Translocation of Poly(ethylene glycol-*co*-hexadecyl)cyanoacrylate Nanoparticles into Rat Brain Endothelial Cells: Role of Apolipoproteins in Receptor-Mediated Endocytosis

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Previous in vivo observations in rats have shown that poly(ethylene glycol) polyhexadecylcyanoacrylate (PEG-PHDCA) nanoparticles could translocate into the brain after intravenous injection, which polyhexadecylcyanoacrylate (PHDCA) nanoparticles did not. Through the detailed analysis of the plasma protein adsorption onto the surface of PEG-PHDCA nanoparticles, the present study aimed at clarifying the mechanism by which nanoparticles could penetrate into rat brain endothelial cells (RBEC). Two-dimensional polyacrylamide gel electrophoresis and Western blotting revealed that, after incubation with rat serum, apolipoprotein E (ApoE) adsorbed more onto PEG-PHDCA than on PHDCA nanoparticles. Adsorption of apolipoprotein B-100 (ApoB-100) onto PEG-PHDCA nanoparticles was demonstrated by capillary electrophoresis experiments. Moreover, only when ApoE or ApoB-100 were preadsorbed onto PEG-PHDCA nanoparticles, nanoparticles were found to be more efficient than control nanoparticles for penetrating into RBEC, suggesting the involvement of a low density lipoprotein receptor in this process. Thus, these data clearly demonstrate the involvement of apolipoproteins in the brain transport of PEG-PHDCA nanoparticles, which may open interesting prospects for brain drug delivery.

Introduction

Drug delivery into the brain is still a major challenge because this tissue is very efficiently protected by the blood−brain barrier (BBB) fashioned by endothelial cells sealed with continuous tight junctions. Hence, this barrier entirely segregates the brain from systemic blood circulation and restricts the molecular exchanges to transcellular transport.[1] This persistent obstacle prohibits the delivery of many potentially effective therapeutic or diagnostic agents. Therefore, one approach to overcome this barrier is the use of colloidal carrier systems with brain translocation properties, to improve the bioavailability of drugs on brain diseases.

Numerous nanoparticle systems have been developed for drug targeting purposes, but only few of them have the ability to diffuse through the BBB. At this point, Kreuter's group has conceived polysorbate 80-overcoated poly(butylcyanoacrylate) (PS 80-PBCA) nanoparticles that could be loaded by a desired drug such as dalargin,[2] loperamide,[3] or doxorubicin.[4] These nanoparticles were found to improve drug transport into the brain. As an alternative, nanoparticles composed of an amphiphilic copolymer consisting of poly(ethylene glycol) (PEG) (hydrophilic chains) and poly(hexadecylcyanoacrylate) (PHDCA) (hydrophobic part) were proposed.[8] After nanoprecipitation of this poly(ethylene glycol)-*co*-polyhexadecylcyanoacrylate (PEG-PHDCA) copolymer, PHDCA was found to compose the particle core, whereas PEG constituted a protective cloud onto their surface.[5] These nanoparticles with a long circulating time in blood[6] have shown an interesting ability to penetrate both into healthy rat brain and into brain glioma.[7,8]

One important factor that determines the biodistribution of particulate carriers after iv administration is the plasma protein adsorption pattern onto their surface. Recently, Kreuter's group reported that apolipoprotein E was detected on the surface of PS 20-, 40-, 60-, or 80-coated nanoparticles by means of two-dimensional polyacrylamide gel electrophoresis (2-D-PAGE) analysis.[9] These authors have hypothesized that the uptake mechanism would be due to apolipoprotein E (ApoE) and B (ApoB)-mediated transport by forming the mimic lipoprotein particle Trojan Horses and resulting in the recognition of these adsorbed apolipoproteins by receptors such as the low density lipoprotein receptor (LDLR) gene family in the brain endothelial cells of BBB.[10]

Now, even if PEG-PHDCA nanoparticles have demonstrated a remarkable ability to translocate into the brain, their mechanism has not yet been clearly elucidated. Some in vitro techniques using a rat BBB model, a fractionation method of rat brain endothelial cell (RBEC) and RBEC uptake experiments, have been performed allowing the demonstration that PEG-PHDCA nanoparticles were internalized by endocytosis in RBEC.[11,12] However, the involvement of apolipoproteins remained unknown. Thus, the aim of this study was to understand more about this mechanism through the detailed analysis of

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plasma protein adsorption by comparing the adsorbed protein distribution on PEG-PHDCA nanoparticles with that on conventional PHDCA nanoparticles and by investigating the influence of the presence of apolipoproteins on the surface of PEG-PHDCA nanoparticles on their cellular uptake in vitro. This has been performed by two-dimensional polyacrylamide gel electrophoresis (2-D-PAGE), matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometric analysis, Western blotting, and capillary electrophoresis. In addition, different apolipoproteins and preadsorbed PEG-PHDCA nanoparticles were prepared, and their uptake by RBEC was investigated.

Experimental Procedures

Materials. The poly(ethylene glycol)-*co*-polyhexadecylcyanoacrylate (PEG-PHDCA 1:4) copolymers were synthesized by condensation of methoxy poly(ethylene glycol) cyanoacetate (MePEG, 2000 Da molecular weight) with *n*-hexadecyl cyanoacetate (HDCA) in ethanol, in the presence of formalin and pyrrolidine. HDCA was also condensed by a similar reaction to obtain the polyhexadecylcyanoacrylate (PHDCA) polymers used as non-PEGylated nanoparticles as described elsewhere.[5,7,13] For the cellular uptake experiments, radiolabeled PEGylated poly(hexadecylcyanoacrylate) ([^{14}C]PEG-PHDCA 1:4) (co)polymers and [^{14}C]poly(hexadecylcyanoacrylate) ([^{14}C]PHDCA) polymers were synthesized by a procedure reported previously[5,7] at the Commissariat à l'Energie Atomique. Their specific activities were 2.8 and 3.8 μCi/mg for [^{14}C]PEG-PHDCA 1:4 and [^{14}C]PHDCA, respectively. All chemicals were of analytical grade.

