A Self-Assembled DNA Origami-Gold Nanorod Complex for Cancer Theranostics

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A self-assembled DNA origami (DO)-gold nanorod (GNR) complex, which is a dual-functional nanotheranostics constructed by decorating GNRs onto the surface of DNA origami, is demonstrated. After 24 h incubation of two structured DO-GNR complexes with human MCF7 breast cancer cells, significant enhancement of cell uptake is achieved compared to bare GNRs by two-photon luminescence imaging. Particularly, the triangle shaped DO-GNR complex exhibits optimal cellular accumulation. Compared to GNRs, improved photothermalysis against tumor cells is accomplished for the triangle DO-GNR complex by two-photon laser or NIR laser irradiation. Moreover, the DO-GNR complex exhibits enhanced antitumor efficacy compared with bare GNRs in nude mice bearing breast tumor xenografts. The results demonstrate that the DO-GNR complex can achieve optimal two-photon cell imaging and photothermal effect, suggesting a promising candidate for cancer diagnosis and therapy both in vitro and in vivo.

1. Introduction

DNA molecules have been used as building blocks for self-assembly into nanomaterials with various complex geometries.[1] Through rational design, DNA strands spontaneously assemble into desired 2D or 3D shapes.[2] Particularly, the origami techniques provide DNA materials with well-defined nanoscale shapes, with uniform sizes, precise spatial addressability, and excellent biocompatibility.[3] With these features, the DNA nanostructures show great potential for biomedical applications; various DNA-based biomedical imaging probes or payload delivery carriers have been developed.

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for the construction of multifunctional delivery and release carriers. The multivalent DNA origami nanostructures have gained special attention because of improved delivery efficacy to targeted cells and reduced susceptibility to nontargeted ones. In particular, hexagonal barrel-shaped DNA origami has been applied to targeting delivery of molecular cargoes, which is achieved by the activation and reconfiguration of the sites specifically functionalized DNA aptamers. In a CpG DNA sequences transport system, hollow 30-helix DNA origami tubes have been successfully used as efficient nanocarriers and triggered strong immune responses of splenocytes. Furthermore, triangular and tubular origami were used for intercalation of the anticancer drug, doxorubicin, and improved cytotoxicity was achieved against drug-resistant tumor cells. For the origami-drug complex, the significant enhancement of cytotoxicity is attributed to increased intracellular internalization of drug with the aid of DNA origami vehicles. In another doxorubicin-origami delivery system, the encapsulation efficiency and the release rate of the drug were tunable by controlled design of the origami nanostructure. Compared to free drug, increased cytotoxicity and lower intracellular elimination rate were achieved. DNA origami has been used to fabricate nanoscale robots that are capable of dynamically interacting with each other in living cockroaches (Blaberus discoidalis). In the tumor bearing mice model, DNA origami served as antitumor drug carriers and successfully transported the payloads to tumor regions. The in vivo biodistribution confirmed that DNA origami realized the effects of enhanced passive targeting and long-lasting accumulation at tumor region. Essentially, further experiments demonstrated that the DNA origami-drug delivery system displayed optimal antitumor efficacy in vivo without inducing observable systemic toxicity.

However, the bare DNA nanostructures cannot provide imaging or therapy functions directly. The construction of the DNA-based platform for diagnostic or therapeutic applications is usually achieved by loading the sorts of molecular cargoes (antibody protein, fluorescent probes, immunostimulatory oligo DNA, or drug molecules) onto DNA nanostructures. Particularly, in all the reported DNA-based carrier system, only one small molecular drug, chemotherapeutics doxorubicin, was used. On the other hand, noble metal nanostructures such as aggregated gold nanospheres, gold nanorods (GNRs), gold nanoshells and nanocages, and hollow gold/silver dendrites showed intriguing optical and photothermal conversion properties due to localized surface plasmon resonances (LSPRs), which offer great potential for simultaneous molecular imaging and photothermal cancer therapy. Specifically, GNRs that can absorb and scatter strongly in the NIR region are the excellent candidate for fluorescent visualization and plasmonic photothermal therapy. Based on these developments, we proposed to combine the photothermal effect of GNRs and optimal delivery effect of DNA origami together to achieve enhanced therapeutic efficacy.

