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The First Step into the Brain: Uptake of NIO-PBCA Nanoparticles by Endothelial Cells in vitro and in vivo, and Direct Evidence for their Blood–Brain Barrier Permeation

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*By using fluorescent polysorbate 80 coated poly(*n*-butylcyanoacrylate) (PBCA) nanoparticles in an in vivo study, direct evidence was found for the presence of nanoparticles entering the brain and retina of rats. The nanoparticles, prepared with a miniemulsion process, were labeled in situ with a fluorescent dye and coated with polysorbate 80. After preparation the particle size, ζ potential, and the molecular weight distribution were determined. BMEC cells were used as an in vitro model for the BBB. The cells showed significant uptake of the particles, but no transcytosis could be observed in vitro. After applying the particles to the animals at two concentrations, cryosections of the brains and retinas were prepared. Regarding the sections of the rats that received the lower dose, co-localization of the applied fluorescent particles and the stained endothelial cells could be detected in the brain and retina, indicating particle internalization in the endothelial cells. Applied at higher doses, the particles could be detected within the brain and retina with few co-localized signals, suggesting passage through the blood–brain and blood–retina barriers.*

Introduction

Even after 30 years of intense research, the transport of drugs to the central nervous system (CNS) is a major challenge in pharmaceutical science. On their way from the bloodstream into the brain or retina, most drugs encounter a nearly insurmountable obstacle: the blood–brain barrier (BBB) or blood–retina barrier (BRB), respectively. The BBB is formed by a dense layer of endothelial cells facing the blood flow. These cells are linked by tight junctions.[1] A basal membrane is interposed and on the other side the endothelial cells are lined by astrocytes and neurons. Small hydrophilic compounds with a molar mass less than 150 g mol^{-1} and hydrophobic compounds with a mass less than 400 g mol^{-1}[2] can pass the cellular barrier by passive diffusion and thereby enter the brain. Compounds that do not meet these requirements are excluded from this path and remain in the bloodstream. Substances internalized by the endothelial cells are effectively removed from the cells by the *p*-glycoprotein efflux pump system if they are not recognized as necessary for brain metabolism. Substances or systems essential for brain metabolism, such as amino acids, glucose, or low-density lipoprotein (LDL) particles are recognized by specific receptors on the endothelial cells and are allowed to permeate the barrier.[1]

The treatment of tumors in the brain, for example, still demands the effective transport of therapeutics into the brain. Although an increase of the dose of the drug slightly enhances the therapeutic effects, the adverse systemic effects caused by these drugs are usually severe, as most of the applied agents, especially those used for the treatment of tumors, show significant toxicity. Thus, an increase of the dose might lead to severe damage to the patient's organs and should be avoided.

Several strategies have been developed to establish a passage for substances across the BBB.[1] The BBB can be temporarily opened by the application of a concentrated solution of, for example, urea or mannitol to the carotid artery. The osmotic pressure created in the capillaries shrinks the endothelial cells and opens a passage from the bloodstream to the brain, thus enabling applied drugs to reach their target in the brain. However, the protection usually offered by the BBB is temporarily disabled, allowing toxins, viruses, or even bacteria to enter the brain. After several hours the normal concentration of urea or mannitol in the blood is reestablished, the cells regain their initial size and the BBB is sealed again.[3,4]

Chemical modification of the drug molecules ("prodrugs") can also result in enhanced BBB permeation.[1,5,6] The conjugation of specific ligands (saccharides or proteins) allows the drug to exploit receptor mediated pathways through this cellular barrier, mimicking endogenous systems. The increase in hydrophobicity increases their ability to diffuse passively across

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the barrier. Despite the success of prodrugs, this approach is very specific and has to be adjusted to every compound.

A more general approach proposes nanoparticulate (colloidal) systems such as lipid or polymeric nanoparticles, liposomes, or micelles as drug carriers.[7–10] The drug, hydrophilic or hydrophobic, is incorporated in or adsorbed onto the transporter systems. Various examples can be found in the literature.[11–17] Regarding polymeric nanoparticles, the surface characteristics, presumably in combination with the polymeric matrix, are responsible for the passage to the brain. In particular poly(*n*-butylcyanoacrylate) (PBCA) based particles coated with polysorbates such as Tween 80 or poloxamers such as Pluronic F68 have been reported to successfully pass the BBB.[13,18] The (bio)degradability of PBCA in the organism makes this polymeric matrix perfectly suitable for sustained release applications. The coating is responsible for increased adsorption of specific plasma proteins of the apolipoprotein family.[19–22] These proteins disguise the particles as LDL particles which are endocytosed by the endothelial cells without immediate efflux. These experiments have been performed, with, for example, dalargin, which is an antinociceptive drug that acts on neurons in the central nervous system but does not cross the intact BBB. Although it has been shown that these dalargin-loaded PBCA particles enhance the antinociceptive effect dramatically, the fate of the particle and the drug after intravenous application is not yet clarified. The fact is that the adsorbed drug reaches the brain whereas elsewhere no therapeutic effect could be observed. Other polymeric matrices such as poly(methyl methacrylate) (PMMA) coated with either of the mentioned surfactants shows increased presence in the brain, but also in other non-reticuloendothelial system (RES) tissues such as heart, kidneys, or muscles.[23] Usually a successful passage through the BBB is indicated in in vivo experiments by the effect of a particle associated drug[24–26] or by radioactive labeling[27–29] but very rarely with fluorescence markers.[30,31]

Most of the in vivo experiments described in the literature were performed with PBCA nanoparticles prepared in an anionic emulsion process. The particles are obtained after adding the butylcyanoacrylate (BCA) monomer to a solution of dextran in hydrochloric acid. The acid inhibits polymerization, dextran serving as a steric stabilizer, is required in large quantities (up to 100% with respect to the monomer). Usually dispersions of ~1% solid content are obtained. As dextran possesses nucleophilic hydroxy groups, the polysaccharide is able to initiate the polymerization reaction of BCA. Thus, the surface of the particles is modified with dextran. After polymerization the particles having sizes of ~200 nm are freeze dried in the presence of a cryoprotectant (for example, mannitol) so that they can be redispersed and incubated with the desired drug and surfactant (polysorbate or poloxamer).