Biodegradable Cyanoacrylate Nanoparticle Preparation and Characterization. Nanoparticles were obtained by the nanoprecipitation method previously described.[7] Briefly, (co)polymers (20 mg) were dissolved in acetone (2 mL), and this solution was mixed with 4 mL of an aqueous solution containing 1% (w/v) Pluronic F68. The nanoparticle precipitation occurred immediately. After the acetone evaporated, nanoparticles were purified by ultracentrifugation (150 000*g*, 1 h, 4 °C, Beckman Coulter, Inc.). The pellet was resuspended in the appropriated volume of water. For the nanoparticle characterization, we measured their sizes and zeta potential using the quasi-elastic light scattering (QELS) at 90° (Coulter N4MD, Beckman Coulter, Inc.) and the Zeta sizer (Zeta sizer 4, 7032 Multi 8 correlator; Malvern Instruments).

Sample Preparation and Quantification of Plasma Protein Adsorption onto Nanoparticles. A total of 350 μL of nanoparticle suspension (20 mg/mL) was incubated in 1.75 mL of Sprague−Dawley OF1 rat serum (Charles River Laboratories) for 20 min at 37 °C. Plasma proteins adsorbed onto nanoparticles were separated from bulk serum by centrifugation at 15 000*g* for 1.5 h at 4 °C. The supernatant serum was discarded, and the pellet was extensively washed with water by centrifugation (15 000*g* for 1.5 h at 4 °C) to remove excess serum. After the centrifugation, the plasma protein adsorbed nanoparticles were resuspended in 100 μL of solution containing 2.5% sodium dodecyl sulfate (SDS) and 30 mM 1,4-dithioerythritol (DTE). The suspension was incubated at 50 °C for 2 h to detach the adsorbed proteins from nanoparticles.[14] After centrifugation at 15 000*g* for 1 h at 4 °C, Commassie staining and a colorimetric Bicinchoninic Assay Kit (Uptima, Interchim) were applied to quantify the amount of proteins in the final supernatant. The protein amounts in the supernatant obtained from each type of nanoparticle were compared as the protein quantities (μg) adsorbed by unit weight (mg) of nanoparticles.

2-D-PAGE Analysis and MALDI-TOF Mass Spectrometry. The previously mentioned protein solutions were diluted with the 2-D-PAGE sample buffer (7 M urea, 2 M thiourea, 4.5% CHAPS, 100 mM DTE, 40 mM Tris, pH 8.8) and treated as follows. For isoelectric focusing (IEF), 350 μL of the adsorbed plasma protein solutions (100 μg) was loaded onto rehydrated immobilized pH 3−10 nonlinear gradient strips (Amersham). IEF was carried out for about 80 000 V h as follows: 500 V for 1 h, 1000 V for 1 h, and 8000 V for 10 h. The gels were incubated for 15 min with equilibration buffer 1 (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and 65 mM DTT) and then with equilibration buffer 2 (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and 260 mM iodoacetamide) for 15 min. The second dimension was applied on 8−16% gradient polyacrylamide gels (180 mm × 200 mm × 1.5 mm) at a constant 40 mA per gel until the dye front reached the lower end of the gel. Silver staining of proteins was performed with the gel,[15] and gel images were obtained from a GS-710 Imaging Densitometer (Bio-rad) and converted into electronic files, which were then analyzed with an Image Master Platinum 5.0 software program (Amersham). The protein spots were identified by matching the gels to the rat serum 2-D-PAGE reference map.[16] Each experiment was performed in triplicate, and the data were represented as the mean ± SD of % vol. Spot numbers 12 and 16 excised from 2-D gels were destained, reduced, alkylated, and then digested with trypsin (Promega).[17,18] For MALDI-TOF MS analysis, the tryptic peptides were concentrated by a POROS R2 and Oligo R3 column (Applied Biosystems) and eluted in α-cyano-4-hydroxy cinnamic acid.[19] Spectra were obtained using a Voyager DE-PRO MALDI-TOF spectrophotometer (Applied Biosystems). Protein database searching was performed with MASCOT (http://www.matrixscience.com) and MS−-FIT (http://prospector.ucsf.edu) to search the protein databases, SWISS−-PROT and GenBank.

Western Blot Analysis. A total of 20 μg of proteins was migrated on a 12% SDS-polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane. Blots were blocked with 10% (w/v) skim milk in Tris buffer saline. Then blots were rinsed and incubated with apolipoprotein specific antisera for 3 h at room temperature, followed by a peroxidase-conjugated anti-goat IgG (DAKO Cytomation) as a secondary antibody. The immunoreactive bands were visualized by an enhaned chemiluminescent system (Amersham Bioscience). The samples for adsorbed plasma proteins onto PEG-PHDCA and PHDCA nanoparticles were performed, and the negative controls without nanoparticles or serum were applied in the same conditions as for the samples to identify the limits of significance and separation efficacy.