We constructed a self-assembled DNA origami-GNR complex (abbreviated to DO-GNR), which is a dual-functional nanotheranostics, by decorating GNRs onto the surface of DNA origami. The hybridized nanoparticle/DNA complex designed as integration of the optical photothermal effects of GNR with passive tumor targeting and long-lasting accumulation of origami. Moreover, DO-GNRs offer two-photon imaging and photothermal ablation in one DNA nanoplatform, suggesting a promising candidate for cancer diagnosis and therapy. We used the structured DO-GNR complex to perform photothermal therapy in vitro and in vivo.

2. Results

2.1. Construction of DO-GNR Complex

The experimental scheme is illustrated in Figure 1. Triangular and tubular shaped origami were folded by annealing the M13mp18 genome DNA strand (scaffold), capture strands, and staple strands in a ratio of 1:10:10 from 95 °C to room temperature, according to Rothemund’s work with several modifications. Each edge of the triangular shaped origami was 120 nm long. The length of tubular origami was nearly 380 nm. DNA capture strands with carefully designed sequences were extended from one arm of the triangular DNA template working as binding sites to precisely organize one GNR (40 × 12 nm) as shown in Figure 1. The binding sites were designed to display linear pattern on the top surface of triangular or tubular DNA templates to match the shape of GNR. The assembled DNA origami nanostructure was subsequently purified with a filter device to remove the extra capture and staple stands. Next, the purified DNA origami and GNRs functionalized with corresponding complementary DNA strands were mixed and annealed from 45 °C to 25 °C in 2 h for 30 cycles. After hybridization, GNR was organized at the desired binding sites on the DNA platform. The DO-GNR complex was characterized and then administrated to human breast tumor cells (MCF7). After two-photon fluorescence imaging, the triangle shaped DO-GNR demonstrated preferable tumor cell accumulation, subsequently it was used for the investigation of the photothermal ablation effects (Figure 1).

2.2. Characterization of DO-GNR Complex

The DNA origami nanostructures were characterized after GNRs loading. For the purification of assembled structures, agarose gel was used. The target products were sliced and extracted from the gel with freeze-squeeze column (Bio-Rad) at 4 °C. The purified nanostructures were then characterized by transmission electron microscope (TEM) and ultraviolet–visible (UV–vis) spectrometer. TEM images of conjugates of triangular DO-GNR and tubular DO-GNR complex are shown in Figure 2a,b and Figures S1 and S2 (Supporting Information). TEM images showed clearly that GNR was attached on origami template at designed location. These results provided direct evidence of the formation of DO-GNR complex and the morphology of the DNA nanostructures was retained after assembled of GNRs. Figure 2c shows UV–vis extinction spectra of unloaded GNR (black line), triangle DO-GNR complex (red line), and tube
DO-GNR complex (blue line). The three spectra nearly coincide with each other except the conjugates’ small peak at ≈260 nm, indicating the presence of DNA origami.

2.3. Internalization of DO-GNR Complex

The property of strong plasmon resonance in the NIR region makes GNRs ideal contrast agents for two-photon luminescence (TPL) imaging of live cells. Efficient and fast conjugation methods of DNA modified GNR have been well studied. After single strand DNA (ssDNA) modification, GNRs can be precisely organized on DNA origami template by addressable DNA hybridization. After incorporation of GNRs on the DNA origami nanostructures, DO-GNR complex was incubated with tumor cells and the internalization of the origami nanostructures was investigated by TPL. MCF7 cells were employed as a cellular model for direct visualization of the internalization of GNRs and DO-GNR complex conjugates. After 24 h incubation with GNR, triangle DO-GNR complex, or tube DO-GNR complex (0.1 × 10⁻⁹ m), the live cells were investigated by two-photon excitation laser-scanning microscopy after removing excess nanoparticles and conjugates. The wheat germ agglutinin conjugates (Alexa Fluor 594 conjugate) staining was used for labeling cell membrane. The TPL excited by two-photon laser (λ<sub>max</sub> = 750 nm) was visible inside cells treated with DO-GNR complex and bare GNR, demonstrating that DO-GNR complex nanostructures and GNR can enter and accumulate in cells. The enhanced internalization
by DO-GNR complex can be directly observed (Figure 3a and Figure S3, Supporting Information). In the TPL images, stronger intensity was detected in tumor cells treated with DO-GNR complex than with bare GNR. The increased TPL phenomenon caused by DO-GNR complex was confirmed by quantitative analysis of these images by using Image J (Figure 3b and Figure S3, Supporting Information). The results demonstrated that DO-GNR complex can accumulate more in tumor cells than bare GNRs. Compared to GNR, DO-GNR complex showed enhanced cell internalization, which may attribute to the size and shape of assembled triangular and tubular origami template. Particularly, the triangle shaped origami showed slightly better internalization effects, therefore it was next used for further photothermal therapy of tumor cells and nude mice bearing breast tumors.

