RSC Advances

ROYAL SOCIETY OF CHEMISTRY

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Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

High-efficient inhibition of recognition in allorejection via pMyD88/liposomes complex

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Abstract: Data are emerging that recognition of foreign antigen by Toll/like receptors (TLRs) was predominant in skin graft rejection. More interestingly, most of the TLRs recruit myeloid differentiation factor 88 (MyD88) as adaptor during signaling transduction. Design of an efficient shRNA/vector complex to suppress expression of MyD88 protein seems to be a potential approach in preventing allorejection of skin. In this work, we prepared a complex composed of a MyD88-shRNA plasmid (pMyD88) and a cationic polymeric vector. The results showed that: The pMyD88 and vectors with an optimal mass ratio (1:2) was selected by agarose gel electrophoresis (AGE) experiments; The cationic liposome vectors increased the transfection efficiency of naked pMyD88; The gene transcription of MyD88 mRNA and expression of MyD88 protein was significantly suppressed by the pMyD88/liposomes complex. Notably, the Lipopolysaccharide (LPS) stimulation test told us recognition of foreign antigen in DCs that treated by the pMyD88/liposomes complex was significantly inhibited. This attempt promised to solve skin allorejection problems in the future.

1. Introduction

The transplantation of skin is the most effective therapeutic option for large-area burns patients. However, rejection always comes to the graft after the operation. Unlike heart, pancreas and kidney allografts, current immunosuppressor has little effect in skin transplantation [1-3]. Though artificial skins are on research, they are not effective in clinic. Therefore, new strategies are on a strong demand to prevent allorejection of skin.

Once the allogeneic skin patch was placed at the injury, toll-like receptors (TLRs) would first response and induced DCs to migrate from skin graft to lymphoid tissue of recipient [4]. TLRs are a family of transmembrane proteins that play an important role in innate immunity and provide a link between innate and adaptive responses to an allograft [5-8]. There at least 11 kinds of TLRs molecules have been known. More interestingly, most of them (TLR 1, TLR 2, TLR 4, TLR 5, TLR6, TLR 7, TLR 9, TLR 11) require myeloid differentiation factor 88 (MyD88) as adaptor during signal transduction [1, 9-12] (as shown in Fig.1). MyD88 protein contains a molecular structure with a Toll/IL-1R (TIR) domain and a death domain [13]. The former is identified to involve in the activation of TLRs, while the latter propagates a signal by capturing downstream signaling intermediates. Data are emerging that TLR signaling through MyD88 adaptor cause downstream effects which include pro-inflammatory cytokines/type□interferon (IFNs) release [2, 14]. Many reports have demonstrated that skin graft in mice with targeted deletion of MyD88 adaptor protein received allograft acceptance without rejection [1,15]. In addition, MyD88 was also required for the induction of T cell proliferation and production of the acute phase proteins [16]. Therefore, MyD88 is an ideal target to prevent the signal transduction of TLRs for recognition and inhibiting immune response.

RNA interference (RNAi) is widely used to introduce an exogenous specific siRNA to cells for degradation of targeted mRNA [17], leading to the relative gene silencing. The shRNA is an enhanced approach of RNAi for inhibiting the expression of unwanted protein [18]. Herein, we anticipated to block the signal transduction of TLRs for recognition results from employing shRNA to suppress expression of MyD88. However, shRNA has a difficulty in reaching targeted cells because of their lack of serum stability and rapid clearance. Thus, a reliable vector holds the significance of protecting shRNA from nuclease, serum protein and RES capture. Conventional viral vectors can trigger insertional mutagenesis and immunological problems, despite they have high delivery efficiency [19-20]. Nonviral delivery systems provide some advantages over viral vectors, including safety of the materials, simplicity of preparation, ability to load plasmid of any size and multifunctional modifiability. In our previous work [21-22], we have successfully synthesized a cationic polymer liposomes-based vector with three advantages: 1) The vectors with high transfection efficiency are easily to be prepared; 2) The cationic polymer can stablely condense genes via electrostatic interactions; 3) Nano-shells formed by octadecyl-quaternized lysine modified Chitosan (OQLCS) with positively charges have a good dispersibility.

In this study, we proposed a new approach on inhibiting immune recognition by delivering a shRNA of MyD88 with a cationic

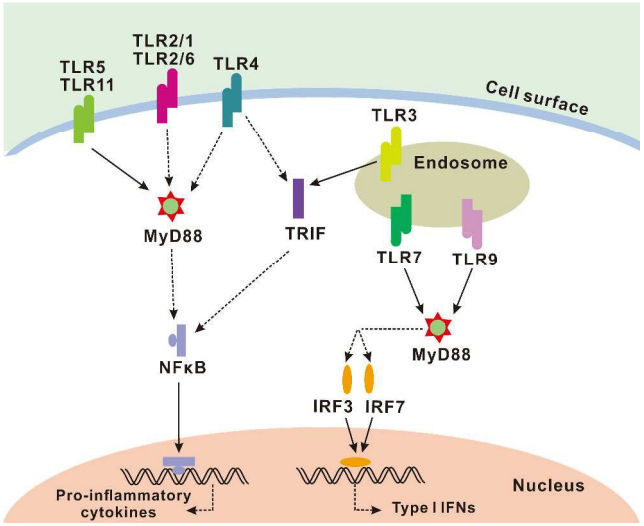

Fig.1 TLR signaling in conventional dendritic cells. TLR2 (in association with TLR1 or TLR6), TLR4, TLR5 and TLR11 are localized on the cell surface for ligand recognition. TLR3, TLR7 and TLR9 are localized in the endosome. All TLRs, except TLR3, recruit MyD88. TLR4 and TLR6 recruit other additional adaptor. Dendritic cells stimulated with TLR 1, TLR2, TLR5, TLR6, TLR11 ligands initiate the MyD88-dependent pathway. MyD88 activated NFκB and IRFs via complicated interactions, respectively. NFκB initiate the transcription of pro-inflammatory cytokines whereas IRFs initiate the transcription of type Ⅰ interferons.

polymeric liposomes vector. We expected that this method can efficiently inhibit expression of MyD88, resulting in failed immune recognition. This attempt promised to be potential in solving immune-related diseases.

2. Materials and methods

2.1 Construction of shRNA of MyD88 plasmid

The MyD88 complementary shRNA was inserted into the Bam HI and Hind III restriction sites of I pGenesil-1 expression vector containing EGFP sequences (Wuhan Genesil Bio-technology, Wuhan, China). The shRNA was cloning specific sequences: GCC TCT CGC TGT TCT TGAA. Primer 1 (5′-AGG ACA AAC GCC GGA ACT TTT-3′) and primer 2 (5′-GCC GAT AGT CTG TCT GTT CTA GT-3′) were set as primers of the target genes and gene of β-actin (primer 1: 5′-GTC GTA CCA CTG GCA TTG TG-3′, primer 2: 5′-CTC TCA GCT GTG GTGGTG AA-3′) was set as reference gene. The formed MyD88-shRNA plasmid was named as pMyD88. In addition, control plasmid pHK was also constructed by cloning a non-specific shRNA sequence into the same restriction sites.

2.2 Preparation of the cationic polymer liposomes system

We have successfully synthesized an excellent cationic polymer liposomes (OQLCS) vector for genes in our previous work [21-22]. Amphiphilic molecules OQLCS (180 ug) and cholesterol (60 ug) dissolved in 3 mL chloroform was assembled to form a nanostructure in 6 mL distilled water under ultrasonic bath. After removed chloroform using a vacuum rotary evaporator, the residual was dispersed in distilled water. Subsequently, 0.5 mg SPDP in 1 mL water was added to the lipid solution. After stirring of the mixture for 2 h, the solution was purified by dialysis with a 10000 MW cutoff membrane for 4 h. Then added 1 mL TAT solution (0.01 M), the mixture was incubated overnight at 4 □. Finally we obtained the liposomes vectors with purification using dialysis.

2.3 Optimization of pMyD88/liposomes complex

The formed pMyD88 were incubated with the liposomes at different mass ratio (1:0,1:0.5,1:1,1:2,1:2.5,1:3,1:3.5), respectively. Plasmids were loaded onto the surface of liposomes via electrostatic interaction. The systems with different mass ratio were detected and screened by agarose gel electrophoresis (AGE) experiment. The optimum mass ratio selected was used in the subsequence experiments.

2.4 Cell culture and cell transfection

Rats' myeloid dendritic cells (DCs, from Cell bank, Chinese academy of medical science) were cultured in complete medium (Dulbecco's minimal essential medium with 10% fetal calf serum, 1% penicillin and streptomycin) at 37 □ in a humidified atmosphere of 5% CO_2. After treating with 0.25% Trysin-EDTA, cells resuspened with complete medium at a concentration of 1×10^5 cells/mL were seeded on the plates. Added 2 μg naked pMyD88, 1 μL Lipofectamine 2000 and the 2 μg pMyD88/liposomes complex to 50μL Opti-MEM respectively, the mixtures were incubated for 30 min. Then mixtures were transferred into the seeded cells. 50 μL Opti-MEM were used for control. Subsequently, 500μL of complete medium were added into all wells. After incubating for 48h, the transfection efficiency was detected by fluorescence microscope and flow cytometry (FAC-Scalibur, Becton Dickinson, Franklin Lakes, NJ, USA).

2.5 Examination of gene silence effect in transfected DCs

The transfected cells were collected to Eppendorf tubes. After added 1 mL Trizol and 0.2 mL CHCL3 to the tube, the mixture was standing for 3min. Next, the mixture was centrifuged and the supernate was washed three times with ethyl alcohol. Then 1 μL mRNA extract was used to reverse transcription to obtain cDNA. Added 1.0 μL cDNA, 9.0 μL SYBR MasterMix, 1.0 μL primer 1 （10 μM）, 1.0 μL primer 2（10 μM）to 2.0 μL double distilled water, then 20 μL mixture was used for amplification with Real Time PCR (Bio-Rad, Hercules, CA). The β-actin was set as reference genes for PCR analysis.

At the same time, MyD88 protein was determined by western blot analysis. The rest collected cells were treated with lysis buffer (RIPA 1.0 mL and PMSF 10 μL) to obtain proteins. The proteins were separated using the SDS-PAGE method. Separated proteins were transferred to a nitrocellulose membrane, which was incubated with blocking buffer for 1 h. The membrane was washed with PBST three times before being incubated with rabbit-anti-rat MyD88 antibody (1:200) at 4 □ overnight. After washing the membrane with PBST three times, goat-anti-rabbit HRP-tagged antibody (1:1000) was added for incubation of 1 h. Then, the membrane was washed and treated with chemiluminescence reagents. Finally, the membrane was used for detection and the images were analyzed with Image-Pro Plus 6.0 software. Rabbit-anti-rat GAPDH antibody was used to test reference protein.

2.6 Investigation on immunogenicity of transfected DCs

1.6 μg LPS was resolved in 80 μL of Opti-MEM to obtain LPS

solution. Transfected cells were collected for resuspension with complete media at 1×10^5 cells/mL. 180 μL resuspened solution was incubated with 20 μL LPS solutions for 3 days. 20 μL Opti-MEM solution was used for control.

After cultured for 3 days, IFN-γ and IL-2 concentrations in supernatant from cultured cells were examined respectively using ELISA kit. Following blank wells, standard wells and testing wells incubated for 2 h, the supernatant was discarded and corresponding antibody was added into each well., The wells were washed with PBST for 5 times after incubating for 1 h. Then 90 μL TMB was added into the well followed by incubating for 30min. Finally, the optical density was read by Safire 2 at a wavelength of 450 nm. Standard curve and sample density was calculated by the applied software of Safire 2. At the same time, the cells were collected for examination of MyD88 protein with western blot.

The expression of MHC II protein was tested by flow cytometry. Phycoerythrin-conjugated mouse-anti-rat MHC II antibody was used to incubate with cells for 30 min in the dark. Then the DCs were analyzed by flow cytometry.

3. Results and discussions

3.1 Optimization of pMyD88/liposomes complex

The MyD88 complementary shRNA was inserted in to the plasmid containing enhanced green fluorescent protein (EGFP) sequences (as shown in Fig. 2). The resulting recombinant MyD88-shNRA pGenesil-1 was named as plasmid shRNA-MyD88 (pMyD88). EGFP can be observed in fluorescence microscope, which was used as a maker of exogenously administered MyD88-shRNA. The same plasmid with a non-specific shRNA (pHK) was used as control.

