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# Development of transferrin functionalized poly(ethylene glycol)/poly(lactic acid) amphiphilic block copolymeric micelles as a potential delivery system targeting brain glioma

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**Abstract** The aim of present study is to conceive a biodegradable poly(ethylene glycol)–polylactide (PEG–PLA) copolymer nanoparticle which can be surface biofunctionalized with ligands via biotin–avidin interactions and used as a potential drug delivery carrier targeting to brain glioma in vivo. For this aim, a new method was employed to synthesize biotinylated PEG–PLA copolymers, i.e., esterification of PEG with biotinyl chloride followed by copolymerization of hetero-biotinylated PEG with lactide. PEG–PLA nanoparticles bearing biotin groups on surface were prepared by nanoprecipitation technique and the functional protein transferrin (Tf) were coupled to the nanoparticles by taking advantage of the strong biotin–avidin complex formation. The flow cytometer measurement demonstrated the targeting ability of the nanoparticles to tumor cells in vitro, and the fluorescence microscopy observation of brain sections from C6 glioma tumor-bearing rat model gave the intuitive proof that Tf functionalized PEG–PLA nanoparticles could penetrate into tumor in vivo.

**Abbreviations**

| | |
|---|---|
| PEG | Poly(ethylene glycol) |
| PLA | Polylactide |
| Tf | Transferrin |
| BBB | Blood–brain barrier |
| TfR | Transferrin receptor |
| DMEM | Dulbecco's modified Eagle's medium |
| FBS | Fetal bovine serum |
| GPC | Gel permeation chromatography |
| DLS | Dynamic light scattering |
| DiI | 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate |
| FCS | Fetal calf serum |

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## 1 Introduction

The treatment of brain cancer by chemotherapy is still one of the most difficult challenges in oncology. Despite combining with surgical resection and radiotherapy, the median survival span of patients (1 year) has not been significantly improved for 30 years. The failure of chemotherapy is mainly attributed to the existence of blood–brain barrier (BBB), which separates the blood from the cerebral parenchyma, restricting the penetration of agents given systemically to brain tumor [1].

Traditional approaches to overcome brain drug delivery obstacles include direct intracerebral drug injection or

disruption of the BBB by infusion of hyperosmotic solutions [2]. Although these procedures can significantly increase drug levels into the brain, all of them are associated with a high risk for the patient. Another approach concerns the modification of drugs to make them more lipophilic, improving their penetration into the brain by passive diffusion. Lipophilic analogs and prodrugs were thus developed [3]. However, despite lipidization, no modified molecule demonstrated an anticancer activity superior to the parent drug.

Polymeric nanoparticles have been proposed as an interesting alternative. These colloidal systems allow the enhancement of therapeutic efficacy and toxicity reduction of a large variety of drugs. Different strategies have been developed to allow the nanoparticles to cross the BBB. Most of these are based on the physical absorption of surfactant polysorbate 80 on the surface of the polyalkylcyanoacrylate nanoparticles [4], but the mechanism of the enhanced BBB transport has not yet been fully elucidated. A promising strategy seems to be that of using PEGylated nanoparticles. Based on their long-circulating characteristics, PEGylated PHDCA (PEG 2000 cyanoacrylate-co-hexadecylcyanoacrylate) nanoparticles made by a PEGylated amphiphilic copolymer penetrate into the brain to a large extent than polysorbate 80-coated nanoparticles [5].

Besides overcoming BBB, targeting of nanoparticles to tumor is necessary for the glioma therapy, thus individual cancer cells can be destroyed by the loaded chemotherapeutic agents without causing diffuse damage to surrounding brain tissues [6]. An approach to obtain the targeting of the nanopartciles to the tumor is to functionalize their surface with biorecognitive ligands such as transferrin (Tf). Tf is a monomeric glycoprotein that can transport one (monoferric Tf) or two (diferric Tf) iron atoms [7]. Transferrin receptor (TfR) is overexpressed on the brain capillary endothelium [8] and at the surface of proliferating cells such as brain tumor cells, especially glioblastoma multiforme [9]. In contrast, a low level of TfR is observed on normal tissue. Receptor-mediated transcytosis of this protein has been demonstrated to occur across both the rat and the human BBB [10–12]. Tf-coupled PEGylated liposomes have also been extensively developed for many years. Pharmacokinetics analysis revealed a significant increase of the brain uptake for small Tf-PEG-liposomes (80 nm) in comparison with PEG-liposomes [13].

