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Transport of Poly(*n*-butylcyano-acrylate) nanoparticles across the blood–brain barrier *in vitro* and their influence on barrier integrity

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ABSTRACT

In previous studies it was shown that polysorbate 80(PS80)-coated poly(*n*-butylcyano-acrylate) nanoparticles (PBCA-NP) are able to cross the blood–brain barrier (BBB) *in vitro* and *in vivo*. In order to explore and extend the potential applications of PBCA-NP as drug carriers, it is important to ascertain their effect on the BBB. The objective of the present study was to determine the effect of PS80-coated PBCA-NP on the BBB integrity of a porcine *in vitro* model. This has been investigated by monitoring the development of the transendothelial electrical resistance (TEER) after the addition of PBCA-NP employing impedance spectroscopy. Additionally, the integrity of the BBB *in vitro* was verified by measuring the passage of the reference substances ^{14}C-sucrose and FITC-BSA after addition of PBCA-NP. In this study we will show that the application of PS80-coated PBCA-NP leads to a reversible disruption of the barrier after 4 h. The observed disruption of the barrier could also be confirmed by ^{14}C-sucrose and FITC-BSA permeability studies. Comparing the TEER and permeability studies the lowest resistances and maximal values for permeabilities were both observed after 4 h. These results indicate that PS80-coated PBCA-NP might be suitable for the use as drug carriers. The reversible disruption also offers the possibility to use these particles as specific opener of the BBB. Instead of incorporating the therapeutic agents into the NP, the drugs may cross the BBB after being applied simultaneously with the PBCA-NP.

1. Introduction

The central nervous system (CNS) is protected by the blood–brain barrier (BBB), which is mainly composed of capillary endothelial cells connected by narrow tight junctions, which prevent paracellular diffusion of polar molecules. Furthermore there are almost no fenestrations present in the cerebral endothelium, which is also characterized by a low pinocytotic activity and a continuous basement membrane. In addition to its protective function, the BBB ensures sufficient nutrient supply of the brain by regulating the transport of endogenous compounds and controlling their selective and specific uptake, efflux and metabolism. Even though the BBB is indispensable to life, it also represents the main obstacle in the treatment of many brain diseases. More than 95% of potentially active (*in vitro*) compounds are not able to overcome the BBB in a pharmacologically sufficient concentration. Thus, many drugs need to be administered in high doses to reach suitable concentrations for an effective treatment of CNS diseases. But this may cause severe side effects in peripheral organs. Consequently various drug delivery and targeting strategies to overcome the BBB are currently under investigation. One possible approach may be the use of surface modified and biologically degradable polymeric nanoparticles as drug carriers. Poly(*n*-butylcyano-acrylate) nanoparticles (PBCA-NP) have shown to be promising candidates for CNS drug delivery due to their ability to encapsulate drugs, to bypass the efflux pumps of the BBB and to reach the brain. PBCA-NP, coated with polysorbate 80 (PS80), have been reported to exhibit a significant anti-tumoral efficacy in a rat glioma model after being loaded with doxorubicin, suggesting that they are able to cross the BBB and release their load in the brain parenchyma [1,2]. The mechanism of transport has not been fully elucidated yet, but most likely, the nanoparticles are transported via receptor-mediated endocytosis [3,4]. In this context absorption of apolipoproteins on the surface of PS80-coated nanoparticles in human plasma has been postulated. Thereby these nanoparticles mimic lipoprotein particles, and reach the brain via low-density lipoprotein receptor-mediated endocytosis. Beyond that, PS80 is known to be a (moderate) inhibitor of p-glycoprotein [5,6]. For this study PBCA-NP were prepared employing a new and efficient mini-emulsion technique that ensures excellent yield and reproducibility. These PBCA-NP were loaded *in situ* with rhodamine-123, a fluorescent compound, which allows the detection of the particles. Furthermore it was selected,

Abbreviations: BBB, blood-brain barrier; PBCA, Poly(*n*-butylcyano-acrylate; TEER, Transendothelial electrical resistance; PS80, Polysorbate 80; PBCEC, Porcine brain capillary endothelial cells.

