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Influence of particle size on transport of methotrexate across blood brain barrier by polysorbate 80-coated polybutylcyanoacrylate nanoparticles

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Abstract

Transports of methotrexate-loaded polybutylcyanoacrylate nanoparticles with different sizes across blood brain barrier were investigated in this experiment. The drug-loaded nanoparticles were prepared by emulsion polymerization method. After coating with polysorbate 80, nanoparticles with the size 70, 170, 220, 345 nm were, respectively, i.v. injected into rats at the dose of 3.2 mg/kg. Uncoated nanoparticles and methotrexate solution were also i.v. injected at the same dosage as controls. 0.5, 1, 1.5, 2, 3, 4 h after injection, cerebrospinal fluids and brain tissues were collected for tests. Drug level in all biological samples was determined by HPLC. It was found out that nanoparticles overcoated by polysorbate 80 could significantly improve the drug level in both brain tissues and cerebrospinal fluids compared with uncoated ones and simple solution. Seventy-nanometer nanoparticles could deliver more drugs into brain while no significant difference was observed among the other three size ranges. In conclusion, polysorbate 80-coated polybutylcyanoacrylate nanoparticles could be used to overcome blood brain barrier especially those whose diameter was below 100 nm.

Keywords: Polybutylcyanoacrylate; Methotrexate; Nanoparticles; Blood brain barrier

1. Introduction

The blood brain barrier (BBB) formed by tight endothelial cell junctions of brain capillary prevents a large number of therapeutic compounds from central nervous system (CNS), including antibiotics, antineoplastic agents and CNS active drugs. Methotrexate (MTX) is a promising antitumor drug. Unfortunately, it is very difficult for MTX to penetrate BBB so that the administration of intrathecal injection is employed in clinical treatment for brain tumors. However, this route subjects patients to operation wounds and high risks.

Several pharmaceutical methods have been tried to overcome BBB. First, drugs with proper chemical structures can be modified to enhance brain uptake usually by introduction of lipophilic groups. Besides, a novel chemical delivery system has also developed (Yoshikawa et al., 1999). However, this strategy is largely limited by the chemical structure of compounds. At the same time, a large number of lipophilic compounds that can pass through BBB are rapidly pumped back into blood stream by multi-drug resistance proteins (MRPs) including multiple organic anion transporter (MRP2) and P-glycoprotein (Pgp). The existence of such efflux pumps proves to be another limitation to this method. Second, sterically stabilized immunoliposomes are used for both chemical drugs and genes delivery to brain (Huwyler et al., 1996; Shi and Pardridge, 2000). However, human derived antibody is too expensive and the efficacy of such technology is not remarkable.

With the advance of nanotechnology, polymer nanoparticles can also be employed as carriers to transport the entrapped or adsorbed drugs across BBB. Since Kreuter etc. first reported that polysorbate 80-coated polybutylcyanoacrylate (PBCA) nanoparticles could deliver the peptide dalargin into CNS to exert its analgesic effect in 1995 (Kreuter et al., 1995), several drugs have been successfully taken into brain by this colloidal carrier (Alyautdin et al., 1997; Schroder et al., 1998; Gulyaev et al., 1999; Friese et al., 2000). It was demonstrated

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that the coat of polysorbate 80 was indispensable. Without the surfactant coating, nanoparticles were supposed to be predominantly engulfed by mononuclear phagocyte system. Meanwhile, in terms of brain targeting, other surfactants for coating was proved to be less effective than polysorbate 80 (Borchard et al., 1994). As far as the mechanism is concerned, endocytosis of endothelial cells of brain capillary may be the most likely one. It is presumed that nanoparticles mimic low-density lipoprotein (LDL) when injected into blood stream. They may adsorb upon ApoE which play an important role in transportation of LDL into brain so that the LDL receptor-mediated endocytosis takes place (Kreuter, 2001). It was reported that both the dalargin-loaded PBCA nanoparticles coated with a serial type of apolipoprotein and polysorbate 80 were able to achieve antinociceptive effects (Kreuter et al., 2002). Although this result partially supports the above mechanism, further researches and more direct evidences are required to draw conclusions.

