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Specific role of polysorbate 80 coating on the targeting of nanoparticles to the brain

Wangqiang Sun[a,b], Changsheng Xie[a,b,*], Huafang Wang[c], Yu Hu[c]

[a] Nano Pharmaceutical Research Center, Huazhong University of Science and Technology, Wuhan 430074, China
[b] Department of Material Science and Engineering, Huazhong University of Science and Technology, Luo-Yu Road 1037, Wuhan, Hubei 430074, China
[c] Institute of Hematology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China

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Abstract

It was reported that nanoparticles with polysorbate 80 (Tween 80, T-80) coating represented tools used for delivering drugs to brain. Nevertheless, disputations were once aroused for some complications. Aimed to have a better understanding of the specific role of T-80 coating on nanoparticles and simplify the problem, the direct observation of brain targeting combined with in vivo experiments was carried out in this work using the model nanoparticles (MNPs). The presence of a complex composed by the model loading, T-80 and nanoparticles was found in the preparation of MNPs. The result was further supported by some surface properties of MNPs. Being bound to nanoparticles that were overcoated by T-80 later, was necessary for the loading to be delivered to brain. Partial coverage was enough for T-80 coating to play a specific role in brain targeting. It seemed that brain targeting of nanoparticles was concerned with the interaction between T-80 coating and brain micro-vessel endothelial cells. Therefore, the specific role of T-80 coating on nanoparticles in brain targeting was confirmed.
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Keywords: Polylactic acid (PLA); Nanoparticles; Polysorbate 80 (Tween 80,T-80); Brain targeting; Brain micro-vessel endothelial cells (BMECs); Fluorescein isothiocyanate-dextran (FITC-dextran)

1. Introduction

Featured with tight continuous circumferential junctions between them, brain micro-vessel endothelial cells (BMECs) mainly build up the blood–brain barrier (BBB) [1–5], which hinders water-soluble molecules and those with molecular weight above 500, such as the therapeutic peptides, proteins, genes and antibiotics, from the circular system to the brain. The applicability of medicines in brain diseases is thus limited.

In pharmaceutics, nanoparticles [4,6] are polymeric particles with a size ranging from 10 to 1000 nm, employed to carry drugs through incorporation or absorption. Loaded by nanoparticles, drugs will be released at right rate and dose at specific sites in body during a certain time to realize the accurate delivery, which will enhance the therapeutic efficacy and reduce the toxicity and the side effect. It was reported that nanoparticles overcoated by polysorbates (especially polysorbate 80 (Tween 80, T-80)) were capable of transporting the loaded drugs across BBB after administration, which supplied tools delivering drugs to brain [4,7–10]. Nevertheless, disputations were once aroused for some complications [11]. Up to now, most works [4,7–11] were focused on nanoparticles of poly(alkylcyanoacrylate) (PACA), a polymer that is not authorized to application in human [12]. Brain targeting was characterized mainly through physiological or pharmacological reactions of testing animals that had administrated model drugs mediated by nanoparticles [4,7–11]. PACA will be rapidly degraded by esterases presented in biological fluid and some toxic products will stimulate or damage the central nervous system (CNS) [11]. Furthermore, possibly due to competitive adsorption, complete desorption of the pre-loaded model drug after surface modification by T-80 was reported [11]. Hence, the doubt on the specific role of T-80 coating on nanoparticles was put forward and a mechanism of a

*Corresponding author. Department of Material Science and Engineering, Huazhong University of Science and Technology, Luo-Yu Road 1037, Wuhan, Hubei 430074, China. Tel.: +86-027-87543840; fax: +86-027-87543776.
E-mail address: csxie@mail.hust.edu.cn (C. Xie).

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non-specific role of the permeabilization of BBB concerned with the toxicity of PACA was surmised [11]. Meanwhile pungent rebutment was also aroused for the design and operation of the experiments supporting the doubt [4]. These disputations reflected that there lied some limitations in the works on nanoparticles in brain targeting.