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because it is known to be a substrate of p-glycoprotein and consequently shows only a low capability to cross the BBB. In this study we determined the influence of PS80-coated PBCA-NP on the barrier integrity of a porcine *in vitro* model of the BBB.

2. Methods

2.1. Preparation and characterization of PBCA nanoparticles

The poly(*n*-butylcyano-acrylate) nanoparticles (PBCA-NP) were kindly provided by Capsulution Pharma AG (Berlin, Germany). The rhodamine-123 labeled PBCA-NP were prepared and characterized as described before [7].

2.2. Preparation and cultivation of porcine brain capillary endothelial cells

Porcine brain capillary endothelial cells (PBCEC) were isolated, cultivated and cryoconserved as described before [8,9]. Briefly, cerebra of freshly slaughtered adult pigs were mechanically homogenized and stepwise digested by two proteases, followed by further purification steps. The cells were seeded on collagen G-coated culture flasks (Nunc, Wiesbaden, Germany) and, 24 h after initial plating, were washed with phosphate-buffered saline (PBS) containing 1 mM Ca^{2+} and 0.5 mM Mg^{2+}. Puromycin (2.5 μg/ml, Sigma–Aldrich, Munich, Germany) was used to remove pericytes [10]. PBCEC were subcultured by fractionated trypsinization on day *in vitro* 2 (DIV 2) to reduce contamination by other cells. Afterwards PBCEC were frozen and stored in liquid nitrogen.

2.2.1. Culture model

We established an *in vitro* model of PBCEC on microporous Transwell® filter inserts (Corning, Wiesbaden, Germany, polycarbonate membrane, 1.12 cm^2 growth area, 0.4 μm pore size). In brief, the PBCEC were seeded (250,000 cells/well) at DIV 2 in plating medium on the apical (upper) compartment of rat tail collagen (0.54 mg/ml)-coated 12-well Transwell® filter inserts. To induce the differentiation of PBCEC and to retain their *in vivo* phenotype, the cells were cultured after DIV 4 with serum free medium (Dulbecco's modified Eagle's medium/Ham's F-10, 4 mM L-glutamine, 100 μg/ml gentamicin, 100 U/ml penicillin and 100 μg/ml streptomycin) and 550 nM hydrocortisone. The apical compartment refers to the blood side *in vivo* and the basolateral side refers to the brain side. The permeability studies (^{14}C-sucrose, FITC-BSA (70 kDa), PBCA-NP), immunocytochemical analysis, and the TEER measurements were performed on DIV 6. The integrity of the PBCEC monoculture was checked by measuring the capacity of the plasma membrane using the cellZscope® device (nanoAnalytics, Muenster, Germany). Only filter inserts containing capacity values between 0.45 and 0.60 μF/cm^2, revealing a confluent PBCEC monolayer, were used.

2.3. TEER measurements

For the quantification of the barrier integrity, the transendothelial electrical resistance (TEER) was monitored by the cellZscope® device (nanoAnalytics, Muenster, Germany) with a module suited for the measurement of 24 Transwell® filter inserts grown with a PBCEC monolayer.

2.4. Permeability studies

2.4.1. ^{14}C-Sucrose permeability

Another technique to analyze barrier integrity is the measurement of the permeability for radiolabeled ^{14}C-sucrose through the endothelial cell layer. Since sucrose is not taken up by endothelial cells either by active or facilitated transport the permeability for sucrose solely depends on the paracellular barrier tightness. Radiolabeled ^{14}C-sucrose was added to the apical side of the Transwell® filter. By measuring the time-dependent amount of ^{14}C-sucrose which passes to the basolateral side, the permeability was calculated as described previously [11].

2.4.2. FITC-BSA permeability

For the detection of macromolecular passage across the monolayer, a tracer solution containing fluorescein isothiocyanate labeled bovine serum albumin (FITC-BSA) was prepared in medium. FITC-BSA was added to the apical side of the Transwell® filter. The concentration of FITC-BSA was measured using a Berthold Mithras LB 940 Luminometer (Berthold, Bad Wildbad, Germany), at emission/excitation wavelengths of 492/535 nm. By measuring the time-dependent amount of FITC-BSA which passes to the basolateral side, the permeability was calculated as described previously for sucrose permeability.