The objective of the present study was to determine whether the size of nanoparticles had any influence on brain targeting. No experiments concerning this aspect have been reported. PBCA nanoparticles with different size could be prepared by adjusting the polymerization conditions such as media pH, concentration of emulsifier and monomer plus stirring rate. MTX was used as the model drugs and in vivo experiments were carried out to obtain some pharmacokinetic results.

2. Materials and methods

2.1. Drugs and reagents

n-Butyl-cyanoacrylate (BCA) was purchased from Zhejiang Jinpeng Chemical Industry Co. Ltd. (China). MTX raw material and MTX sodium lyophilized powder were, respectively, obtained from Zhejiang Wanma Pharmaceutical Co. Ltd. and Shanghai Hualian Pharmaceutical Co. Ltd. (China). Pluronic F68 was a generous gift from BASF Corp. (Shanghai, China). Dextran 70 was purchased from Shanghai Glucose Manufactory (China). Polysorbate 80 was bought from Shanghai Dazhong Pharmaceutical Co. Ltd. (China). HPLC grade methanol from Shanghai Chemical Reagents Research Institution was used as one component of HPLC mobile phase. All other reagents were commercially analytical grade.

2.2. Preparation of MTX-loaded PBCA nanoparticles

The MTX-loaded PBCA nanoparticles were prepared by the literature methods of emulsion polymerization (Gulyaev et al., 1999; Couvreur et al., 1979). Reaction condition was changed according to the expected particle size. Pluronic F68 or Dextran 70 at different concentration was firstly dissolved in the HCl media with certain pH ranged from 2 to 3. After the above solution was saturated with MTX raw material, certain amount of BCA was added in droplet into it under constant magnetic stirring with different rate. Four hours after the beginning of reaction, the media pH was adjusted to 6–7 by 0.1N NaOH solution and the polymerization continued for another 2 h. The milky suspension was then filtered by a 0.45 μm membrane to remove agglomerates.

2.3. Particle size and encapsulation efficiency measuring

After the agglomerates were removed by filtration, the size of nanoparticles in suspension was measured by dynamic laser scattering (NICOMP 380ZLS, Santa Barbara, CA, USA).

Ten percent in volume of the resulting suspension after filtration was used for encapsulation efficiency measuring. The separated suspension was ultracentrifuged at 4 °C, 6700 × *g* for 3 h. MTX concentration in the supernatant was determined by UV spectrophotometry at 313 nm and the whole volume of supernatant was also measured. Thus the quantity of the unbound MTX (M_1) could be calculated. The encapsulation efficiency was the ratio of the quantity of bound drug to that of the originally added drug (M_0). So it could be indirectly estimated by the following equation:

$$E = \frac{0.1M_0 - M_1}{0.1M_0} \times 100\%$$

2.4. Factorial designs

Since preparation conditions greatly affected the characteristics of drug-loaded nanoparticles, uniform design was carried out to evaluate the influence of these conditions on nanoparticle diameter and drug encapsulation efficiency. A design consisting four factors (media pH, monomer concentration, stabilizer concentration and stirring rate) at nine levels was performed (Tian and Fang, 1998; McCarron et al., 1999) Average diameter (d) and encapsulation efficiency (E) were mainly observed as two responses. Nine observations were required to fulfill such a uniform design. Each observation was triplicate and the mean of each response was calculated for regression. Linear regression by SPSS software was employed to establish equations describing the relationship between the factors and the responses. Since there were two different types of stabilizer, two separate designs were taken for Pluronic F68 as emulsifier and Dextran 70 as stabilizer. Tables 1 and 2 illustrate the detailed arrangement of the two designs.