Recently we developed a miniemulsion polymerization process for the preparation of PBCA nanoparticles which allows precise control over the particle size, the molecular mass distribution of the polymer, and the particle surface functionalization. Additionally the solid content of the resulting dispersion could be increased to 10% and the surfactant concentration decreased to as low as 1% (with respect to the monomer) in comparison with the conventional preparation process.[32] A miniemulsion is prepared from a solution of hexadecane (and, if required, fluorescent dye) in BCA and a solution of the surfactant sodium dodecyl sulfate (SDS) in 0.1 molar hydrochloric acid by ultrasound homogenization. Hexadecane serves as hydrophobic agent suppressing diffusion processes, the so-called Ostwald ripening between the droplets after homogenization. Thus, all of the droplets share the same composition, which is not changed during polymerization. Additionally their initial size and size distribution is preserved during polymerization which is initiated by the addition of an aqueous solution of a water soluble nucleophilic initiator. The choice of the initiator determines the particles' surface functionalization. Unfunctionalized particles are obtained after initiation with NaOH solution, whereas amines, amino acids, or poly(ethylene glycols) used as initiators lead to surface functionalized particles. Regarding the OH-initiated system, the molecular weight distribution can be adjusted by the pH of the continuous phase during polymerization, whereas the size (100–350 nm) depends on the amount of SDS (1–10% with respect to the monomer) used for stabilization. The size of the functionalized particles can be adjusted by the amount of active initiator used for initiation (60–350 nm).[32] The typical solid content is 10%. Still, polysorbates or poloxamers can be used for coating in flexible amounts after completed preparation.

In this paper we present the direct preparation of fluorescent dye labeled Tween 80 coated PBCA nanoparticles (NIO-particles, derived from nonionic surfactant decorated particles) with the miniemulsion polymerization technique. We were able to prepare particles consisting of only a PBCA matrix with encapsulated fluorescent dye and polysorbate functionalized surface, thus eliminating a possible influence on the passage through the BBB of dextran and cryoprotectants (such as mannitol) necessary for the conventional approach. The particles are characterized physicochemically, with respect to size, ζ potential, and molecular weight distribution.

The particles' ability to pass the BBB/BRB was evaluated using an in vitro BBB model (human brain microvascular endothelial cells (BMEC)) and an in vivo model using Sprague–Dawley rats. The BMEC were evaluated in a transwell assay with a confluent BMEC layer as a cell barrier.

In vivo experiments were performed to provide evidence that the particles are internalized by the endothelial cells of rat brain/retinal capillaries and conveyed to the brain/retina tissue after intravenous injection of a particle dispersion in the rats' tail vein. Brains and eyes of the rats were cryosectioned and the endothelial cells were stained with a fluorescent antibody (von Willebrand factor primary and anti-IgG secondary antibody with Alexa fluor 594 fluorescent label) with a different emission spectrum than the particles.

Results and Discussion

In contrast to the conventional preparation method for PBCA nanoparticles it was possible to prepare nanoparticles consisting only of PBCA (with payload) and a Tween 80 coating, thus

eliminating the possible influence of any other chemicals used during the conventional preparation process. Because of the absence of diffusion during the polymerization process the miniemulsion technique additionally provides a way to incorporate a fluorescent dye (PMI) into the particles as a marker for flow cytometry and fluorescence microscopy. These particles were used for in vitro and in vivo experiments.

Dispersion and particles

In the previous formulation sodium dodecyl sulfate (SDS) has been used as electrostatic stabilizer for the miniemulsion droplets and the polymer nanoparticles.[32,33] Stabilization of BCA droplets with nonionic surfactants in dilute hydrochloric or phosphoric acid is impossible as the polymerization reaction is initiated immediately by the surfactant. The reason is the chemical structure of the nonionic surfactant: polysorbates possess one or more nucleophilic hydroxy groups capable of initiating the polymerization reaction. An application of these surfactants can be realized by using a concentrated solution containing at least 1 mol L^{-1} of the respective acid. Under these conditions polymerization is inhibited long enough for the formation of droplets during homogenization. As the surfactant also takes the role of the initiator, no further initiator is required for particle formation.

For in vivo administration the particles of the batch T80 were brought to pH 7 and diluted to the appropriate concentration (45 and 200 mg particles in 1.2 mL dispersion) with PBS. With the application of 1 mol L^{-1} phosphoric acid solution as continuous phase for miniemulsification, a buffered solution formed. The FDA-approved soybean oil was used as hydrophobic agent to stabilize the monomer droplets against Ostwald ripening instead of the previously used hexadecane.

The as-prepared dispersion T80 is stable for about one month before coagulation can be noticed. Removal of excessive electrolytes from the continuous phase by dialysis enhances the stability to at least three months. The solid content of the dispersion was measured as ~23% wt, which is higher than the theoretical value for the polymer only (19% wt). The presence of stabilizer and electrolytes is responsible for the deviation from the theoretical value.

