Smart Therapeutic Nanoparticles

DOI: 10.1002/ange.201403846

Angiopep-Conjugated Electro-Responsive Hydrogel Nanoparticles: Therapeutic Potential for Epilepsy**

Xiaoying Ying, Yi Wang, Jiao Liang, Jiaxing Yue, Cenglin Xu, Lina Lu, Zhenghao Xu, Jianqing Gao, Yongzhong Du, and Zhong Chen**

Abstract: *A safe and effective therapy for epilepsy requires a drug delivery system that can penetrate the blood–brain barrier and subsequently release antiepileptic drugs rapidly to suppress neuronal discharges in a timely manner. We have developed electro-responsive hydrogel nanoparticles (ERHNPs) modified with angiopep-2 (ANG) to facilitate the delivery of the antiepileptic drug phenytoin sodium. The resulting ANG-ERHNPs had an average diameter of (102.3 ± 16.8) nm and were electro-sensitive with regard to particle size and drug release in vitro. ANG-ERHNPs have the characteristics of penetrate the BBB easily, resulting in a higher distribution in the central system. The improved antiepileptic effects were investigated with the amygdala kindling model. The results demonstrate that the ANG-ERHNPs were able to transport antiepileptic drugs into the brain and release them under electroencephalograph epileptiform abnormalities to greatly improve the therapeutic index of existing drugs in clinical use.*

Epilepsy is one of the most prevalent neurological disorders, affecting nearly 1% of the population. For most patients, antiepileptic drugs (AEDs) are the mainstay of the management of epilepsy.[1] However, the relatively narrow therapeutic window and low permeability across the blood–brain barrier (BBB) for conventional AEDs, such as phenytoin sodium (PHT), result in intolerable side effects, and drug resistance, and further the inability of completely controlling the seizures.[2,3] Thus, a safe and effective therapy for epilepsy requires a drug delivery system that can penetrate the BBB and subsequently release antiepileptic drugs rapidly to suppress neuronal discharges in a timely manner.

In the last few years, a number of nanostructured drug delivery carriers have been developed and explored to transport drugs to the brain, including polymeric nanoparticles,[4–6] liposomes,[7,8] polymeric micelles,[9] and dendrimers.[10] Although numerous drug delivery carriers were developed to transport antiepileptic drugs to the brain,[11–13] only a few of these carriers advanced due to the physiological and pathological characteristics of epileptic seizures. Stimuli-responsive "smart" hydrogels have attracted great interest in the fields of biotechnology and biomedicine over the last two decades.[14] Among these materials, electro-responsive polymers can transform electrical energy into mechanical energy and have been successfully used to trigger the release of molecules from polymeric bulk materials or implantable electronic delivery devices.[15] The variation of osmotic pressure based on the voltage-induced directional motion of ions in the solution caused the gel to be deformed and the drug to be released.[16,17] Epileptic seizure is a paroxysmal abnormality in the synchronization of the electrical activity in brain neurons, which reflect voltage fluctuations resulting from ionic current flows within the neurons of the brain.[18] Therefore, constraining the paroxysmal abnormality in the synchronization of the electrical activity to the local site and further suppressing it in a timely manner are the key points in preventing epileptic seizures.

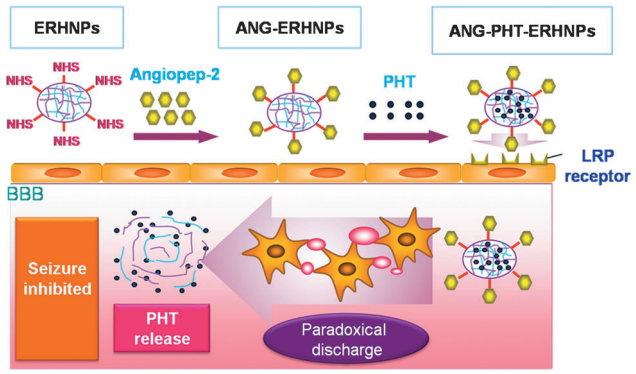
In this study, electro-responsive hydrogel nanoparticles (ERHNPs), which are characterized by the stimuli-sensitive drug release upon external electrical fields and their size in the nanometer range, were designed and modified with the brain-targeting angiopep-2 peptide, a ligand of the low-density lipoprotein receptor-related protein (LPR),[19] for antiepileptic therapy (Scheme 1).

