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Research paper

Delivery of nanoparticles to the brain detected by fluorescence microscopy

Isolde Reimold [a], Diana Domke [b], Joe Bender [b], Christoph A. Seyfried [b], Hans-Eckhard Radunz [b], Gert Fricker [a,*]

[a] Institute of Pharmacy and Molecular Biotechnology, Ruprecht-Karls-University, Heidelberg, Germany
[b] NanoDel Technologies GmbH, Magdeburg, Germany

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ABSTRACT

This study aimed to explore and extend the application potential of poly(*n*-butylcyano-acrylate) (PBCA) nanoparticles to cross the blood–brain barrier (BBB) and to deliver their content into the central nervous system. PBCA particles were prepared by a new and efficient mini-emulsion method with excellent yield and reproducibility. These nanoparticles were loaded with 1.5% (w/v) fluorescein-isothio-cyanate-dextran (FITC-dextran), 1.5% rhodamine-123 or 7.3% doxorubicin. Particles were characterized by dynamic light scattering determining particle size, polydispersity index and ζ-potential. They were coated with 10% w/v polysorbate 80 and administered to rats. Cryosections of the brain were prepared and time-dependent distribution of fluorescence was studied. After the administration of polysorbate 80-coated particles by carotic injection, fluorescence could first be detected in capillary lumina with a progressive shift to capillary endothelial cells at 30 min and a rather evenly spread distribution across the brain tissue at 60 min after administration. 60 min after administration into the tail vein, fluorescent particles could be assigned to endothelial cells, whereas after 2 h a rather evenly spread distribution across the brain tissue was seen. These observations indicate that surface-coated PBCA nanoparticles are able to cross the blood–brain barrier and to serve as a drug-delivery system to the central nervous system.

1. Introduction

The central nervous system is mainly protected by the blood–brain barrier (BBB), which acts as a firewall to defend the brain versus toxins, bacteria and viruses. In addition, this barrier regulates the transport of endogenous compounds by controlling their selective and specific uptake, efflux and metabolism. Unfortunately, more than 95% of potentially active (in vitro) compounds for the treatment of brain diseases are not able to cross the BBB in a pharmacologically sufficient concentration. As a consequence, various drug delivery and targeting strategies are currently being developed to cope with this challenge. In spite of this limitation, there are many CNS drugs on the market that are administered in very high doses to reach suitable brain concentrations with the consequence of severe side effects in peripheral organs.

Specifically, brain cancer is one of the most difficult malignancies to treat mainly because of the difficulty in getting diagnostic and therapeutic agents beyond the blood–brain barrier. Most cytostatics are useless for the treatment of cerebral metastases or primary brain tumors as long as the blood–brain barrier remains intact. Primary reason is the recognition of these drugs by export proteins located in the luminal membrane of brain capillaries, such as p-glycoprotein (p-gp) or breast cancer resistance protein [1–3]. Several strategies have been exploited to deliver drugs to the brain, e.g., retrometabolic prodrug approaches [4,5] or the use of vector-coupled liposomes, which were able to bypass the export pumps [6,7]. In this context, surface modified and biologically degradable nanoscaled polymeric carriers have also been shown to be promising carriers for CNS drug delivery due to their potential in encapsulating drugs, their ability to escape p-glycoprotein in the blood–brain barrier and to target the brain. Recently, paclitaxel has been entrapped in cetyl-alcohol/polysorbate nanoparticles, which significantly increased the drug brain uptake and its toxicity toward p-glycoprotein expressing tumor cells. It has been hypothesized that the nanoparticles could mask paclitaxel characteristics and thus limit its binding to p-gp, which consequently would lead to higher brain and tumor cell uptake of the otherwise extremely low permeable drug [8]. In another study, polysorbate 80-coated and drug-loaded poly(*n*-butylcyano-acrylate) nanoparticles have been shown to exert a significant anti-tumoral efficacy in a rat gliomas model suggesting that they are able to release their content beyond the blood–brain barrier [9,10]. However, it became not yet clear whether the particles themselves were able to cross the blood–brain barrier. Nevertheless, the most likely transport of drugs across the BBB using nanoparticles is receptor-mediated

* Corresponding author. Institute of Pharmacy and Molecular Biotechnology, Ruprecht-Karls-University, Im Neuenheimer Feld 366, D-69120 Heidelberg, Germany. Tel.: +49 6221 548336; fax: +49 6221 545971.
E-mail address: gert.fricker@uni-hd.de (G. Fricker).