The particles' *z*-average hydrodynamic diameter has a value of 144 nm with a PDI of 0.2 before dialysis. The value measured after dialysis (146 nm) has not significantly changed and is within the experimental error. Measured before dialysis, the particles' ζ potential has a value of about −1 mV which drops to a value of about −5 mV after dialysis. Figure 1 shows a TEM micrograph of the PBCA nanoparticles. It is clearly visible, that because of the soft nature of the polymer,[34] the particles flatten upon drying and eventually form a polymeric film. Thus, the average diameter of about 190 nm obtained from TEM analysis is larger than the hydrodynamic diameter obtained from dynamic light scattering measurements. The particle characteristics are summarized in Table 1.

The PMI content was determined as 0.08 μg (g polymer)$^{-1}$, meaning that nearly the entire fluorescent dye was encapsulated (theoretically predicted 0.1 μg (g polymer)$^{-1}$). The rest of the dye could not be dissolved in the monomer and precipitated during homogenization. Furthermore, the filtrate obtained after dialysis did not show any sign of free dye as no fluorescence can be detected. The encapsulation of the dye by the miniemulsion process is advantageous in comparison with the application of conventionally used fluorescent labeled stabilizer (for example, FITC-dextran), as it does not alter the particles' surface characteristics which are essential for the interaction of cells and particles and eventually for the internalization of the particles into cells. Furthermore, the possibility of leaking dye is minimal. The hydrophobic nature of the polymer excludes particle swelling by water, thus an exchange of material entrapped inside the particle and the aqueous medium. Additionally the dye shows an extremely low solubility in an aqueous environment. Thus, the presence of free dye in the aqueous phase of the dispersion is negligible.

Recently, we have shown that the molecular weight of the polymer is crucial for the toxicity of the particles after internalization into cells. Particles composed of PBCA polymer with low molecular weight induce cell death at an earlier stage after incubation than particles composed of broadly distributed polymer.[33] As the nanoparticles prepared for the studies presented

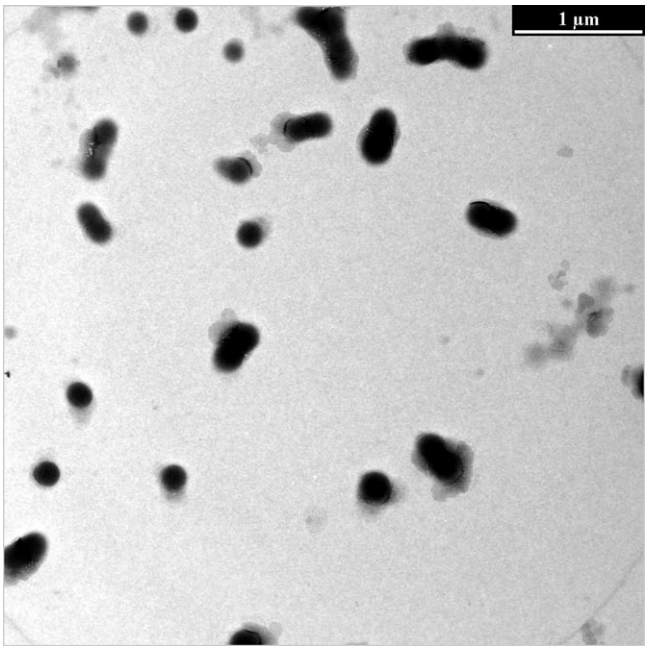

Figure 1. TEM image of air-dried PBCA nanoparticles. The magnification is 6000×.

Table 1. Summarized characteristics of NIO-PBCA nanoparticles.

Diameter[a]		ζ potential		Solid content of the dispersion [%]
Before dialysis [nm]	After dialysis [nm]	Before dialysis [mV]	After dialysis [mV]	
144	146	−1	−5	23

[a] Determined by dynamic light scattering.

herein were composed of high molecular weight polymer (see Figure 2), minor cytotoxic effects during the course of the in vivo experiments were expected (see below).

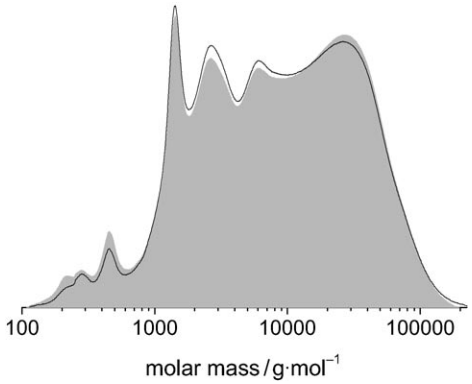

Figure 2. Molar mass distribution of the polymer PBCA obtained from sample T80 (gray area: before dialysis, black line: after dialysis).

In vitro experiments

Transwell assay

For nanoparticles in the bloodstream, brain endothelial cells represent the first line of the BBB. The BMEC chosen for the transwell assay provide characteristics similar to primary brain microvascular cells as determined for example, by trans-endothelial electrical resistance,[35] and have already served as a model for BBB permeation studies.[36] Transwell systems are common settings to study trans-endothelial processes. The tightness of the cell layer is critical and different methods exist to prove it. A fluorescein permeation test was established to avoid radiolabeled inulin which is commonly used for permeation studies.[28] Data from parallel experiments with both tracers were similar (not shown).

A transwell assay was established as an in vitro model of the BBB. The particle concentration in the basolateral compartment was calculated from the detected fluorescence against a calibration curve, shown in Figure 3.