Hydrogel nanoparticles were obtained from the soap-free emulsion copolymerization using 2-dimethylamino ethyl methacrylate (DMAEMA), sodium 4-vinylbenzene sulfonate (NaSS), styrene (ST), and acrylate-poly(ethylene glycol)-*N*-hydroxysuccinimidylester (ACLT-PEG-NHS) as the monomers and *N*,*N*′-methylene bisacrylamide (MBA) as the cross-linker (Figure 1 A). The obtained hydrogel nanoparticles have characteristics both of a hydrogel system and of a nanoparticle. Under high concentrations (above 100 mg mL^{-1}), a gel was formed (see the dish in Figure 1 B); however, under dilute conditions (10 mg mL^{-1} and 50 mg mL^{-1}), a nanoparticle dispersion was observed (two bottles in Figure 1 B). The average diameter of the obtained nanoparticle in 10 mg mL^{-1} was (96.8 ± 12.2) nm, as deter-

[*] Dr. X. Ying,[+] Dr. Y. Wang,[+] Dr. J. Liang, Dr. J. Yue, Dr. C. Xu, Dr. Z. Xu, Prof. Dr. Z. Chen
Department of Pharmacology, Neurobiology of the Ministry of Health of China and Zhejiang Province Key Laboratory of Neurobiology, College of Pharmaceutical Sciences, School of Medicine
Zhejiang University, Hangzhou (China)
E-mail: chenzhong@zju.edu.cn
Dr. X. Ying,[+] Dr. L. Lu, Prof. Dr. J. Gao, Prof. Dr. Y. Du
Institute of Pharmaceutics, College of Pharmaceutical Sciences
Zhejiang University
866 Yuhangtang Road, Hangzhou 310058 (China)
E-mail: duyongzhong@zju.edu.cn
[+] These authors contributed equally to this work.

[**] This study was financially supported by the National Natural Science Foundation of China (81202478, 91332202, 81221003), the Program for Zhejiang Leading Team of S&T Innovation (2011R50014), the Public Welfare Project of the Zhejiang province (2013C33G2010274), and the Fundamental Research Funds for the Central Universities, China (2013QNA7013).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201403846.



Scheme 1. PHT-loaded electro-responsive hydrogel nanoparticles modified with angiopep-2 (ANG-PHT-ERHNPs) for antiepileptic therapy.

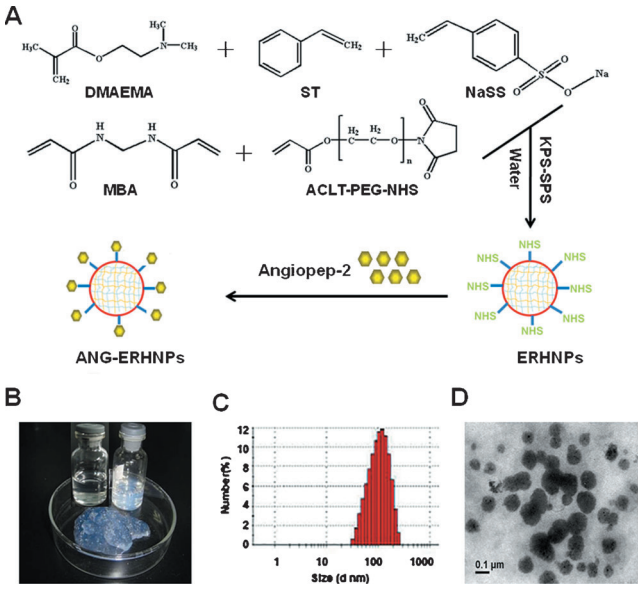


Figure 1. Synthesis and characterization of the ANG-ERHNPs. A) The synthesis of the ANG-ERHNPs. B) Photograph of the ERHNPs dispersion. C) Particle size distribution of the ERHNPs. D) TEM images of the ERHNPs

mined by dynamic light scattering (DLS) (Figure 1C). This result was close to that from transmission electron microscopy (TEM) imaging (Figure 1D).