Table 1
Arrangement of uniform design experiments using Pluronic F68 as emulsifier

No.	Factors			
	pH, *A*	Concentration of PF68, *B* (%)	Stirring rate, *C* (rpm)	Concentration of BCA, *D* (%)
1	2.0	0.4	500	0.5
2	2.0	0.5	500	0.7
3	2.0	0.6	500	0.9
4	2.5	0.7	700	1.1
5	2.5	0.8	700	1.3
6	2.5	0.9	700	1.5
7	3.0	1.0	900	1.7
8	3.0	1.1	900	1.9
9	3.0	1.2	900	2.1

Table 2
Arrangement of uniform design experiments using Dextran 70 as stabilizer

No.	Factors			
	pH, *A*	Concentration of Dex70, *B* (%)	Stirring rate, *C* (rpm)	Concentration of BCA, *D* (%)
1	2.0	1.0	500	0.5
2	2.0	1.5	500	0.7
3	2.0	1.5	500	0.9
4	2.5	2.0	700	1.1
5	2.5	1.0	700	1.3
6	2.5	1.0	700	1.5
7	3.0	1.5	900	1.7
8	3.0	2.0	900	1.9
9	3.0	2.0	900	2.1

2.5. Purification of MTX-loaded nanoparticles and drug level determination in suspension

The filtered suspension was then ultrafiltered to be purified under the N_2 pressure of 0.1 MPa through a membrane whose retention molecular weight was 50,000. The nanoparticles were rinsed three times with distilled water to guarantee the thorough removing of unbound MTX. After that, 100 μL purified suspension was taken and diluted accurately by 5 mL DMSO. Thus, both the MTX and nanoparticles were dissolved in DMSO so that the MTX concentration in the purified suspension could be determined by UV spectrophotometry at 313 nm where PBCA had no UV absorption.

2.6. Nanoparticle surface coating and animal testing

Immediately before administration, the MTX-loaded nanoparticles were coated with 1% polysorbate 80 under constant magnetic stirring at 37 °C. The European Community guidelines are accepted as principles for the use of experimental animals. Male Sprague–Dawley rats (200–230 g) were divided into six groups. One to four groups was, respectively, treated by polysorbate 80-coated MTX-loaded nanoparticles with the size of 70, 170, 220 and 345 nm in purified suspension. The group 5 was treated by uncoated MTX-loaded nanoparticles of 170 nm in suspension, while the group 6 received commercially available MTX sodium lyophilized powder solution (see Table 3). In all groups the formulations were administered i.v. in to the tail vein at the dose of 3.2 mg/kg. The injection volume of purified suspension was determined according to the MTX concentration in it to make sure the i.v. dosage. After selected time intervals post injection the animals (four rats per time point) were anesthetized with ethyl ether. The cisterna magna was exposed and cerebral spinal fluid (CSF) was obtained through puncture at this place (Wang and Jiang, 2001). Then the animals were sacrificed by decapitation. The cerebrum and cerebellum were separately collected after removal of visible brain blood vessels.

Table 3
Arrangement for in vivo test of MTX-loaded PBCA nanoparticles for brain delivery

Group	Preparation
1	cNP 70
2	cNP 170
3	cNP 220
4	cNP 345
5	ucNP170
6	Injection

cNP represents coated nanoparticles all the above numbers represent nanoparticle size (nm), ucNP means uncoated nanoparticles, injection is resuspension of MTX sodium lyophilized powder.

2.7. Biological sample analysis by HPLC

MTX concentrations in both CSF and brain tissues were determined by HPLC. Fifty microliters of CSF samples were directly injected to HPLC system simply after centrifuging at 10,000 rpm for 5 min. No special treatment was needed. Brain tissues were firstly weighed and then homogenized in glass homogenizer with distilled water of twice the tissue weight. To 300 mg homogenized matter, 300 μL 10% perchloric acid (v/v) was added, the mixture was vortex-mixed violently for 2 min and then centrifuged at 10,000 rpm for 10 min to precipitate the tissue proteins. The whole process was done in a 2 mL polyethylene conical centrifuge tube. Fifty microliters supernatant was injected to HPLC system which consisted of a LC-10A VP solvent pump and SPD-10A UV spectrophotometric detector (Shimadzu, Kyoto, Japan), a C_{18} column (Diamosil, 20 cm × 4.6 mm, 5 μm, Dikma, USA) and HS2000G chromatographic integrator (HS Empire, Hangzhou, China). A mixture of 50 mM ammonium acetate buffer (pH 6.0) and methanol (77:23, v/v) was used as mobile phase with the flow rate 1.0 mL/min. The wavelength of ultraviolet absorbance for detection was monitored at 313 nm and the column temperature was kept at 45 °C (Wang et al., 2003).