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endocytosis [11,12]. It is proposed that apolipoproteins (e.g. ApoE, ApoB) get adsorbed on the surface of polysorbate-coated nanoparticles in human plasma. This concept suggests that nanoparticles coated with such ligands mimic lipoprotein particles, and thus can be endocytosed via a lipoprotein receptor-mediated mechanism transporting the loaded drug preferentially into the brain. Apart from this, the used surfactant polysorbate 80 is a moderate inhibitor of p-glycoprotein [13,14].

In this study, we prepared polysorbate 80-coated PBCA nanoparticles by a mini-emulsion technique. The particles were loaded in situ with either rhodamine-123, FITC-dextran or doxorubicin as easily detectable fluorescent marker compounds. The fluorescent compounds rhodamine-123 and doxorubicin were selected as they are both p-glycoprotein substrates with a very low capability to cross brain capillary endothelial cells, FITC-dextran was chosen as it is normally used as a large non-permeable paracellular marker. The nanoparticles were administered in an in vivo study to target the brain and to deliver their content beyond the blood–brain barrier.

2. Materials and methods

2.1. Materials

n-Butylcyano-acrylate was from Henkel-Loctide (Dublin, Ir). The monomer is on the market under the trade name Indermil™ as tissue adhesive (FDA-admission). Fluorescein-isothiocyanate bovine serum albumin (FITC-BSA) was purchased from Sigma–Aldrich, Taufkirchen, FRG. Unless otherwise stated, all materials were obtained from the usual commercial sources at the highest purity available.

2.2. Preparation of PBCA nanoparticles

2.2.1. Preparation of FITC-dextran labelled PBCA nanoparticles

Two solutions were prepared: (S1): 33.75 mg FITC-dextran (MW 70000, Sigma–Aldrich, Taufkirchen, FRG) and 0.513 g polysorbate 80 (Tween 80, Roth, Karlsruhe, FRG) were filled into a vial. Then 9 ml 0.8 M phosphoric acid was added. After 30 min the clear solution was placed in ice water. (S2): 90 mg soybean oil was filled into a reaction tube, then 2.05 ml (2.255 g) of *n*-butyl-α-cyano-acrylate was added and carefully mixed. S2 was transferred into S1. The two-phase mixture was sonicated with a sonifier [SONOPLUS HD 70, (Bandelin, Berlin, FRG) with SH 70 resonator and titan plate TT13, performance 70%] for 2 min under ice cooling. Polymerization was started by pouring the mini-emulsion into 9 ml of 0.8 M ammonium hydroxide solution under stirring. After 5 min the pH was adjusted to 7.0 by adding 0.8 M ammonium hydroxide solution. The suspension was kept at 4 °C under protection from light.

2.2.2. Preparation of rhodamine-123 labelled PBCA nanoparticles

Two solutions were prepared: (S1): 18 mg rhodamine-123 (Sigma–Aldrich, Taufkirchen, FRG), 60 mg Lutrol F68 (BASF, Ludwigshafen, FRG) and 24 mg sodium dodecylsulfate (SDS) were filled into a 20 ml vial. Then 4.8 ml of 0.1 M phosphoric acid was added. After 30 min the clear solution was placed in ice water. (S2): 72 mg soybean oil was filled into a 1.5 ml reaction tube and 1.09 ml (1.2 g) of *n*-butyl-α-cyano-acrylate was added and mixed. S2 was transferred into S1. The two-phase mixture was sonicated for 4 min under ice cooling. Polymerization was started by pouring the mini-emulsion into 4.8 ml of 0.1 M ammonium hydroxide solution under stirring. After 5 min of stirring the pH was adjusted to 7.0 by adding 1 M ammonium hydroxide solution. The suspension was kept at 4 °C under protection from light.