To facilitate the brain delivery of the hydrogel nanoparticles, angiopep-2 was used as a brain targeting peptide to further modify the hydrogel nanoparticles by coupling the *N*-hydroxysuccinimidylester on the surface of the ERHNPs to the amino groups of the peptide (Figure 1A). The composition of the synthesized angiopep-2 modified ERHNPs (ANG-ERHNPs) was analyzed by FTIR spectroscopy. The IR spectra of ANG-ERHNPs (Figure S1) show a new peak at 1668 cm^{-1} that indicates the presence of a carboxyl group in the peptide. After the modification with the angiopep-2 peptide, the average diameter of the ANG-ERHNPs was (102.3 ± 16.8) nm similar to that of the ERHNPs. When loaded with PHT, the average diameter of ANG-PHT-ERHNPs and PHT-ERHNPs increased to (130.8 ± 22.4) and (142.8 ± 18.2) nm, with encapsulating efficiencies of (81.2 ± 6.4) % and (80.1 ± 5.8) %, respectively (see the Supporting Information Table S1).

Because of the presence of the polyelectrolyte poly(sodium 4-vinylbenzene sulfonate) (PSS) in the hydrogel nanoparticles, their conductivity accelerated the ionization of the sulfonate groups in the polymer chains. The increased degree of ionization of the polyelectrolyte hydrogel under an electric field has been reported to be responsible for the higher swelling ratio.[20] We investigated the particle size variation of hydrogel nanoparticles versus an applied electric field (50–500 μA) using two platinum electrodes separated by a distance of 1 cm. The current in the present study was chosen to be 5–50 $mV\,m^{-1}\,m^{-2}$, similar to the values measured during a seizure onset, in which the extracellular field potential can reach as high as 20 $mV\,m^{-1}\,m^{-2}$. The results show that the particle size increased to up to (388.0 ± 20.4) nm when the current of the external electric field increased from 50 to 500 μA for 1 min (Figure 2A). The size increase occurred within 1 min in

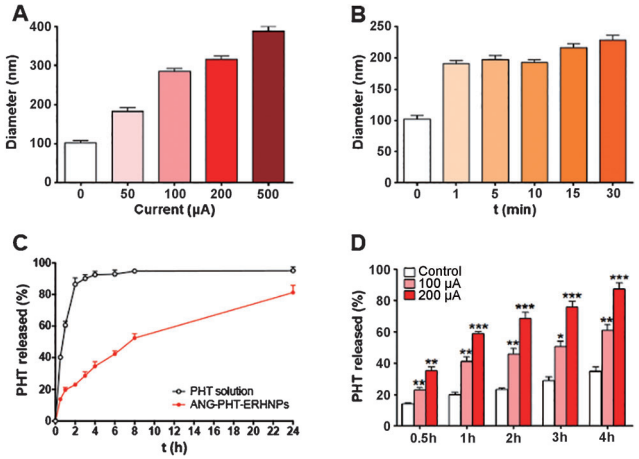


Figure 2. The electric-field-responsive behavior of the hydrogel nanoparticles with regard to drug release and diameter. A) The diameter of the ERHNPs changes after the application of various currents for 1 min. B) The diameter of the ERHNPs changes after the application of a 100 μA current for various times. C) The percentage of released PHT versus the release time of a PHT solution and ANG-PHT-ERHNPs in pH 7.4 PBS buffer. D) The percentage of PHT from ANG-PHT-ERHNPs with (100 μA and 200 μA) or without an external electric field for 4 h. $*P<0.05$, $**P<0.01$, $***P<0.001$ compared to a control group. A one-way ANOVA with Tukey's t-test was used for (D).

response to the electric field (Figure 2B), indicating the practicality of this approach for the clinical prophylaxis of epilepsy. The in vitro release showed that a sustained release of PHT from the ANG-PHT-ERHNPs was achieved for 24 h, whereas almost all was released within 2 h from the PHT solution (Figure 2C). The triggered release capabilities of ANG-PHT-ERHNPs increased significantly under an electric field. The percentage of accumulated PHT release increased from 34.6 % to 60.8 % and 87.3 % (nearly 1- and 1.5-fold), when currents of 100 and 200 μA were applied, respectively (Figure 2D). These results indicated that the ANG-PHT-ERHNPs can quickly release PHT to produce an antiepileptic effect upon detecting the onset of epileptic seizures.