Capillary Electrophoresis. Capillary electrophoresis (CE) was performed on a P/ACE system 5510 (Beckman Instruments) equipped with a diode array detector. The System Gold Chromatography software (Beckman) was used to control the instrument functions and to analyze the electropherograms. A fused silica capillary tube of 75 μm diameter was used. The total length of the capillary tube was 57 cm, and the distance from injection point to detector was 50 cm. The capillary was first preconditioned by consecutive rinsing as follows: distilled water (5 min), 1 N NaOH (5 min), 0.1 N NaOH (5 min), and distilled water (5 min). The CE buffer solution containing 20 mM phosphate, 25 mM borate, and 25 mM SDS (pH 8) was used as cathode and anode electrolytes. For sample preparation, 10 μg of apolipoprotein B-100 was incubated in the PEG-PHDCA suspension (4 mg/mL) for 1 h at 37 °C. After three washing steps by centrifugation at 15 000*g* for 1.5 h at 4 °C with water, the final pellet containing nanoparticles and proteins adsorbed on their surface was resuspended in the CE buffer and directly applied in the CE system without using the previous procedure of protein separation. The sample was injected by pressure for 15 s into the capillary. The migration was run in reversed polarity and at 20 kV constant voltages for 25 min at 30 °C using a liquid cooling system, and the UV detector was operated at 214 nm. Between subsequent runs, the capillary was rinsed with 0.1 N NaOH (3 min) and CE buffer (3 min).

Rat Brain Endothelial Cell Culture. The rat brain endothelial cells (RBEC) were obtained by the primary culture method as described previously[11] with slight modifications. Briefly, the 2-week-old Sprague−Dawley rat brain cortex was used. After dissection of the gray matter, the first enzyme digestion was carried out in the collagenase type II (270 U/mL, Worthington Biochemical Corp.) solution supplemented with 20 U/mL DNase I (deoxyribonuclease I) and 3.64 μg/mL TLCK

(tosyl-lysine-chloromethyl-ketone, Sigma-Aldrich) for 2 h at 37 °C with gently shaking. The digested suspension was centrifuged (1500*g*, 15 min, 4 °C) with 25% BSA (bovine serum albumin, Sigma-Aldrich) for density dependent separation. The pellet was resuspended for the second enzyme digestion in the collagenase/Dispase (0.1% neutral protease Dispase II, Roche Diagnostics) solution supplemented with 20 U/mL DNase I and 3.64 μg/mL TLCK and incubated for 1.5 h at 37 °C with occasional shaking. The suspension of the second enzyme digestion was filtered through a 10 μm pore size nylon mesh. Finally, the capillary fragments were seeded in the 60 mm petri dish (Corning Costar, Mass., USA) coated by collagen type IV (100 μg/mL, Sigma-Aldrich) and were grown in a humidified atmosphere with 5% CO_2 at 37 °C. The culture medium was the EBM-2MV basal medium containing 10% fetal bovine serum (FBS) and antibiotics (1/1000 dilution of gentamycin/amphotericin in SingleQuot kit) supplemented with 3 μg/mL puromycin (Sigma-Aldrich) for 3 days.[20] From the fourth day, the culture medium was changed by the EBM2-MV Bullekit every other day for a week until confluence. The EBM2-MV Bullekit contained manufacturer's supplements such as basal medium, 5% FBS, antibiotics, epidermal growth factor (hEGF), insulin-like growth factor (R3-IGF-1), endothelial growth factor (VEGF), human fibroblast growth factor (hFGF-B), hydrocortisone, and ascorbic acid. For uptake experiments, at first passage, RBEC were seeded onto collagen-type IV-coated six well plates (Corning Costar), and the cell medium, EBM-2MV Bullekit, was changed routinely to make a cell confluence.

Uptake Experiment. Two uptake experiments have been performed. First, to investigate the influence of the PEG coating onto the nanoparticle cell capture, 20 μg/mL PEG-PHDCA (1:4) and PHDCA nanoparticles as well as a physical mixture of PEG (MW = 2000) and PHDCA nanoparticles (1:4 of molecular ratio) were resuspended in the transport medium (TM) containing 5% FBS and basal medium supplied by EBM2-MV Bullekit and then immediately incubated with cells. Second, to study the influence of apolipoprotein adsorption on the nanoparticles, 1 nM each apolipoprotein A, B-100, C−-III, and E (Calbiochem) was incubated with 20 μg/mL nanoparticles in PBS for 1 h in the presence of protease inhibitor cocktail (Sigma-Aldrich, final dilution factor is 100) under gentle stirring. These apolipoprotein preadsorbed [^{14}C]PEG-PHDCA nanoparticles were resuspended in the TM (final concentration 20 μg/mL) and then immediately used for uptake experiments. As the endothelial cells suffered with lower concentrations of FBS, the 5% FBS was constantly maintained during the cellular uptake experiments. In both experiments, cells were washed with 2 mL of PBS, and the 20 μg/mL various nanoparticle suspensions were added to the cells for 20 min or 1 h according to the experimental conditions at 37 °C in a humidified atmosphere with 5% CO_2. Nanoparticle incubation was stopped by discarding the supernatant, followed by washing the cells 3 times with 2 mL of cold PBS (4 °C). Cell lysis occurred by treatment with 0.2 M NaOH containing 1% SDS, and the radioactivity was counted (BECKMAN model LS 6000TA). The protein content in the cell lysate was measured using the colorimetric Bicinchoninic Assay Kit (Uptima, Interchim). Cellular uptake was represented as the amount (μg or ng) of nanoparticles per unit weight (mg) of cellular proteins. The experiments were performed in triplicate, and the results are the mean ± SD.

Results

Nanoparticle Characterization. Nanoparticle mean diameters were 135 ± 41 and 140 ± 42 nm, and the zeta potentials were −22 ± 2 and −20 ± 2 mV for [^{14}C] PHDCA and [^{14}C] PEG-PHDCA, respectively. For non-labeled PHDCA and non-labeled PEG-PHDCA (1:4), the mean sizes of the nanoparticles were 165 ± 33 and 171 ± 35 nm, and their zeta potentials were −23 ± 1 and −20 ± 2 mV, respectively.