2.2.3. Preparation of doxorubicin-loaded PBCA nanoparticles

Two solutions were prepared: (S1): 75 mg Lutrol F68 (BASF, Ludwigshafen, FRG) and 30 mg SDS were dissolved in 6 ml of 0.1 M phosphoric acid. After 15 min 150 mg doxorubicin–HCl (141.5 mg doxorubicin base) (H.G. and C. Blau GmbH, Hamburg, FRG) was added. The solution was kept under ice cooling. (S2): 90 mg soybean oil was mixed with 1.2 g *n*-butyl-α-cyano-acrylate and kept under ice cooling. (S2) was mixed with (S1) and the mixture was sonicated with a sonifier for 2 min under ice cooling. The polymerization was initiated by dropwise addition of 8.75 ml of 0.1 M ammonium hydroxide. The final suspension was kept at 4 °C under protection of light.

2.2.4. Coating of the nanoparticles

Polysorbate 80-coated particles with doxorubicin or fluorescence marker were prepared by adding 1 mg polysorbate 80 per 1 mg nanoparticles administered. The mixture was used after an incubation time of 30 min.

2.3. Particle characterization

2.3.1. Particle size and ζ-potential

The particle size distribution (*z*-average-size), the polydispersity index (PDI) and the ζ-potential of the labelled PBCA particles were determined with a Zetasizer NanoZS (Malvern Instruments GmbH, Karlsruhe, FRG).

2.3.2. Determination of the binding ratio of fluorescence marker/doxorubicin

In order to separate the non incorporated fluorescence markers (FITC-dextran or rhodamine-123) and the non incorporated doxorubicin from the particle-bound compounds, 5 ml of the freshly synthesized particle preparations was mixed with 5 ml of demineralized water and transferred into an Amicon™ Ultra 15 centrifuge tube (membrane 100,000 Da, Millipore, Schwalbach, FRG). The suspension was concentrated by centrifugation (∼15 min at 100,000*g*) to such a degree, that the retenate on the filter containing particles could easily be resuspended with another 10 ml of water. After recentrifugation, FITC-dextran, rhodamine-123 and doxorubicin were quantified in the combined filtrates by UV spectroscopy. Interestingly, and in contrast to other compounds, no free rhodamine-123 could be detected in the filtrates. Thus, the amount of bound markers or bound doxorubicin results in the difference in the amounts found in the filtrates and the amounts originally employed in the polymerization procedure.

2.4. Animal studies

All experiments were performed with male Wistar rats (body weight 230–250 g). Animals were maintained under standard diurnal conditions and were allowed access to food (V1534-000 R/M-H, Ssniff, Soest, FRG) and water ad libitum in accordance with animal protection standards.

2.4.1. Administration of FITC-dextran labelled nanoparticles

Prior to carotic administration [15], the animals were anesthetized by intraperitoneal injection of ketamine and xylazine (100 mg/kg and 5 mg/kg, respectively). The nanoparticles were suspended in 0.9% saline to a final concentration of 12.6 mg/ml and 0.5 ml of the suspension was administered, resulting in a dose of 25.2 mg/kg body weight and a volume of 2.5 ml/kg body weight. Either uncoated nanoparticle suspensions were used or particles, which had been surface modified by incubation for 30 min with 1 mg polysorbate 80 per mg nanoparticles. For intravenous injections, 1 ml of particle suspension, i.e., twice the dose and volume as above, was administered into the tail vein. After specific time

intervals the animals were sacrificed by cervical dislocation and the brains were removed and further studied after cryosection. Usually, 3 animals were used for each experimental group.

2.4.2. Administration of rhodamine-123 labelled nanoparticles

For this particle-bound fluorescent marker only i.v. injections were used. The dose was 80 mg/kg body weight in a volume of 4 ml/kg body weight. Dilutions were again done in saline and either uncoated or polysorbate 80-coated nanoparticles were used as described above. As a control, a mixture of pure, non-particle bound rhodamine-123 and polysorbate 80 was given.

2.4.3. Co-treatment with rhodamine-123 or doxorubicin labelled nanoparticles and FITC-BSA

Rats were treated with the fluorescent nanoparticles as described under Section 2.4.2. In addition, 15 min before sacrifice, a solution of 2% (w/v) FITC-BSA in saline was given intravenously (80 mg/kg, 4 ml/kg body weight). As a control, rhodamine-123 or doxorubicin mixed with polysorbate 80 was given in combination with FITC-BSA 15 min before sacrifice.