The efficiency and low toxicity are essential requirements for therapeutic nanoparticles.[21,22] The cytotoxicity of the synthesized ERHNPs and ANG-ERHNPs was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) method in bEnd3 cells (mouse brain microvascular endothelial cells). The half-maximal inhibitory concentration (IC_{50}) values of the ERHNPs and ANG-ERHNPs were determined as 449.0 $\mu g\,mL^{-1}$ and 414.9 $\mu g\,mL^{-1}$, respectively (Figure S2), suggesting a relatively low cytotoxicity. To investigate the ability of the ERHNPs to cross the BBB after modification with angiopep-2, we first examined the transport ratio of hydrogel nanoparticles labeled with fluorescein isothiocyanate (FITC) across an in vitro BBB model. As shown in Figure 3A, the transport of the FITC-ERHNPs and

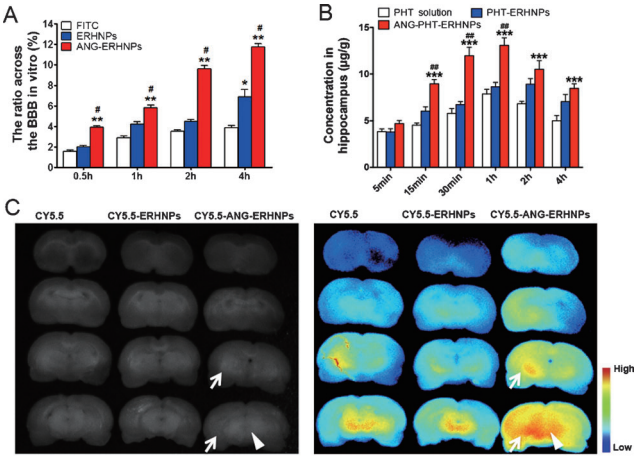


Figure 3. A) The transport percentage of FITC, ERHNPs, and ANG-ERHNPs across an in vitro BBB model at various times. $*P<0.05$, $**P<0.01$ compared to FITC, $\#P<0.05$ represents the difference between the ERHNPs and ANG-ERHNPs. B) PHT concentrations in the hippocampus after the administration of PHT, PHT-ERHNPs, and ANG-PHT-ERHNPs at the dose of 50 $mg\,kg^{-1}$ for various times. C) Ex vivo fluorescence imaging of coronal brain slices. Arrows indicate the hippocampus and triangles indicate the brainstem.

FITC-ANG-ERHNPs across the bEnd3 BBB models was time-dependent and the transport percentage in 4 h increased from $(6.90\pm1.21)\%$ to $(11.71\pm0.65)\%$ after the ERHNPs had been modified with ANG. FITC-ERHNPs and FITC-ANG-ERHNPs were further used to investigate the cellular uptake of the nanoparticles. A time-dependent uptake was observed for both types of nanoparticles in both qualitative and quantitative investigations, and the cellular uptake of the FITC-ANG-ERHNPs was significantly higher than that of the FITC-ERHNPs without angiopep-2 modification (Figure S3A–C). This might be due to the enhanced BBB transport ability mediated by angiopep-2. It should be mentioned that after pretreatment with free angiopep-2, the cellular uptake of FITC-ANG-ERHNPs was significantly reduced within the following 2 h (Figure S3A,C); this reduced uptake was similar to that observed with FITC-ERHNPs.

To further investigate the brain targeting ability of the ANG-ERHNPs in vivo, Cy5.5 labeled ANG-ERHNPs were prepared and injected (intraperitoneal, i.p.) and the fluorescence intensity of the brains was determined using an in vivo imaging system. The stronger fluorescence intensity in the hippocampus and brainstem regions of the brain were found in the ANG-ERHNPs group (Figure 3C). To analyze the brain targeting ability of ANG-PHT-ERHNPs quantitatively, we used an assay measuring PHT concentrations in the brain. Higher concentrations of PHT were found in the hippocampus, amygdale, cerebellum, and brainstem regions of the brain (Figures S4 and 3B); these regions are thought to play an important role in seizure initiation and spread.[23,24] The difference of the PHT levels in the brain became evident 15 min after treatment with PHT-ERHNPs and ANG-PHT-ERHNPs, at which time an increase by 1.49- and 1.97-fold, respectively, was observed compared to the PHT control (Figure 3B). This may be due to the angiopep-2 brain targeting effect as well as the nanoparticle characteristics, i.e. their small size and their prolonged blood circulation time owing to PEGylation.[25]

