Macromolecular Research, Vol. 21, No. 4, pp 435-441 (2013)
DOI 10.1007/s13233-013-1029-2

www.springer.com/13233
pISSN 1598-5032 eISSN 2092-7673

Cell-Penetrating Peptide-Modified PLGA Nanoparticles for Enhanced Nose-to-Brain Macromolecular Delivery

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Received April 1, 2012; Revised May 21, 2012; Accepted May 23, 2012

Abstract: Macromolecular drugs become an essential part in neuroprotective treatment. However, the nature of ineffective delivery crossing the blood brain barrier (BBB) renders those macromolecules undruggable for clinical practice. Recently, brain target *via* intranasal delivery have provided a promising solution to circumventing the BBB. Despite the direct route from nose to brain (*i.e.* olfactory pathway), there still are big challenges for large compounds like proteins to overcome the multiple delivery barriers such as nasal mucosa penetration, intracellular transport along the olfactory neuron, and diffusion across the heterogeneous brain compartments. Herein presented is an intranasal strategy mediated by cell-penetrating peptide modified poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles for the delivery of insulin to the brain, a potent therapeutic against Alzheimer's disease. The results revealed that the cell-penetrating peptide can potentially deliver insulin into brain *via* the nasal route, showing a total brain delivery efficiency of 6%. It could serve as a potential treatment for neurodegenerative diseases.

Keywords: cell-penetrating peptide, intranasal delivery, brain target, insulin, PLGA nanoparticles.

Introduction

Peptide and protein drugs have become an essential part of modern medicine, owing to the high activity and specificity, and unique benefits to clinical practice. As a case in point, macromolecular agents are commonly used for neurodegenerative diseases. However, ineffective penetration through the blood brain barrier (BBB) is the primary challenge for the clinical application of these macromolecules, due to their large size and the unstable structure. It therefore generally requires the most invasive method for drug administration–intracranial injection. Thus, the therapeutic values of protein drugs have been restricted because of the inability to transport into brain *via* convenient dose administration.[1]

Nose-to-brain route has attracted much attention for its unique benefit for bypassing the formidable BBB and delivering macromolecules into brain *via* non-invasive pathway, where there is the direct association between nasal cavity and central nervous system in anatomical physiology. Other advantages of nasal drug delivery include the reduced drug degradation and side effects, due to the minor systemic exposure. However, on account of the mucous membrane permeability and cilia clearance system, the bioavailability of protein drugs with nasal administration was about 0.01%-0.1%, which is difficult to achieve the therapeutic effective concentrations.[2]

Cell-penetrating peptides (CPPs) are short cationic peptides with specific conserved sequence, and CPPs have been widely used in drug delivery for the capacity of transporting cargoes into cells.[3] The most commonly used CPP is Tat peptide, derived from the protein transduction domain of the human immunodeficiency virus Tat protein. The 11-amino acid YGRKKRRQRRR of Tat was the minimal sequence responsible for cell penetration. Not only can Tat itself penetrate cell membrane highly efficiently, but it also could carry the macromolecular drugs that are even 100-fold bigger than Tat in size to cross the membrane.[4,5] This property is potential used for the delivery of large molecular such as peptide or protein drugs. More interestingly, Tat peptide can mediate protein delivery in a non-covalent manner.[6,7]

Insulin has been long since confirmed to play an important role in neurodegenerative diseases.[8,9] Intranasal inhaling insulin has been demonstrated its ability to improve memory and slow decline of cognitive function in Alzheimer's disease, and the positive results from the clinical trial have garnered a great deal of attention recently.[10] In this study, a Tat-mediated poly(lactic-*co*-glycolic acid) (PLGA) nano-

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particles were developed for brain targeting through intranasal delivery of insulin.

Experimental

Materials, Cells and Animals. PLGA (50/50, M_w=11,000 Da, COOH terminated) was purchased from Daizhi Co. (Jinan, China), poloxamer188 (P188) from BASF Co. (USA), insulin from Wanbangjinaqiao Co. (Xuzhou, China). Caco-2 cells were kindly provided by Fudan University. Cell culture media included Dulbecco's Modified Eagle's medium (Gibico Co., USA), certified fetal bovine serum (FBS), non-essential amino acids (Hyclone Co., USA) and 0.25% trypsin-EDTA (Sigma-Aldrich Co., USA). Methanol and acetonitrile were from Merck.