2.5. Tissue preparation

Brain tissue was cut on a cryostat CM 3050 S Leica, Bensheim, FRG) in 25 μm sections and the tissue slices were thaw-mounted onto surface-treated glass slides (Superfrost plus, Fisher, Pittsburgh, PA, USA). The sections were embedded with paraffin. In general, samples were taken from frontal and/or parietal cortex.

2.6. Microscopic studies

Brain slices were studied by confocal laser scanning microscopy with a Leica DM IRBE microscope using the Leica TCS-SP software.

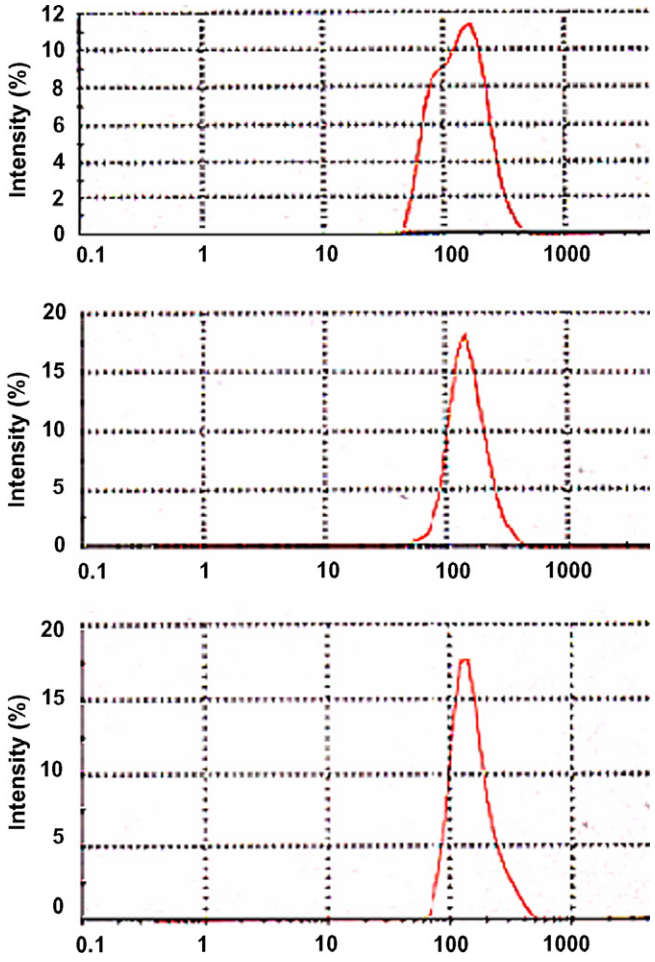

Fig. 1. Size distribution of PBCA nanoparticles determined by dynamic light scattering. (a) FITC-dextran-loaded nanoparticles, (b) rhodamine-123-loaded nanoparticles, (c) doxorubicin-loaded nanoparticles.