Aimed to have a better understanding of the specific role of T-80 coating on nanoparticles in an easy way, we tried to introduce new idea to investigate those problems in this elementary work. A new kind of model nanoparticle (MNP) was designed based on the surfactant-free polylactic acid (PLA) nanoparticles. As a typical biodegradable polyester currently employed in clinic approved by Food and Drug Agency of US, PLA [12–15] was employed instead of PACA to avoid the toxic effects induced by the matrix material. Since the suspensions of surfactant-free nanoparticles (SFNPs) of biodegradable polyesters were relatively stable during storage [16], the suspension of SFNPs of PLA was adopted as a reference system to physically characterize MNPs. The preparation of MNPs was formed through incubation of the model loading with SFNPs, followed by the treatment of overcoating with T-80. Fluorescence microscopy is a physical method, once employed to support some findings in brain targeting of nanoparticles [7,8]. To further avoid the potential toxicity possibly induced by some compositions of the drug carriers, which had led to complications in experiments related to physiological or pharmacological reactions of testing animals [11], the direct observation of brain targeting combined with in vivo experiments was carried out using fluorescence microscopy. Having been widely applied in in vivo or in vitro experiments observed with fluorescence microscopy [7,8,17], fluorescein isothiocyanate-dextran (FITC-dextran, dextran labelled by fluorescein isothiocyanate) was used as the model loading to probe the distribution of MNPs in brain. Vascular perfusion fixation was performed instead of immersion fixation once used in the experiments of PACA nanoparticles [7], because it is the best method for the study of CNS morphology with light or electron microscopy in the small laboratory animal, which can maintain the tissue in as near a life-like state as possible, both morphologically and chemically [18].

2. Experiments

2.1. Materials, reagents and animals

PLA (racemic, 5000 in molecular weight) and FITC-dextran (77,000 in molecular weight) were purchased from Shandong Province Institute of Medical Equipments, China and Sigma, respectively. Reagents were analysis-grade chemicals made in China mainly including acetonitrile, anhydrous ethanol, T-80, cobalt nitrate hexahydrate ($Co(NO_3)_2 \cdot 6H_2O$), ammonium thiocyanate (NH_4SCN), chloroform, acetone, sodium chloride (NaCl) and paraformaldehyde. These materials and chemicals were applied as obtained without further treatment. Experimental animals were inbred Kunming mice (20–30 g in weight) supplied by the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology, China.

2.2. Preparation of nanoparticles

SFNPs were firstly prepared by a nanoprecipitation method [15,19] with some modifications. In brief, PLA was dissolved in acetonitrile and slowly added into 50% (v/v) ethanol aqueous solution. The pre-formed suspension was slowly added to distilled water with shaking and then treated in a rotative vacuum evaporator to remove organic solvents and surplus water. Finally, the suspension with a concentration of 1% (w/v) was produced.

Based on the suspension of SFNPs as described above, MNPs were formed at 20–25°C as below. FITC-dextran was added into the preparation of SFNPs and incubated for 24 h. Thereafter T-80 was added and stored for another 24 h. The ratio among nanoparticles, FITC-dextran and T-80 in weight was 10:1:10. Free FITC-dextran and T-80 in the final preparation were not further treated as those free model drugs and T-80 in the preparations of model drug loaded nanoparticles of PACA [7–11].

Two controls, T-80 coated nanoparticles (TNPs) and FITC-dextran loaded nanoparticles (FNPs), were prepared similar as MNPs, respectively, only without adding FITC-dextran or T-80.

These suspensions of different nanoparticles (listed in Table 1) were used in characterization of nanoparticles and preparation of some injections administrated to the testing animals thereinafter. When necessary, some chemicals would be added and dissolved in the suspensions therein.

2.3. Characterization of nanoparticles

The morphology of SFNPs was observed in a JEM-10CX II transmission electron microscope (TEM)

Table 1
Components added in different suspensions

Type of suspensions	Components added in suspensions (w/v)
SFNPs	SFNPs (1%)
TNPs	SFNPs (1%) + T-80 (1%)
FNPs	SFNPs (1%) + FITC-dextran (0.1%)
MNPs	SFNPs (1%) + FITC-dextran (0.1%) + T-80 (1%)

(JEOL, Japan) at 80 kV using the sample taken from the suspension of SFNPs by C-coated Cu grips.