KM mice were housed under standard conditions of temperature (22-24 ºC), humidity (40%-60%), and 12 h light/dark-cycles. Food and water supply were freely accessed for animals. All animal experiments were carried out in accordance with a protocol approved by the Laboratory Animal Management Committee, SIMM.

Preparation of Insulin-Loaded Nanoparticles (NPs). Insulin-loaded PLGA NPs were prepared by a modified double emulsion method.[11] Briefly, a predetermined amount of insulin was dissolved in Tris buffer as internal phase, and PLGA in acetone as oil phase. Internal phase was slowly added to external oil phase. The dispersion was then vigorously stirred to obtain the primary emulsion, which was subsequently poured into the P188 solution to achieve the secondary W/O/W emulsion. The organic solvent was removed by vacuum evaporation, and the insulin-loaded NPs were obtained. Blank NPs were prepared by the same method.

Development of the Insulin-Loaded NPs Assay. The encapsulation efficiency of insulin in the NPs was determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology, China). The standard protein (1 mg/mL) was diluted to different concentrations, protein standards are processed in the same manner by mixing them with assay reagent and using a spectrophotometer to measure the absorbance, thus a regression equation was obtained.

The insulin released from the NPs was examined by high-pressure liquid chromatography (HPLC, Agilent-1260, Agilent Technologies, USA) with a Grace C18 column (4.6 mm ×250 mm, 5 μm) at 40 ºC (mobile phase: sodium sulfate buffer (pH 2.3)/acetonitrile, 73.5:26.5), and the detection wavelength of 214 nm.

Measurement of Encapsulation Efficiency. After ultracentrifugation, the concentration of the non-entrapped insulin in the supernatant was determined by BCA method. The encapsulation efficiency X was calculated as the formula below:

$$X(\%)=(C-C_{free})/C\times 100\%$$

where C was the amount of added insulin, and C_{free} was the amount of free insulin in the supernatant.

Table I. Factors and Levels of $L_9(3^4)$ Orthogonal Experiment

Levels	Factors			
	A P188 (%)	B PLGA (mg)	C Acetone: Ethyl Acetate	D Oil: Water Phase
1	0.2	20	2:1	1:10
2	0.5	25	1:1	1:15
3	1	30	1:0	1:20

Optimization Process with Orthogonal Experiment. The process parameters, including concentration of emulsifier, PLGA concentration, oil phase composition, and phase volume ratio were optimized by orthogonal design $L_9(3^4)$ using entrapment efficiency and particle size as indexes (Table I). Then after analysis of variance using weighted scoring method, main factors and optimal prescription were determined.

Synthesis of Tat-Conjugated Insulin Loaded PLGA-NPs. NPs were mixed with equal amount of Tat (YGRKKRRQRRR, 4 mg/mL), and then reacted on a rotary shaker (80 r/min) for 4 h in dark. The reaction solution was purified by dialysis (M_w=12,000 Da) and the Tat-modified NPs were obtained.

Characterization of the NPs. The morphology of the NPs was observed using transmission electron microscopy (TEM). Samples were prepared by dropping onto a copper grid that pre-coated with a layer of formvar film, and then stained by uranyl acetate solution before measurement.

The particle size, size distribution and ζ-potential were measured by Nano-ZS ZEN3600 (Malvern Instruments) at 25 ºC.

***In vitro* Release Study.** The NPs containing 1 mg of insulin were placed into 20 mL of phosphate buffer saline (PBS) pH 7.4 at 37 ºC with gently constant shaking. At predetermined time intervals, aliquots were withdrawn with replenishment of equal volume of PBS. The insulin concentration was determined by the HPLC method.

Cellular Uptake Study. The cells were cultured with DMEM containing 10% FBS, 1% NEAA, 1% *L*-glutamine at 5% CO_2, 37 ºC. To study the kinetics of cellular uptake, the PLGA-NPs were incubated with Caco-2 cells for 4 h. After washing with PBS three times, the cells were fixed with 4% paraformaldehyde, stained with DAPI, mounted with glycerol. The fluorescence was observed with a confocal laser scanning microscope (Nikon C1 Plus, Japan).