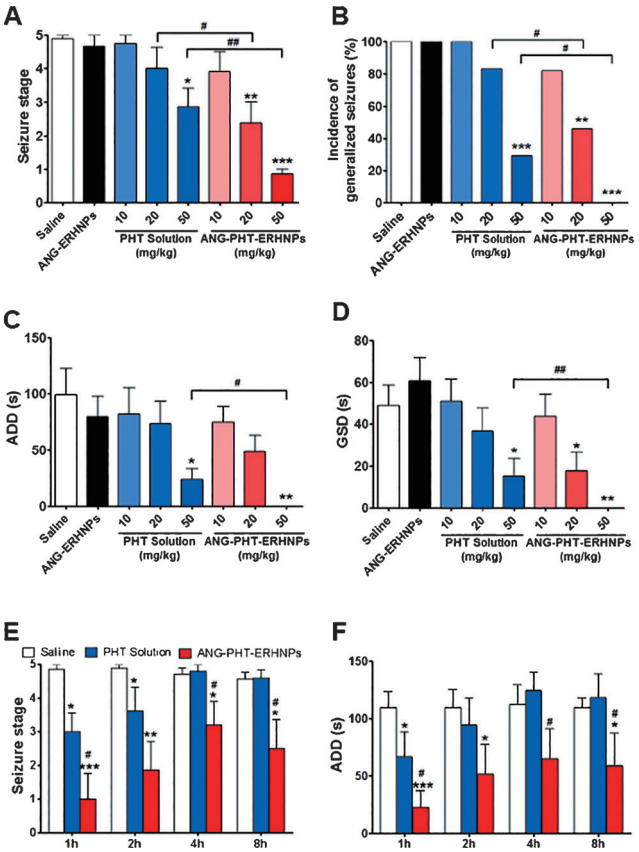


Figure 4. Antiepileptic effects of the ANG-PHT-ERHNPs on amygdala-kindled seizures. A) The behavioral stages, B) the incidence of generalized seizures, C) the afterdischarge duration (ADD), and D) the generalized seizure duration (GSD) during amygdala-kindled seizures in rats; E) the behavioral stages and F) the ADD at various time points after drug delivery. $*P<0.05$, $**P<0.01$, $***P<0.001$ compared to the solvent; $\#P<0.05$ and $\#\#P<0.01$ represent the difference between the PHT solution and PHT preparation. A one-way ANOVA with Tukey's t-test was used for (A) and (C–F); the chi-square test was used for (B).

Finally, we evaluated the antiepileptic efficacy of ANG-PHT-ERHNPs in the amygdala kindling seizure model, which resembles complex partial seizures with secondary generalization in clinical manifestation.[26] Both the PHT solution and the ANG-PHT-ERHNPs greatly reduced the kindling-stimulation-induced seizures compared to the saline group as shown by the seizure stage, the incidence of generalized seizure, the afterdischarge duration (ADD), and the generalized seizure duration (GSD). At the dose of 50 mg kg^{-1}, the ANG-PHT-ERHNPs significantly lowered seizure stages and shortened the ADD and GSD, whereas the PHT solution showed a relatively weak effect at the same dose (Figure 4 A–C). The ANG-PHT-ERHNPs even lowered the severity of the seizure behavior at a dose of 20 mg kg^{-1}, whereas the PHT solution did not have a protective effect at the lower dose (Figure 4 A,B,D). Representative afterdischarges and their corresponding energy spectra are shown in Figure 5 A,B. In addition, the EEG spectrum analysis further showed that ANG-PHT-ERHNPs treatment greatly reduced the severity of the afterdischarges onset (Figure 5 C). We further analyzed the long-acting effect and found that the protective effect of ANG-PHT-ERHNPs can last as long as 8 h whereas the PHT solution only had a narrow therapeutic window of approximately 2 h (Figure 4 E,F).

In conclusion, we have developed an electro-responsive hydrogel nanoparticle modified with angiopep-2 as the targeting ligand for antiepileptic drug delivery. Rapid PHT release from our hydrogel nanoparticles could be readily achieved upon the application of an electric field. The long-circulating property and angiopep-2 ligand of the ANG-PHT-ERHNPs allowed for a rapid, long-term, and accurate in vivo brain targeting and improved the antiepileptic efficacy of PHT in amygdala kindling models. Here we have shown that angiopep-conjugated ERHNPs are a promising targeted drug delivery system for the treatment of epilepsy.