The assay was validated by measuring linearity, recovery and precision with CSF and brain tissue containing MTX at a series of concentration. The linearity ranged from 25 ng/mL to 2 μg/mL. The limitation of quantitation and detection for MTX were found to be 1.25 and 0.8 ng, respectively. The recovery of both CSF and brain tissue was between 94 and 100%. The between-day and within-day RSD for both SCF and brain tissue measuring were less than 5%. The above HPLC analysis was accurate and sensitive to determine MTX level in CSF and brain tissue.

2.8. Data analysis

The in vivo data were analyzed by one-way ANOVA to compare C_{max} and AUC in both CSF and brain tissues of the six groups. For this purpose, the software of SPSS was employed.

3. Results

3.1. Factorial design

MTX-loaded PBCA nanoparticles with different sizes were obtained by adjusting the media pH, the concentra-

Table 4
Preparation conditions and the size of resulting nanoparticles measured by laser diversity

Group	pH	Stabilizer type and concentration (%)	Monomer quantity (%)	Stirring rate (rpm)	d_c (nm)	d_m (nm)	S.D. (nm)
1	2.2	Pluronic F68 1.2	1.0	800	87	70	14.5
2	2.0	Pluronic F68 1.0	1.1	700	155	170	26.1
3	2.0	Pluronic F68 0.5	2.0	600	203	220	37.3
4	2.0	Dextran 70 1.4	1.5	700	318	345	56.8

d_c and d_m represent nanoparticle size calculated by the equations and measured by DLS, respectively.

tion of emulsifier or stabilizer, the quantity of monomer and the stirring rate. When Pluronic F68 was used as emulsifier, the relationship between the two responses and the four factors could be described as following linear equations: d (nm) = 260.4 − 27.2A − 60.6B − 0.099C + 38.89D, E (%) = −41.85 + 22.2A − 5.2B + 0.012C + 28.5D. When Dextran 70 was employed as stabilizer, the equations were as follows: d (nm) = 290.6 − 45.9A + 21.2B − 0.136C + 104D, E (%) = −41.35 + 32.4A − 3.5B − 0.031C + 27.3D. All the above equations were statistically significant tested by ANOVA ($P < 0.05$). According to the above equations, increasing the media pH and stirring rate, or decreasing the quantity of stabilizer and monomer appropriately could decrease the nanoparticle size. Thus, the size of nanoparticles could be controlled while the encapsulation efficiency remained at about 40%. Meanwhile, in order to prepare nanoparticles larger than 300 nm, Dextran 70, instead of Pluronic F68, should be used. Table 4 shows the average diameter of nanoparticles prepared under certain conditions for animal testing. The nanoparticle size predicted by the regression equations was highly consistent with that measured by dynamic laser scattering.

3.2. In vivo tests

Figs. 1–3 show MTX levels in CSF, cerebrum and cerebellum, respectively, after i.v. administration of six formulations. In CSF samples, MTX reached its peak concentration 1.0 h after administration for each formulation. Polysorbate-coated nanoparticles could double the maximum drug concentration (C_{max}) and the area under the concentration–time curve (AUC) compared with MTX sodium solution. In cerebrum and cerebellum tissues, C_{max} came 1.5 h post administration. C_{max} and AUC of MTX delivered by coated nanoparticles were about three times as high as those by MTX sodium resuspension. In