The amount of T-80 coating on TNPs or MNPs was determined based on a quantitative test for poly(ethylene oxide) with ammonium cobaltothiocyanate ($NH_4[Co(SCN)_3]$) [20]. Briefly each suspension with a known volume was centrifuged at 16,850*g* at −4°C for 30 min in a TLL-C table centrifuge (Beijing Sihuan Instrument Plant, China), respectively. The supernatant in each sample was discarded to eliminate unadsorbed T-80. The pellets were rinsed with distilled water twice and the washing solution was eliminated by another centrifugation as described above. The nanoparticles thus purified were resuspended in $NH_4[Co(SCN)_3]$ solution (dissolving $Co(NO_3)_2 \cdot 6H_2O$ (30 g/l) and NH_4SCN (200 g/l) in distilled water) with vigorous shaking. The complex between T-80 coating on nanoparticles and $NH_4[Co(SCN)_3]$ that might be produced was then extracted into chloroform. The adsorption of the chloroform solution was determined in a UV-2102PC spectrophotometer (Unico (Shanghai) Instrument Co., Ltd., China) at a wavelength of 318.5 nm, respectively. The amount of T-80 coating in each sample was calculated according to the determination.

To estimate the amount of FITC-dextran absorbed on FNPs or MNPs, high-speed centrifugation and rinse as described above were carried out to eliminate unadsorbed FITC-dextran in the suspensions with a known volume. Each sample thus purified was lyophilized in a LGJ table lyophilizer (Beijing Sihuan Instrument Plant, China) and acetone was added then. To further eliminate dissolved PLA, another high-speed centrifugation was performed and washed twice with acetone. The washing solution was discarded by centrifugation again as described above. The final precipitations were then dissolved in distilled water and measured in an RF-540 fluorescence spectrophotometer (Shimadzu, Japan) at excitation and emission wavelengths of 495 and 520 nm, respectively. The amount of FITC-dextran absorbed on FNPs or MNPs was estimated according those determinations at last.

To test the size and the zeta potential (ζ) of those nanoparticles therein before, samples from those suspensions were diluted with distilled water by 50 folds, respectively, and then each triply measured at 15°C in a Zeta Pals zetasizer (Brookhaven Instruments, US). Using SFNPs as a reference system, the hydrodynamic thickness (δ) of the layer adsorbed on different nanoparticles was calculated as follows [20]:

$$\delta = \frac{d_a - d_o}{2},$$

where d_a is the diameter of nanoparticles with adsorbed FITC-dextran and/or T-80 and d_o is the diameter of SFNPs.

2.4. In vivo experiments and fluorescence microscopy analysis

Healthy mice were divided into seven groups (three mice per group) in in vivo experiments. In each group each mouse was intravenously injected with the same injection at a dose of 0.2 ml in tail vein, respectively. Those injections were suspensions or solutions containing some components of the preparation of MNPs and 0.9% (w/v) NaCl, a component of physiological saline (see Table 2). In Group 1, the injection contained MNPs. The other groups were controls, just prepared before injection, including a mixture of FNPs and T-80, a mixture of TNPs and FITC-dextran, a suspension of FNPs, a mixture of SFNPs, FITC-dextran and T-80, a mixture of FITC-dextran and T-80, as well as a solution of FITC-dextran.

The vascular perfusion fixation with 4% paraformaldehyde solution was performed in those experimental animals after intravenous injection for 45 min. Before the perfusate was infused, vessels were rinsed to eliminate residual blood. The prefixed brain tissues were

Table 2
Main components in injections

Group	Type of injections	Main components in injections
1	A suspension of MNPs in saline	MNPs suspension (FITC-dextran, 0.1% (m/v), 8 mg/kg; T-80, 1% (m/v), 80 mg/kg) + NaCl (0.9% (m/v), 72 mg/kg)
2	A mixture of FNPs and T-80 in saline	FNPs suspension (FITC-dextran, 0.1% (m/v), 8 mg/kg) + T-80 (1% (m/v), 80 mg/kg) + NaCl (0.9% (m/v), 72 mg/kg)
3	A mixture of TNPs and FITC-dextran in saline	TNPs suspension (T-80, 1% (m/v), 80 mg/kg) + FITC-dextran (0.1% (m/v), 8 mg/kg) + NaCl (0.9% (m/v), 72 mg/kg)
4	A suspension of FNPs in saline	FNPs suspension (FITC-dextran, 0.1% (m/v), 8 mg/kg) + NaCl (0.9% (m/v), 72 mg/kg)
5	A mixture of SFNPs, FITC-dextran and T-80 in saline	SFNPs suspension + FITC-dextran (0.1% (m/v), 8 mg/kg) + T-80 (1% (m/v), 80 mg/kg) + NaCl (0.9% (m/v), 72 mg/kg)
6	A mixture of FITC-dextran and T-80 in saline	FITC-dextran (0.1% (m/v), 8 mg/kg) + T-80 (1% (m/v), 80 mg/kg) + NaCl (0.9% (m/v), 72 mg/kg)
7	A solution of FITC-dextran in saline	FITC-dextran (0.1% (m/v), 8 mg/kg) + NaCl (0.9% (m/v), 72 mg/kg)