Influence of Covalent PEGylation on PHDCA Nanoparticles in RBEC Uptake. The cellular uptake by RBEC of PEG-PHDCA nanoparticles, of PHDCA nanoparticles, and of the physical mixture of PEG with PHDCA nanoparticles was examined after 1 h incubation (Figure 1). The result showed that the uptake of PEG-PHDCA nanoparticles (2569 ± 453 ng/mg of protein) was about 3-fold higher than that of PHDCA nanoparticles (937 ± 84 ng/mg of protein). To study the particular influence of PEG per se on cellular uptake, incubation of RBEC was further performed with the physical mixture of PEG (1 mmol) and PHDCA nanoparticles (4 mmol) corresponding to the same molecular ratio as covalent PEGylated PHDCA (1:4) nanoparticles. Interestingly, the presence of free PEG in solution did not influence the uptake of PHDCA nanoparticles.

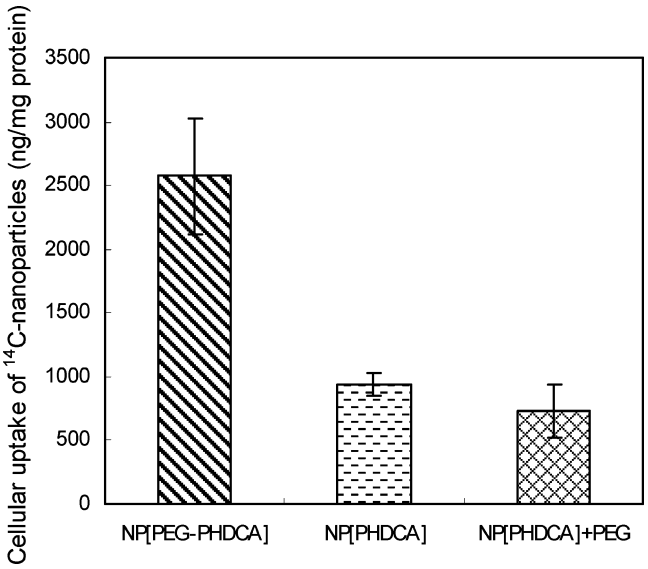

Figure 1. RBEC uptake of 20 μg/mL [^{14}C]PEG-PHDCA nanoparticles, [^{14}C]PHDCA nanoparticles, and the physical mixture of PEG and [^{14}C]PHDCA nanoparticles (1 h incubation).

Plasma Protein Adsorption on Nanoparticles. To certify the validity of the procedure used for protein desorption by SDS/DTE treatment, we monitored the total amount of adsorbed plasma proteins onto the surface of the nanoparticles before and after the last centrifugation, aiming at separating the adsorbed proteins from the nanoparticles. The Commassie staining demonstrated that almost all of the adsorbed proteins have been desorbed from the nanoparticles after incubation for 2 h at 50 °C with SDS and DTE, as shown by the low staining of the pellet lanes in Figure 2A. The BCA colorimetric assay of these fractions showed that 99% of the total protein content was detected in the supernatants and that less than 1% still remained in the pellet after the last centrifugation. Then, the total amount of adsorbed plasma proteins was measured after incubation in rat serum of the different nanoparticles and compared (Figure 2B). The amount of adsorbed plasma proteins on the PHDCA nanoparticles (56 ± 2 μg/mg of nanoparticles) was, according to expectations, higher than for PEG-PHDCA nanoparticles (35 ± 1 μg/mg of nanoparticles).

After 20 min incubation of nanoparticles with rat serum, the adsorbed plasma proteins were separated from nanoparticles, purified by the validated SDS/DTE treatment, and then analyzed by 2-D-PAGE with silver staining. Figure 3A,B shows that the protein adsorption patterns are roughly similar for PEG-PHDCA and PHDCA nanoparticles. Quantification of adsorbed proteins is presented in Table 1 as % vol of proteins on gels (i.e., representing the fraction of each protein from the total amount of proteins adsorbed onto the nanoparticles). Albumin, the most abundant protein in bulk serum, was predominant and represented almost the same fraction on the surface of both types of