2.4. In Vitro Photothermal Therapy of DO-GNR Complex

For the in vitro photothermal therapy, triangle DO-GNR complex was utilized for photothermal ablation of tumor cells by NIR laser. The MCF7 cells were treated with $0.1 \times 10^{-9}$ M purified triangle DO-GNR complex or GNR alone for 24 h incubation. After visualization of DO-GNR complex inside the tumor cells with TPL, the photothermal therapy was applied by laser confocal microscopy or NIR laser. After exposure to the two-photon laser, the obvious photoinduced cell membrane blebbing appeared in the MCF7 cells treated with DO-GNR complex, indicating the photothermal damages generated by the DO-GNR complex (Figure 4a). Under the same condition, the GNR-treated cells did not show significant morphological change in the bright field (Figure 4a). Exposure to continuous NIR laser was performed to confirm the improved photothermolysis efficacy of DO-GNR complex. Cell viability assay was conducted after administration with GNR and DO-GNR complex for 24 h, respectively, and then exposure to continuous NIR laser (12 W cm$^{-2}$, 3 min and 6 W cm$^{-2}$, 3 min). For the two groups of GNR and DO-GNR complex, induced tumor cell death was observed. MCF7 cells treated with DO-GNR complex displayed significantly lower cell viability compared to the unloaded GNR-treated ones (Figure 4b). These results indicated that enhanced photothermal therapy efficacy is presumably due to increased intracellular accumulation of DO-GNR complex (Figure 3 and Figure S3, Supporting Information). Together with the TPL property, the DO-GNR system also provided an appealing candidate for imaging-guided photothermal therapy.

2.5. In Vivo Photothermal Effects of DO-GNR Complex

For the in vivo photothermal therapy, the MCF7 xenograft tumor bearing mice were used and were intravenously injected through tail with 0.9% saline (150 µL), GNRs ($3 \times 10^{-9}$ M, 150 µL), and DO-GNR complex ($3 \times 10^{-9}$ M, 150 µL), respectively. The NIR irradiation (1.5 W cm$^{-2}$, 8 min) was performed on the tumor xenografts 24 h postinjection. The temperature of tumors was measured before and after NIR laser irradiation. Similar to the saline treatment in the blank group, DNA origami itself did not negatively impact on the temperatures of tumor regions (Figure S4, Supporting Information). As shown in Figure 5, the thermographic maps demonstrated that the DO-GNR complex induced the highest increase of temperature compared to the saline and GNR-treated groups. The results suggested that DO-GNR complex can increase the optical and photothermal effects compared with plain GNRs, which was due to the enhanced tumor passive targeting and long-lasting properties at tumor region of DNA origami.[12] After the NIR laser irradiation and thermal imaging, the images...
showed that the tumors with saline treatment and laser irradiation grew rapidly, indicating that the tumor itself was not impaired by NIR laser irradiation. In GNR treatment group, the tumors were burned after irradiation and formed scars on the surface after laser irradiation. For the nude mice treated with DO-GNR complex, the tumors were burned obviously after irradiation, leaving the original site with black scars (Figure S5a, Supporting Information). The mouse death was recorded and the survival rate was calculated. Thirty days after NIR irradiation, the mouse survival rate was highest in the DO-GNR complex treatment group compared to the saline and GNR-treated ones (Figure S5b, Supporting Information). The data indicated that DNA origami facilitated the photothermal effects of GNR on tumors and therefore effectively prolong survival time of the tumor bearing mice.