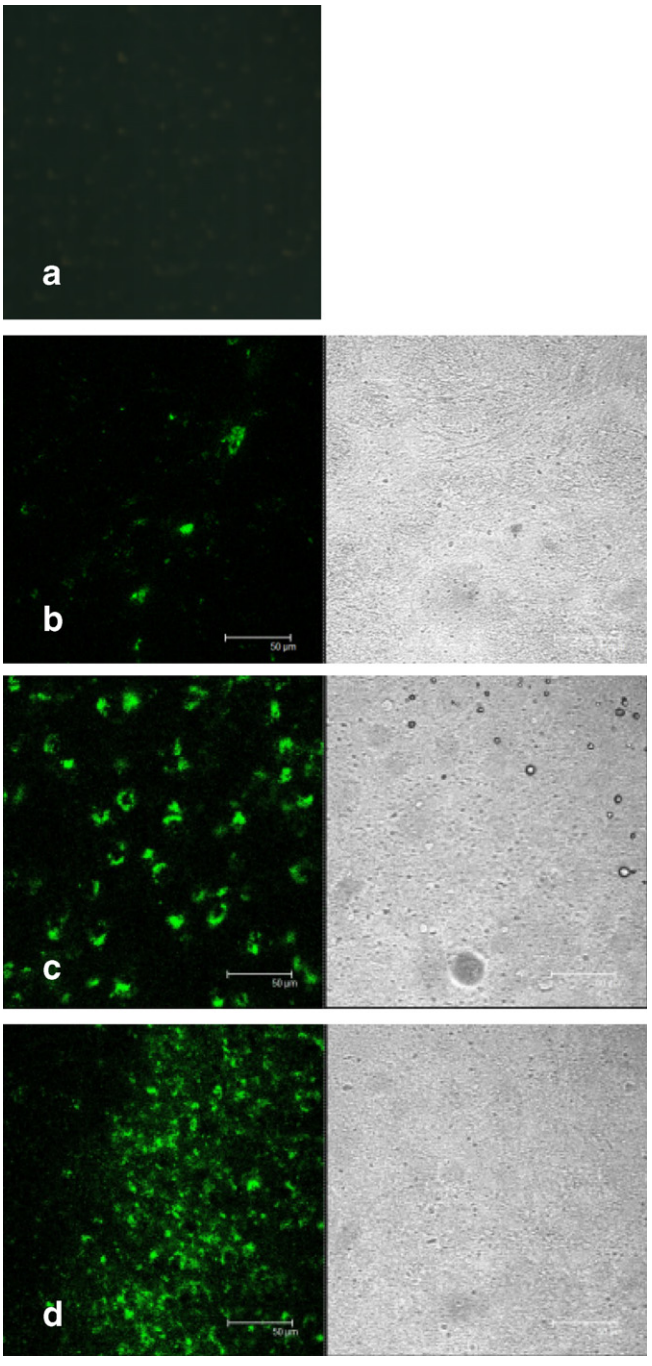

Fig. 2. Cerebral distribution of FITC-dextran-loaded nanoparticles in rat brain after administration via the carotic artery: 0.5 ml nanoparticle suspension (12.6 mg/ml) in 0.9% saline was administered. (a) Uncoated nanoparticles (b) 20 min after administration of Tween 80-coated PBCA nanoparticles fluorescence predominantly be detected in the lumina of brain capillaries. (c) 30 min after administration coated nanoparticles fluorescence can mainly be assigned to brain capillary endothelial cells. (d) 60 min after administration the fluorescence shows a more evenly spread distribution across the brain tissue.

3. Results

3.1. Particle characterization

All suspensions of fluorescence labelled and doxorubicin-loaded nanoparticles were stable at 4 °C and under protection from light for at least 60 days.

3.1.1. FITC-dextran PBCA nanoparticles

The *z*-average-size of nanoparticles was 120–130 nm (Fig. 1a) with a PDI of 0.2; the ζ-potential was −10 mV (20 μl suspension diluted with 980 μl H_2O) at a solid content of 12%. The size of the purified particles was 130 nm with a PDI of 0.25. The incorporation ratio was 50% of the originally employed amount of FITC-dextran.

3.1.2. Rhodamine-123 PBCA nanoparticles

The *z*-average-size of nanoparticles was 140–160 nm (Fig. 1b) with a PDI of 0.17 at a solid content of 10%; the ζ-potential was −43.8 mV. The incorporation ratio of the originally employed rhodamine-123 was 100%.

3.1.3. Doxorubicin nanoparticles

The *z*-average-size was 140–150 nm (Fig. 1c) with a PDI of 0.16 and a ζ-potential of 6.8 mV. The nanoparticle suspension contained 7.3% of doxorubicin after separation of the nonincorporated drug.

3.2. Animal studies

3.2.1. Administration of FITC-dextran labelled nanoparticles

Nanoparticles were administered to rats by injection into the carotic artery or (preferentially) into a tail vein. In order to see whether the uncoated nanoparticles distribute into brain, an initial experiment was performed in which 0.5 ml uncoated nanoparticles in saline was administered to rats via the carotic route. In this case no fluorescence could be detected in brain tissue 20, 30 and 60 min after injection, suggesting that the uncoated nanoparticles were not able to permeate the brain capillary endothelial cells (Fig. 2a, only one time point shown).