The uptake efficiency of the fluorescein-labeled NPs was quantitatively measured by fluorescence-activated cell sorting (FACS). In brief, Caco-2 cells were exposed to the culture medium containing the NPs for 4 h. After washing with PBS, the cells were collected by trypsinization and centrifugation (1000×*g*, 10 min), and the cell pellets were washed with PBS, alternating wash and centrifugation for three time. The cells were subjected to fluorescence analysis with

flow cytometer (BD, USA) using 480 nm laser excitation and 520 nm emission filter.

Brain Delivery *via* Intranasal Administration. For intranasal administration, mice were administered 20 μL of the fluorescein-labeled NPs into each nostril. At 4 h post-treatment, cardiac perfusion was conducted, and the cryosection of the brain tissues was carried out and the slides were observed under fluorescence microscope (Olympus IX70, Japan). In the meanwhile, to quantitatively evaluate the brain delivery efficiency, the fluorescence intensity of the homogenized brain tissues was determined by a fluorescence plate reader (Bio-Rad, USA), and the brain delivery efficiency calculated as follows.

$$\text{Brain delivery efficiency (\%)}=\frac{\text{Dose}_{(brain)}}{\text{Dose}_{(administration)}}\times 100$$

Statistical Analysis. Statistical analysis was performed using a student's *t*-test. The data from the experiments express the mean±S.D.

Results and Discussion

The double emulsion solvent evaporation method was used for preparation of the PLGA NPs, due to its compatibility with hydrophilic drugs, and easy process. Particle size, zeta potential and encapsulation efficiency were measured for evaluation of the PLGA NPs. With orthogonal experiment, the levels of each factor (PLGA amount, P188, the ratio oil components and oil phrase and external phrase volume ratio) were optimized (Tables II and III). The elevating concentration of P188 (>0.5%) directly contributed to the reduction in particle size. Another contributing factor was the ratio of oil phrase and external phrase, and when it was > 1:15 v/v, particle size decreased. It should be noted that despite the difference in size, particles possessed similar zeta potential, approximately -10 mV. In addition, high stirring speed (> 600 r/min) was favorable to the formation of small particles. Hence, the P188 concentration, oil phrase and external phrase volume ratio as well as stirring speed were optimized for obtaining small particle size.

Based on the orthogonal experiments, the prescription and process were optimized as follows: PLGA was 30 mg, oil phase acetone, oil phrase/external phrase ratio 1:15 v/v, and the P188 concentration of 0.5%. Three batches of the NPs were then prepared, in which the encapsulation efficiency of insulin was measured to be 76.8%, and drug loading effi-

Table II. Results of Orthogonal Design

Factors / No.	A	B	C	D	Particle Size (nm) *y*1	EE (%) *y*2	*y*
1	1	1	1	1	209.7	64.74	79.32
2	1	2	2	2	218.5	72.61	80.62
3	1	3	3	3	205.4	66.42	84.01
4	2	1	2	3	197.1	63.14	83.36
5	2	2	3	1	175.6	68.27	95.74
6	2	3	1	2	200.8	75.38	88.68
7	3	1	3	2	199.1	70.75	87.20
8	3	2	1	3	224.7	64.05	72.89
9	3	3	2	1	216.3	69.89	79.78
K1	81.317	83.293	80.293	84.947			
K2	89.260	83.080	81.253	85.500			
K3	79.953	84.157	88.983	80.083			
R	9.307	1.007	8.690	5.417			

Table III. Variance Analysis of Orthogonal Design

Source of Variance	Square of Deviance	Degree of Freedom	F Ratio	P Value	Source of Variance
A	151.569	2	77.728	19.000	*P<0.05
B	1.950	2	1.000	19.000	
C	136.191	2	69.842	19.000	*P<0.05
D	53.298	2	27.332	19.000	*P<0.05
Error	1.950	2			

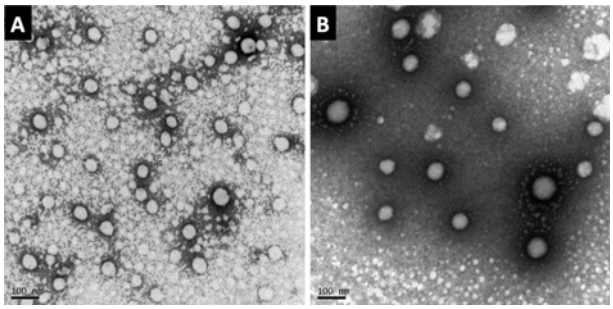

Figure 1. TEM photograph of the insulin-loaded NPs (A) and insulin-loaded Tat-NPs (B).

ciency 13.2%.