The liposome vectors with sufficient positively charges condensed genes to form a pMyD88/liposomes complex as shown in Fig. 3(1). Charge density influenced the electrostatic interaction between pMyD88 and cationic polymeric liposomes. So in order to obtain stable complexes, AGE was used to screen the optimal mass ratio between the two components in their assemble attachment. As shown in Fig. 3(2), when the mass ratio comes to 1:2 or higher than 1:2, no separated plasmid was observed. Thus, the mass ratio of 1:2 was selected as the optical ratio and was used in all subsequent experiments.

The morphology of the optimal complex was characterized by TEM. From the Fig. 3(3), we can see a uniform distribution and a diameter less than 100 nm, most of which were about 70 nm. Results from DLS and surface zeta potential analysis showed the average size and zeta potential of the liposomes and the complexes. In Fig. 3(4), the average size of pMyD88/liposomes complexes were about 95nm, while the liposomes had a much larger size of 400 nm. There was sufficient positive charge on the surface of the liposomes since surface zeta potential was 54 mV as shown in Fig. 3(5). The zeta potential was decreased with combination of negatively charged pMyD88. The addition of shRNA condensed the liposomes via electrostatic interaction, which caused the complex with much smaller size in the DLS analysis. Meanwhile, decreased zeta potential confirmed the formation of the pMyD88/liposomes complexes.

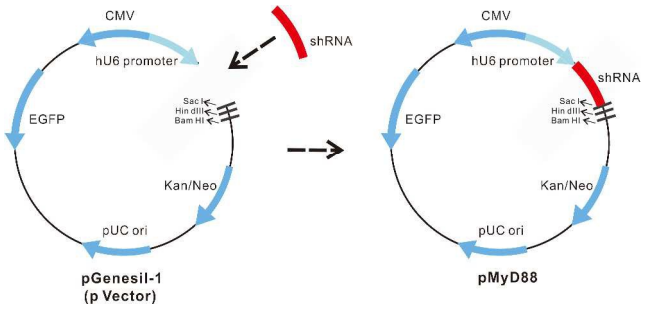

Fig.2 Schematic illustration of pMyD88 design. The shRNA-pMyD88 was inserted into the plasmid.

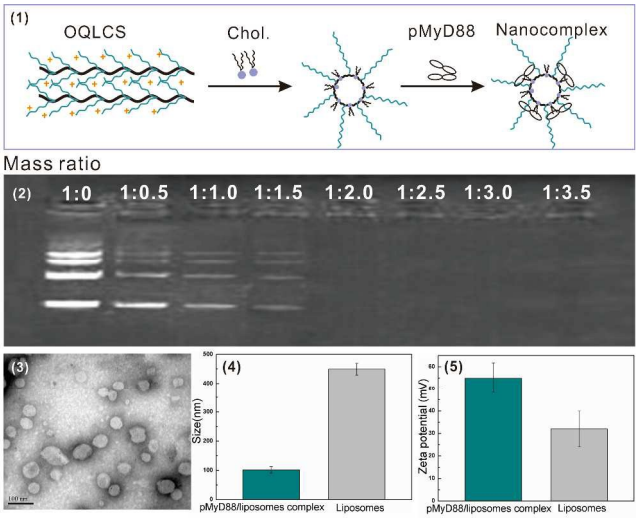

Fig.3 Preparation and Characterization of the optimal pMyD88/liposomes complex. (1) The formation of pMyD88/liposomes complex. (2) The complex with different weight ratio of pMyD88 to liposomes was optimized by AGE. (3) TEM image of the optimal complex. (4) Average size of the liposomes and optimal complex. (5) Zeta potential of the liposomes and the optimal complex.

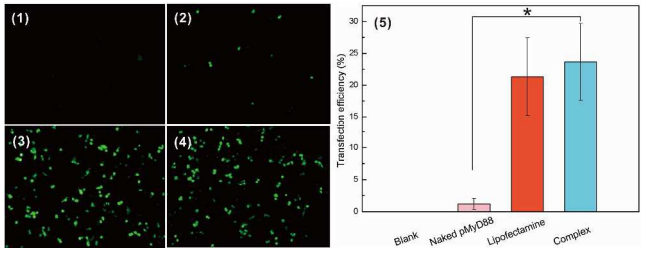

Fig.4 Transfection efficiency of the complex. (1)-(4) were fluorescence (1) Blank media. (2) Media with pMyD88. (3) Media with lipofectamine. (4) Media with pMyD88/liposomes complex. (5) Flow cytometry analysis of intracellular delivery efficiency. The data are the mean ± SE (n = 6). *P < 0.01 as compared with the cells treated with naked pMyD88 (ANOVA, analysis of variance).