3. Discussion

Nanoscale systems are emerging as a class of cancer diagnostic and therapeutic tools. Drug delivery systems have been widely reported, including the applications of liposomes, micelles, dendrimers, carbon nanotubes, and nanoparticles.[17] Many nanoscale delivery systems have demonstrated tumor-targeting imaging and increased antitumor efficiency, owing to enhanced permeability and retention (EPR) effects in tumor regions, and size and shape-dependent cellular uptake.[18] One promising trend is the development of nanotheranostics, integrating multiple functional groups with diagnostic and therapeutic effects at the nanoscale.[19] In the present work, GNRs were attached to DNA origami at designated locations, forming DO-GNR hybrids complex with uniform shapes and sizes. With LSPR peaks in NIR region, DO-GNR complex exhibited a broad two-photon photoluminescence (450–650 nm) when excited by two-photon laser source. The highly efficient TPL excited by NIR laser makes DO-GNR complex ideal optical agents for two-photon imaging, which has great potential in early and noninvasive diagnosis of cancers. Two-photon imaging
demonstrated that hybrid DO-GNR exhibited much stronger TPL intensity compared with GNRs. These results indicated the enhancement of cell accumulation by DO-GNR complex, which was presumably induced by the optimal size and shape-dependent internalization effect of DNA origami (Figure S6, Supporting Information). Specifically, the triangular DO-GNR complex demonstrated better cellular internalization and was used in next-step photothermal therapy in vitro and in vivo.

Integrated with the TPL property, the DO-GNR system provided an appealing platform for imaging-guided photothermal therapy. The TPL-guided photothermal ablation was performed in MCF7 cells and triangular DO-GNR complex demonstrated significant improved photothermolysis efficacy compared with GNRs. After exposure to the two-photon laser, obvious heat-induced cell membrane blebbing appeared in the MCF7 cells treated with DO-GNR complex, suggesting the enhanced photothermal damages generated by the enriched GNRs inside the cells. On the other hand, GNR and DO-GNR complex induced tumor cell death were both observed after continuous NIR irradiation. MCF7 cells treated with DO-GNR complex displayed significantly lower cell viability compared to the GNRs treated ones, at both 12 W cm$^{-2}$ and 6 W cm$^{-2}$ laser exposure. Interestingly, the hybrid DO-GNR complex required only 6 W cm$^{-2}$ laser exposure for 3 min to induce more than 80% cell death through photothermal ablation, which was about two times photothermal damage effects compared with GNR-treated group under the same intensity irradiation. Actually, high-temperature hyperthermia can generate systemic and local side effects.$^{[20]}$ Considering application in vivo and further clinical use, relative lower power of NIR laser is essential, which could decrease injure of hyperthermia for surrounding healthy tissues.$^{[20,21]}$ These in vitro experiments demonstrated the enhanced photothermal therapy efficacy induced by DO-GNR complex, which is attributed to increased intracellular accumulation of GNRs (Figure 3 and Figure S3, Supporting Information).

The preferable photothermal effects were then confirmed in the MCF7 xenograft tumor bearing mice. After NIR laser irradiation at 24 h postinjection intravenously, the DO-GNR complex induced the highest increase of temperature with comparison of saline and GNR treatments. The survival time of DO-GNR complex treated group was also the longest among the three different groups. The enhanced heat-generating and improved photothermal therapy in vivo were realized by DO-GNRs complex, due to the optimal passive targeting and long-lasting retention effects at tumor region.$^{[12]}$ Gold signals detected by inductively coupled plasma mass spectrometry (ICP-MS) in tumor cells or tumor tissues treated with DO-GNR were significantly higher than GNR-treated ones (Figure S7, Supporting Information, $^{*}P < 0.05$, $^{***}P < 0.0001$). The results of ICP-MS were consistent with the imaging data, revealing that DO-GNR complex can accumulate more in tumor cells than GNRs. The in vivo data showed DO-GNR enhanced accumulation and retention in tumors, indicating that the DNA origami carriers with superior EPR effects induced DO-GNR accumulation at the tumors. The in vitro and in vivo photothermal therapy data demonstrated that the hybrid DO-GNR complex was a promising candidate for cancer therapy.

4. Conclusion

In this work, a self-assembled biological DNA origami-inorganic GNR complex serving as a successful dual-functional nanotheranostics was reported. The hybrid nanoplatform integrated the advantages of self-assembly DNA origami nanostructures and GNRs, demonstrated a promising candidate for cancer diagnosis and therapy in vitro and in vivo. Optimal intracellular TPL of DO-GNR complex was observed. Significant enhancement of photothermolysis in vitro was achieved by two-photon laser or NIR laser irradiation compared to GNR. Moreover, the DO-GNR complex exhibited enhanced antitumor efficacy compared with GNRs in nude mice bearing breast tumor xenografts. In this nanotheranostics system, the DO-GNR complex integrates preferable delivery capability with two-photon cell imaging and photothermal ablation therapy.