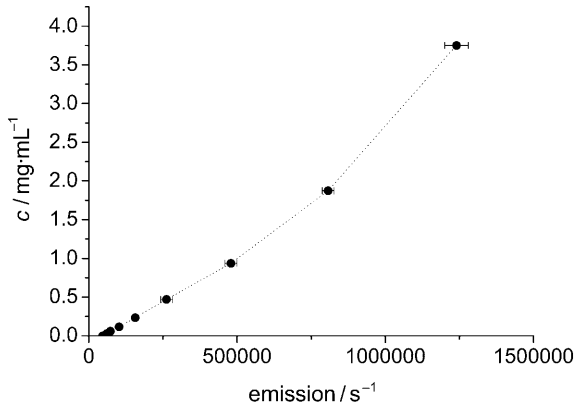

Figure 3. Calibration of T80 particle concentration in the culture medium.

The setup of the transwell assay also included diffusion controls in which particles were added to the apical compartment of a membrane without any cells, to evaluate if an increase in the fluorescence intensity originates in simple diffusion from the apical to the basolateral compartment. Both diffusion controls show an increase in fluorescence intensity during the course of the experiment (see Figure 4). Already after 30 min, particle amounts well above the background were measurable in these diffusion controls. However, when the compartments were separated by a tight BMEC layer as in the parallel batches, the particle concentrations in the basolateral compartments remained at background level for both concentrations evaluated in the experiment.

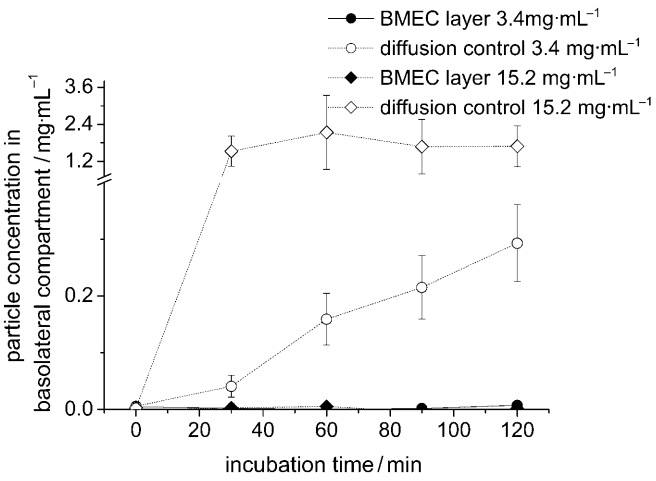

Figure 4. Transwell assay as an in vitro model of the BBB. Fluorescent PBCA nanoparticle batch T80 (3.4 and 15.2 mg mL^{-1}) were added to the apical compartment of a transwell system with or without (diffusion control) a confluent BMEC layer. Samples were taken from the basolateral compartment at the indicated times, fluorescence was measured, and the particle concentration was calculated from Figure 3.

In summary, Figure 4 shows that a tight BMEC layer prevents the passage of T80 nanoparticles to the basolateral compartment. It can be concluded that the cells might take up the particles (see next paragraph) but do not transcytose them within the course of 2 h in a way described for several other substances, such as insulin, for example.[38]

Kinetics of cellular uptake

Based on the results of the in vivo experiments, the uptake kinetics of the particles into the cells was recorded. Similar to all of our previous experiments with different kinds of particles and cells, a particle concentration of 75 μg mL^{-1} was chosen for uptake studies.[33,39–41] The data presented in Figure 5 shows the relative fluorescence intensity versus incubation time determined by flow cytometry.

The measured fluorescence intensity of the BMEC increased with time. Within the first 30 min, the fluorescence intensity increased faster than during the rest of the time course but did not reach a plateau during the 240 min monitored. This fact in-

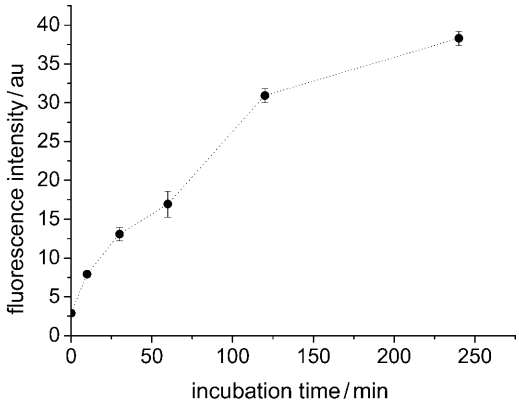

Figure 5. Uptake kinetics determined by flow cytometry. BMEC were incubated with 75 μg mL^{-1} PBCA particle batch T80 for the indicated times (dotted line is provided only as a visual guide).

dicates that the internalization process is not finished after this time. Internalization of PBCA nanoparticles with MePEG or phenylalanine-functionalized surfaces as well as unfunctionalized PBCA nanoparticles prepared in a miniemulsion process employing an ionic surfactant could be shown using HeLa, Jurkat, and mesenchymal stem cells.[32,33] Thus we assume, that T80 particles also enter the cells, although we cannot exclude that the particles adhere tightly at the cellular membrane.

Using particle concentrations of 75 μg mL^{-1}, 3.4, and 15.2 mg mL^{-1}, the particles' cytotoxic effects were evaluated by 7-AAD staining. After 90 min, the maximum time chosen for the in vivo experiments, more than 75% of the cells were still viable following incubation with 75 μg mL^{-1} of PBCA nanoparticles. Additionally the cytotoxic effects of the particle concentrations used for the in vivo experiments (see below) were assayed. Even with these significantly higher concentrations, more than 70% of the cells were viable after 90 min.

In vivo experiments

To evaluate the capability of the nanoparticles to permeate the BBB or BRB, 45 and 200 mg of particles dispersed in 1.2 mL were intravenously applied resulting in a total particle concentration in the rats' circulation of 3.4 and 15.2 mg mL^{-1}, respectively, based on a total blood volume of 12 mL.[37]

Regarding the BBB or BRB, the first obstacle the particles encounter on their way from the circulation to CNS tissue is the dense layer of endothelial cells lining the blood vessels. As soon as the particles enter these cells and are not removed by the efflux system protecting the CNS, the particles have made the first step into the CNS. Endothelial cells were identified by immunostaining with a specific antibody for the von Willebrand factor.

In several in vivo studies (rats and mice), using the effect of a drug adsorbed to polysorbate 80 coated PBCA particles as an indicator for BBB permeation, a maximum drug effect was observed after circulation times as low as 5 min[42] to 90 min.[16,27] The onset, maximum, and the duration of the effect was found to be dependent on the amount of nanoparticles administered to the animals. As the particles were prepared by the classic emulsion polymerization process they have diameters of more than 200 nm and an undefined amount of dextran on their surface. As there is no other reference, the data obtained from these studies suggested that the circulation time we report herein (90 min) is sufficiently long to detect BBB permeation.

Figure 6 shows the cryosections of the rats' brains. All of the sections were performed in the same area of the brain, the ventricles, as observed in the transmission images. The first row shows the brain of a rat which was sacrificed without prior application of fluorescent nanoparticles, which served as control experiment. This ensures that the treatment, including anesthesia and sacrifice does not leave any green fluorescing traces, which could be mistaken as fluorescing nanoparticles. The green channel, visualizing the fluorescence originating from PMI encapsulated in the particles, does not detect any signal. The red channel, representing the stained endothelium of the brain microvasculature is clearly visible. In the second row, the images obtained from the brain sections of the rat which was given a dose of 45 mg of the fluorescent PBCA nanoparticles are presented. Distinct signals can be seen in the green channel images (Figure 6, 45 mg, nano) which coincide with the pattern of the stained endothelial cells, which can be clearly observed in yellow in the images showing the combination of the two fluorescent channels (Figure 6, 45 mg, merge 40× and merge 63×). Thus, it can be concluded that the particles had been either internalized into or firmly attached to the endothelial cells. Sun et al.[43] observed a similar co-localization of the endothelial cells of the brain microcapillaries and polysorbate 80 coated PLA nanoparticles. The third row shows the micrographs obtained from the rat brain sections after the application of 200 mg of the fluorescent nanoparticles. In contrast to the application of 45 mg of particles, the green fluorescent signals of the nanoparticles are distributed throughout the observed area (Figure 6, 200 mg, nano). Compared with the arrangement of the endothelial cells represented by the red fluorescent signal, no co-localization can be observed (Figure 6, 200 mg, merge 40× and merge 63×). This suggests that the particles are located in the brain tissue and have thus passed the BBB.

In addition to the brains, the rats' retinas were cryosectioned. The images are presented in Figure 7. The upper row (Figure 7, control) shows the sections of the untreated rat retina. No signal is visible in the green fluorescent channel (Figure 7, control, nano). In the second row (Figure 7, 45 mg), after the application of 45 mg of nanoparticles to the rat, a localized signal can be detected. Combined with the red fluorescence of the stained endothelial cells, a well defined co-localization of the two signals can be observed. The lower row (Figure 7, 200 mg) shows the images obtained from a rat retina after the application of 200 mg of nanoparticles. In addition to the particles' fluorescent signal, co-localized with the endothelial cells (Figure 7, 200 mg, merge 40× lower left), green signals can be found in areas where no endothelial cells are located (Figure 7, 200 mg, merge 40× and merge 63×, indicated by arrows).

Although it has not been directly observed, these findings suggest that the particles are first internalized in the endotheli-

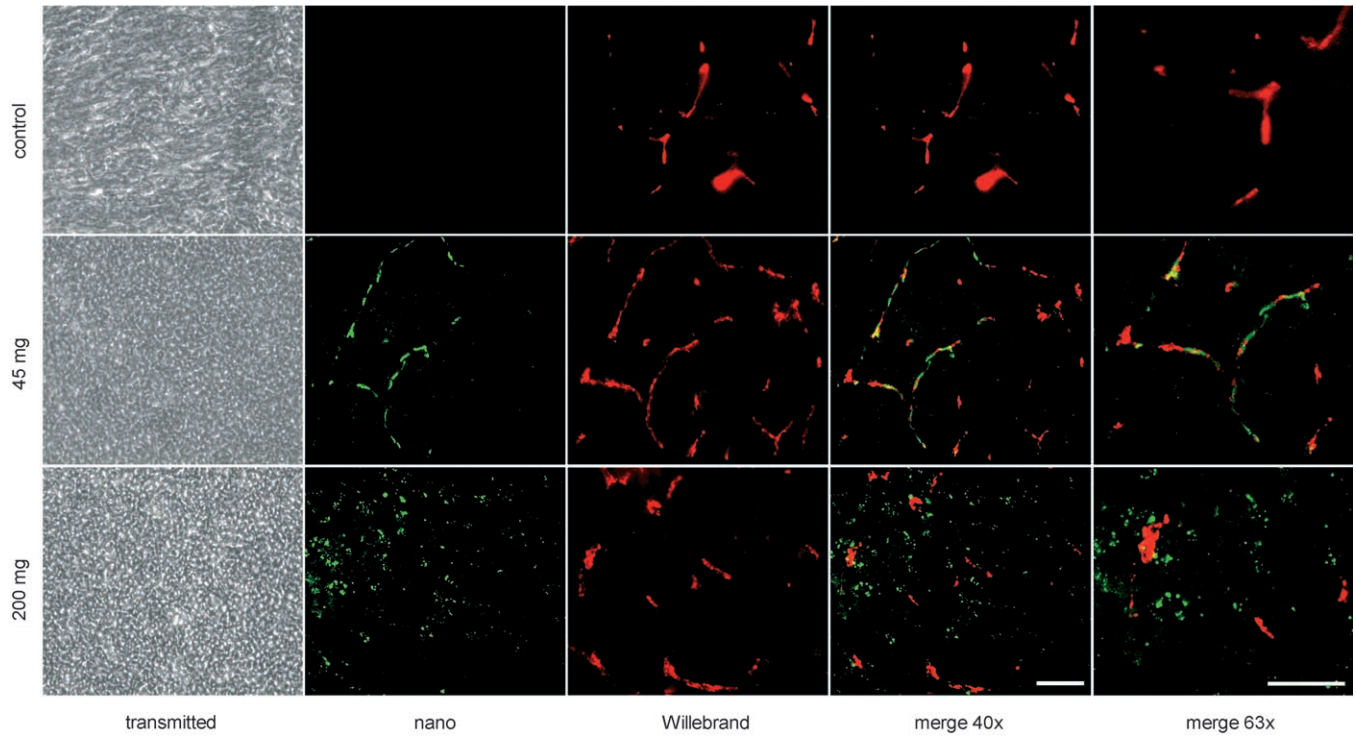

Figure 6. Cryosections of the rat brains. The left image (transmitted) shows the optical transmission image of the section (40×). nano: fluorescence created by the PMI-labeled nanoparticles. Willebrand: endothelial cells stained with fluorescent antibody (von Willebrand factor primary and anti-IgG secondary antibody with fluorescent label). Merge 40× and 63×: image merged from the green and red channel. The scale bars represent 100 μm.

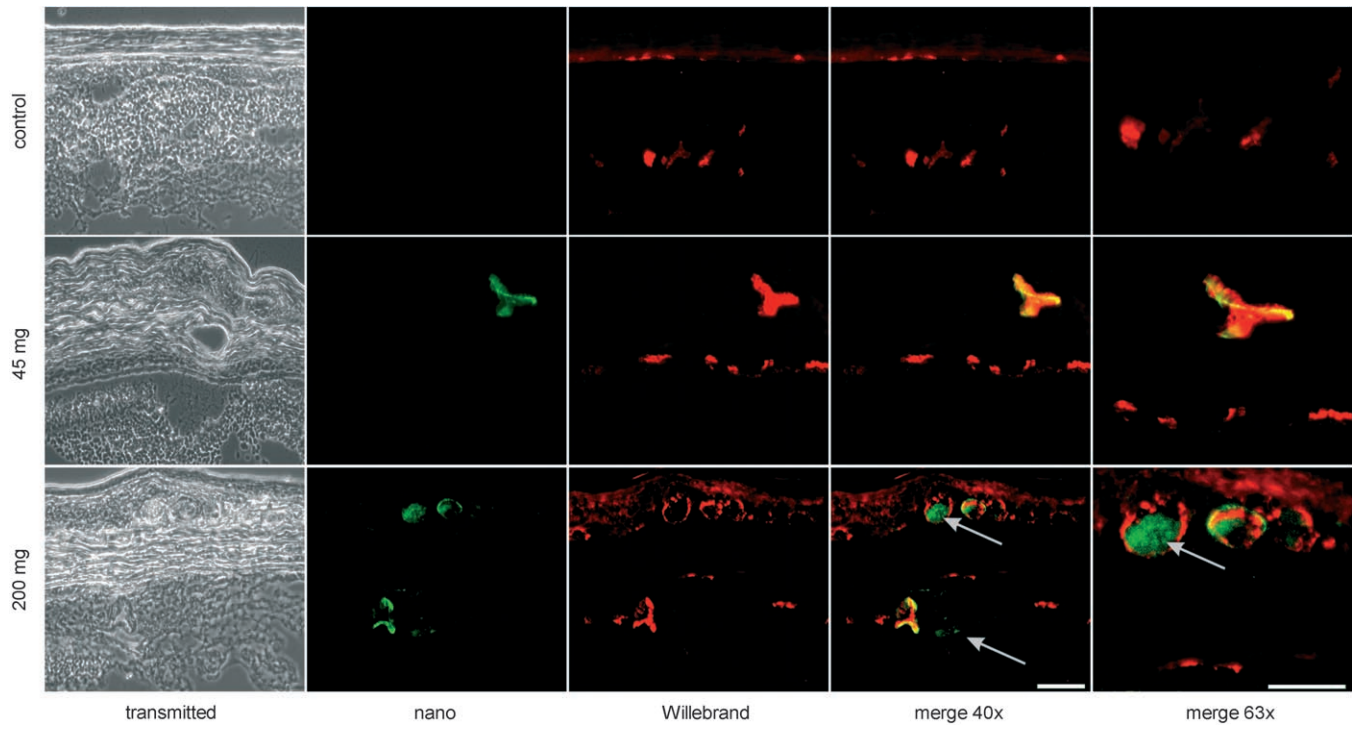

Figure 7. Cryosections of the rat retinas. The left image (transmitted) shows the optical transmission image of the section (40×). nano: fluorescence created by the PMI-labeled nanoparticles. Willebrand: endothelial cells stained with fluorescent antibody (von Willebrand factor primary and anti-IgG secondary antibody with fluorescent label). Merge 40× and 63×: image merged from the green and red channel. The arrows indicate particles not co-localized with endothelial cells. The scale bars represent 100 μm.

um and subsequently released on the basolateral side of the endothelial cells into the brain and retina. Additionally, this experiment clearly shows that the amount of particles applied to the rat plays a crucial role for their distribution. After the administration of a 200 mg dose, the particles can be observed in the brain tissue, whereas the particles applied at the lower dose are localized in the endothelium. As the experiments have been terminated after an incubation time of 90 min, there is still the possibility that the particles localized in the endothelium are conveyed to the brain after a longer incubation time. Still, it might be possible that the actual transport of the nanoparticles themselves to the brain is not necessary for a successful delivery of a drug to the brain as the drug may diffuse from the particles.

Comparing the in vitro and in vivo results, it can be stated that endothelial cells possess the ability to internalize the polysorbate 80 coated PBCA nanoparticles. This is valid for immortalized BMEC as well as the endothelial cells of the rat brain microcapillaries. We found clear evidence that the nanoparticles can permeate the BBB in vivo. Still, no sign of translocation could be found in the established transwell system. This difference may be explained in that BMEC in the transwell system may have changed their behavior because of immortalization or that the cells have not yet established their ability for transcytosis of nanoparticles, as this is a directed process for which further signals, for example from the endoglial cells or the basal membrane, may be important. Both other components of the BBB were not included in the present model. Therefore transwell experiments require a more complex BBB in vitro model and underline the necessity to investigate questions concerning the BBB with in vivo models as they are the more relevant system.

The results of the toxicity assay indicate that the particle concentration of 15.2 mg mL^{-1} (200 mg dose) does not result in higher cell mortality than the lower concentration of 3.4 mg mL^{-1} (45 mg dose). Although the cells behaved differently than the tissues regarding the translocation of the particles, it is likely that the appearance of nanoparticles in the brain is not induced by an increased cytotoxic effect of the increased particle dose.

Conclusions

To summarize, we reported the preparation of fluorescent polysorbate 80 coated PBCA nanoparticles with a size of about 150 nm and a ζ potential of close to 0 mV. Although the particles were internalized by human BMEC, the cells did not show any sign of particle transcytosis in a transwell assay using these cells as a confluent layer. Fluorescence microscopy of thin sections, obtained from in vivo experiments with rats, clearly showed co-localization of the nanoparticles with the endothelial cells of the microcapillaries in the brain and the retina after perfusion, which clearly indicates that the PBCA nanoparticles were internalized by the these cells. Applying a higher dose of particles to the rats led to an appearance of the particles in the brain and the retina of the animals, thereby verifying successful permeation of the BBB/BRB. The data clearly shows the particles' capability to permeate the rat BBB and BRB.

The encapsulation of the fluorescent marker and its appearance in the brain sections shows that it is possible to transport payload to the brain. Moreover, as the particles show relatively low toxicity in the in vitro experiments and very good tolerance in vivo, even at high doses, the particles are very suitable as drug carrier systems for the brain.

Experimental Section

Materials

n-Butylcyanoacrylate (BCA, Indermil, Henkel Loctite) was used without further purification. Phosphoric acid (reactant grade) was purchased from Merck, polysorbate 80 (Tween 80) was purchased from Aldrich, soybean oil (analytical grade) was purchased from Roth. *N*-(2,6-diisopropylphenyl)-perylene-3,4-dicarbonacidimide (PMI) was obtained from BASF. All chemicals were used as received.

Preparation of the PBCA particles

Dispersion T80: Polysorbate 80 (2.25 mg) was dissolved in phosphoric acid (36 g, 1 mol L^{-1}). After the addition of a solution of PMI (0.9 mg) and soybean oil (0.375 g, hydrophobic agent) in BCA (9.0 g), a two phase mixture formed. Immediately after the formation, the mixture was sonicated with a Branson Sonifier 450 D for 150 s (90%, $^1/_2''$ tip, ice cooling). A milky orange miniemulsion formed, which was left for 1 day at ambient temperature for polymerization. A milky, orange colored dispersion could be obtained. Two identical batches were prepared for in vivo experiments and uptake studies, and for the transwell assay, respectively.

Dialysis

The surfactant was removed by using Amicon Ultra membrane centrifuge filters (50 mL, MWCO 30 000). The respective dispersions (8 mL) were placed in the upper compartment and dialyzed against water for 10×30 min at 2500 min^{-1} in a Sigma 2–5 centrifuge.

Characterization of the particles

The particle size and the ζ potential were determined with a Malvern Zetasizer Nano ZS. For the photon correlation spectroscopy (PCS) measurements, 35 μL of the dispersion were pipetted into a single use polystyrene cuvette and diluted with 1.5 mL distilled water.

For the ζ potential measurements, 50 μL of the dispersion was diluted with 10^{-3} M KCl solution to a total volume of 5 mL. The diluted dispersion (0.8 mL) was placed in a single use U-tube cuvette equipped with electrodes.

UV-Vis spectra of the dissolved latexes confirmed the amount of encapsulated fluorescent dye. A sample (~20 mg) of the freeze dried dispersion was dissolved in chloroform. The UV-Vis spectra were recorded with a PerkinElmer Lambda 16 UV/Vis spectrometer. With a calibration curve, the acquired absorbance ($\lambda = 488$ nm) value was calculated into the amount of μg dye per mg polymer.

This amount was used for normalizing the fluorescence intensity obtained from flow cytometry.