The Tat modification of the PLGA NPs was conducted *via* charge affinity. Surface modification with cationic hydrophilic biomaterials (*e.g.*, chitosan,[12] PEI,[13] and poly(*L*-lysine)[14]) through charge interaction is widely used in PLGA-based delivery. The introduction of hydrophilic property on the surface of the PLGA NP would be helpful. Due to the strong ionic affinity, it is enough to ensure a stable polyelectrolyte-coating process. A unique benefit of the coating method is that chemical processes are not required, and the efficiency and reproducibility can be monitored by zeta potential and size measurement. In this system, the free carboxyl end groups on the PLGA NPs rendered high net negative charge, and thus were electrostatically bound with the cationic Tat. Homogeneous morphology of the Tat-NPs and unmodified NPs was observed by TEM, as showed in Figure 1. The particle size and zeta potential of the NPs were determined by DSL. The size of unmodified NPs was 189 nm, and zeta potential -10.4 mV (Figure 2(A) and (B)). With modification of Tat, the particle size slightly increased and the zeta potential flipped over to positive charge, +11.3 mV (Figure 2(B)).

Due to the unstable nature of insulin, the *in vitro* release experiment was conducted in a period of 24 h, showing a cumulative release of 23% (Figure 2(C)).

The cellular uptake efficiency in Caco-2 cells is shown in Figure 3. The intracellular delivery efficiency of insulin by the Tat-NPs was greatly enhanced, displaying 4.5-fold higher according to the FACS analysis compared to the unmodified NPs. The confocal imagings show significant intracellular accumulation of insulin delivered by the Tat-NPs. The capacity of CPPs for mediating cellular uptake of nanocarriers has been widely confirmed.[15] Echoed with the already reported findings, our results showed that Tat modification was a useful strategy for mediating cellular uptake of nanocarriers. It should be pointed out that the mechanisms of CPP-