In a second series of experiments also with FITC-dextran the same dose of nanoparticles as mentioned above was injected into a carotic artery. The nanoparticles were treated on the surface with 1 mg polysorbate 80 per mg nanoparticles. At specific time intervals the animals were sacrificed and brain samples were withdrawn. Whereas uncoated nanoparticles showed no brain uptake (Fig. 2a), a different pattern of distribution could be observed after surface modification by polysorbate 80-incubation. Within the first minutes the highest fluorescence intensity was associated with capillaries (Fig. 2b), 30 min after administration the fluorescence was mainly located inside capillary endothelial cells (Fig. 2c), and 1 h after administration a rather ubiquitous distribution (Fig. 2d) of fluorescence could be observed in brain tissue. This observation strongly indicates that the superficial modification of PBCA particles is essential for an effective passage across the blood–brain barrier.

3.2.2. Administration of rhodamine-123 labelled nanoparticles

In a third study rhodamine-123 labelled nanoparticles were injected into the tail vein of rats and the animals were again sacrificed at specific time intervals and brain samples were withdrawn. In this case the sections were Nissl stained. When only the pure fluorescent dye with polysorbate 80 or PBCA nanoparticles was injected in the absence of polysorbate 80, no fluorescence could be detected in brain at 60 and 120 min after administration

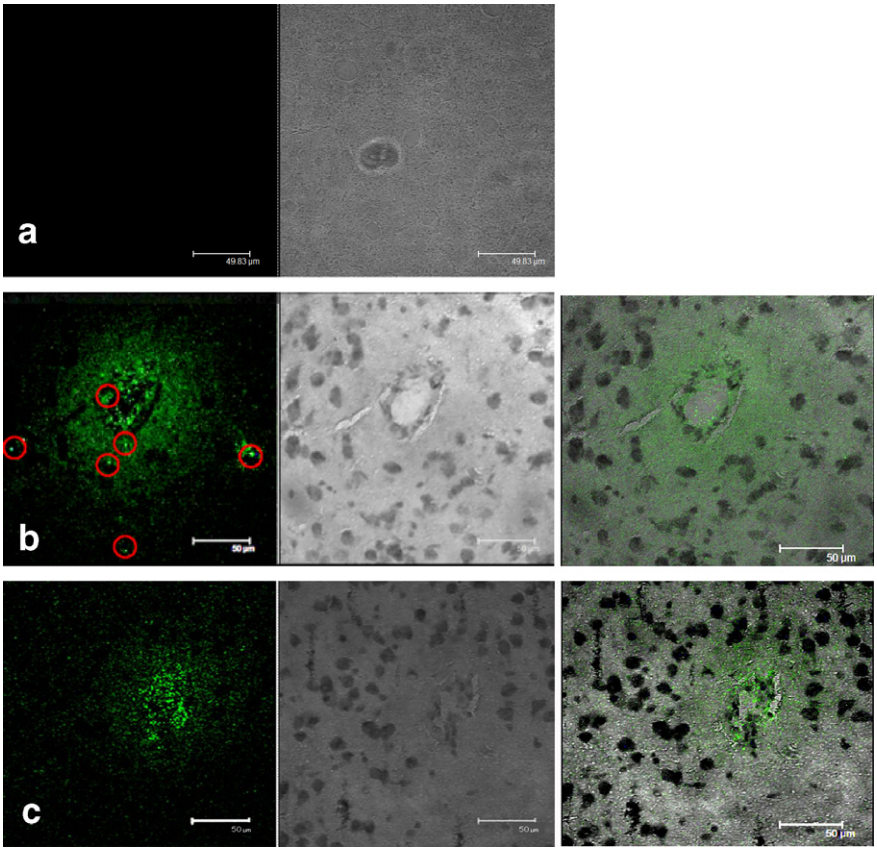

Fig. 3. Cerebral distribution of rhodamine-123 labelled nanoparticles in rat brain after administration via a tail vein. (a) At 60 and 120 min after administration of uncoated PBCA nanoparticles no fluorescence can be detected in brain (Only one time point shown here). (b) 60 min after administration of polysorbate 80-coated nanoparticles particle-associated fluorescence can be assigned to the brain capillary lumen, the endothelial cells and the perivascular brain tissue. Red circles indicate identified particles (c) 2 h after administration the fluorescence has started to spread throughout the brain tissue.

