonstrating that opening of the BBB enables paracellular brain entry of normally excluded compounds (9). Increasing barrier permeability, however, also allows brain entry of toxins and unwanted molecules (10). For this report, we addressed this potentially toxic route of brain entry by evaluating [^{14}C]-sucrose (an integrity marker as it does not appreciably cross an intact BBB) (24) movement *in vivo* and *in vitro* in the presence of either NP formulation. *In vivo* increases of [^{14}C]-sucrose vascular volume would represent increased permeability of the BBB, as the paracellular transport of [^{14}C]-sucrose is increased. Figures 3 and 2B demonstrate that *in vivo* vascular volume, as measured by [^{14}C]-sucrose, did not significantly change in the presence of either NP with the exception of cerebellum volume decrease in the presence of NPE72.

Olivier *et al.* (5), measured the flux of [^{14}C]-sucrose and [^{3}H]-inulin across BBMECs (co-cultured with astrocytes) in the presence of PBCA-polysorbate 80 NPs. At NP concentrations of less than 1 μg/ml no significant flux alteration of [^{14}C]-sucrose or [^{3}H]-inulin occurred. At NP concentrations between 10–500 μg/ml, however, a greater than 10-fold flux increase occurred with both impermeable markers. This data was also supported by an observed 6.5-fold increase of [^{14}C]-sucrose across BBMECs at a PBCA-polysorbate 80 NP concentration of 10 μg/ml (13). Other investigators however, have shown PBCA-polysorbate 80 NP concentrations of 10 and 20 μg/ml had no significant effect on apical to basal permeation of [^{14}C]-sucrose or [^{3}H]-inulin in a BBMECs system (4).

Based on previous concentrations used, and possible therapeutic *in vivo* NP concentrations, we chose to incubate

Table I. BBMEC Permeability Coefficients for Markers of BBB Parameters in the Presence (200 μg/ml) and Absence of Each NP Formulation

	Control	NPE72	NPE78
Sucrose	$8.6 \pm 0.43 \times 10^{-4}$	$9.3 \pm 0.37 \times 10^{-4}$	$11.1 \pm 1.1 \times 10^{-4}$
Choline	$6.4 \pm 0.6 \times 10^{-4}$	$6.7 \pm 0.3 \times 10^{-4}$	$7.6 \pm 0.6 \times 10^{-4}$
Thiourea	$7.9 \pm 0.9 \times 10^{-4}$	$7.6 \pm 0.3 \times 10^{-4}$	$8.6 \pm 0.6 \times 10^{-4}$

Note: Units are cm/min. Data is reported as mean ± SEM (n = 6). No significant differences in permeability coefficients were observed in the presence of either NP ($p > 0.05$).