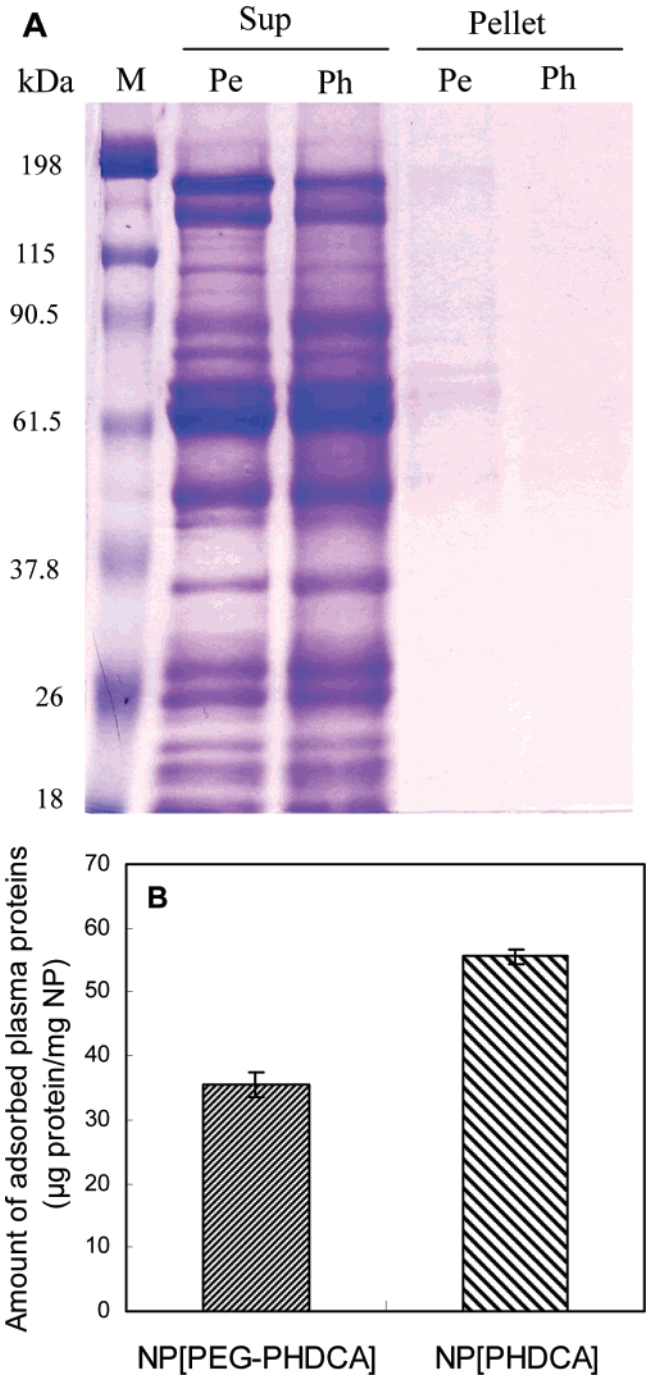

Figure 2. Plasma proteins released from the surface of PEG-PHDCA and PHDCA nanoparticles after incubation in rat serum (20 min). Supernatants and pellets were analyzed in 10% acrylamide gel and stained by Commassie Blue. M, Pe, and Ph indicate markers of molecular weight, PEG-PHDCA, and PHDCA nanoparticle samples, respectively (A). The total adsorbed amount of plasma proteins onto the surface of nanoparticles in rat serum was measured by the colorimetric BCA kit for PEG-PHDCA and PHDCA nanoparticles (B).

nanoparticles (i.e., 17.83 ± 2.05 % vol for PEG-PHDCA nanoparticles and 18.28 ± 2.14 % vol for PHDCA nanoparticles). Transferrin, a protein recognized by brain endothelial cell receptors, had also a nearly similar portion: 4.21 ± 0.98 % vol for PEG-PHDCA nanoparticles and 4.04 ± 1.66 % vol for PHDCA nanoparticles. Immunoglobulin G, known as an opsonizing protein, was preferentially adsorbed on the PHDCA nanoparticles as expected. Spot 16 was found only on PHDCA nanoparticles and was identified by mass spectrometry as a cardiotrophin-like cytokine with a 20 406 Da molecular weight.