(Fig. 3a, only one timepoint shown here). However, 60 min after administration of polysorbate 80-coated nanoparticles a particle-associated fluorescence could be assigned to the brain capillary lumina, inside endothelial cells and in the perivascular brain tissue Fig. 3b). Two hours after administration the fluorescence had started to spread throughout the brain tissue (Fig. 3c).

3.2.3. Co-treatment with rhodamine-123 or doxorubicin labelled nanoparticles and FITC-BSA

Finally, 1 ml polysorbate 80-coated nanoparticles loaded with 1.5% rhodamine-123 or 7.3% doxorubicin in saline were administered by injection into the tail vein. 15 min prior to sample collection the animals received 1 ml saline containing 2% w/v FITC-labelled bovine serum albumin (FITC-BSA). Controls received no nanoparticles, but the same amount of rhodamine-123 or doxorubicin dissolved in 0.9% saline as well as 2% w/v FITC-BSA. Another control received FITC-BSA only. Because FITC-BSA is not able to cross the blood–brain barrier it was used as non-permeable marker compound. Consequently, when FITC-BSA alone or together with the non-particle-bound fluorescent compounds was given, fluorescence could only be detected in brain microvessels but not in the surrounding tissue (Fig. 4a). However, when FITC-BSA was administered together with rhodamine-123 (Fig. 4b) or doxorubicin (Fig. 4c and d)-loaded PBCA nanoparticles, an evenly distributed pattern of fluorescent dots could be detected in the cerebral cryosections, indicating that particles had crossed the blood–brain barrier. On closer inspection of cross-sected microvessels, it became obvious that albumin-associated green fluorescence was predominantly located in brain capillary endothelial cells, whereas the red fluorescence – associated to doxorubicin – exhibited a punctuate pattern of distribution clearly across endothelial cells, suggesting that particles had passed the blood–brain barrier, but not FITC-albumin.

4. Discussion

Development of strategies to deliver drugs to the central nervous system is highly important as many drug candidates, particularly chemotherapeutics, are not able to permeate the blood–brain barrier. Colloidal carrier systems and particularly polymeric nanoparticles have been used to overcome the blood–brain barrier and to deliver their content into the central nervous system. Recently, polylactide- and PBCA nanoparticles have been used as colloidal carrier systems and the efficacy of that approach has been demonstrated [9–12,16]. PBCA nanoparticles were loaded with loperamide or dalargin and intravenously injected into mice. The results indicated that nanoparticles that provided an antinociceptive effect required coating with polysorbate 80 and/or apolipoprotein. It has been hypothesized that these nanoparticles adsorb apolipoproteins from the bloodstream, and therefore could undergo receptor-mediated endocytosis into the brain capillary endothelial cells like lipoproteins via the LDL receptor family [17]. But, in none of these studies could it be shown whether particles just adhere to the luminal membrane of brain microvessels, whether they are internalized by capillary endothelial cells or whether they are able to cross the blood–brain barrier.

Therefore, fluorescent polysorbate 80-coated PBCA nanoparticles were prepared and the capability of these nanoparticles to overcome the blood–brain barrier was studied in an in vivo approach in rats. Careful analysis by confocal laser scanning microscopy gave clear evidence of a localization of particle-associated fluorescence within microvessel endothelial cells as well as beyond the brain capillaries. Thus, it can be stated that brain capillary endothelial cells are able to internalize polysorbate 80-coated PBCA nanoparticles and to process their content across the barrier in vivo.