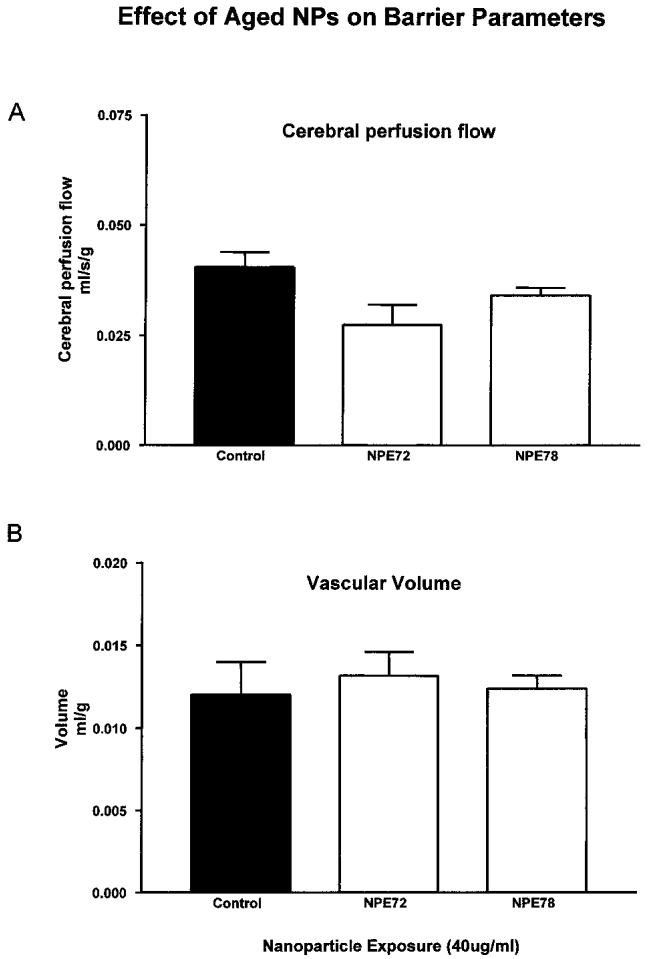

Fig. 5. (A) Cerebral Perfusion Flow: A comparison of frontal cortex [^{3}H]-diazepam PA (ml/s/g) in the absence (control) and presence (40μg/ml; total BBB NP exposure 200 μg) of either NP formulation after aging for 1 week at 4°C. (B) Vascular Volume: Analysis of frontal cortex vascular volume ([^{14}C]-sucrose) (ml/g) in the absence (control) and presence (40 μg/ml; total BBB NP exposure 200 μg) of either NP formulation after it has been aged for 1 week at 4°C. (control, n = 6; NP experimental groups n = 3). Data are mean ± SEM. No significant differences (compared to control) were found for vascular volume in the presence of either NP at the concentrations evaluated ($p > 0.05$).

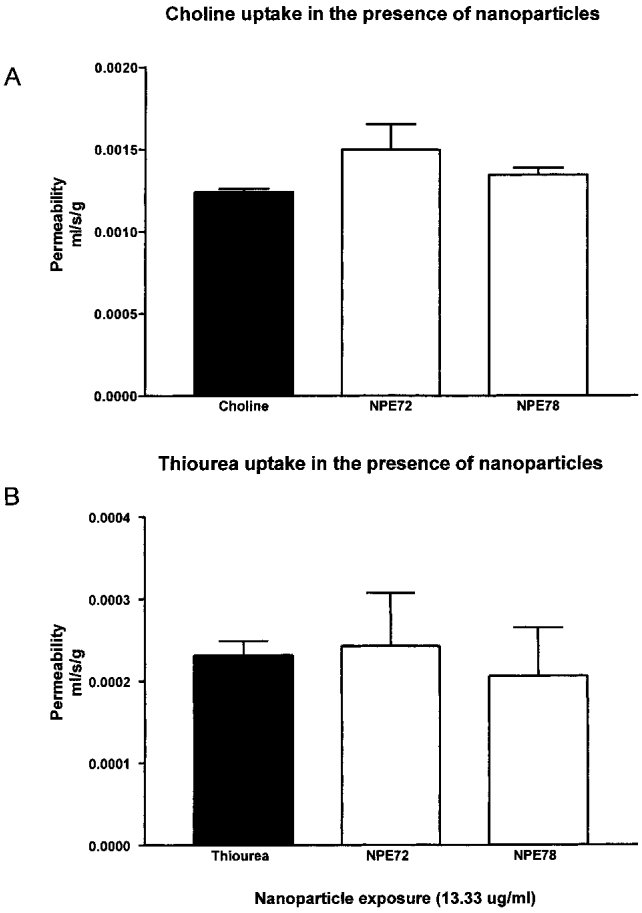

Fig. 6. (A) Comparison of frontal cortex [^{14}C]-thiourea (a small permeability marker) PA (ml/s/g) and (B) frontal cortex [^{3}H]-choline (a transported compound at the BBB) PA (ml/s/g) in the absence (control) and presence (13.33 μg/ml; total BBB NP exposure 200 μg) of either NP formulation (control, n = 5; NP experimental groups n = 3-4). Data are mean ± SEM. No significant differences (compared to control) were found for PA in the presence of either NP at the concentrations evaluated ($p > 0.05$).

the BBMECs in the donor chamber with significantly higher doses of 200 μg/ml. Figure 4 represents the movement of [^{14}C]-sucrose from the donor chamber to the receiver chamber in the presence and absence of either formulation. At all times no significant difference in [^{14}C]-sucrose accumulations was observed. Control permeability coefficients calculated (Table I), agree with previously published data (25) and is not statistically altered experimentally. Given an increased movement of [^{14}C]-sucrose from the donor to receiver chamber would represent decreased integrity of the BBMECs tight junctions (paracellular transport of [^{14}C]-sucrose is increased), we suggest the NP formulations have little effect on BBMECs tight junction integrity and paracellular permeability in the time frames evaluated.

The development of colloidal NP's for large-scale manufacturing would partially depend on NP size stability. *In vitro* stability of NPs in aqueous suspension was tested over a period of one week at room temperature, –20°C (data not shown) and 4°C. Both preparations showed superior size stability at 4°C over room or freezing temperatures remaining well within the nm requisite for brain delivery. Given the approximate size doubling we reevaluated cerebral perfusion flow and vascular volume *in vivo* with NPs that have aged for 1 week at 4°C. Figure 5 shows at 40 μg/ml of NPs no significant changes in [^{3}H]-diazepam PA or [^{14}C]-sucrose volume were observed. Considering the stability data and lack of parameter effects after 1 week of NP aging, clinical investigation of the NP products may be further warranted.