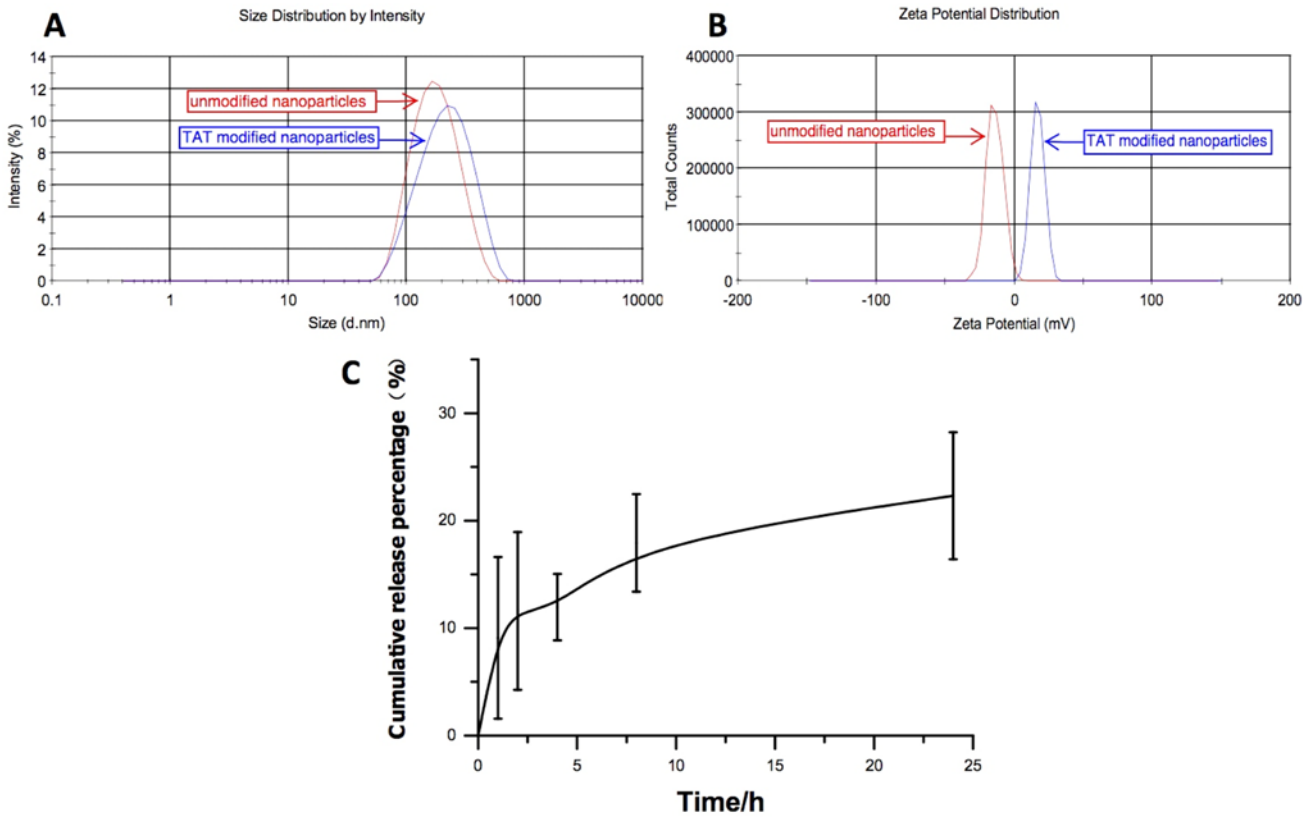

Figure 2. Characterization of the insulin-loaded NPs: (A) particle size distribution, (B) zeta potential, and (C) the *in vitro* release profile of the Tat-NPs.

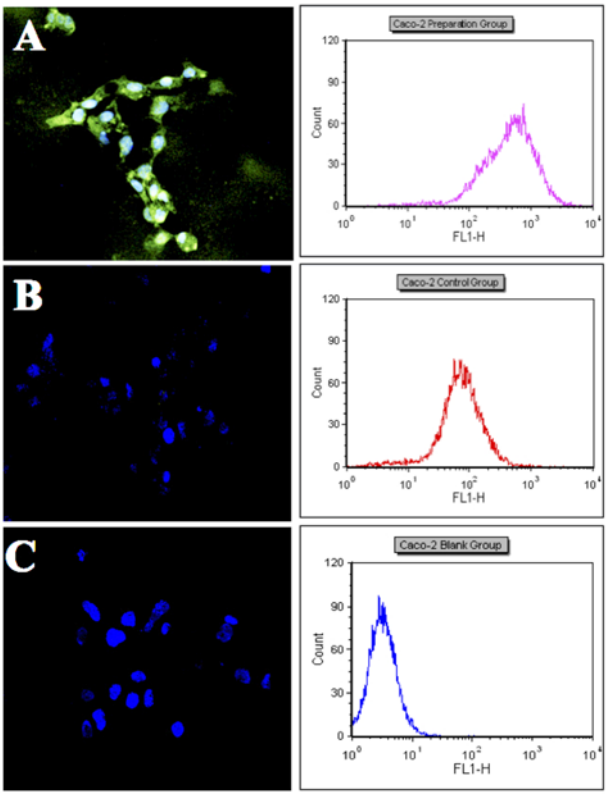

Figure 3. Cellular uptake efficiency in Caco-2 of (A) the Tat-NP group, (B) the unmodified NP group, and (C) the control group.

based delivery are complicated, and largely remain unknown. A common explanation is that the cell penetration probably simultaneously involves several pathways, such as endocytosis, energy-independent direct translocation,[16] and pore formation.[17]

Further, *in vivo* experiments were conducted to investigate the feasibility of intranasal transport of the insulin-loaded