In the present study, we present a protocol for surface engineering of PEG–PLA nanoparticles with Tf, and evaluate their ability of targeting the brain tumor following intravenous administration. To achieve this goal, asymmetric biotinylated PEG (PEG-B) was first synthesized through the reaction of biotin chloride with PEG, then biotinylated PEG–PLA (PLA–PEG-B) block copolymers were obtained via ring opening polymerization of D, L-lactide, using stannous octanoate as catalyst and terminal hydroxyl group of PEG-B as initiator. PLA–PEG-B nanoparticles were prepared by nanoprecipitation evaporation. The resulting nanoparticles were then functionalized with Tf by taking advantage of the biotin–avidin complex formation. The targeting of the functionalized nanoparticles to glioma was assessed both in vitro and in vivo, by flow cytometry and fluorescence microscopy observation of brain sections, using a lipophilic fluorescence probe, compared with the unmodified nanoparticles.

## 2 Experiments

### 2.1 Reagents and materials

Biotin was purchased from Shanghai Desano C. Ltd. Polyethylene glycol (PEG) and methoxy polyethylene glycol (mPEG) with molecular weight (MW) of 2000 g/mol was purchased from Fluka. Stannous octanoate, avidin, biotin amidohexanoic acid N-hydroxysuccinimide ester (BCNHS), transferrin (Tf), BCA protein assay kit, and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) were purchased from Sigma-Aldrich. D,L-Lactide was synthesized and purified as described [14]. The C6 glioma cell lines were gift from NIH(USA). Fetal bovine serum (FBS) was purchased from GIBCO. Dulbecco's modified Eagle's medium (DMEM) was obtained from Sigma-Aldrich. Sprague–Dawley rats were purchased from Animal Center of the Chinese Military Medical Science Institute (Beijing, China).

### 2.2 Synthesis of B-PEG

3 ml thionyl chloride was added to 2% biotin anhydrous chloroform suspension. The reaction was carried out at 40°C until biotin was dissolved. The resultant mixture was evaporated with rotary evaporator to remove chloroform and unreacted thiony chloride, and then dissolved in 20 ml anhydrous chloroform.

6 g PEG was dissolved in 30 ml anhydrous chloroform and 1.5 ml triethylamine was added. After well mixing under electromagnetic stirring, biotinyl chloride (molar ratio of hydroxyl groups is 3:2) was added dropwise and the reaction system was stirred at 30°C under nitrogen for 12 h. The product was isolated and purified by chromatography column employing chloroform/methanol (5:1) as eluant. Then the resultant products were dried overnight under vacuum at 40°C. The polymer structure was confirmed via $^{1}$H NMR and $^{13}$C NMR measurements with a

500 MHz Bruker B-ACS 60 spectrometer by using deuterated chloroform (>99.8%) as a solvent.

### 2.3 Synthesis of B-PEG–PLA block copolymers

B-PEG–PLA block copolymers were synthesized with ring opening polymerization using stannous octanoate as the catalyst [15]. Briefly, D,L-lactide and B-PEG were mixed at a ratio of 50/1 (mol/mol) in a dried polymerization tube and then stannous octanoate catalyst (0.008% w of lactide) was added as a solution in chloroform. The reactants were dried under vacuum at 50°C for 2 h. Then the tube was sealed and the copolymerization was carried out at 140°C for 50 h. B-PEG-PLA was obtained by dissolving the product in chloroform and then precipitating in methanol followed by drying under vacuum for 24 h. As a control, mPEG-PLA copolymer was synthesized using the same procedure as mentioned above.

The copolymer structure was analyzed by $^{1}$H-NMR with a 500 MHz Bruker B-ACS 60 spectrometer, using deuterated chloroform (>99.8%) as a solvent. The molecule weight and distribution were measured through gel permeation chromatography (GPC). The samples were dissolved in analytical-reagent grade tetrahydrofuran. The number-average and weight-average molecular weights of the polymers were determined by universal calibration obtained from a polystyrene reference.

### 2.4 Preparation of B-PEG–PLA nanoparticles

Nanoparticles were prepared by nanoprecipitation method [16]. 10 mg B-PEG-PLA copolymer was dissolved in mixture of 0.8 ml acetone and 0.2 ml ethanol. The solution was injected dropwise into 10 ml deionized water, and then the solvent was evaporated under electromagnetic stirring for 6 h. The mean diameter and the size distribution of the nanoparticles in aqueous dispersion were measured by dynamic light scattering (DLS) using BI-90 Pluslaser particle-size analyzer (Brookhaven Instruments Corporation). The morphology of the prepared nanoparticles was observed with an H-600 transmission electron microscope (Hitachi, Tokyo) after negative staining with phosphotungstic acid solution (2% w/v). Zeta potentials were measured by JS94 J Microelectrophoresis (five runs per sample).