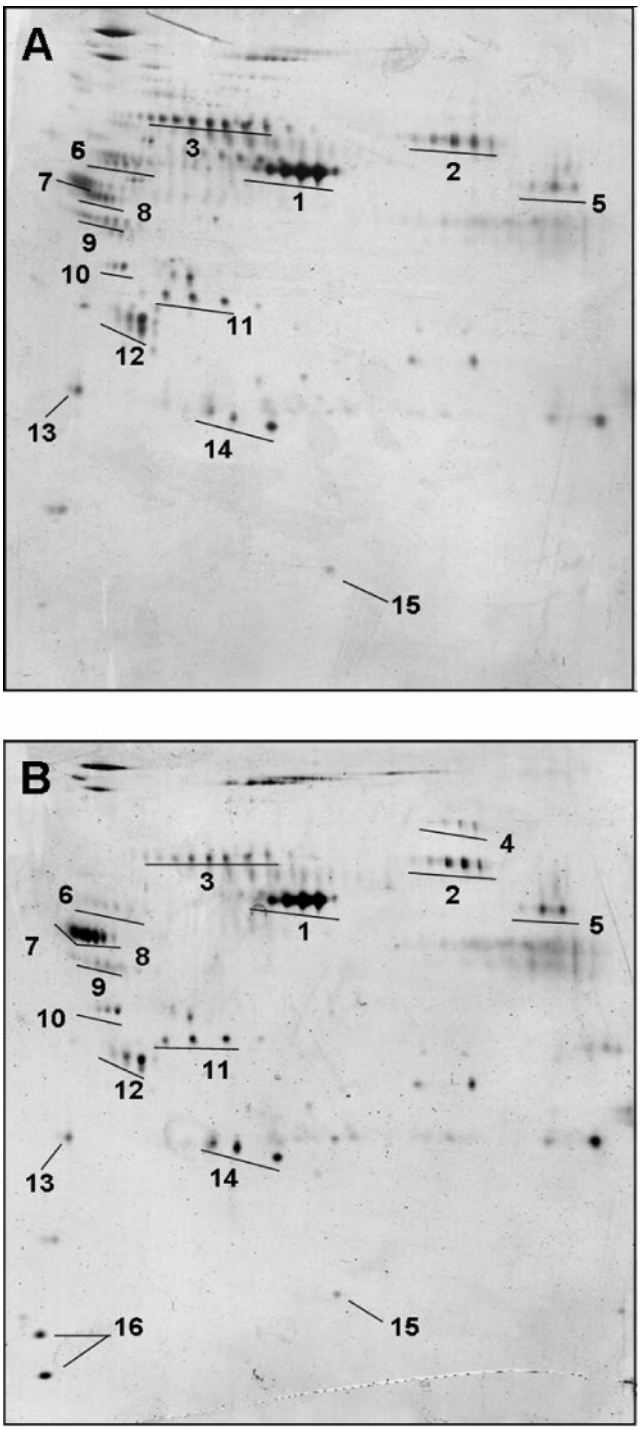

Figure 3. Silver stained 2-D-PAGE gels of adsorbed proteins on the PEG-PHDCA (A) and PHDCA (B) nanoparticles after 20 min incubation in rat serum. The gel size is 180 mm × 200 mm with ranges of molecular weights from 220 to 10 kDa (top to bottom) and of isoelectric points from pH 3 to 10 (left to right). Indicated spots: (1) albumin; (2) transferrin; (3) hemopexin; (4) Ig G; (5) IgG H chain; (6) AT III; (7) α2-HS-glycoprotein; (8) Kallikren binding protein; (9) α1-antitrypsin (10) Apo A-IV; (11) α1-macroglobulin; (12) ApoE; (13) IgL chain; (14) ApoA-I; (15) transthyretin; and (16) cardiotrophin-like cytokine. ApoE and cardiotrophin-like cytokine were identified by MALDI-TOF mass spectrometry.

Only for five proteins (ApoA-I, ApoE, hemopexin, IgG, and cardiotrophin-like cytokine) was % vol of adsorbed proteins onto both nanoparticles different (Table 1).

To allow for comparison of the apolipoprotein adsorption pattern on both types of nanoparticles from rat serum, the %

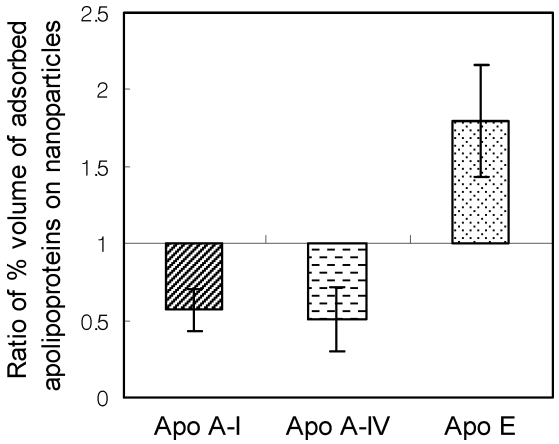

Figure 4. Ratio of % vol of adsorbed apolipoproteins on PEG-PHDCA nanoparticles to PHDCA nanoparticles. Ratios are calculated from the % vol determined from apolipoproteins (indicated in bold in Table 1) by 2-D-PAGE analysis.

Table 1. Proteins Adsorbed onto the Surface of Nanoparticles as Represented by Their Fraction (% vol) of the Total Amount of Proteins Adsorbed, as Calculated from 2-D-PAGE Analysis (Figure 3)

	% vol	
adsorbed proteins	nanoparticles [PEG-PHDCA]	nanoparticles [PHDCA]
ApoA-I	**1.84 ± 0.40**	**3.34 ± 0.63**
Apo A-IV	**0.75 ± 0.18**	**1.70 ± 0.78**
ApoE	**3.82 ± 0.61**	**2.17 ± 0.44**
Albumin	17.83 ± 2.05	18.28 ± 2.14
Hemopexin	6.48 ± 1.32	4.01 ± 0.94
ATIII	1.28 ± 0.44	1.70 ± 0.89
α2-HS-glycoprotein	3.87 ± 1.19	5.37 ± 2.05
Kallikren binding protein	2.76 ± 1.21	4.27 ± 1.29
α1-antitrypsin	1.83 ± 0.46	2.62 ± 0.72
α1-macroglobulin	1.84 ± 0.37	1.28 ± 0.40
Transferrin	4.21 ± 0.98	4.04 ± 1.66
Transthyretin	0.22 ± 0.10	0.61 ± 0.36
IgG H chain	1.50 ± 0.36	2.05 ± 0.41
IgG		1.09 ± 0.22
IgL chain	0.84 ± 0.19	0.53 ± 0.20
cardiotrophin-like cytokine		2.70 ± 0.60

vol data of Table 1 were calculated as the ratio of % vol for PEG-PHDCA/% vol for PHDCA nanospheres (see Figure 4). Therefore, a ratio superior to 1 means that the corresponding protein was more adsorbed onto PEG-PHDCA nanoparticles than on PHDCA ones. Interestingly, ApoA-1 (0.57 ± 0.14) and A-IV (0.51 ± 0.21) were clearly more adsorbed onto the surface of PHDCA nanoparticles, whereas ApoE (1.8 ± 0.4) was the only protein remarkably more adsorbed onto PEG-PHDCA nanoparticles (Figure 4). To confirm 2-D-PAGE analysis, we performed a Western blotting experiment with the anti-ApoE antibody. Figure 5 distinctly exhibited that ApoE was more adsorbed on PEG-PHDCA than on PHDCA nanoparticles from rat serum.

Although 2-D-PAGE analysis and Western blot analysis were convenient and beneficial techniques to identify and detect protein adsorption onto nanoparticles, ApoB-100 was not displayed in the 2-D gel because its molecular weight (550 kDa) exceeded the range of the protein size of the gel and was not detected in the Western blot (data not shown). Finally, capillary electrophoresis (CE) was employed for ApoB-100 detection on the surface of PEG-PHDCA nanoparticles after incubation with

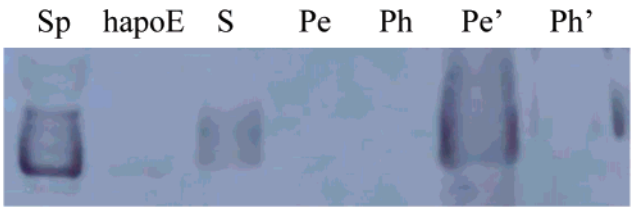

Figure 5. Western blot image (A) using anti-ApoE antibody of the adsorbed plasma proteins after incubation of PEG-PHDCA (Pe′) and PHDCA (Ph′) nanoparticles in rat serum for 20 min. Sp corresponds to pure rat serum and hapoE to human apolipoprotein E without sample preparation. The antibody recognizes specifically rat ApoE. The negative controls are samples of rat serum without nanoparticles (S) and PEG-PHDCA (Pe) and PHDCA (Ph) nanoparticles without rat serum that were prepared according to sample preparation procedures.