3.2 Evaluation of transfection efficiency of the complex in DCs

After characterized the optical complex formation, we investigated the pMyD88/liposomes complex transfection ability in rats' DCs, with naked pMyD88 and Lipofectamine as the controls. Firstly, the uptake of exogenously administered pMyD88 was observed under fluorescence microscope with the help of enhanced green fluorescent protein (EGFP). The strong green fluorescence came out from the transfected cells indicated that the plasmid was successfully delivered to the cells. It was

clear in Fig. 4 that the liposomes vector enhanced the transfection efficiency of the naked pMyD88. Flow cytometry was used for quantitative analysis of transfection efficiency. Compared the values in four groups, the transfection efficiency of the pMyD88/liposomes complex was the strongest (28.19±5.38), which indicated that the liposomes system significantly enhanced the transfection efficiency of naked pMyD88 (1.25±0.86).

3.3 Gene silence effect of pMyD88/liposomes complex

In order to assess gene silence effect of pMyD88/liposomes complex, we investigated MyD88 gene transcription level and protein expression level. We had confirmed the vectors indeed enhanced transfection efficiency of naked pMyD88, in the subsequent experiments, non-specific pHK was used as control. The Real Time PCR results showed that there was a significant decrease of MyD88 mRNA in the cells treated with pMyD88/liposomes complex. As depicted in Fig. 5(1), the pMyD88/liposomes complex group was at a much lower value of 0.11±0.04, whereas the controls were all about to be 1. The results indicated a significant inhibition of MyD88 gene transcription in the cells treated with pMyD88/liposomes complex. Moreover, western blot revealed the obvious suppressed expression of MyD88 protein in the pMyD88/liposomes complex group (0.11±0.05), but not in the other groups as shown in Fig. 5(2). These results suggested the pMyD88/liposomes complex worked well in DCs to make gene MyD88 silence.

3.4 Investigation on immunogenicity of the gene silenced DCs

We expected that the DCs transfected by pMyD88/liposomes complex would fail in recognizing exogenous antigen. Lipopolysaccharide (LPS), a major cell wall constituent of gram-negative bacteria, was used as external stimuli. The LPS stimulation test was carried to mimic the initial contact of DCs with foreign antigen, in which process TLRs recruit MyD88 to produce cytokines and induce immune response.

TLRs signaling recruited MyD88 as adaptor and induced more MyD88 protein to be synthesized. So, we first investigated the MyD88 gene expression of transfected DCs incubated with LPS. The Real Time PCR and western blot analysis showed that there was a significant suppressed expression of mRNA (Fig. 6(1)) in the cells treated with pMyD88/liposomes complex as well as the MyD88 protein (Fig. 6(2)), while there was no difference in the other groups. Downstream effect of TLRs signaling concluded cytokines (IFN-r and IL-2) release and up-regulation of MHC Ⅱ antigen on the cells surface. Cytokines and MHC Ⅱ were associated with subsequent immune response, such as T cells activation and proliferation. So, we investigated the secretion of cytokines (IFN-r and IL-2) in culture media of transfected DCs. As shown in Fig. 6(3) and (4), ELISA results revealed that cytokines secretion in pMyD88/liposomes complex group was remarkably suppressed. No difference occurred in the control groups. Finally, we tested the MHC Ⅱ protein expressed on the cell surface. In Fig. 7, flow cytometry results showed a similar expression level of MHC Ⅱ protein in pMyD88/liposomes complex group to that of negative control. These results indicated that immune recognition via TLRs signaling was successfully blocked in DCs transfected by pMyD88/liposomes complex.

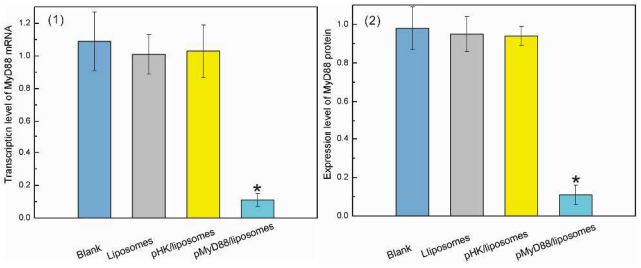

Fig.5 Gene expression of MyD88. (1) Transcription level of MyD88 mRNA. (2) Expression level of MyD88 protein. The data are the mean ± SE (n = 6). *Significantly lower than other groups (P < 0.01, ANOVA).

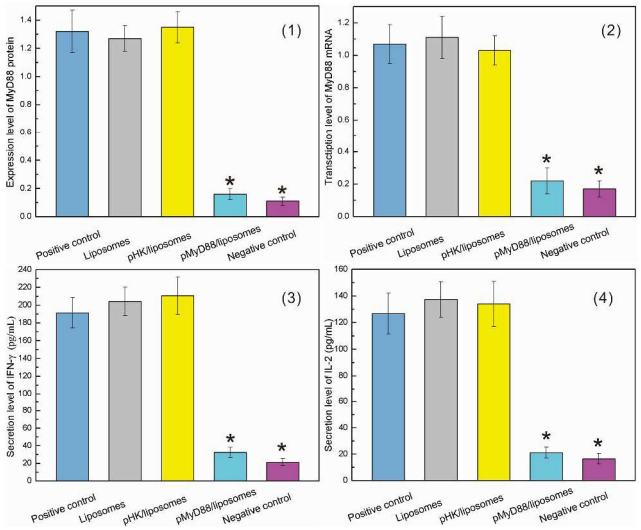

Fig.6 Expression of MyD88 protein and secretion level of cytokines in deficiency-MyD88 DCs treated with LPS. (1) Transcription level of MyD88 mRNA. (2) Expression level of MyD88 protein. (3) Secretion of IFN-γ. (4) Secretion of IL-2. The data are the mean ± SE (n = 6). *P < 0.01 as compared with cells in positive control, liposomes and pHK/liposomes groups (ANOVA).

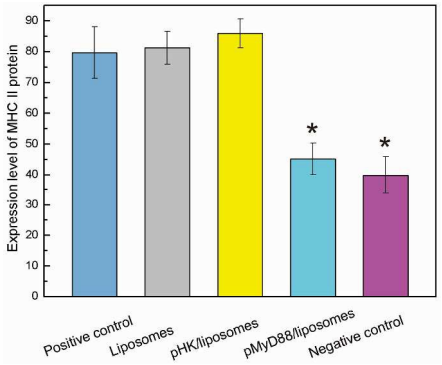

Fig.7 Expression of MHC□protein in deficiency-MyD88 DCs treated with LPS. The data are the mean ± SE (n = 6). *P < 0.01 as compared with cells in positive control, liposomes and pHK/liposomes groups (ANOVA).

4. Conclusions

In this study, we demonstrated the pMyD88/liposomes complex made MyD88 gene silence with high efficiency. Moreover, immune recognition via TLRs signaling was significantly inhibited in DCs transfected by the pMyD88/liposomes complex. AGE experiment was utilized to obtain the optimal mass ratio of

1:2 between plasmids and liposomes for the complex. The exciting transfection results revealed the liposomes system significantly enhanced the transfection efficiency of naked pMyD88. Gene and protein detection proved that the plasmid/liposomes complex produced deficiency-MyD88 DCs with high efficiency. Furthermore, the LPS stimulation experiment confirmed that deficiency of MyD88 protein blocked the signal transduction of immune recognition, resulting in low immunogenicity of transfected DCs. In conclusion, our results indicated that pMyD88/liposomes complex could be developed as an effective approach for inhibiting immune recognition. Above the results, the pMyD88/liposomes complex seems to be potential in solving skin graft rejection where TLRs signaling was predominant.

Acknowledgement

The authors gratefully acknowledge National Natural Science Foundation of China (81102246, 51373117, 51303126), Tianjin Natural Science Foundation (13JCQNJC11900), Tianjin Municipal Education Commission Science Foundation (20090126), Key Project of Tianjin Natural Science Foundation (13JCZDJC33200), and Doctoral Base Foundation of Educational Ministry of China (20120032110027).

Notes and references

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† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/
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