For the tumor targeting study, nanoparticles loaded with fluorescent probe DiI were prepared using the same method as mentioned above, except that DiI (0.6% w of PLA) was added to the B-PEG-PLA solution before the nanoprecipitation. The unloaded DiI were removed with a Sephadex G-25 column.

### 2.5 Surface-biofunctionalization of B-PEG-PLA nanoparticles

#### *2.5.1 Preparation of avidin-modified nanoparticles*

An aliquot (2.0 mg) of avidin was dissolved in 0.2 ml of PBS buffer (pH 7.2). Then the resulting solution was added to 1.0 mL PBS buffer containing 1 mg B-PEG–PLA nanoparticles. The mixture was incubated under shaking for 40 min at room temperature. The non-conjugated avidin was removed by Millipore Filter-Centrifugation technique. In brief, the mixture was shifted into the sample reservoir of the Microcon$^{TM}$ centrifuge filter (Molecular Weight Cut-off 100 kDa, Millipore Corporation), and then submitted to centrifugation for 5 min at 4000r/min. The mixture was kept 0.2 ml left in the reservoir to avoid the aggregation of the nanoparticles; the protein solutions filtered to the bottom of the tube were collected. After that, 0.6 ml PBS solution was added to up tube and the centrifugation was performed again. This procedure was repeated six times. The residual liquids in the reservoir were carefully collected. The reservoir was washed three times and every time the eluent was collected to be added to the collected liquid of nanoparticles. The solution volume was constant to 0.5 ml, then stored at 4°C until use.

#### *2.5.2 Transferrin functionalization of avidin-modified B-PEG–PLA nanoparticles*

10 mg transferrin was dissolved in 1 ml PBS (pH = 7.4) and incubated with 50 μl BCNHS solvent (2 mg/ml) at room temperature for 4 h, then the solution was subjected to Sephadex G-50 gel chromatography in a column equilibrated with PBS buffer to remove the free BCNHS. The obtained 0.2 ml biotinylated Tf solution (4 mg/ml) was mixed with 0.5 ml solution of avidin-modified B-PEG–PLA nanoparticles prepared previously. The mixtures were incubated for 40 min, and then subjected to centrifugation with Millipore Filter to remove unbound Tf.

The binding of Tf on the nanoparticles was analyzed qualitatively and quantitatively by polyacrylamide gel electrophoresis and BCA protein assay kit, respectively.

### 2.6 Evaluation of glioma tumor targeting of Tf-PEG–PLA nanoparticles

#### *2.6.1 Glioma cell targeting in vitro*

The C6 glioma cell line was a gift from NIH (USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37°C with 5% $CO_2$. Cells ($1 \times 10^5$) were plated in 60 mm cell culture dishes and grown overnight until they were

50–80% confluent. Then the cells were trypsinized with 0.125% (w/v) trypsin/EDTA (pH 8.0) and plated into each well of a 12-well plates to allow growth overnight.

0.1 ml DiI-loaded Tf-PEG–PLA and mPEG–PLA nanoparticles (1 mg/ml) were filtered through 0.22 μm membrane, and then added into each well. After incubating nanoparticles with tumor cells for 8 h, the cells were washed with PBS solution to remove the unbound nanoparticles. The uptake of nanoparticles by tumor cells was assessed by FACSCalibur flow cytometry. The cell populations were gated based on forward/side scatter and propidium iodide fluorescence to remove debris and dead cells from analysis. A total of at least 10,000 gated events were obtained for each sample.

#### 2.6.2 *Tumor animal model and tumor targeting of Tf-PEG–PLA nanoparticles in vivo*

All experimental procedures were carried out according to the regulations and internal biosafety and bioethics guidelines of Tianjin Medical University and the Tianjin Municipal Science and Technology Commission. Six male Sprague–Dawley rats, each weighing 200–250 g, were anesthetized with intraperitoneal 10% chloraldurat (300 mg/kg) and placed in a stereotactic device. Each rat was then inoculated, using a Hamilton microliter syringe, with $10^6$ cultured C6 glioma cells into the cerebrum in the anterior fontanel region, 3 mm to the right of the midline and 5 mm in depth from the surface of the dura. The rats bearing well-established cerebral gliomas, demonstrated by MRI imaging at 6 days post-implantation (Electronic supplementary material), were divided into two groups. Each group consisted of 3 rats. Group 1 received 100 μl DiI-labeled Tf-PEG–PLA nanoparticles suspension via tail vein, while Group 2 received 100 μl DiI-labeled mPEG–PLA nanoparticles. The animals were anaesthetized with 20% urethane, and then were humanely killed at 24 h after intravenous injection. The brains were removed and fixed in a PBS solution of 4% parafornaldehide for 12 h. After that, samples were placed in 20% sucrose for 24 h. The brains were embedded in OCT compound and frozen at −60°C in isopentane. Frozen sections of 5 μm thickness were prepared with cryo-cut Cryostat microtome (DIS, France). The samples were observed by Olympus IX70 Inverted Fluorescence Microscope.