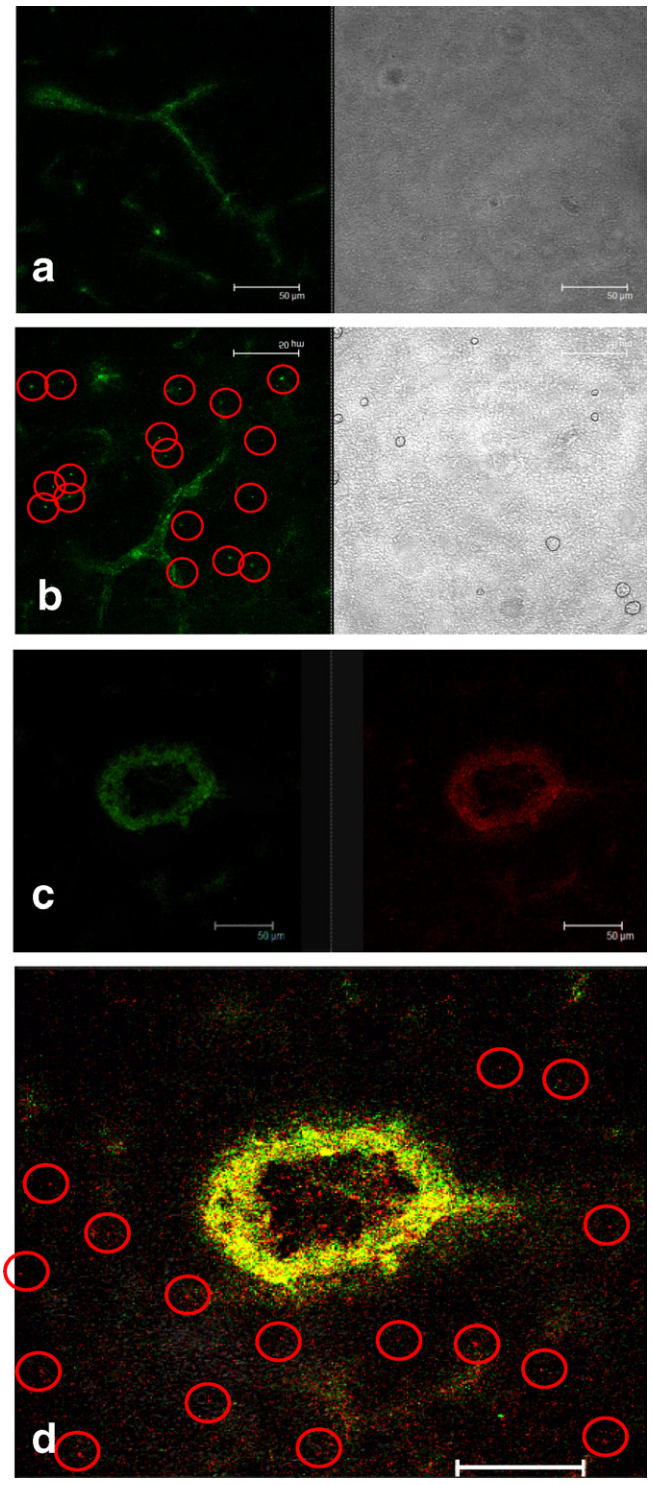

Fig. 4. Fluorescence distribution after i.v. administration of 2% FITC-BSA (a) or 2% FITC-BSA and rhodamine-123-loaded PBCA nanoparticles (b) or FITC-BSA an doxorubicin-loaded nanoparticles (c and d) in 0.9% saline. (a) Fluorescence is exclusively located in capillary lumina. (b) In addition to luminal fluorescence a punctuate green rhodamine-123 fluorescence within the tissue can be recognized. Red circles indicate identified particles (c) cross-section of a brain microvessel. FITC-BSA associated green fluorescence can be detected in the capillary wall, doxorubicin associated red fluorescence is also found in tissue beyond the capillary vessel. (d) Overlay of the 2 images from (c).

The finding of FITC-BSA associated fluorescence within brain capillary endothelial cells was surprising, because it could be assumed that no FITC-albumin related fluorescence can be found in-

side endothelial cells. Therefore, it may be speculated that the cytotic mechanism induced by polysorbate 80-coated nanoparticles also triggers unspecific cellular uptake of otherwise excluded compounds.

The present data indicate that colloidal polymeric systems represent a promising strategy to overcome the blood–brain barrier. However, further efforts are required to clarify in more detail the uptake mechanism, the fate of the polymer after drug release as well as clinical efficacy of the system.

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