2.5. Immunocytochemistry

For immunocytochemical analysis, the cells were seeded as described above. The medium was exchanged on DIV 4 to serum free medium, and the immunocytochemical analysis was performed on DIV 6 like described previously [12]. The primary antibody (1 μg/ml anti-occludin, Zytomed, Berlin, Germany) was incubated for 1 h at 37 °C in a 0.5% (wt./vol.) BSA solution. The fluorophore-labeled secondary antibody (2 μg/ml Alexa Fluor® 546 goat anti-mouse, Invitrogen, Paisley, UK) was diluted in 0.5% (wt./vol.) BSA and incubated for 1 h at 37 °C. The sample filters were thoroughly washed, cut out from the inserts, and mounted in Aqua Poly/Mount (Polysciences, Washington, USA). After drying for at least 24 h, the microscopy analysis was carried out.

2.6. Statistical analysis

The data are presented as mean ± standard error (SE); *n* equals the number of experiments which were performed each with a different cell preparation. Sets of data were compared performing a one-way ANOVA analysis. The differences were considered significant at $p < 0.05$. The significance levels of the data were determined using a Tukey range test in conjunction with an ANOVA. Employing this test, it was possible to determine if the sets of data were considered significant at $p < 0.05^{(*)}$, $p < 0.01^{(**)}$ or $p < 0.001^{(***)}$.

3. Results

The impact of PBCA-NP on barrier integrity was quantified by monitoring the TEER development of PBCEC cultured on microporous Transwell® filter inserts for 24 h (Fig. 1A). It was found that PBCA-nanoparticles disrupt the barrier integrity in a dose-dependent manner. Incubation of the PBCEC with PBCA-NP in a solids content of 26,62 μg/mL led to an irreversible decrease of the TEER to almost zero within 4 h. The application of PBCA-NP in a lower solids content of 13,31 μg/mL also induced a complete decrease of TEER within 3–4 h, but allowed the reconstitution of the barrier in the following hours. After 24 h the TEER of the NP-treated cells recovered to about 80% of the starting value. The described decrease became less pronounced when PBCA-NP in a solids content smaller than 13,31 μg/mL were applied. At 3,33 μg/mL a decrease by approximately 80–85% and at 1,66 μg/mL by only about 45–50% of the starting value as well as an almost complete reconstitution of barrier integrity was achieved after 24 h. Non-treated control cells maintained a TEER of at least 90–95% of the starting value during the whole experiment.