To further develop a smart nanotheranostic system, DNA origami nanostructures can serve as the template to integrate multiple functional elements into one platform. The organization of multiple photothermal conversion nanoparticles at pre-designed locations of origami template could achieve enhanced photothermal effects. Tumor-targeting biomolecules (such as peptides and aptamers) and DNA intercalated drugs (doxorubicin) can also be assembled into the same template. The mechanical reconfiguration of DNA origami through appropriate structural design promises controlled release of the loaded cargos. Using DNA origami as the nanoplatform, combinatorial diagnosis and therapy could be realized. After loading other functional elements, such as iron oxide nanoparticles on origami template, the hybrid DNA-iron oxide nanocomplex could play a very important role to understand the distribution and fate of the formulation via magnetic resonance imaging. These will result in a multifunctional DNA-based platform, which will extend the applications of the self-assembled DNA nanostructures in cancer diagnosis and therapy. Our study here will not only offer new insights into the understanding of multifunctional nanotheranostics but also encourage the development of DNA origami platform as efficient and biocompatible candidates for novel antitumor drug delivery systems.

5. Experimental Section

Materials: All oligonucleotides were purchased from Invitrogen (Shanghai, China). The origami staple strands were all diluted to 100 × 10$^{-6}$ M and used without further purification. The 3’-thiol-modified DNA strands were purified by high-performance liquid chromatography and the concentration of each strand was estimated by measuring the UV absorbance at 260 nm. M13mp18 phage DNA (N4040S) was purchased from New England Biolabs (USA). Auric acid (HAuCl$_4$), cetyltrimethylammonium bromide (CTAB), sodium borohydride (NaBH$_4$), silver nitrate (AgNO$_3$), and tris(carboxyethyl) phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich (USA).
Synthesis of GNRs and Modification of GNRs with Thiolated DNA: Seed-mediated growth was performed to synthesize GNRs according to the method developed by Nikoobakht and El-Sayed.[23] Next, GNRs were modified with oligo-DNA at low pH value according to the method of Ding.[23]

DNA Origami Assembling and Purification: Triangle and tube shaped DNA origami structures were assembled according to Rothemund’s and Yan’s work with several modifications. See Schemes S1 and S2 (Supporting Information) for the details of origami design scheme. A 1:10:10 molar ratio between the M13mp18 ssDNA (5 × 10^−9 mol), the short staple strands, and capture strands were used. The DNA origami was assembled in 1 × TAE-Mg2+ buffer (40 × 10^−3 M Tris, 20 × 10^−3 M acetic acid, 2 × 10^−3 M ethylenediaminetetraacetic acid (EDTA); 12.5 × 10^−3 M magnesium acetate; pH 8.0) in an Eppendorf thermocycler (Eppendorf China) by slowly cooling from 90 °C to room temperature over 12 h. DNA origami was then filtered with 100 kDa MWCO centrifuge filters (Amicon, Millipore, USA) to remove extra DNA staple strands.

Self-Assembly of DO-GNR Complex: Purified DNA origami was mixed with GNRs with a ratio of two GNRs for one DNA origami. The mixture was annealed from 45 °C to 25 °C in 2 h for 30 cycles.

Purification of DO-GNR Complex with Agarose Gel Electrophoresis: For DO-GNR complex purification, EtBr-free agarose gel was used (running buffer 0.5 × tris-boric acid-EDTA (TBE) buffer with 11 × 10^−3 M Mg2+, loading buffer 50% glycerol, 15 V cm⁻²). Selected bands were cut off and the DO-GNR complex was extracted from the gel with Freeze-Squeeze column (Bio-Rad, USA) at 4 °C.

TEM Characterization of DO-GNR Complex: 7 µL of DO-GNR solution was deposited onto carbon-coated grids and incubated for 10 min. For negative staining of DNA nanostructures, the grid was treated with a drop of 0.7% uranylacetate for 40 s and kept at room temperature for 2 h. TEM imaging was carried out by using a Tecnai G2-20S TWIN, operated at 80 kV in the dark-field mode.