## 3 Results and discussion

Amphiphilic polymers nanoparticles have been gaining increasing attention as drug carriers for the treatment of tumor due to their biodegradability and biocompatibility [17, 18]. Among them, functionalized PEGylated nanoparticles are regarded as a new perspective for the therapy of brain tumor [19, 20] and different strategies have been developed for the functionalization of PEGylated nanoparticles, especially for PEG-PLA nanoparticles. For the synthesizing of functionalized copolymers, biotinylation strategy received major attention due to the high-efficiency and stable coupling between biotin and avidin [21], which permits, usually protein, to be conjugated to nanoparticles under non-denaturing condition [22].

Several papers have reported the synthesis of biotinylated PEG–PLA (B-PEG–PLA) [21, 23–25]. In the present study, a new method was developed for the synthesis of B-PEG–PLA based on the use of the biotin chloride derivative as shown in Scheme 1. Biotin chloride was obtained via thionyl chloride and covalently linked to PEG using TEA as catalyst. B-PEG–PLA was obtained by ring opening polymerization starting from hetero-biotinylated PEG (B-PEG) and lactide. The product was precipitated out from methanol. The byproduct PEG with two biotin endgroups (B-PEG-B) was removed from the final copolymer since it did not react with lactide and was soluble in aqueous media, in contrast to B-PEG–PLA.

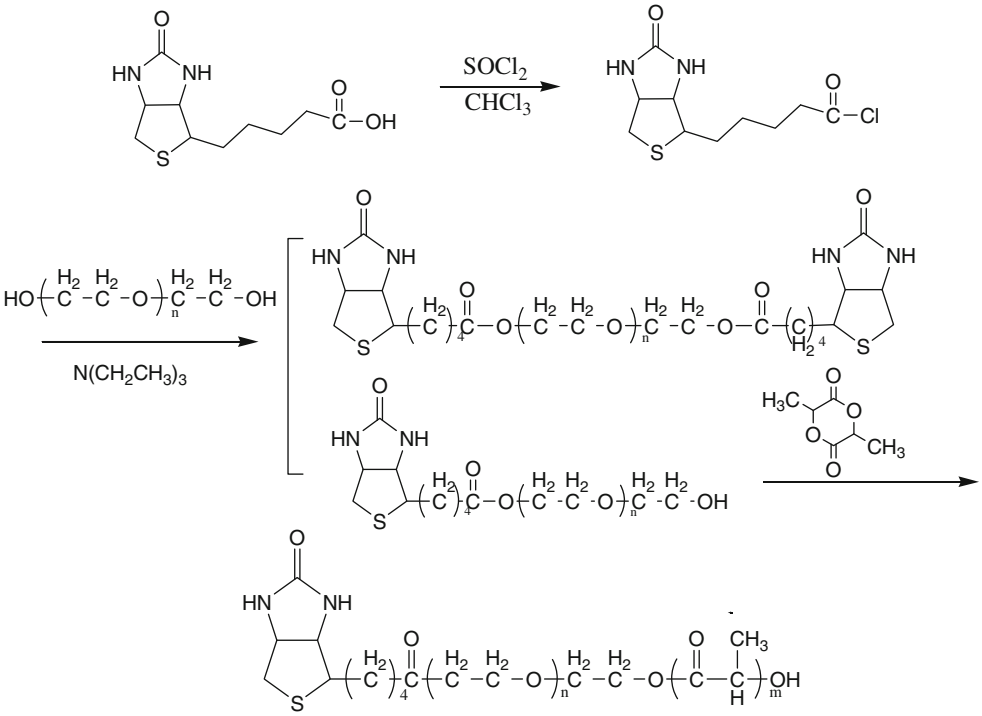
The conjugation of biotin onto PEG was confirmed by analysis of $^{1}$H-NMR and $^{13}$C-NMR spectra. As shown in Fig. 1a, the peak at 3.64 ppm assigns to PEG methine, while the peaks at 4.3 and 4.2 ppm correspond to the methine protons (H-3, H-4) of the biotin cyclic structure. Furthermore, peaks at 5.5 and 5.8 ppm correspond to urea protons of biotin. Figure 2 was $^{13}$C-NMR spectra of PEG-B, the peak at 173.19 ppm assigns to carboxyl of biotin, while the peaks at 161.80 ppm correspond to the acyl of biotin cyclic structure. Furthermore, peaks at 70.32 ppm correspond to the carbon of PEG.

The synthesis of B-PEG–PLA was confirmed by $^{1}$H NMR measurement too. Figure 1b shows the $^{1}$H-NMR spectrum of the B-PEG–PLA copolymer. The peaks at 1.56 ppm ($CH_3$) and 5.18 ppm (CH) attributed to PLA blocks, and the peaks at $\delta = 3.64$ ppm ($CH_2$) should be assigned to the main-chain methylene units in the PEG blocks. The biotin signal appeared at $\delta = 4.3$, 4.2, 5.5 and 5.8 ppm.

The average molecular weight and molecular weight distribution of B-PEG–PLA were measured by GPC chromatogram (Fig. 3). Only a single peak at retention times corresponding to a molecular weight of 8562 (determined by polystyrene calibration standards) with a polydispersity of 1.36 can be found, suggesting the high purity of the B-PEG–PLA product.

Nanoparticles of block copolymers were prepared by the nanoprecipitation method. Binary mixture of acetone and alcohol was used as the solvent of copolymer to increase the yields of nanoparticles, because the adding of alcohol to acetone could accelerate the phase separation rate of the

**Scheme 1** Synthesis of biotinylated PEG–PLA copolymers



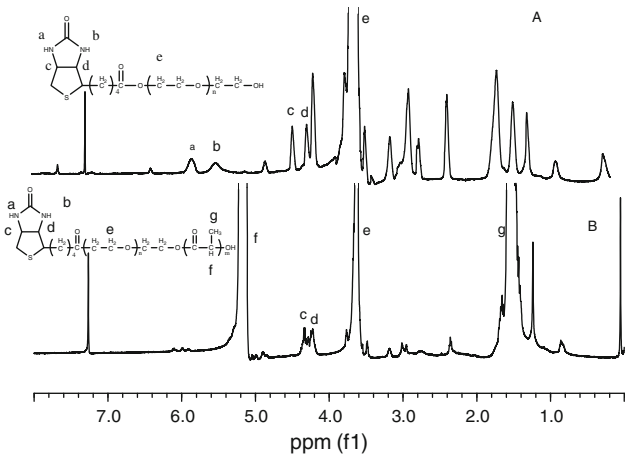




**Fig. 1** **a** $^{1}$H-NMR spectra of PEG-B, and **b** B-PEG–PLA copolymers

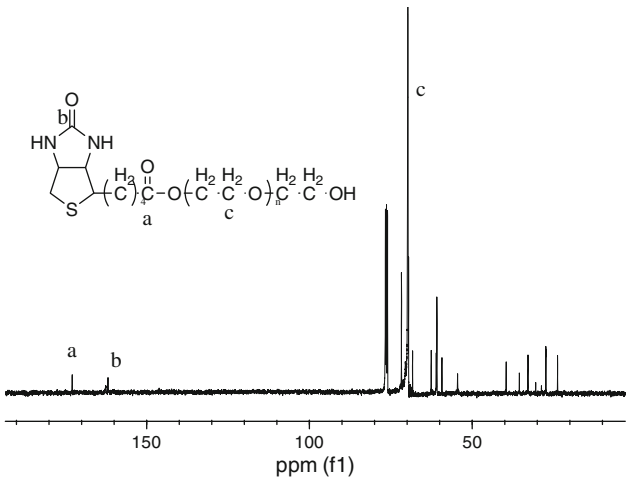



**Fig. 2** $^{13}$C-NMR spectra of PEG-B

copolymers during the dispersion process [26]. A representative TEM photograph of the B-PEG–PLA nanoparticles is shown in Fig. 4. The nanoparticles possessed a spherical shape and a rather broad size distribution ranging from 85 to 100 nm. The average hydrodynamic diameter measured by DLS was about 87.03 nm. The nanoparticles exhibited negative surface charge with zeta-potential values of −13 mV, which is very close to that of mPEG–PLA nanoparticles (−14 mV) but much higher than that of PLA nanoparticles prepared through simple dialysis method (−35 mV) [27], suggesting that the cover of PEG moieties orients itself toward the water phase. Assuming that all PEG chains migrated to the surface to form the coating "brush", it is possible to calculate the distance between two terminally attached PEG chains [28]. In the case of the control PEG–PLA nanoparticles studied here, the calculated distance between two PEG was close to 2 nm, which was shorter than the minimum dimension of avidin(4.5 nm) [29]. Thus, it was impossible that all of the biotin end-groups bound to avidin (in theory the bonding amount of protein could not reach to 100%). In fact, the results of BCA protein assay kit showed that the amount of avidin bound on the nanoparticles surface was 1.22 ± 0.03 mg/μg nanoparticles. That means there were about 15.6 avidin proteins bound on per 100 biotin endgroups. On control PEG–PLA surfaces, the absorption of avidin to the

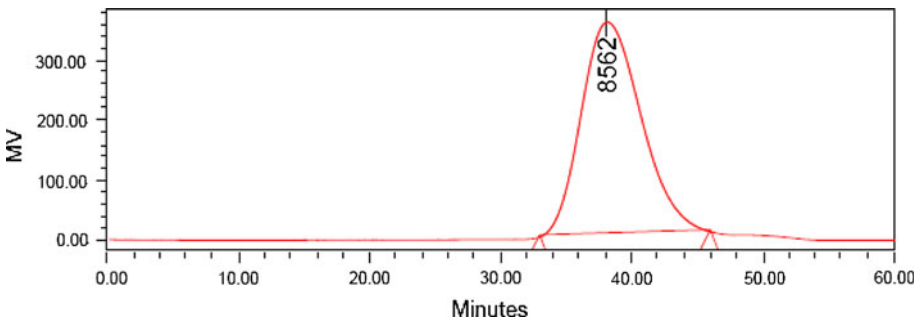



**Fig. 3** Gel permeation chromatograms of B-PEG–PLA copolymer

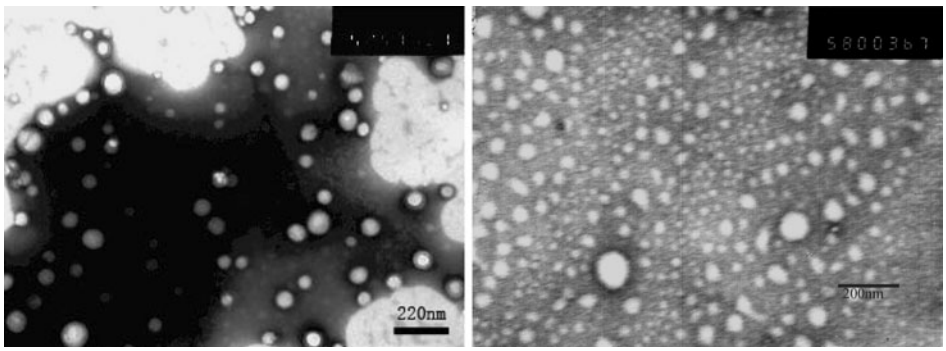



**Fig. 4** TEM micrographs of B-PEG–PLA nanoparticles negatively stained with phosphotungstic acid solution (2%, w/v)

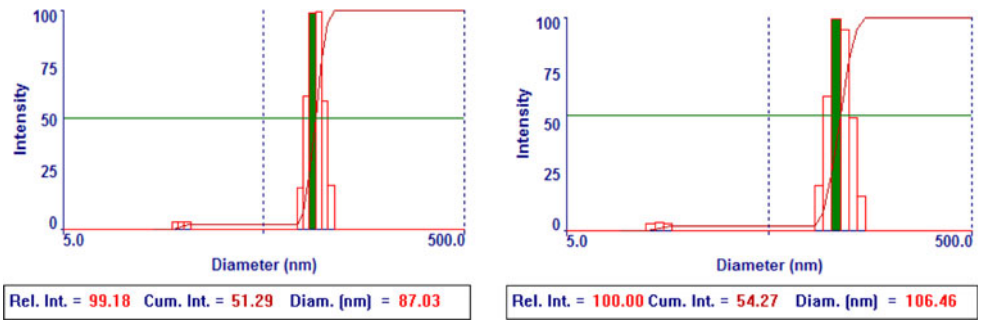



**Fig. 5** Histograms of the size distribution of B-PEG–PLA nanoparticles obtained by DLS measurement

nanoparticles was only 0.070 ± 0.003 mg/μg nanoparticles under identical incubation conditions, indicating that only a small degree of nonspecific protein (6.35%) adsorption to the polymer surface occurred. This data indicates a large amount of specific binding of avidin occurred to the PLA-PEG-biotin surfaces with very little nonspecific adsorption Fig. 5.

After esterified by biotinamidohexanoic acid N-hydroxysuccinimide ester, Tf were coupled onto the nanoparticles by taking advantages of interaction between biotin and avidin. The biotinamidohexanoic acid was used as a spacer to overcome the possible steric hindrance effect of nanoparticles on the binding of Tf to targeting cells or tissues. The binding of Tf on the nanoparticles were qualitatively analyzed by Western blot. As shown in Fig. 6, the amount of protein was obviously reduced after the incubation with avidin-binding B-PEG–PLA nanoparticles. The quantitative measurement indicated that there was 1.081 ± 0.012 mg Tf bound per

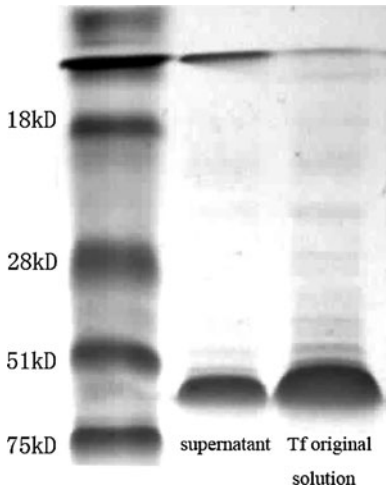



**Fig. 6** Western blot of Tf before and after binding to avidin-modified nanoparticles

microgram of nanoparticles, which means that 79.2% of avidin on the surface of particles was bound with Tf.

A slight increase in diameter (10–20 nm) was observed after the functionalization of nanoparticles. This increase may be attributed to, on the one hand, the size of the protein (3 nm for Tf, and 4.5 nm for avidin, respectively), and on the other hand, the merger of the nanoparticles probably arose from the multi-conjugation site of avidin, as the size distribution of nanoparticles was slightly broadened (Fig. 4b). After removed the aggregates by filtration, the final diameters of the nanoparticles were 95–110 nm. This reduced size is advantageous for cell interaction and tumor uptake. In fact, the fluorescence microscopy observation indicated that both the mPEG–PLA and Tf-coated nanoparticles were easily internalized into glioma tumor cells due to their small size. However, the Tf-coated nanoparticles showed higher uptake in comparison to PEG-coated nanoparticles. The flow cytometry measurement demonstrated that the C6 cells' uptake of Tf-coated nanoparticles (92.8 ± 2.5%) was significantly higher than PEG-coated nanoparticles (68.3 ± 3.1%) (Fig. 7) ($P < 0.01$, $t$-test).

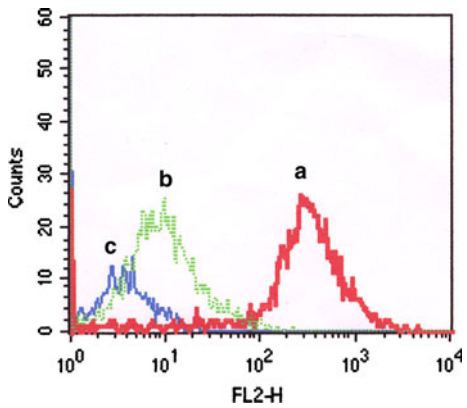



**Fig. 7** Flow cytometry analysis of C6 cells incubated with Tf-PEG-PLA (**a**), mPEG–PLA (**b**), or PBS control (**c**)

The Tf coating endowed the nanoparticles with the ability to targeting the glioma tumor in vivo. Figure 8 indicates the strong fluorescence in the groups of the rats treated with Tf-PEG-NPs. However, no obvious fluorescence was detected in the group treated with PEG-NPs. Such discrimination from Tf-coated nanoparticle groups illustrated that brain tumor targeting corresponded to the Tf functionalization on the nanoparticles. It seems that the Tf-coated nanoparticles prefer to accumulate in the tumor. According to Fig. 8a and b, it can be recorded that the fluorescence remains confined to the tumor, and no fluorescence could be found in the normal brain tissue. These results seem inconsistent with the previous reports that Tf can mediate the nanoparticles penetrating through BBB and enter the brain parenchyma [30, 31], because the receptor of Tf expressed not only on the surface of tumor cells but also on that of capillary endothelial cells of BBB. In fact, there are two Tf receptors exist in vivo, Tf receptor 1 (TfR1) and receptor 2 (TfR2), but the affinity of TfR2 for