immersed in the same perfusate, further fixed at 4°C in a refrigerator overnight and cut into sections by a vibratome thereafter. Those sections were observed in an FM fluorescence microscope (Nikon, Japan) at excitation and emission wavelengths of 495 and 520 nm, respectively.

3. Results and discussion

3.1. Characterization of model nanoparticles

As a reference system, the properties of SFNPs were investigated at first in this work. As shown in Fig. 1, SFNPs were solid and nearly spherical particles. From Fig. 2, the effective diameter was 162.1 nm with a narrow polydispersity index of 0.108 at the confidence level of 95%.

From Table 3, both FITC-dextran and T-80 were located on the surface of MNPs. The result suggested that the preparation of MNPs was not just a mixture of FITC-dextran, T-80 and nanoparticles and a complex composed by FITC-dextran, T-80 and nanoparticles was presented indeed. Compared with the two controls the total amount of the layer adsorbed on MNPs was the highest, while both FITC-dextran and T-80 bound on MNPs were each lower than the situation of FITC-dextran or T-80 lonely adsorbed on nanoparticles, respectively. The dextran employed in this work is a typical water-soluble polymer with high molecular weight. Because of the large size of polymer chains, it is not easy to displace adsorbed high molecular weight molecules [22]. Hence, desorption of FITC-dextran pre-adsorbed on nanoparticles due to competitive adsorption was unobvious. Since FITC-dextran was previously bound to MNPs, the steric effect [22] could not be ignored. The adsorption of T-80 on MNPs was undoubtedly influenced. Consequently, T-80 only partially covered MNPs.

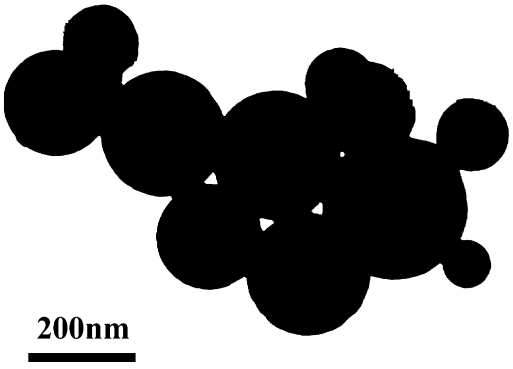

Fig. 1. TEM image of SFNPs.

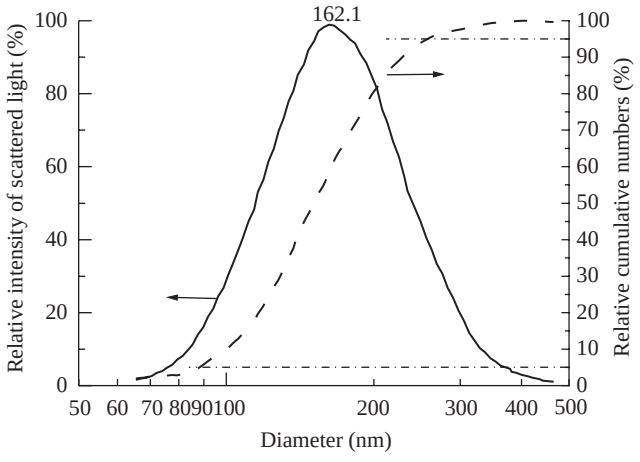

Fig. 2. Size distribution of SFNPs: — density distribution and –– cumulative distribution.