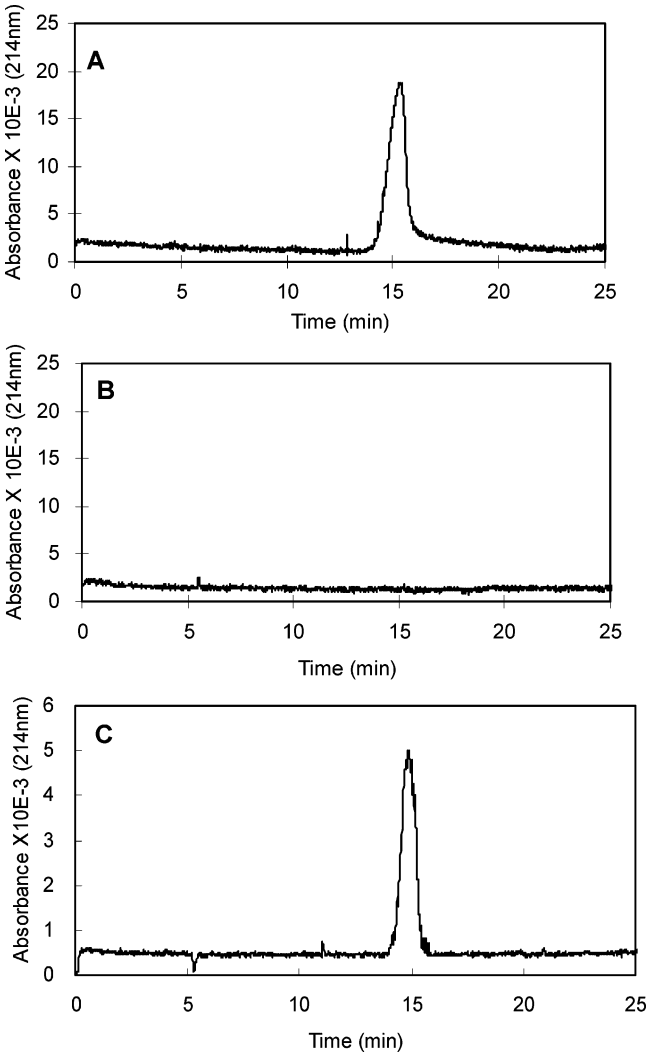

Figure 6. Electropherograms of capillary electrophoresis after incubation (1 h) of PEG-PHDCA nanoparticles with (A) and without (B) pure ApoB-100. (C) Represents the injection of 100 μg/mL ApoB-100 only.

the ApoB-100 solution (Figure 6A). The negative control of nanoparticles without protein was carried out in the same conditions (Figure 6B), showing that PEG-PHDCA nanoparticles did not absorb at 214 nm, the wavelength used in the CE system. As demonstrated in Figure 6, ApoB-100 had the capability to adsorb on the surface of PEG-PHDCA nanospheres, displaying a peak at 15 min, corresponding to the elution time of the control ApoB-100 (Figure 6C).

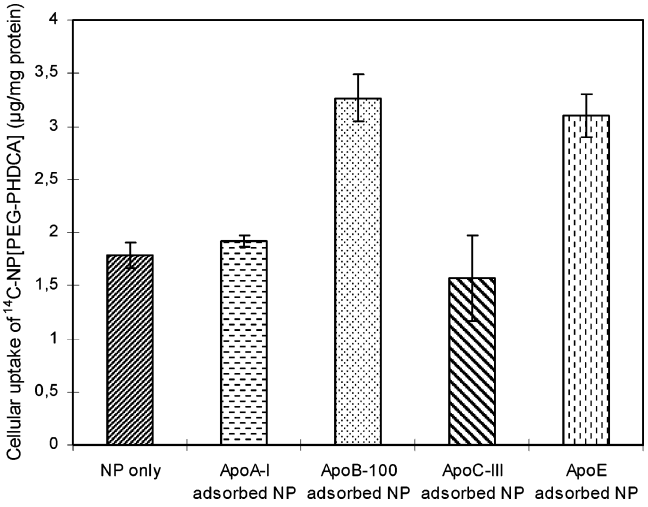

Figure 7. RBEC uptake of [^{14}C]PEG-PHDCA nanoparticles coated with various apolipoproteins (20 min incubation with 5% FBS transport medium).

Cellular Uptake of the Different Apolipoproteins Preadsorbed PEG-PHDCA Nanoparticles in RBEC. To investigate the contribution of apolipoprotein adsorption on the cell uptake of PEG-PHDCA nanoparticles by RBEC, various apolipoproteins preadsorbed PEG-PHDCA nanoparticles were prepared and incubated with cells for 20 min (Figure 7). The preadsorption of ApoA-1 and ApoC-III did not affect the uptake of the PEG-PHDCA nanoparticles by RBEC. On the contrary, the cellular uptake of ApoB-100 and ApoE preadsorbed nanoparticles was clearly increased in comparison with PEG-PHDCA nanoparticles (without the preincubation step).