Gel permeation chromatography (GPC) was used to determine the molecular weight of the poly(*n*-butylcyanoacrylate) of the nanoparticles. After the polymerization had been completed, the dispersions were frozen at −22 °C and subsequently freeze-dried. The resulting powders were dissolved in 1 mL THF, the solutions were filtered through a 0.45 μm syringe filter. The setup consisted of a Thermal Separations Products P2000 pump with Waters Styragel 5 μm particles, 10^3 Å pore size, PSS SDV 5 μm particles, 10^4 Å pore size, PSS SDV 10^5 Å pore size columns, and a Thermal Separations Products AS100 autosampler. The eluent was THF p.a. with a flow rate of 1 mL min^{-1}. The signal was detected with a Waters 2410 RI-detector. As there is no molar standard for PBCA, the molar masses were calculated with respect to PS standards and can therefore not reflect the exact values for PBCA.

The solid content of the dispersion was calculated from the mass difference of the dispersion before freeze drying and the powder obtained after freeze drying the dispersion.

Cell culture experiments

With an estimated blood volume of the rats equaling 12 mL (rat of ~200 g),[37] we calculated the particle concentration in the bloodstream as 3.4 and 15.2 mg mL^{-1} for the particle doses of 45 mg and 200 mg, respectively. Thus, these concentrations were used for the in vitro experiments.

Human brain microvascular endothelial cells (BMEC) were immortalized with the Simian virus 40 large T antigen and were a kind gift from Joachim Clement, Department for Internal Medicine of the University of Jena.[35] They were kept in RPMI (Invitrogen, Karlsruhe, Germany), supplemented with 20% FCS, 100 U penicillin and streptomycin (100 μg mL^{-1}), L-glutamine (2 mM, all from Invitrogen, Karlsruhe, Germany), and 1 mM pyruvate (Sigma, Seelze, Germany). Cells were grown in a humidified incubator (Heraeus, Hanau, Germany) at 37 °C and 5% CO_2.

In vitro BBB model

A transwell assay was established as an in vitro BBB model. BMEC were grown on 8 μm porous membranes (BD Falcon, Heidelberg, Germany) until confluence. The tightness of a cell layer was demonstrated by a fluorescein permeation test in a parallel batch: Fluorescein (2 μg mL^{-1}) was added to the apical (upper) compartment and after 1 h of incubation, the amount of fluorescein in the basolateral (lower) compartment was calculated measuring the fluorescence signal using a spectrofluorometer (FluoroMax-3, HORIBA Jobin Yvon). If the fluorescence signal was less than 6×10^5 s^{-1} at the chosen wavelength of 515 nm when a control batch without cells gave a signal of larger than 2×10^6 s^{-1}, the BMEC layer was regarded as confluent. If the BMEC layers were proven to be confluent, 3.4 and 15.2 mg mL^{-1} of T80 particles were added to the apical compartment and samples were taken from the basolateral compartments at the beginning of the experiment and after incubation times of 30, 60, 90, and 120 min. The fluorescence of the samples was detected at a wavelength of 530 nm which had been estimated as the maximum fluorescence of T80 particles suspended in culture medium. The particle concentration in the samples was calculated with a calibration curve, shown in Figure 3.

Flow cytometry

For uptake studies, BMEC were seeded at a density of 50 000 cells cm^{-2} and allowed to attach overnight in 6-well plates (Nunc, Wiesbaden, Germany). T80 particles were added for the indicated incubation times. After particle incubation, BMEC were trypsinized (Gibco, Karlsruhe, Germany), washed with PBS, centrifuged, and the pellet was resuspended in PBS. Vitality was estimated by incubation with 28.6 μg mL^{-1} 7-aminoactinomycin (7-AAD) for 15 min in the dark at RT. Particle concentrations of 75 μg mL^{-1}, 3.4, and 15.2 mg mL^{-1} were used.
Flow cytometric measurements were performed using a FACSScan equipped with CellQuest 3.3 software (Becton Dickinson, Heidelberg, Germany). BMEC were gated by forward scatter versus sideward scatter (FSC/SSC) plots. Fluorescence measurements were performed in the FL1 channel.

In vivo experiments

All experiments were performed with female adult Sprague-Dawley rats (180–250 g). Care and maintenance of the animals conformed to the statement for the use of animals in research and were approved by the local authorities (Regierungspräsidium Tübingen).
Rats were anesthetized by intraperitoneal injections of ketamine (60–80 mg kg^{-1}) and xylazine (10–15 mg kg^{-1}). For systemic nanoparticle application, a dispersion of 45 and 200 mg in 1.2 mL PBS (pH 7.4) was injected into the tail vein of each rat. After 90 min animals were killed with a lethal overdose of anesthetic and perfused through the heart with cold saline followed by 4% paraformaldehyde. Rats serving as control were treated in the same way with the exception of particle administration.

Immunohistochemistry

All rats (control and with administered particles) were treated according to the following procedure: After perfusion and tissue preparation tissues were post-fixed overnight in 4% paraformaldehyde, transferred to 30% sucrose overnight (4 °C), and frozen. Tissue was cut on a cryostat, thaw-mounted onto coated glass slides (Superfrost plus, Fisher, Pittsburgh, PA), and stored at −20 °C until further use. Immunohistochemical staining was performed according to standard protocols. A mouse anti-von Willebrand factor (Serotec) was used at a dilution of 1:100 to stain capillary blood vessels. A goat anti-mouse IgG antibody carrying the fluorophore Alexa fluor 594 (Molecular Probes) served as a secondary antibody and was used at a dilution of 1:500. Fluorescent sections were covered using Mowiol (Merck, Darmstadt, Germany) and analyzed under a fluorescent microscope.

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