Since SFNPs were uniform and nearly spherical, δ of the adsorbed layer on different nanoparticles could be estimated. The results listed in Table 4 demonstrated that the adsorbed layer on MNPs was thicker than those of the controls, which was in good agreement with the results therein before (Table 3). These results gave a hint to support that a complex composed by FITC-dextran, T-80 and nanoparticles was presented indeed in the preparation of MNPs. As the surface become more crowded when more surfactants or polymers are adsorbed, the adsorbed are probably more laterally compressed and trend to stretch in the direction perpendicular to the surface, which will lead to δ of adsorbed layer increased [21,22].

The extent of positive shift in ζ of different nanoparticles was listed in Table 5. The results were content with those of δ of the adsorbed layer (Table 4) as described above, which further supported the presence of a complex composed by FITC-dextran, T-80 and nanoparticles. As a very important parameter on the electrical properties of surface, ζ is defined as the potential at the shear plane in the Stern model. It is generally assumed in tests of double-layer theory that ζ and the Stern potential (ψ_s) are the same except some situations, e.g. in the presence of absorbed non-ionic species or polymers that force the shear plane further away from the surface, reducing ζ relative to ψ_s [22]. PLA carries the negative charge for its end group of carboxylic acid [23], while FITC-dextran is a non-ionic surfactant and T-80 is a kind of non-ionic polymer. Therefore, the more the adsorbed, the thicker the adsorbed layer and the more positive shift in ζ.

3.2. Direct observation of brain targeting

In vivo experiments were carried out to investigate the specific role of T-80 coating in brain targeting. Contrary to the data of PACA nanoparticles [11], Table 6 showed no case of mortality was found in each group, which

Table 3
Amount of T-80 or FITC-dextran adsorbed on different nanoparticles

Nanoparticles	Amount of T-80 (μg/mg)	Relative amount of T-80 (%)[a]	Amount of FITC-dextran (μg/mg)	Relative amount of FITC-dextran (%)[b]	Total amount of the adsorbed layer (μg/mg)[c]
TNPs	30.7	100	0[d]	0	30.7
FNPs	0[e]	0	15.4	100	15.4
MNPs	25.6	83.4	14.0	90.9	39.6

[a] Taking TNPs as the standard.
[b] Taking FNPs as the standard.
[c] Calculated by summing both the amount of T-80 and FITC-dextran.
[d] Since TNPs were not treated with FITC-dextran, the value was supposed as zero.
[e] Since FNPs were not treated with T-80, the value was supposed as zero.

Table 4
δ of the layer adsorbed on different nanoparticles

Nanoparticles	Diameter (nm)	δ (nm)
SFNPs	162.1	0
TNPs	174.8	6.4
FNPs	194.2	16.1
MNPs	202.6	20.3

Table 5
ζ of different nanoparticles

Nanoparticles	ζ (mV)	Positive shift in ζ (mV)	Relative positive shift in ζ (%)
SFNPs	−29.52	0	0
TNPs	−26.32	3.20	10.8
FNPs	−13.40	16.12	54.6
MNPs	−10.17	19.35	65.5

demonstrated the security of the compositions of the preparation of MNPs. In body scissions of PLA will take place and lactic acid will be produced by further biodegradation. The degraded product, lactic acid, enters the tricarboxylic acid cycle and is metabolized as CO_2 and H_2O at last [13]. It seemed that the effects of toxicity of the carrier on CNS might be ignored in this work.

From Table 6, fluorescence only appeared in Group 1 that injections contained MNPs. These indicated that MNPs owned the property of brain targeting. Fluorescence was neither found in groups (Groups 4 and 7, also see Table 2) without T-80 in injections nor observed in Group 2 (also see Table 2) without T-80 coating on nanoparticles. Such discriminations from Group 1 illustrated that brain targeting corresponded to the T-80 coating on nanoparticles. The experiments of Oliver et al. [11] suggested that the preparation of T-80 coated nanoparticles was actually a simple mixture of model drug, T-80 and nanoparticles. In groups (Groups 3, 5 and 6, also see Table 2) that FITC-dextran was just mixed with T-80, however, no fluorescence was found. The results contradicted those of Oliver et al. and illustrated that brain targeting was related to the complex composed by FITC-dextran, T-80 and nanoparticles. These data supported the findings of Kreuter et al. [4,7–10], i.e. the coating of T-80 plays a specific role in brain targeting. The experiments also suggested that partial coverage was enough for T-80 coating to play a specific role in brain targeting taken together with the results therein before (Table 3).