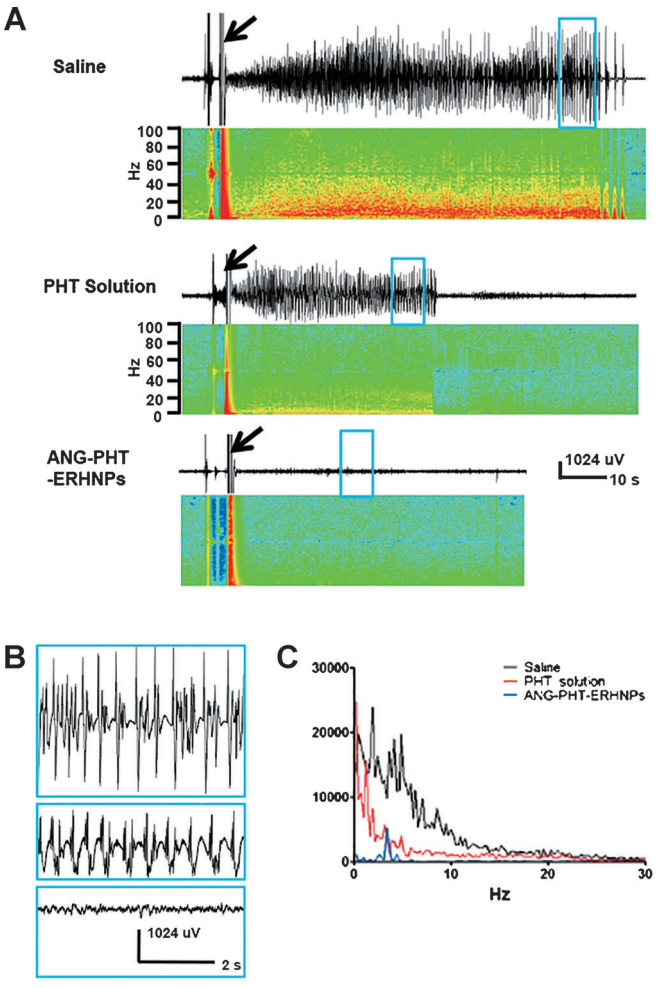


Figure 5. A) Representative EEGs recorded from the right amygdala and the corresponding energy spectra of the solvent, PHT solution and PHT preparation at the dose of 50 mg kg^{-1}. The black arrows denote the kindling stimulation artifacts. B) Enlarged views of the EEGs in the boxes in (A). C) The power spectra analysis of the EEGs.

Experimental Section

Experimental details: See the Supporting Information for the synthesis and characterization of ERHNPs, ANG-ERHNPs, and drug-loaded ANG-ERHNPs, in vivo antiepileptic therapy, etc. All animal experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals with the approval of the Scientific Investigation Board of Zhejiang University, Hangzhou, China.

Received: March 31, 2014
Published online: July 18, 2014

Keywords: blood–brain barrier · drug delivery · epilepsy · hydrogels · nanoparticles