Discussion

The data obtained in this study have clearly shown that the uptake by RBEC of [^{14}C]PEG-PHDCA nanoparticles was 3-fold more effective than that of [^{14}C]PHDCA nanoparticles (Figure 1), although these particles are of the same size and zeta potential (140 ± 42 and -20 ± 2 mV and 135 ± 41 nm and -22 ± 2 mV for [^{14}C]PEG-PHDCA and [^{14}C]PHDCA nanoparticles, respectively). These results confirm previous observations done in vitro and in vivo using rat species.[7,8] Two hypotheses may explain the competence of PEGylated nanoparticles to be captured by RBEC. First, PEG could be responsible on its own for nanoparticle cell uptake, as a consequence of an enhancement of cell membrane permeability[21] through the partial dehydration of phospholipid bilayers by PEG[22] and/or by stimulating endocytosis. Second, the different adsorption patterns of plasma proteins on nanoparticles owing to their different surface characteristics would result in an enhancement of cellular uptake of [^{14}C]PEG-PHDCA nanoparticles. As it was shown (Figure 1) that PEG physically mixed with PHDCA nanoparticles did not influence cell association of PHDCA nanoparticles, the hypothesis of PEG acting as a promoter of absorption seems unlikely. Therefore, this study has focused on the second hypothesis related to the adsorption pattern of rat plasma proteins on the nanosphere surface. Thus, PEG-PHDCA and PHDCA nanoparticles have been incubated in rat serum, and the adsorbed proteins have been separated and analyzed by different techniques.

As PEG-PHDCA nanoparticles showed smaller amounts of adsorbed serum proteins than PHDCA nanoparticles (Figure 2B), these results have a good coherence with the previously described long circulating properties of these PEGylated nanoparticles.[6] The silver staining images of 2-D-PAGE analysis (Figure 3) showed apparently similar protein adsorption profiles for PEG-PHDCA and PHDCA nanoparticles except concerning IgG and cardiotrophin-like cytokine (CLC), both proteins only appearing in the gel corresponding to proteins adsorbed on PHDCA nanoparticles. CLC is known to be a member of the interleukin-6 family of cytokines[23] involved in B cell proliferation and immunoglobulin production in the immune response.[24] CLC and IgG adsorption seems to accelerate the clearance of PHDCA nanoparticles from the blood, which is consistent with previous biodistribution studies in vivo.[7,8] As albumin and transferrin adsorption showed similar % vol in two types of nanoparticles (Table 1), these proteins are unlikely to contribute to the mechanism of prominent brain translocation of PEG-PHDCA nanoparticles.

This 2-D-PAGE investigation showed that ApoE is likely involved in brain translocation of PEG-PHDCA nanoparticles (Figure 4), which was confirmed by Western blotting (Figure 5). ApoE is implicated in the lipid transport metabolism and is a component of high density lipoprotein (HDL) with ApoA-1[25] and of low density lipoprotein (LDL) or very low density lipoprotein (VLDL) with ApoB-100 in blood.[26] Whereas ApoA-1 did not adsorb more onto PEGylated nanoparticles, capillary electrophoresis demonstrated the ability of ApoB-100 to adsorb on their surface (Figure 6). The interaction between LDL and nanoparticles will be necessary to study as a perspective. It will be interesting to discuss the reason why ApoB-100 was not nearly detected even in pure serum by the Western blot. Two possibilities were proposed. First, this result could be due to the very low amount of ApoB-100 (2.8−8.2 mg/dL) in bulk plasma, which falls below the limit of detection. However, it is instantly ruled out by the fact that the concentration of ApoE, one of very low components, is 1.6−9.2 mg/dL in plasma, which was detected by Western blotting. Alternatively, it was possible that the Western blot technique could not be appropriate to detect too high molecular weight molecules like ApoB-100.

Besides, in the cellular association experiment carried out to gain knowledge of the contribution of apolipoprotein adsorption for the internalization of nanoparticles in the RBEC, both ApoB-100 and ApoE preadsorbed onto PEG-PHDCA nanoparticles were uptaken more by cells as compared to other nanoparticles in the 5% FBS experiment condition (Figure 7). The result suggests that not only ApoE adsorption onto the surface of PEG-PHDCA nanoparticles but also ApoB-100 adsorption could be implicated in brain translocation of nanoparticles. As suggested by Kreuter's group,[9] the involvement of LDLR in this mechanism has been investigated by cell culture experiments that demonstrated that PEG-PHDCA nanoparticles were internalized into RBEC by a LDLR-mediated endocytosis.[27] Taken together, these data allow us to suggest that the conjugation of ApoE or ApoB-100 to the PEG chains at the surface of the nanoparticles should help to design a new carrier for brain LDLR targeting. In vivo experiments on the biodistribution and cerebral accumulation of these new carriers coupled with ApoE or ApoB-100 should be performed in the near future.

Thus, the present study suggests that a similar mechanism may be responsible for the brain translocation of different types of nanoparticles by adsorption of ApoE and perhaps ApoB-100 on their surface and by mimicking lipoproteins. Kreuter's group has, indeed, observed that ApoE adsorbed on the surface of nonionic surfactants PS 20-, 40-, 60-, or 80-coated poly(butylcyanoacrylate) nanoparticles.[10] The results obtained on

both types of nanoparticles (PS 80 and PEGylated nanoparticles) suggest that the key parameters for brain targeting are not only a long residence time in blood—either by protected surfactant or by grafted polymer—but also Apo E affinity on nanoparticle surfaces. The complementary relevant balance of both parameters would also be important. To explore this argument, it will be interesting to determine the influence of the length and density of PEG chains at the surface of PEG-PHDCA nanoparticles on the efficacy to be transported by brain endothelial cells.

In summary, this study is a detailed analytical investigation on the nature of the proteins adsorbed at the surface of PEG-PHDCA nanoparticles after incubation with serum. The data obtained demonstrate the involvement of Apo E and B-100 in the brain transport of PEG-PHDCA nanoparticles. This study may open interesting prospects for the conception of more efficient nanocarriers for brain drug delivery.

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