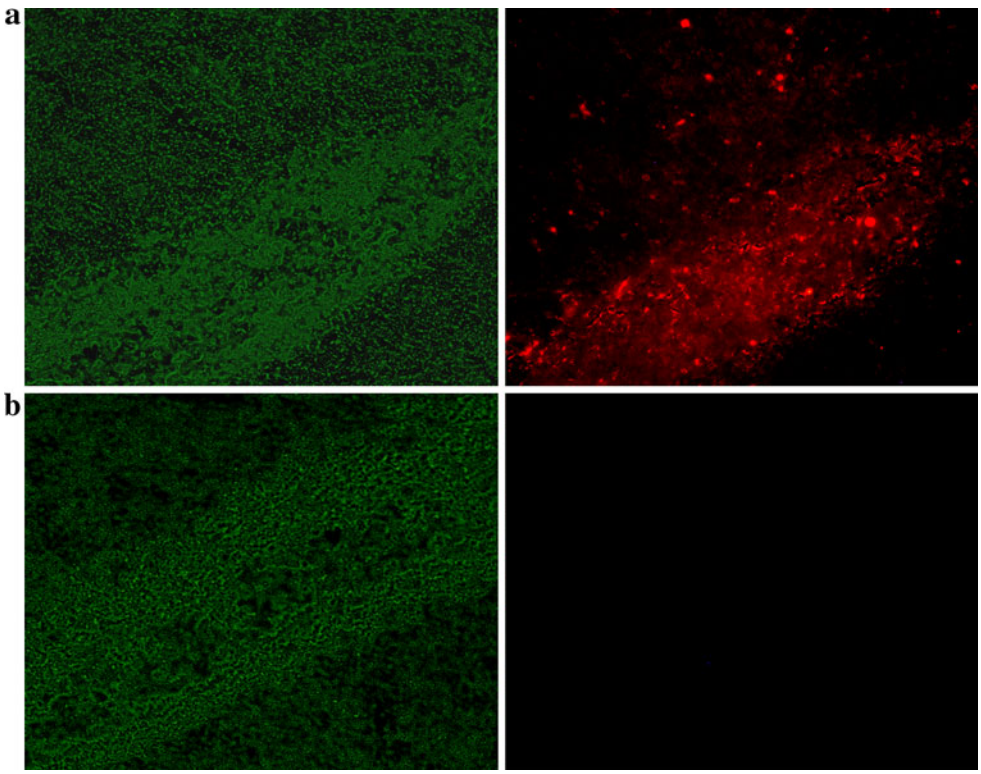



**Fig. 8** Phase contrast and fluorescence micrographs of SD rats' brain frozen sections after nanoparticles injection via tail vein for 24 h. **a** Tf-PEG–PLA nanoparticles. **b** B-PEG–PLA nanoparticles. The zone with heavy cell distribution was glioma tumor

iron-loaded Tf is 25-fold lower than that of TfR1 for Tf [32]. The TfR1 appears to be expressed in all nucleated cells in the body, such as red blood cells, erythroid cells, hepatocytes, intestinal cells, monocytes (macrophages), brain, BBB, blood–testis and blood–placenta barriers, etc., but it differs in levels of expression [33, 34]. It is over-expressed on rapidly dividing cells, such as brain tumor cells, especially glioblastoma multifore. In contrast, in nonproliferating cells, expression of TfR1 is lower or frequently undetectable.

For long-circulating PEGylated nanoparticles, their accumulation in tumors is mainly ascribed to the enhanced permeability and retention (EPR) effect, which depended on the leakiness of the tumor microvasculature. The proliferation and invasion of glioma tumoral cells generally cause a local disruption of the BBB, leading to the improvement of the permeability of the blood-tumor interface, and consequently, the penetration of the nanoparticles. There have been a few papers about the retention of nanoparticles in brain tumors due to the EPR effect, and representative samples are polysorbate 80-coated or PEGylated PHDCA nanoparticles [35, 36]. But the fluorescence observation of present study seems to disagree with these reports, because nearly no fluorescence of the PEG-coated nanoparticles was detected in the tumor area. The reason might be due to the lower efficiency of the EPR effect, compared with the ligand–receptor-mediated endocytosis.

## 4 Conclusion

Biotinylated PEG–PLA copolymers were synthesized. The resultant copolymers were employed to prepare the nanoparticles with biotin groups on the surface through nanoprecipitation method. Avidin, followed by biotinylated transferrin, was coupled onto the nanoparticles via biotin-avidin complex formation, endowing the nanoparticles with ability to targeting the glioma tumor both in vitro and in vivo. The present results strongly suggest that the biotinylated PEG–PLA nanoparticles can be applied as a promising carrier for the drug delivery targeting to the glioma tumor.

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