[1] S. D. Shorvon, *Epilepsia* **2009**, *50*, 93 – 130.
[2] M. Bialer, H. S. White, *Nat. Rev. Drug Discovery* **2010**, *9*, 68 – 82.
[3] W. Löscher, H. Klitgaard, R. E. Twyman, D. Schmidt, *Nat. Rev. Drug Discovery* **2013**, *12*, 757 – 776.
[4] D. Brambilla, R. Verpillot, B. Le Droumaguet, J. Nicolas, M. Taverna, J. Kona, B. Lettiero, S. H. Hashemi, L. De Kimpe, M. Canovi, M. Gobbi, V. Nicolas, W. Scheper, S. M. Moghimi, I. Tvaroska, P. Couvreur, K. Andrieux, *ACS Nano* **2012**, *6*, 5897 – 5908.
[5] Y. M. Lu, J. Y. Huang, H. Wang, X. F. Lou, M. H. Liao, L. J. Hong, R. R. Tao, M. M. Ahmed, C. L. Shan, X. L. Wang, K. Fukunaga, Y. Z. Du, F. Han, *Biomaterials* **2014**, *35*, 530 – 537.
[6] C. K. Kim, T. Kim, I.-Y. Choi, M. Soh, D. Kim, Y.-J. Kim, H. Jang, H.-S. Yang, J. Y. Kim, H.-K. Park, S. P. Park, S. Park, T. Yu, B.-W. Yoon, S.-H. Lee, T. Hyeon, *Angew. Chem.* **2012**, *124*, 11201 – 11205; *Angew. Chem. Int. Ed.* **2012**, *51*, 11039 – 11043.
[7] J. Q. Gao, Q. Lv, L. M. Li, X. J. Tang, F. Z. Li, Y. L. Hu, M. Han, *Biomaterials* **2013**, *34*, 5628 – 5639.
[8] Q. Lv, L. M. Li, M. Han, X. J. Tang, J. N. Yao, X. Y. Ying, F. Z. Li, J. Q. Gao, *Int. J. Pharm.* **2013**, *444*, 1 – 9.
[9] Y. Miura, T. Takenaka, K. Toh, S. Wu, H. Nishihara, M. R. Kano, Y. Ino, T. Nomoto, Y. Matsumoto, H. Koyama, H. Cabral, N. Nishiyama, K. Kataoka, *ACS Nano* **2013**, *7*, 8583 – 8592.
[10] L. Xu, H. Zhang, Y. Wu, *ACS Chem. Neurosci.* **2014**, *5*, 2 – 13.
[11] M. Yusuf, R. A. Khan, M. Khan, B. Ahmed, *Int. J. Nanomed.* **2012**, *7*, 4311 – 4321.
[12] M. F. Bennewitz, W. M. Saltzman, *Neurotherapeutics* **2009**, *6*, 323 – 336.
[13] J. Darius, F. P. Meyer, B. A. Sabel, U. Schroeder, *J. Pharm. Pharmacol.* **2000**, *52*, 1043 – 1047.
[14] Y. Qiu, K. Park, *Adv. Drug Delivery Rev.* **2001**, *53*, 321 – 339.

[15] J. Ge, E. Neofytou, T. J. Cahill 3rd, R. E. Beygui, R. N. Zare, *ACS Nano* **2012**, *6*, 227 – 233.
[16] M. Hamidi, A. Azadi, P. Rafiei, *Adv. Drug Delivery Rev.* **2008**, *60*, 1638 – 1649.
[17] D. Roy, J. N. Cambre, B. S. Sumerlin, *Prog. Polym. Sci.* **2010**, *35*, 278 – 301.
[18] J. O. McNamara, *J. Neurosci.* **1994**, *14*, 3413 – 3425.
[19] H. Xin, X. Sha, X. Jiang, L. Chen, K. Law, J. Gu, Y. Chen, X. Wang, X. Fang, *Biomaterials* **2012**, *33*, 1673 – 1681.
[20] H. S. Lim, J. H. Lee, J. J. Walish, E. L. Thomas, *ACS Nano* **2012**, *6*, 8933 – 8939.
[21] J. Kim, L. Cao, D. Shvartsman, E. A. Silva, D. J. Mooney, *Nano Lett.* **2011**, *11*, 694 – 700.
[22] Q. Tian, J. Hu, Y. Zhu, R. Zou, Z. Chen, S. Yang, R. Li, Q. Su, Y. Han, X. Liu, *J. Am. Chem. Soc.* **2013**, *135*, 8571 – 8577.
[23] M. Avoli, M. D′Antuono, J. Louvel, R. Kohling, G. Biagini, R. Pumain, G. D′Arcangelo, V. Tancredi, *Prog. Neurobiol.* **2002**, *68*, 167 – 207.
[24] Y. Wang, Z. H. Xu, H. Cheng, Y. Guo, C. L. Xu, S. Wang, J. M. Zhang, M. J. Ding, Z. Chen, *Brain Struct. Funct.* DOI: 10.1007/s00429-013-0594-7.
[25] J. Frigell, I. Garcia, V. Gomez-Vallejo, J. Llop, S. Penades, *J. Am. Chem. Soc.* **2014**, *136*, 449 – 457.
[26] K. Morimoto, M. Fahnestock, R. J. Racine, *Prog. Neurobiol.* **2004**, *73*, 1 – 60.