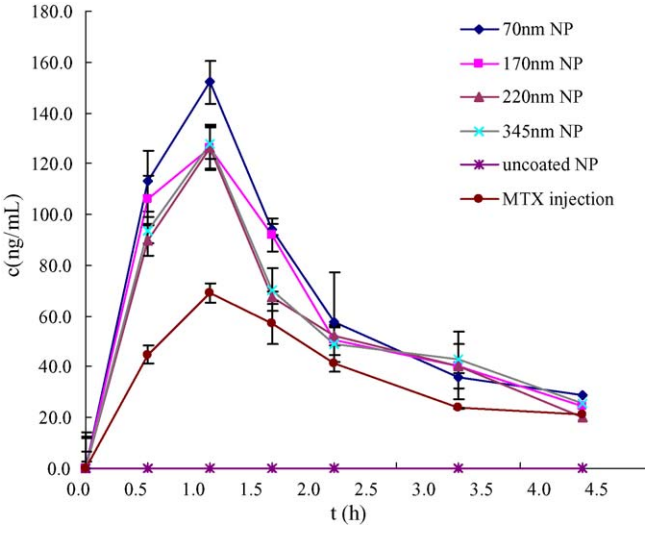

Fig. 1. Methotrexate level in cerebrospinal fluid following i.v. administration of the six preparations at the dose of 3.2 mg/kg. Data represents the mean ± S.D. ($n = 4$).

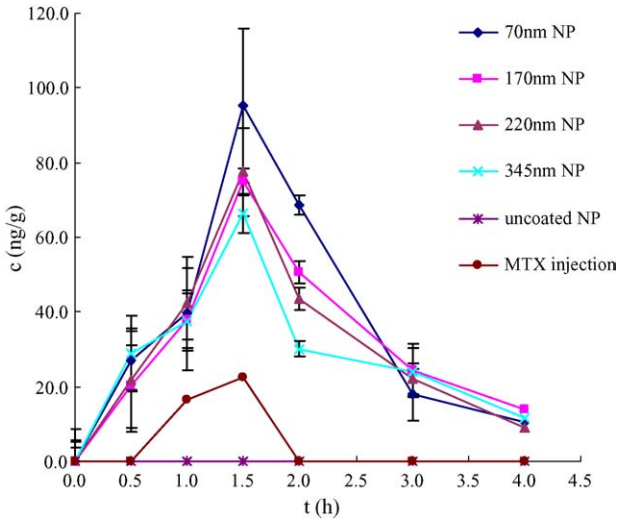

Fig. 2. Methotrexate level in cerebrum following i.v. administration of the six preparations at the dose of 3.2 mg/kg. Data represents the mean ± S.D. ($n = 4$).

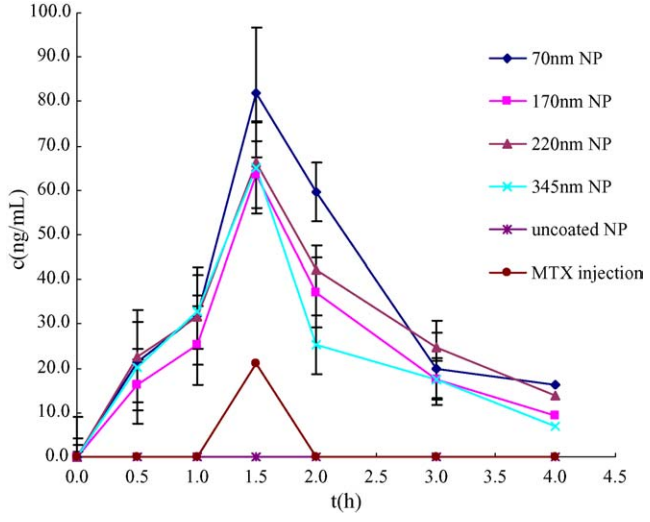

Fig. 3. Methotrexate level in cerebellum following i.v. administration of the six preparations at the dose of 3.2 mg/kg. Data represents the mean ± S.D. ($n = 4$).

Table 5
Average C_{max}, $AUC_{0\rightarrow t}$ and the relative value of methotrexate following i.v. administration of six preparations at the dose of 3.2 mg/kg

	1	2	3	4	5	6
CSF						
C_{max} (ng/mL)	152.01 a	126.28b	126.43b	128.06b	0.00 c	69.12 d
Relative value	2.20	1.83	1.83	1.85	0.00	1.00
$AUC_{0\rightarrow t}$ (ng h/mL)	273.02 a	249.21 b	231.56 b	238.54 b	0.00 c	150.35 d
Relative value	1.82	1.66	1.54	1.59	0.00	1.00
Cerebellum						
C_{max} (ng/g)	95.36 a	74.96 b	77.44 b	66.30 b	0.00 c	22.41 c
Relative value	4.26	3.34	3.46	2.96	0.00	1.00
$AUC_{0\rightarrow t}$ (ng h/g)	155.50 a	134.96 b	132.82 b	121.10 b	0.00 c	19.51 c
Relative value	7.97	6.92	6.81	6.21	0.00	1.00
Cerebellum						
C_{max} (ng/g)	82.03 a	63.49 b	66.35 b	65.16 b	0.00 c	21.00 c
Relative value	3.91	3.02	3.16	3.10	0.00	1.00
$AUC_{0\rightarrow t}$ (ng h/g)	134.29 a	99.04 b	118.35 b	110.01 b	0.00 c	10.5 c
Relative value	12.79	9.43	11.27	10.48	0.00	1.00