Comparing with (a) and (b) in Fig. 3, one can further find that fluorescence mainly located at the wall of brain micro-vessels. Although it was very hard to discriminate whether MNPs were adhered to the lining of the BMECs or taken up by BMECs from fluorescence microscopy, one thing seemed to be confirmed that brain targeting of nanoparticles was concerned with the interaction between T-80 coating and BMECs.

According to literatures, several mechanisms of nanoparticle-mediated drugs across BBB had been proposed [4,7,8,11]. Among them the mechanism of endocytosis [4,7,8] was supported by many experiments. It is very possible that polysorbates on the surface of nanoparticles anchor apolipoprotein E (apo E) that plays an important role in the transport of the low-density lipoprotein (LDL) into brain. After apo E being bound to the surface, nanoparticles mimic LDL-particles to interact with LDL receptors on BMECs, which makes them up-taken by BMECs [4]. On the contrary some workers suggested that in the situation of PACA nanoparticles a non-specific opening of the tight junctions between BMECs was first induced by the carrier matrix and the unloaded model drug was then penetrate into the CNS with the help of T-80 [11]. From this work the result illustrated that being bound to nanoparticles that were overcoated by T-80 later, was necessary for the loading to be delivered to brain. Therefore, it seemed that the mechanism of endocytosis was more reasonable than the latter.

4. Conclusions

A complex composed by the loading, T-80 and nanoparticles was found in the preparation of MNPs.

Table 6
Comparison of fluorescence microscopy analysis on brain tissues

Group	Type of injections	Complex presented in injections	Mortality before vascular perfusion	Fluorescence in brain tissues
1	A suspension of MNPs in saline	FITC-dextran-T-80-nanoparticles	None	+
2	A mixture of FNPs and T-80 in saline	FITC-dextran-nanoparticles	None	−
3	A mixture of TNPs and FITC-dextran in saline	T-80-nanoparticles	None	−
4	A suspension of FNPs in saline	FITC-dextran-nanoparticles	None	−
5	A mixture of SFNPs, FITC-dextran and T-80 in saline	None	None	−
6	A mixture of FITC-dextran and T-80 in saline	None	None	−
7	A solution of FITC-dextran in saline	None	None	−

Note: +, green fluorescence appeared in the view under a fluorescence microscope; −, no fluorescence was observed, completely dark in the view under a fluorescence microscope.

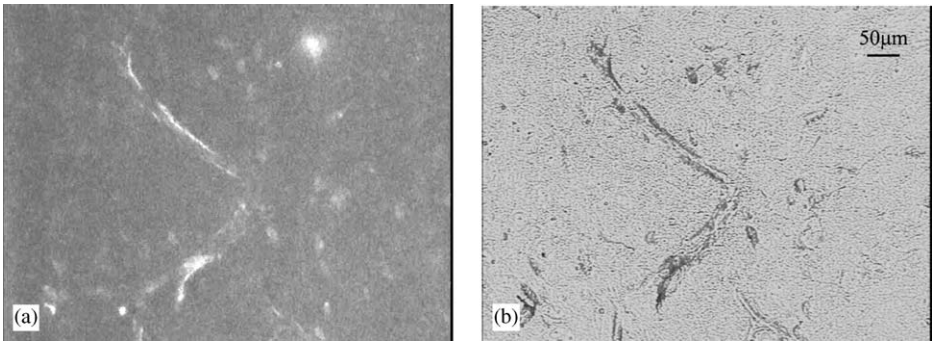

Fig. 3. Fluorescence distributed in the brain tissue of the mouse from Group 1 after 45 min intravenous injection: (a) viewed under a fluorescence microscope and (b) viewed under a photo microscope.

The result was further supported by surface properties of MNPs. Being bound to nanoparticles that were overcoated by T-80 later, was necessary for the loading to be delivered to brain. Partial coverage was enough for T-80 coating to play a specific role in brain targeting. It seemed that brain targeting of nanoparticles was related to the interaction between the T-80 coating and BMECs. The mechanism of endocytosis was more reasonable for nanoparticle-mediated drugs across BBB. The specific role of T-80 coating on nanoparticles in brain targeting was thus confirmed.

Acknowledgements

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