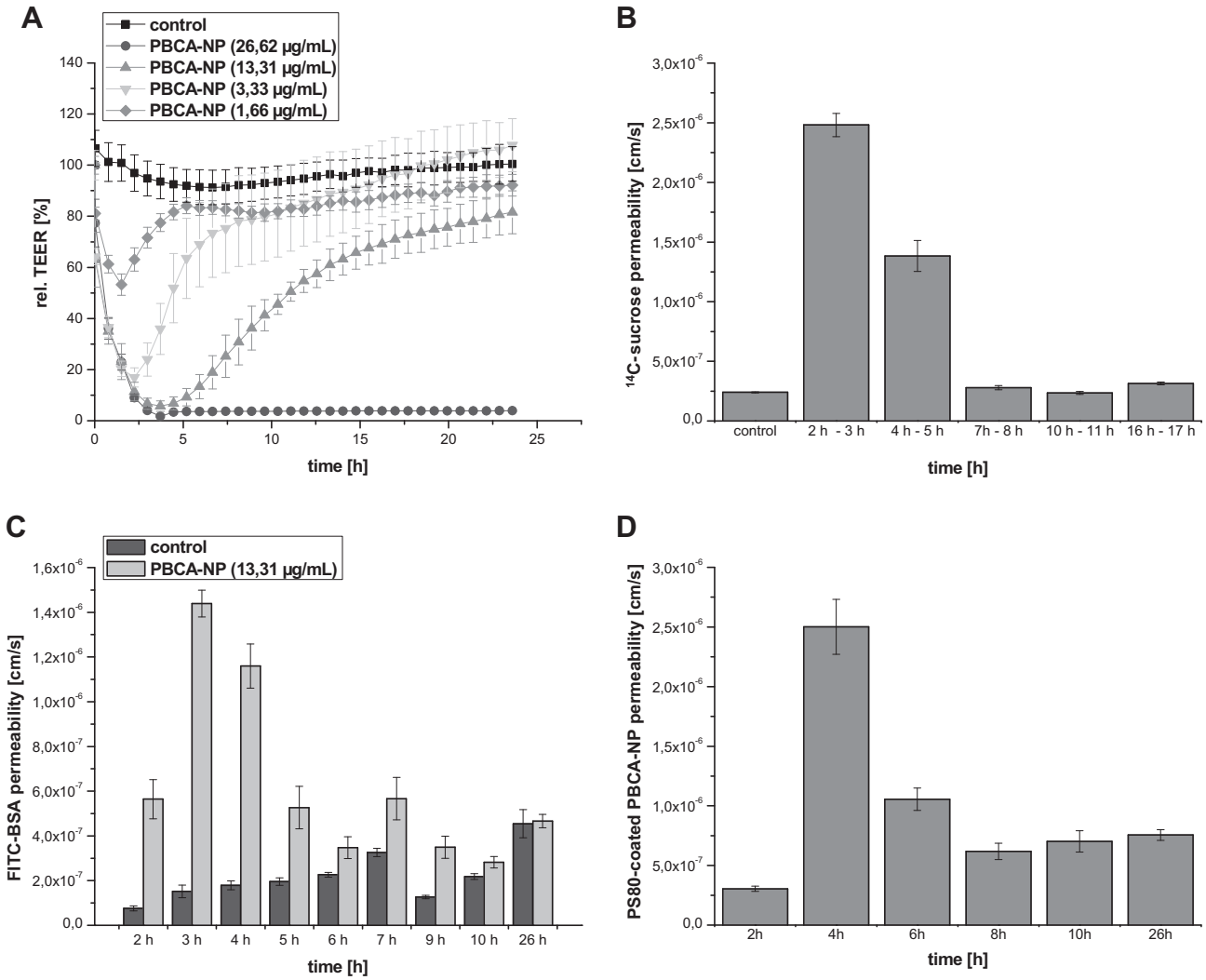

Fig. 1. Impact of PBCA-NP on the barrier integrity of PBCEC. All data are presented by a representative single measurement. For each condition the means of at least three filter devices are shown (±SE). (A) Relative TEER development after addition of PBCA-NP with different solids contents $n = 3$. (B) ^{14}C-sucrose permeability after addition of PBCA-NP [13.31 µg/mL] $n = 3$. (C) FITC-BSA permeability after addition of PBCA-NP [13.31 µg/mL] $n = 4$. (D) Permeability studies of PS80-coated PBCA-NP with a solids content of 13.31 µg/mL $n = 3$.

To verify the results of TEER studies, the permeability of ^{14}C-sucrose was determined. The BBB expresses no specific transport mechanism for sucrose hence the transendothelial passage of the substance can be assumed to be due to passive flux [13]. Fig. 1B shows the results of ^{14}C-sucrose permeability studies conducted 2, 4, 7, 10, and 16 h after the addition of PBCA-NP. The permeation of ^{14}C-sucrose was raised within a time frame of 2–5 h compared to the control. The highest rate of permeation was found between 2 and 3 h, otherwise sucrose permeability decreased and remained on control level.

To further evaluate the impact of PBCA-NP on the barrier integrity, the permeability of a high-molecular weight marker (FITC-BSA) was examined as well (Fig. 1C). The results are consistent with both the TEER measurements (Fig. 1A) and the ^{14}C-sucrose permeability studies (Fig. 1B), revealing the highest FITC-BSA permeability under the influence of PBCA-NP between 3 and 4 h. The FITC-BSA permeability of the control remained unchanged.

Additionally the permeability of PBCA-NP (Fig. 1D) was measured. The results were consistent with the permeability studies described above (Fig. 1A–C). The highest permeation of the PBCA-NP was observed after 4 h. After 6 h the permeability was still slightly elevated, but in the following hours it stayed on a baseline level.