Evaluation of BBB permeability was accomplished by analysis of [^{14}C]-thiourea PA. [^{14}C]-thiourea is considered an *in situ* perfusion reference solute for low brain passive permeation, which agrees with standard IV injection values (26). The *in vivo* [^{14}C]-thiourea PA values we obtained are consistent with published data (26). Figure 6 illustrates no significant changes in brain permeability of [^{14}C]-thiourea PA in the presence of either NP. NP dosing was lowered to accommodate the longer perfusion time needed to ensure half of the slowly penetrating [^{14}C]-thiourea had passed into brain (C_{tot}/

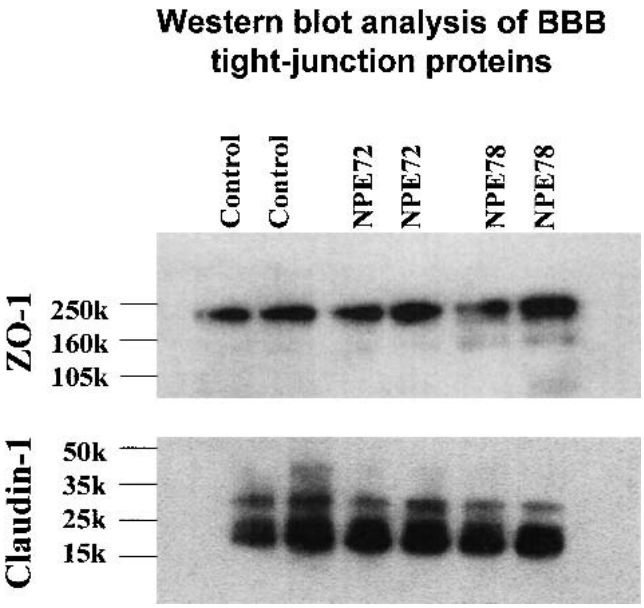

Fig. 7. Western blot analysis of Z0-1 and claudin-1 expression in BBMEC's after a two-hour exposure to either NP type (200 μg/ml). n = 4. Lanes 1 and 2, control; lanes 3 and 4, NPE72 exposure; lanes 5 and 6, NPE78 exposure. No significant differences were noted between percent control and experimental groups using imaging software (data not shown).

$C_{pf} \geq 2 \times V_v C_{pf}/C_{pf}$) (15). Given that [^{14}C]-thiourea is considered a perfusion reference solute it should behave similarly *in vitro*. Table I, illustrates that both NP formulations have a lack of effect on *in vitro* [^{14}C]-thiourea permeation.

The BBB is a strict physical barrier that not only limits drug movement but also may limit passive permeation of essential nutrients. Required compounds rapidly gain access, however, through BBB transporter. For example cationic choline transport at the BBB has been demonstrated *in vivo* to be carrier-mediated and saturable (27). To evaluate if an interaction between the NP constituents (notably the nonionic surfactants Brij 72, Brij 78 and tween 80 used in manufacturing) and the anionic binding site (28) of the choline transport protein is present; [^{3}H]-choline PA values (ml/s/g) were determined (Fig. 6) with control values being consistent with published values (28). In the presence of either NP, no significant difference for the transport of [^{3}H]-choline was observed. Similar to the [^{14}C]-thiourea experiment NP concentrations were lowered. Lack of effect on choline transport was also observed *in vitro* (Table I**).** This combined data indicates NPs using nonionic surfactants do not associate appreciably with the cationic choline transporter and allows physiologic BBB choline transport.

To further explore NP effects on tight junctions western blot analyses were performed with purpose to detect the expression of claudin-1 and ZO-1. Claudin-1 is a major component of tight junction proteins given that: (a) it interacts with separate cellular claudin-1 and (b) claudin-1 and -2 are mainly responsible for tight junction strand formation (integral in cell to cell contact). Occludin appears to be an accessory protein that produces tight junction strands to a lesser extent (29). The cytoplamsic, tight junction accessory proteins, ZO-1, has been shown to play a crucial role of connecting tight junctions to the cytoskeleton (30). If NPs disrupt the tight junction integrity of BBMECs, a decreased expression of these two integral proteins may be recognized after NP exposure. Figure 7 illustrates that there is no apparent change in protein expression after NP exposure. Whereas this data does not account for possible translocation of the tight junction proteins into the cytoplasmic domain, considering the absence of [^{14}C]-sucrose *in vitro* permeability coefficient changes or *in vivo* PA changes, it is unlikely junction integrity is altered in the presence of NP formulation.

The combined *in vivo* and *in vitro* data presented here support: (a) the authors hypothesis that the NP formulations appear to have minimal effect on primary BBB parameters and (b) these NP formulations should be explored as brain drug delivery carriers.

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