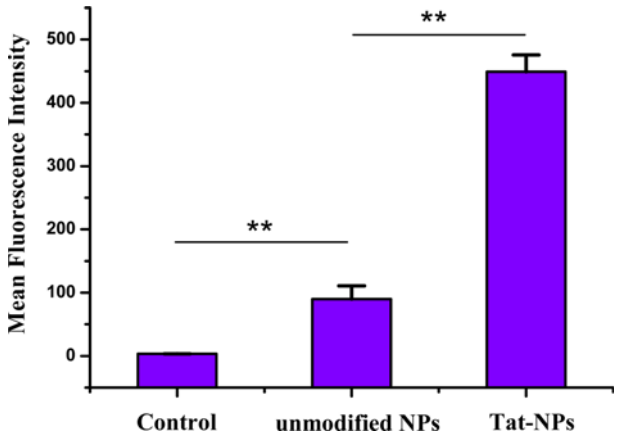

Figure 4. Quantitative analysis of Caco-2 cellular uptake (Represented by mean fluorescence intensity, $^{**}P<0.01$, n=3).

Tat-NPs into the brain. Figure 4 shows the drug deposition of the NPs in the brain after intranasal NPs administration to the mice. The unmodified NPs fluorescence was hardly observed, indicating its poor penetration from nose to brain, while the Tat-NPs showed a gradient penetration across the brain compartments (olfactory bulb and cerebrum), with visible fluorescence (Figure 5(A)-(D)).

Compared to 0.405% of drug disposition in olfactory bulb for the unmodified NPs, the Tat-NPs showed a significant increase (2.64%, Figure 5(E)). Similar pattern of drug disposition was observed in cerebrum, where the Tat-NPs accumulated at significantly higher concentration than the unmodified NPs (3.36% *vs.* 0.95%), indicating the promise of the Tat-NPs for brain delivery of macromolecules. A total accumulated dose in the brain (olfactory bulbs and cerebrum) reached 6%.

The delivery efficiency was characterized by the NPs, instead of insulin itself. However, the penetration along olfactory

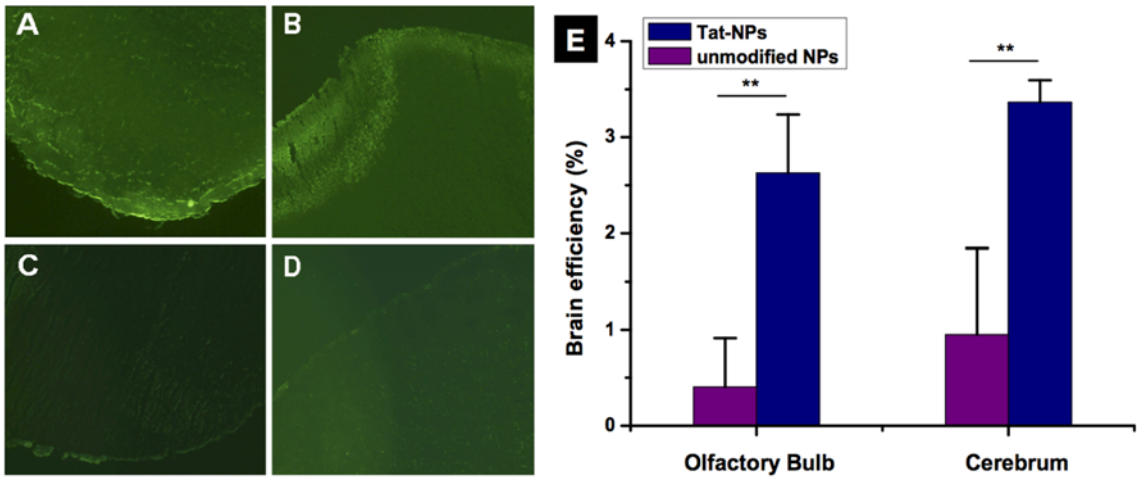

Figure 5. Fluorescence Imaging of the Tat-NP group in olfactory bulb and cerebrum (A and B) and the unmodified NP group (C and D), and the efficiency of brain uptake *via* intranasal pathway (E) ($^{**}P<0.01$, n=5).

pathway to brain occurred merely within a few hours. Over this duration, because the NPs are exposed to relatively less liquid environment (not the typical sink condition), nonspecific leakage of insulin from NP should be very limited in the olfactory pathway, and the delivery efficiency of NPs could represent that of its loaded drug. A subsequent fast drug release would be triggered once entering the brain where the polymer biodegradation is stimulated by the abundant enzymes, because of the major role of enzymatic degradation.[18,19]

Poor bioavailability of peptides and proteins is typically found in drug development. Such problem is primarily due to the low permeability to biomembrane, largely limiting the intracellular delivery. CPPs have been widely applied for assisting intracellular delivery, and already achieved great success in many aspects. However, the *in vivo* application hardly echoes with the success made in cellular experiments. As a case in point, the ability of CPP-mediated penetration through the BBB has been well documented.[20,21] However, the non-selective nature of CPP renders the *in vivo* penetration going wide everywhere, leading to a helplessly low efficiency of brain target. In addition, the nonspecific distribution poses risk of causing severe side effects. In this regard, the intranasal administration for brain delivery is ideal for limiting the CPP-mediated nonselective distribution by minimizing its systemic exposure. Also, other benefits of nose-to-brain delivery include non-invasive dosing, no liver first-pass effect and the consequent improved bioavailability, and rapid onset of action.

Nose-to-brain delivery confronts multiple bio-barriers, where there are heterogeneous compartments needed to be penetrated through before reaching the destination of the brain. Under this circumstance, in order to overcome the multi-leveled biobarriers, a versatile drug carrier is needed. As mentioned, CPPs, displaying all-round penetration ability, ideally provide a solution to develop such a versatile delivery system. Due to charge interaction, direct conjugation of insulin and CPP is difficult, and indeed we previously found the charge-governed binding between the cationic low molecular weight protamine peptide and insulin readily induced large aggregation.[22] Furthermore, it is also a big challenge for purification to separate the chemical conjugates of insulin-CPP from the physical complex.[23] Hence, in this study, we incorporated insulin into the PLGA NPs, which were subsequently modified with Tat. The encapsulation not only can physically prevent direct interaction of Tat/insulin, but also protect the susceptible insulin from enzymatic degradation in the biological fluids.

It should be pointed out that in this study, we carried out a new attempt to modify the PLGA NPs with Tat *via* electrostatic binding. Our resulted showed that Tat successfully facilitated the PLGA NPs for intranasal delivery, echoing with the recent findings that Tat was even able to mediate transdermal protein delivery in non-covalent manner.[6,7] Another typical case is that CPP/gene complexes *via* electronic binding for intracellular delivery. Although the mechanisms are not clear, in these cases the association patterns between Tat and its cargo may not play the determined role in delivery. An advantage of the non-covalent modification is its easy and simple process, consequently avoiding the undesired protein denature caused by chemical reaction.

For *in vitro* release study, the cumulative release reached 23% in a course of 24 h. Owing to the unstable nature of insulin and its inclination of aggregating with Tat, indeed precipitations were found after 24 h, which could be the denatured insulin. Thus, further observation of its release profile could not be achieved. Despite of the relatively low release rate *in vitro*, the drug release *in vivo* is expected to be higher because the decomposition of the NPs would accelerate by the enzymes and other lipid components in the body.

The biodegradability of PLGA is well documented,[24] and due to its excellent biocompatibility, PLGA is the FDA-approved medical materials for human use. PLGA nanoparticles for brain delivery application has also been demonstrated to be safe.[25,26] Although further studies with optimized experimental procedures should be carried out, our results provide a proof-of-concept of intranasal delivery of insulin to the brain, indicating its potential application in treating Alzheimer's disease.

Conclusions

CPP-mediated macromolecular drug delivery has been well documented for its eminent capacity of intracellular delivery, as well as its ability to penetrate through various biobarriers. A Tat-modified PLGA NP system was developed in the work for brain delivery of insulin. The efficiency of intracellular delivery and brain delivery was investigated, and the results showed that this system is potential for delivering macromolecules into brain. It could be a novel treatment for combating neurodegenerative diseases.

Acknowlegdgments. This work was supported by the National Natural Science Foundation of China (NSFC) (91029743, 81172996), and partially funded by Shanghai Pu-jiang Scholarship and School of Pharmacy, Fudan University & The Open Project Program of Key Lab of Smart Drug Delivery (Fudan University), Ministry of Education & PLA, China.

References

(1) L. T. Loftus, H. F. Li, A. J. Gray, C. Hirata-Fukae, B. A. Stoica, J. Futami, H. Yamada, P. S. Aisen, and Y. Matsuoka, *Neuroscience*, **139**, 1061 (2006).
(2) J. D. Suman, *Expert Opin. Biol. Ther.*, **3**, 519 (2003).
(3) Y. Huang, Y. S. Park, J. Wang, C. Moon, Y. M. Kwon, H. S.