Significant difference was observed between each pair of a, b, c, and d group, but there was no significant difference within any of a, b, c and d group.

both CSF and brain tissues, when MTX was bound to polysorbate 80-coated nanoparticles regardless of their size, significant higher drug levels were detected compared with MTX sodium resuspension. No MTX was detected when bound to uncoated nanoparticles. In vivo tests indicated that a significant transport of MTX across BBB facilitated by polysorbate 80-coated nanoparticles occurred.

Different particle size had some influences on drug delivery to the brain. Although there was no significant difference on the function of overcoming BBB among nanoparticles whose average diameter was from 100 to 400 nm, significant difference was observed when nanoparticle size was below 100 nm. More drugs could be delivered into both CSF and brain tissues by coated nanoparticles smaller than 100 nm, according to the value of C_{max} and AUC. Table 5 displays the C_{max}, AUC of MTX after i.v. administration of six formulations and the relative value of the above two pharmacokinetic parameters compared with those of MTX sodium resuspension. The result of ANOVA is also shown in it.

4. Discussion

Polysorbate 80-coated polyalkylcyanoacrylate nanoparticles have successfully transported several compounds across the BBB, including hexapeptide dalargin, dipeptide kytorphin, loperamide, tubocurarine, doxorubicin and the NMDA receptor antagonist (Kreuter et al., 1995; Alyautdin et al., 1997, 1998; Gulyaev et al., 1999; Friese et al., 2000). Although nowadays the application of polyalkylcyanoacrylate as a nanoparticle material is quite limited due to its possible toxicity, the above reports indicated that polymer nanoparticles were a promising drug carrier for BBB penetration. Our research again demonstrated that such a vector could deliver the compound which could not pass through the BBB itself into the brain. Since uncoated nanoparticles failed to have such a function, surface coating of nanoparticles was proved to be indispensable. However, when MTX was used as a model drug, the efficacy was not so remarkable as other reported drugs. It was mainly because of the very low drug loading capacity for polybutylcyanoacrylate nanoparticles to entrap MTX. First, the solubility of MTX in the pH 2.0 HCl solution was very limited, which was only about 0.8 g/L. Second, the encapsulation efficiency was not high enough, which was only about 40%. These two reasons led to the fairly low drug loading capacity. Thus, although plenty of nanoparticles might have entered the central nervous system, only a limited quantity of MTX was transported across the BBB with its vectors. In facts, different compounds have different affinity to the material polybutylcyanoacrylate. Most of those previously reported model drugs, such as doxorubicin and dalargin, had such high encapsulation efficiencies that no purification process was needed before administration and the efficacy of overcoming BBB was outstanding.

MTX is a kind of hydrophilic compound, so when it was intravenously administered, the drug which permeated the BBB in a limited quantity tended to distribute to the CSF rather than brain tissues. Such a distribution of MTX in the central nervous system proves to be another limitation for it to inhibit the tumor inside the brain tissue. In group 6, after MTX sodium solution was injected, only in the CSF could MTX be detected during the whole time course. In cerebrum or cerebellum samples, no drug was detected except at certain time point, such as 1.0, 1.5 h post injection. Most probably, when the drug level in the CSF was high enough (near the peak concentration), the distribution of MTX between the CSF and brain tissues led to the detectable drug level in the cerebrum and cerebellum. However, when the drug was being eliminated from the CSF, MTX level in the brain tissues soon decreased below the detection limit. In the groups 1–4, after the surface-coated nanoparticles were administered, MTX concentrations in the brain tissues were significantly increased compared with group 6. Although still lower than in CSF, the drug levels in brain tissues were all above the detection limit at each preset time point. Even though MTX in the brain tissues might again distribute to the CSF due to its hydrophilic characteristic, such a tissue deliv-

ery turned out to be another advantage of this nanosized drug carrier.