In order to examine whether the temporary opening of the BBB might be caused by a differentiated expression pattern of tight junction proteins, an immunocytochemical analysis of the occludin expression was conducted 2, 4, 7, and 24 h after application of PBCA-NP. Fig. 2 shows the immunocytochemical staining of the untreated control cells (Fig. 2A) and the PBCA-NP-treated cells (Fig. 2B) after 4 h, respectively. In both pictures the cell borders are mostly straight and not serrated. Only small areas showing serrated cell borders could be observed. Apparently, the application of PBCA-NP results in a change of cell morphology of the PBCEC, from the typical spindle-like to a more cobblestone shape. In addition, the organization of the cells within the monolayer seemed to decline. For verification, the area and perimeter of the cells (at least 50 cells/condition) were measured using the software ImageJ (National Institutes of Health). The ratio of the obtained values was calculated (Table 1), revealing that the area of the cells is significantly increased while the perimeter stays unchanged. This results in an increased ratio of area/perimeter. In accordance with previous results the increase in cell area is strongest after 4 h and decreases afterwards.

Next, it was examined whether a subsequent second application of PBCA-NP after first reconstitution of the barrier integrity

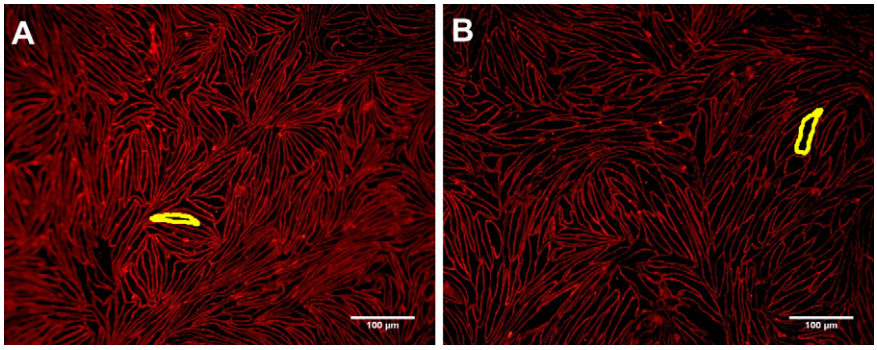

Fig. 2. Immunostaining analysis of PBCEC cultivated on filter inserts. The immunocytochemical staining of occludin was performed on DIV 6, 4 h after application of PBCA-NP. (A) Untreated PBCEC. (B) PBCEC after application of PBCA-NP.

would result again in a reversible disruption of the BBB. For that purpose the development of the TEER was measured under different conditions (Fig. 3). First, all filters were treated with 13,31 µg/mL of PBCA-NP, after 24 h different solids contents were applied. While a renewed application of 13,31 µg/mL was followed by a complete and irreversible breakdown of the BBB, the application of 8,87 mg/mL or 5,32 µg/mL allowed a reconstitution of the barrier. Again, the application of a lower solids content leads to a faster and more pronounced recovery of the BBB *in vitro*. The TEER of the control cells remained constant during the experiment.

Finally, the influence of Polysorbate 80-coating was tested by analyzing the development of the TEER after application of coated and uncoated particles. As depicted in Fig. 4 a decrease in the TEER after 4–5 h and a subsequent reconstitution was found for both PBCA-NP. In comparison, the mentioned effect is much more pronounced for PS80-coated PBCA-NP than for uncoated PBCA-NP. PS80-coated PBCA-NP led to a decrease in TEER to about 20% of the initial value, while uncoated nanoparticles were responsible for a decrease to 50% of the initial value. Additionally, the final TEER value for the uncoated NP only increased to about 75% of the initial value, while coated NP allowed a complete restoration of the barrier integrity.

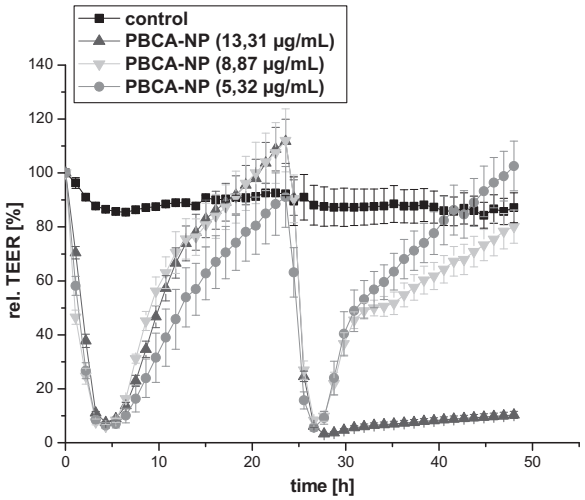

Fig. 3. Relative TEER development after repeated addition of PBCA-NP with different solids contents. First, 13.31 µg/mL PBCA-NP were applied to all filters, after 24 h different solids contents of PBCA-NP were added (see legend). Shown are the means of at least three filter devices for each condition of a representative measurement (±SE). $n = 3$.

4. Discussion

PS80-coated PBCA-nanoparticles might be promising candidates for drug delivery due to their biodegradability and their ability to overcome the BBB. For potential applications in drug delivery it is a matter of great importance to characterize the effects of these nanoparticles on the BBB and to understand the underlying mechanisms. In previous studies it was reported that PS80-coated PBCA-NP were able to overcome the BBB both *in vivo* and *in vitro* and deliver their load into the CNS [1,14]. However, existing *in vitro* studies only examine effects induced within the first hours after application of PBCA-NP. The objective of this study was to investigate the impact of PBCA-NP on the barrier integrity of a porcine *in vitro* model of the BBB within a timeframe of 24 h. In

Table 1
Area, perimeter and ratio of area/perimeter from PBCEC after application of PBCA-NP.

(h)	Area	Perimeter	Area/perimeter
2	1.25 ± 0.04***	1.02 ± 0.03	1.22 ± 0.03*
4	1.49 ± 0.03***	1.01 ± 0.02	1.48 ± 0.02***
7	1.17 ± 0.04***	1.03 ± 0,03	1.12 ± 0,03***
24	1.25 ± 0.03***	0.95 ± 0.02***	1.31 ± 0.02***

PBCEC were cultivated on filter devices, the cell borders were manually determined ($\geqslant$50 cells/condition) and the values calculated via the software ImageJ. All values were normalized on the values of the untreated controls. Significance analysis was performed via one-way ANOVA with tukey-test to determine significance level of the difference from the untreated controls. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; $n = 3$. Data are mean ± SE.

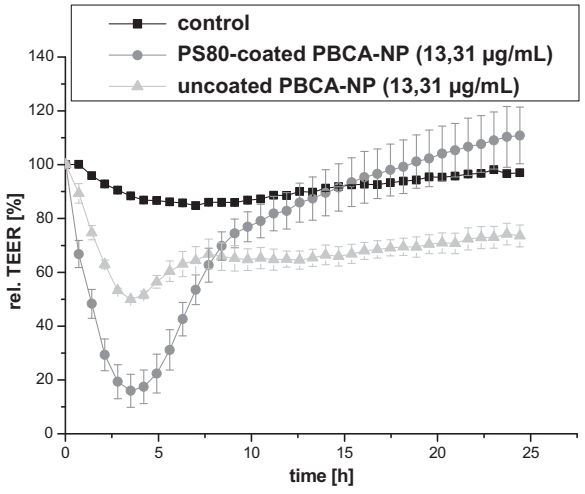

Fig. 4. Influence of Polysorbate 80 coating on the barrier integrity of PBCEC. Relative TEER development after addition of coated and uncoated PBCA-NP with a solids content of 13.31 µg/mL. Shown are the means of at least three filter devices for each condition of a representative measurement (±SE) $n = 3$.

addition the influence of the PS80-coating of the PBCA-NP on the integrity of the BBB *in vitro* should also be investigated. At present, only a few studies have investigated the impact of nanoparticles on the TEER. Koeneman et al. investigated the influence of titanium oxide nanoparticles of the TEER on a human intestinal epithelium cell line [15] and observed a reversible change in TEER only after applying a very high concentration of nanoparticles. Makhlof et al. were able to show that polyelectrolytic nanoparticles, build up from spermine and poly(acrylic acid), induce a reversible decrease of TEER using the same cell culture model [16]. For PBCA-NP, a study examining the effect of these particles on the TEER was not found. In the present study the application of PS80-coated PBCA-NP led to a reversible disruption of the barrier after 4 h, supporting the findings of Makhlof et al. Employing TEER measurements it was also revealed that 13,31 μg/mL constitutes the maximum solids content that still allows the reconstitution of the barrier. Additionally, it was shown that the cells are more sensitive to a second application of nanoparticles since a complete and irreversible breakdown is already observed at 13,31 μg/mL instead of 26,62 μg/mL for the first addition (see Fig. 1A and Fig. 3). Nevertheless, it is not clear whether this is caused by an altered cell phenotype, by nanoparticles which have been taken up by the cells (about 30% of added nanoparticles, data not shown) or by the nanoparticles remaining on the cell surface from the first addition.

The results obtained during the TEER experiments were confirmed by the permeability studies which revealed an elevated permeability of ^{14}C-sucrose and FITC-BSA about 4 h after nanoparticle application. In the literature, conflicting data concerning the influence of PBCA-NP on the ^{14}C-sucrose permeability can be found. Whereas Olivier et al. published an increased permeability of ^{14}C-sucrose induced by PBCA-NP in an *in vitro* model of bovine brain capillary cells (BBCEC) [17], Kreuter et al. [14] and Lockmann et al. [18] did not corroborate this findings also using BBCEC. However, all studies mentioned above observe ^{14}C-sucrose permeability for only a few hours. In this context, the opening of the paracellular pathway by the PBCA-NP is consistent with the study of Olivier et al. who found a maximum in the permeability of ^{14}C-sucrose after 20 min but did not observe a decrease in the following hours, maybe due to a too short observation.

Up to now many studies have reported the uptake of PBCA-NP into the CNS [1,14]. Reimold et al. were able to show that PBCA-NP provided by Capsulution® permeated into the brain of mice [7]. In the present study it was also observed, that PBCA-NP were able to cross the BBB *in vitro* (Fig. 1D). Again, the highest rate of permeation was found after 4 h indicating that there is, at least to some extent, paracellular diffusion of particles due to loss of barrier integrity. Nevertheless some permeation was observed throughout the experiment. Thus, several mechanisms could be involved including endocytosis, transcytosis and inhibition of efflux systems as postulated by Kreuter [19].

In literature conflicting data exist concerning the effect of PBCA-NP on tight junction integrity. While Olivier et al. postulated an opening of tight junctions after PBCA-NP application [17], Kreuter et al. could not confirm a disruption of tight junction integrity [14]. To examine if the temporary opening of the BBB originates from a changed tight junction expression pattern, an immunocytochemical analysis of the occludin expression was conducted. Here, mainly straight and not serrated cell borders could be found after application of PBCA-NP which would not be expected for a monolayer with disrupted barrier integrity. Only small areas of the monolayer showed serrated cell borders. Nevertheless a change in the cell morphology of PBCEC was observed. The typical spindle-like morphology of PBCEC shifted to a more cobblestone shape under the influence of nanoparticles. In addition the organization of the cells within the monolayer seemed to decline. It has already been shown that a shift in morphology of brain endothelial cells is caused by different stimuli [20]. In this context it was observed that a decrease of barrier integrity may be due to changed cell morphology. Taken together, the decline in cell organization, the change in morphology and the areas of serrated cell borders might be an explanation for the temporary opening of the BBB.

For brain targeting employing PBCA-NP as drug carriers, nanoparticles are mostly coated with polysorbate 80, because it has been shown that a coating with this surfactant increases the uptake of PBCA-NP to the brain. In addition, surfactants influence the body distribution and the time of circulation of the particles [21]. Ramge et al. found an increased uptake of PS80-coated PBCA-NP compared to uncoated nanoparticles in an *in vitro* model of BBCEC [22]. To the best of our knowledge there are no studies concerning the effect of PS80-coated and/or uncoated PBCA-NP on the TEER. In the present study the influence of both coated and uncoated nanoparticles on the BBB integrity was determined. Although, both particles induced a reversible opening of the BBB, the described effect was much more pronounced for PS80-coated PBCA-NP. This supports the study of Ramge et al. which reports the greatest brain uptake for PS80-coated PBCA-NP.

Due to the observed reversible disruption of the BBB *in vitro* PS80-coated PBCA-NP might not only be employed as drug carriers but also offer the possibility to be used as specific openers of the BBB. Instead of incorporating drugs into the NP, drugs may cross the BBB after being simultaneously applied with NP.

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