Chung, Y. J. Park, and V. C. Yang, *Curr. Pharm. Des.*, **16**, 2369 (2010).
(4) U. Langel, *Cell-Penetrating Peptides*, Springer, New York, 2010.
(5) J. H. Liu, Y. X. Zhao, Q. Q. Guo, Z. Wang, H. Y. Wang, Y. X. Yang, and Y. Z. Huang, *Biomaterials*, **33**, 6155 (2012).
(6) Y.-H. Wang, C.-P. Chen, M.-H. Chan, M. Chang, Y.-W. Hou, H.-H. Chen, H.-R. Hsu, K. Liu, and H.-J. Lee, *Biochem. Biophys. Res. Commun.*, **346**, 758 (2006).
(7) Y.-W. Hou, M.-H. Chan, H.-R. Hsu, B. R. Liu, C.-P. Chen, H.-H. Chen, and H.-J. Lee, *Experimental Dermatology*, **16**, 999 (2007).
(8) E. Cohen and A. Dillin, *Nat. Rev. Neurosci.*, **9**, 759 (2008).
(9) Z. Laron, *Arch. Physiol. Biochem.*, **115**, 112 (2009).
(10) S. Craft, L. D. Baker, T. J. Montine, S. Minoshima, G. S. Watson, A. Claxton, M. Arbuckle, M. Callaghan, E. Tsai, S. R. Plymate, P. S. Green, J. Leverenz, D. Cross, and B. Gerton, *Arch. Neurol.*, **69**, 29 (2012).
(11) C. E. Astete and C. M. Sabliov, *J. Biomater. Sci. Polym. Ed.*, **17**, 247 (2006).
(12) R. Yang, W. S. Shim, F. D. Cui, G. Cheng, X. Han, Q. R. Jin, D. D. Kim, S. J. Chung, and C. K. Shim, *Int. J. Pharm.*, **371**, 142 (2009).
(13) G. F. Liang, Y. L. Zhu, B. Sun, F. H. Hu, T. Tian, S. C. Li, and Z. D. Xiao, *Nanoscale Res. Lett.*, **6**, 447 (2011).
(14) S. H. Kim, J. H. Jeong, K. W. Chun, and T. G. Park, *Langmuir*, **21**, 8852 (2005).
(15) V. P. Torchilin, *Adv. Drug Deliv. Rev.*, **60**, 548 (2008).
(16) G. Ruan, A. Agrawal, A. I. Marcus, and S. Nie, *J. Am. Chem. Soc.*, **129**, 14759 (2007).
(17) A. Som, A. O. Tezgel, G. J. Gabriel, and G. N. Tew, *Angew. Chem. Int. Ed. Engl.*, **50**, 6147 (2011).
(18) Q. Cai, G. Shi, J. Bei, and S. Wang, *Biomaterials*, **24**, 629 (2003).
(19) H. Pan, H. Jiang, and W. Chen, *Biomaterials*, **29**, 1583 (2008).
(20) S. R. Schwarze, A. Ho, A. Vocero-Akbani, and S. F. Dowdy, *Science*, **285**, 1569 (1999).
(21) G. P. Dietz and M. Bahr, *Brain Res. Bull.*, **68**, 103 (2005).
(22) H. He, A. David, J. Zhang, Y. Park, J. Wang, Y. Huang, J. Wang, and V. Yang, *Macromol. Res.*, **19**, 1224 (2011).
(23) J. F. Liang and V. C. Yang, *Biochem. Biophys. Res. Commun.*, **335**, 734 (2005).
(24) R. C. Mundargi, V. R. Babu, V. Rangaswamy, P. Patel, and T. M. Aminabhavi, *J. Control. Release*, **125**, 193 (2008).
(25) Z. Wen, Z. Yan, R. He, Z. Pang, L. Guo, Y. Qian, X. Jiang, and L. Fang, *Drug Deliv.*, **18**, 555 (2011).
(26) B. Semete, L. Booysen, Y. Lemmer, L. Kalombo, L. Katata, J. Verschoor, and H. S. Swai, *Nanomedicine*, **6**, 662 (2010).