One indispensable property for nanoparticles to cross the BBB is the surface coating. In our experiments, when uncoated nanoparticles was administered, no drug could be detected in both CSF and brain tissues during the whole time course. It was mainly because uncoated nanoparticles whose diameter was about 100 nm were prone to be devoured by the mononuclear phagocyte system (MPS). Thus, the drug-loaded nanoparticles were rapidly cleared from the circulation system and targeted to organs where MPS was chiefly located, such as liver and spleen. Although it was more convincing to choose 70 nm uncoated nanoparticles to evaluate this necessity, the preparation of 70 nm was under some extreme conditions such as high pH, high concentration of Pluronic F68 and high stirring rate. In contrast, 170 nm nanoparticles could be prepared under common and mild conditions. So, 170 nm uncoated nanoparticles were employed in this experiment. Quite a few surfactants had been tested as surface-coating material for the brain targeting effect of nanoparticles, such as polysorbate 20, 40, 60, 80, poloxamer, poloxamine and Cremopor EZ, RH-40 (Kreuter et al., 1997). Consequently, polysorbate 80 proved to be the most effective surface-coating reagent in facilitating brain delivery of nanoparticles. The rest polymers of polysorbate system had only slight effects while other surfactants failed to exert any efficacy. For a period of time, polysorbate 80 was the only effective surfactant employed in this field. However, polysorbate 80 also had its own limitations. Its toxicity and haemolytic effects greatly restricted its application in drug delivery system, especially in intravenous administration. Recent studies of long-circulating PEGylated polycyanoacrylate nanoparticles offered an alternative drug carrier for brain delivery. In vivo tests illustrated that PEGylated polycyanoacrylate nanoparticles could also pass through the BBB in a significant amount (Calvo et al., 2001). Further investigations, especially concerning drug-loaded PEGylated polycyanoacrylate nanoparticles, should be carried out to testify whether such a new carrier really worked for brain delivery.

One hypothesis about the mechanism for surface-coating nanoparticles to overcome the BBB is that they mimic LDL after being intravenously administered. Polysorbate 80 plays a special role as an anchor between nanoparticles and the apolipoprotein, especially ApoE. Nanoparticles combined with the apolipoprotein are considered as LDL and LDL receptor-mediated transcytosis brings drug-loaded nanoparticles across the BBB. If this hypothesis proves to be true, nanoparticles that have the similar size to LDL should be more effective than any other ones. It was reported that the size range of LDL was from 20 to 25 nm (Chen, 1999). Unfortunately, such an emulsion polymerization process could hardly prepare such small drug-loaded nanoparticles. In our experiments, among the tested nanoparticles with four different size ranges, those whose diameter was below 100 nm led to the highest drug level in the brain. One possible reason for this result was that these nanoparticles might be more similar to LDL than others. On the other hand, since the receptor-mediated transcytosis existed, the smaller the particle size was, the easier it was for it to enter the vascular endothelial cells of BBB by endocytosis. Moreover, it was also possible that smaller particles once inside the endothelial cells might degrade more rapidly so that the MTX was released more rapidly into the brain. However, it was quite strange that no significant difference was observed among nanoparticles larger than 100 nm. The most probable reason still might be the low MTX loading capacity of PBCA nanoparticles. Although the quantity of nanoparticles entering the brain might be different, the quantity of MTX passing through the BBB together with the carrier showed no statistically significant difference. The results of our experiments concerning the particle size affecting the brain delivery, although indirect, supported the above hypothesis in a certain degree.

5. Conclusions

Our experiments again demonstrated the possibility of polysorbate 80-coated nanoparticles to facilitate model drugs to overcome the BBB. It was a promising carrier for brain delivery. The size of nanoparticles did have some influence on the function of brain targeting. When the diameter of nanoparticles was below 100 nm, the effects were more obvious.

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