Cell Culture: MCF7 is a human breast adenocarcinoma cancer cell line, which was kindly provided by Prof. Xingjie Liang (NCNST). MCF7 cells were cultured in Dulbecco’s Modified Eagle Medium (HyClone, Thermo Scientific) supplemented with 10% fetal bovine serum (HyClone, Thermo Scientific) and with L-glutamine, penicillin, streptomycin (GIBICO, Invitrogen) was used for cell membrane staining. The cells were incubated with WGA (1 × 10^−6 mol) for 5 min at 25 °C. After being washed with phosphate-buffered saline (PBS), the living cells were visualized by two-photon laser confocal fluorescence microscopy (Olympus, Japan).

Cellular Internalization of GNRs and DO-GNR Complex: MCF7 cells were seeded in confocal dishes, cultured overnight, and then incubated with GNRs (0.1 × 10^−9 mol) and purified conjugates of DO-GNR complex (0.1 × 10^−9 mol, diluted by culture medium) for 24 h. The wheat germ agglutinin (WGA) conjugates (Alexa Fluor 594 conjugate, red fluorescence, Invitrogen) was used for cell membrane staining. The cells were incubated with WGA (1 × 10^−6 mol) for 5 min at 25 °C. After being washed with phosphate-buffered saline (PBS), the living cells were visualized by two-photon laser confocal fluorescence microscopy (Olympus, Japan).

Photothermal Ablation of Tumor Cells after Two-Photon Laser Exposure: MCF7 cells were seeded in confocal dishes, cultured overnight, and then incubated with GNRs (0.1 × 10^−9 mol) and purified conjugates of DO-GNR complex (0.1 × 10^−9 mol, diluted by culture medium) for 24 h. After being washed with PBS, the living cells were exposed under two-photon laser (laser power: 2%; scanning area: 100 × 100 µm², 100 frames).

Cell Viability Assay after NIR Laser Exposure: The photothermal-induced tumor cell death after GNRs or DO-GNR complex incubation and laser irradiation were assessed with a cell-counting kit (ckc-8, Dijindo, Japan) containing a highly water-soluble tetrazolium salt (WST-8) [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt]. After seeded in 96-well plates and cultured overnight, the MCF7 cells were incubated with GNRs and DO-GNR complex for 24 h. Thereafter, the cells were irradiated with NIR laser (750 nm, 4 W or 2 W) for 3 min. Finally, the cells were incubated with fresh serum-free medium containing 0.5 mg mL⁻¹ WST-1 for 1 h at 37 °C for the cytotoxicity assay. Absorbance values at 450 nm were measured using a microplate reader (TECAN, infinite M200, Switzerland).

Establishment of Tumor Xenograft Mouse Model and Photothermal Therapy In Vivo: 5- to 6-week-old athymic female BALB/c nude mice were purchased from the Department of Experimental Animals, Peking University Health Science Center, and all animal procedures were performed in accordance with the guidelines of the local Institutional Animal Care and Use Committee Peking University (Permit Number: 2011-0039). The subcutaneous tumors were established by injecting 1 × 10^6 MCF7 cells into the right upper flanks of the BALB/c nude mice. Mice with tumor volumes around 100 mm³ were randomly divided into different groups (four mice per group) including saline, origami, GNR, and DO-GNR complex groups. For the in vivo photothermal therapy, the MCF7 tumor-bearing mice were intravenously injected with control saline, origami (3 × 10^−9 mol, 150 µL), GNR (3 × 10^−9 mol, 150 µL), and DO-GNR (3 × 10^−9 mol, 150 µL), respectively. 24 h after injection, the NIR irradiation (1.5 W cm⁻², 8 min) was performed on the tumor xenografts.[24] The temperature of tumors was measured before and after NIR laser irradiation, and the thermographic maps were made. The mouse death from each group was recorded for 30 d and the survival rate was calculated.

Statistical Analysis: One-way analysis of variance (ANOVA) and Turkey multiple comparisons test or Student’s t-test was used to determine the statistical differences. *P < 0.05 and ***P < 0.001 were considered statistically significant. Statistical analysis was conducted using Prism4.0 (San Diego, CA, USA).

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements
Q.J. and Y.S. contributed equally to this work. The authors are grateful for the financial support from the National Science Foundation of China (21173059, 91217021, 21222311, 21273053, 81227901, and 81470083), the National Basic Research Program of China (973 Program, 2012CB934000, 2015CB755500, 2014CB748600, and 2011CB707702), the 100-Talent Program of Chinese Academy of Sciences (B.Q.D.), and the Beijing Natural Science Foundation (L140008).


Received: May 5, 2015  
Revised: June 26, 2015  
Published online: