Cooperative binding and self-assembling behavior of cationic low molecular-weight dendrons with RNA molecules†‡

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Received 3rd November 2005, Accepted 2nd December 2005
First published as an Advance Article on the web 9th January 2006
DOI: 10.1039/b515667j

Tri(ethylene glycol) derived, low molecular-weight dendrons with various amine end groups were synthesized and characterized for their properties of binding and self-assembling with RNA using the *Candida* ribozyme as a model RNA molecule. These dendritic compounds form stable complexes and well-defined nanoscale particles with RNA molecules *via* electrostatic interactions and self-assembly process, while leaving the other terminal of the tri(ethylene glycol) chain accessible for targeting. This suggests that dendrimers of this type hold great promise for specific RNA targeting and RNA delivery.

Introduction

Dendrimers are perfectly structured molecules with large numbers of cascade-branched units emanating from a focal point, resulting in densely packed end groups at the molecular surface.[1] This feature of dendrimers has been widely used to generate multivalent interactions and cooperative effects in order to amplify weak interactions and obtain special functions and properties.[1–4] One of the main biological applications of dendrimers is dendrimer-based gene transfer, which involves polycationic dendrimers such as polyamidoamine (PAMAM),[5] polylysine[6] and poly(propylene imine) (PPI).[7] Under physiological conditions these dendrimers have positively charged amine end groups at the dendrimer surface. They self-assemble with DNA *via* electrostatic interactions, which result in cooperative binding and dense packing between the dendrimers and DNA molecules. In general, DNA binding and delivery are more effective with dendrimers of higher-generation or structurally fractured systems.[5,8] Low molecular-mass dendrons were recently reported to have a high binding affinity with DNA and to be capable of efficient gene delivery.[7,9] Polycationic dendrons may be used in a similar way with RNA, another class of biologically relevant nucleic acids which are involved in a wide range of important biological functions such as protein synthesis, post-transcriptional RNA processing, regulation of gene expression and retroviral replication, and are emerging as both important drug targets and versatile therapeutic agents.[10] However, far fewer efforts have been made so far with RNA.[11] We recently established that polycationic PAMAM dendrimers interact strongly with RNA ribozymes *via* cooperative electrostatic interactions, thus strongly inhibiting the catalytic activities of ribozymes.[11] This finding opens up new perspectives for using polycationic PAMAM dendrimers for both RNA targeting and RNA delivery purposes.[12]

The possibility of closely controlling the size, shape and surface chemistry of dendrimers[1] gives us an opportunity of creating a repertoire of structure-, size- and shape-tailored dendrimers binding to various RNA molecules as required. In our ongoing project focusing on RNA targeting and RNA delivery, we are interested in developing low molecular-mass dendrons with well-defined structures that can bind and assemble with RNA molecules *via* cooperative electrostatic interactions, as well as being able to conjugate a ligand for eventual specific targeting purposes. Systems of this kind can help to throw light on structure–activity relationships and may have better chances of being used for cell- or organ-specific delivery of nucleic acids.[13]

Tri(ethylene glycol) is a low molecular-weight compound of poly(ethylene glycol)s, known to be highly water-soluble, non-immunogenic, and biocompatible.[14] Tri(ethylene glycol) is widely used as a linker to connect two distinct functionalities in bioconjugated molecules. By promoting dendrimer growth at only one end of tri(ethylene glycol), we can obtain fan-shaped PAMAM dendrimers with various amine end groups for RNA binding (Scheme 1), while the other terminal of the tri(ethylene glycol) chain can be coupled with a specific ligand for targeting purposes. The tri(ethylene glycol) part is neutral and so will not create electrostatic interactions and may also have steric hindrance effects on RNA binding. It is therefore necessary to demonstrate whether these tri(ethylene glycol) derived PAMAM dendrons still hold strong RNA binding properties. Here we report on the synthesis of tri(ethylene glycol) derived PAMAM dendritic constructs and on studies in which we assessed their binding properties and self-assembling behavior with RNA molecules.

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† Electronic supplementary information (ESI) available: Detailed experimental procedures for dendrimer synthesis and characterization as well as studies on RNA/dendrimer complexes using different methods. See DOI: 10.1039/b515667j

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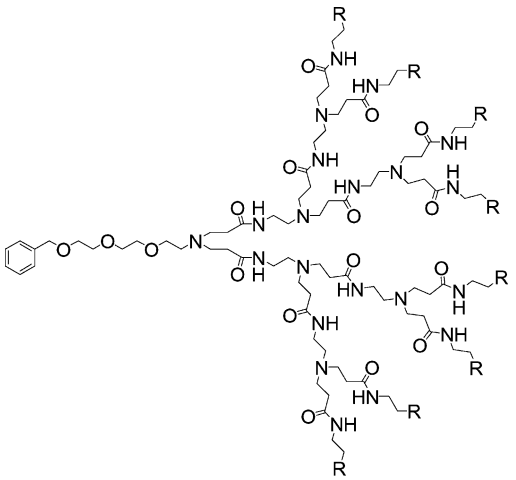


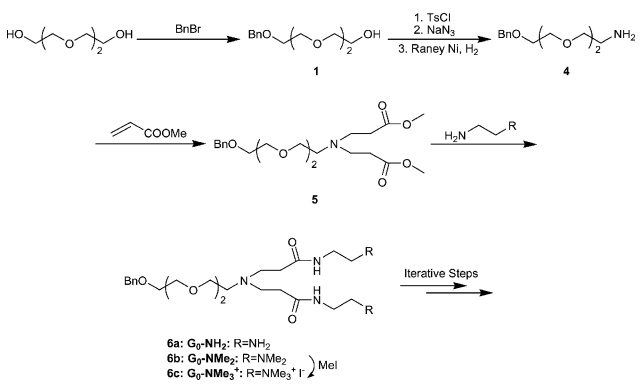
G_2-NH_2: R=NH_2
G_2-NMe_2: R=NMe_2
G_2-NMe_3^+: R=$NMe_3^+I^-$

Scheme 1 Tri(ethylene glycol) derived dendritic constructs with various amine end groups. For clarity, only dendrimers of second generation are drawn.

Results and discussion

Synthesis

Scheme 2 shows the synthesis of the tri(ethylene glycol) derived dendrimers. While one terminal hydroxyl group of tri(ethylene glycol) was protected selectively with a benzyl group, the other end was transformed to amine *via* successive tosylation, azidation and catalytic hydrogenation. The resulting compound **4** was then served as the starting material for dendrimer growth. The subsequent Michael addition of **4** with methyl acrylate gave the diester **5**, which was further treated with diamine, giving **6**, the generation zero dendrimer. We obtained dendrimers **6a** and **6b** with primary and tertiary amine end groups, respectively, using ethylenediamine and *N*,*N*-dimethylethylenediamine as the diamine components. Further dendrimers, from generations one to four, were obtained using the conventional two-step iterative synthesis procedure:[15] (a) branching double alkylation of terminal NH_2 groups with methyl acrylate followed by (b) amidation of the terminal ester with the corresponding diamine. The $–NMe_3^+$ terminating dendrimers were obtained by methylating the $–NMe_2$ terminating dendrimers with MeI, where not only the end amine groups but also some of the interior amine groups are transformed to quaternary amines. The protective benzyl group at the end of the tri(ethylene glycol) chain can be removed by hydrogenation. However, we did not remove the benzyl group because we wanted to use it as a model ligand to study its relationships with the dendritic constructions at the other end of the tri(ethylene glycol) chain. The synthesized dendrimers are referred to here as G_n–NH_2, G_n–NMe_2, G_n–NMe_3^+, which indicate a $–NH_2$, $–NMe_2$, and $–NMe_3^+$terminated dendrimer, respectively (*n* is the generation number). All final compounds and intermediates were fully characterized with ^{1}H- and ^{13}C-NMR, IR and MS (see the electronic supplementary information, ESI†).



Scheme 2 Synthesis of the tri(ethylene glycol) derived dendrimers.

Dendrimer inhibition on the *Candida* ribozyme activity

The binding properties of these dendrimers to RNA were assessed using the *Candida* ribozyme[16] which was used as a model RNA here. Ribozymes[17] provide a unique opportunity for correlating RNA binding with the ribozyme activity: valuable information about RNA/dendrimer binding behavior can be obtained by analyzing the inhibitory effects of dendrimers on the ribozyme activity. The *Candida* ribozyme is a self-splicing group I intron from the 26S rRNA of the opportunistic fungal pathogen *Candida albicans.*[16] The inhibitory capacity of the dendrimers on the *Candida* ribozyme was assessed[16] and the IC_{50} values are listed in Table 1.

Similar to our observations with PAMAM dendrimers having triethanolamine as a core,[11] dendrimers with various amine end groups synthesized in this work were found to have strong inhibitory effects on the *Candida* ribozyme. The inhibitory efficiency of these dendrimers increased with generation (Table 1), whereas no inhibitory activity was observed with first generation dendrimers or dendrimers with ester end groups (data not shown). Neither was any inhibitory effect observed with small cationic ammoniums such as NH_4Cl or NMe_4Cl up to 1 mM (data not shown). The strong inhibitory effects of the dendrimers are therefore attributable to local cooperative interactions between the positively charged amine groups located at the dendrimer surface and the negatively charged phosphate groups present in ribozymes.

Furthermore, no significant differences in inhibition were observed among dendrimers of the same generation with different amine end groups such as $–NH_2$, $–NMe_2$, and $–NMe_3^+$ (Table 1), which can be explained by the fact that the electrostatic forces are the major factors responsible for the interactions between the dendrimers and the RNA ribozymes.[11] However, protonated $–NH_2$ and $–NMe_2$ groups at dendrimer ends might also form strong H-bonds with the branching units inside the dendrimers, generating back-folding effects[18] and making the end groups of the $–NH_2$ and $–NMe_2$ terminating dendrimers less accessible for RNA binding.

Table 1 Inhibitory effects of dendrimers with various generations and various amine end groups on the self-splicing activity of the *Candida* ribozyme

	Inhibition on ribozyme self-splicing (IC_{50}/[μM])		
End group	$–NH_2$	$–NMe_2$	$–NMe_3^+$
Generation 2	2.23	3.94	1.27
Generation 3	0.64	0.84	0.30
Generation 4	0.29	0.48	0.16

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Therefore, slightly weaker inhibition was observed with the $-NH_2$ and $-NMe_2$ terminating dendrimers (Table 1).

Dendrimers compete with Mg^{2+} and affect the ribozyme conformation

We know that RNA ribozymes have a well-defined three-dimensional structure and specific Mg^{2+} and GTP binding sites.[19,20] Mg^{2+} plays an important role in the folding of the RNA into a catalytically active conformation,[19] while GTP contributes importantly to initiating the catalytic reaction of a group I ribozyme.[20] Since the dendrimers are positively charged under physiological conditions, it seems reasonable to suggest that the dendrimer may affect the *Candida* ribozyme folding by competing with Mg^{2+} *via* electrostatic interactions. Our Mg^{2+} displacement data are consistent with this idea (Fig. 1). Further results of GTP displacement experiments (see Fig. S2 in the ESI†) indicate that these dendrimers did not interact with the GTP binding sites. This may be attributable to the fact that the bulkiness of the dendrimers may have prevented them from binding to the well defined GTP site. These findings support the idea that dendrimers bind to the ribozyme mainly *via* charge compensation mechanisms.

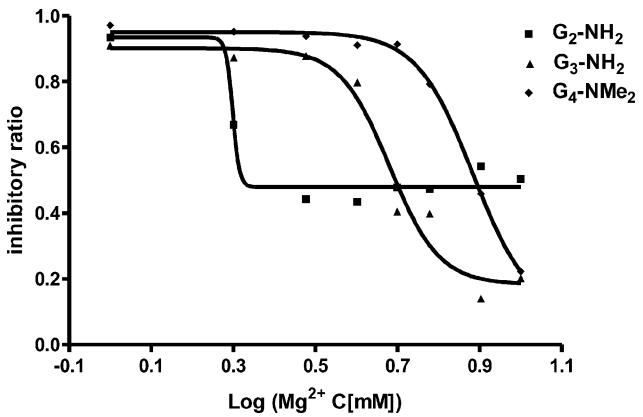

Fig. 1 Mg^{2+} displacement experiments with the *Candida* ribozyme. The indicated concentrations of $MgCl_2$, varying from 1 to 10 mM, were the corresponding pre-incubated concentrations with dendrimers. The final concentrations of Mg^{2+} were kept as 10 mM. The concentrations of different dendrimers G_2–NH_2, G_3–NH_2 and G_4–NMe_2 were 3.6, 1.4 and 0.9 μM, respectively. Two independent experiments were performed and plotted.

CD spectroscopic studies were also performed to characterize the ribozyme/dendrimer complexes. The spectrum of the uncomplexed ribozyme showed the positive band centred near 270 nm characteristic of the active conformation of the *Candida* ribozyme (Fig. 2). None of the dendritic compounds showed any significant optical activity (see Fig. S3 in the ESI†). Increasing addition of the dendrimer to the ribozyme gradually reduced the intensity of the 270 nm band, which suggests that the RNA/dendrimer complex affected the conformation of the active ribozyme. These data are consistent with the results obtained in the ribozyme activity assays and with complex formation between dendrimers and RNA in agarose gel (see below).

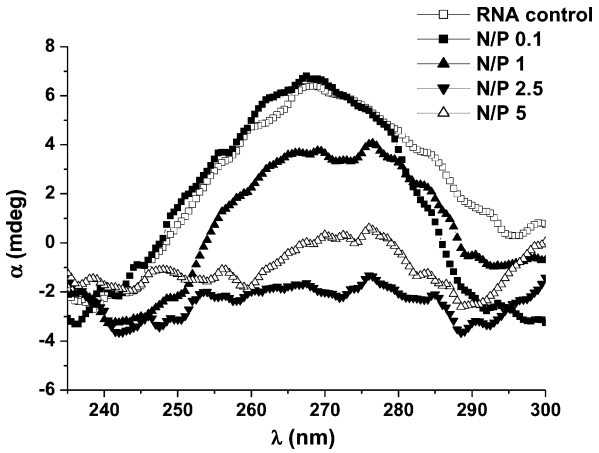

Fig. 2 CD spectral analysis of RNA/G_4–NMe_2 complexes at various N/P ratios: (■) N/P = 1 : 10, (▲) N/P = 1 : 1, (▼) N/P = 2.5 : 1, (△) N/P = 5 : 1 and (□) RNA control.

Dendrimers and RNA form stable complexes and assemble into well-defined nanoparticles

The binding properties and assembly behavior of the dendrimers with the RNA ribozymes were also investigated by electrophoretic gel mobility shift in native agarose gel (Fig. 3). Dendrimers with various amine end groups were able to completely prevent the ribozymes from moving in the gels. In addition, the gel retardation of the ribozyme was found to depend on the generation of dendrimers involved: the higher the generation of the dendrimers, the stronger the interactions between the dendrimer and the RNA were, and therefore the greater the gel retardation of the RNA became. These results agree with those obtained with PAMAM dendrimers having triethanolamine as core,[11] indicating that strong binding and efficient self-assembly occurred between the RNA molecules and the dendritic molecules with cationic amine end groups.

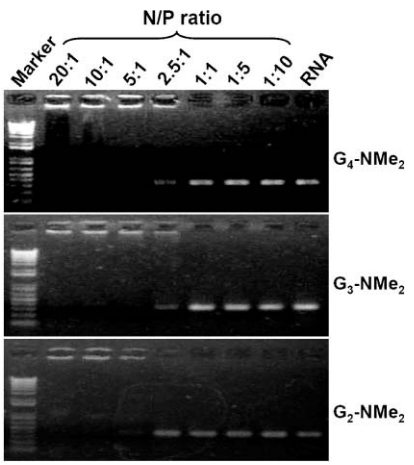

Fig. 3 Agarose gel analysis of the formation of RNA/dendrimer complexes with dendrimers G_2–NMe_2, G_3–NMe_2 and G_4–NMe_2, respectively, at charge ratios N/P varying from 1 : 10 to 20 : 1 in buffer solution (pH 7.6).

Furthermore, transmission electron microscopy (TEM) was used to image the RNA/dendrimer complexes (Fig. 4). The

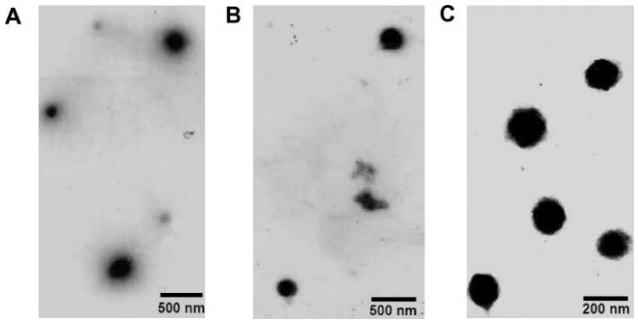

Fig. 4 TEM images of Ca.L-11 RNA/dendrimer complexes at N/P = 10 : 1. (A) RNA/G_2–NH_2 (B) RNA/G_3–NH_2 (C) RNA/G_4–NH_2. The complexes were prepared at a final RNA concentration of 2.0 ng μL^{-1} in 30 mM Tris-HCl buffer, pH 7.6.

addition of dendrimer to the *Candida* ribozyme gave rise to well-defined nanosize particles, indicating that the dendrimers efficiently bind to RNA and condense it into spherical nanosize particles. In addition, the formation of a nanoparticle between the RNA and dendrimer depends on the dendrimer generation: the higher the generation of the dendrimer, the stronger the interaction between the dendrimer and the RNA, and the more compact, well-defined and uniform the nanosize particles formed. Therefore, these dendrimers not only bind strongly to RNA as demonstrated by the ribozyme activity assay and gel shift experiments, but also assemble with RNA into stable nanosize particles, similar to those observed with DNA/dendrimer complexes.[9]

The tri(ethylene glycol) chain terminal is accessible for targeting

The protective benzyl group at the end of the tri(ethylene glycol) chain was used here as a model ligand to study its relationships with the dendritic constructions at the other end of the tri(ethylene glycol) chain. In the ^{1}H-NMR spectra, the chemical shifts observed with the benzyl group did not change with increasing dendrimer generations (Fig. 5), which suggests that the benzyl group at the terminal of tri(ethylene glycol) is not buried in the interior of the dendritic structure. Therefore, the short tri(ethylene glycol) unit could act as a linker to connect two distinct functionalities with a view to developing ligand conjugated dendrimers for specific targeting.

We also would like to know whether the benzyl moiety at the end of the tri(ethylene glycol) chain of the dendritic molecules is still accessible after formation of a complex with RNA molecules. This may help to throw light on the potential uses of these molecules for specific receptor-mediated targeting purposes. We therefore performed NMR studies on the RNA/dendrimer complexes. We have used poly(rU) for the NMR study, because the characteristic NMR signals of the benzyl group in the dendrimers fall in the NMR signals of the *Candida* ribozyme, while not interfering with those of the poly(rU) (Fig. 6). When the dendrimer was mixed with the poly(rU), there are obvious spectral changes for the dendrimer (chemical shifts around 2.5–3.2 ppm which represent moieties connected to amine functionalities in dendrimer) and for the poly(rU) (chemical shifts around 5.8 and 5.7 ppm which represent sugars and bases in poly(rU), respectively). These spectral changes are indications of the interaction between dendrimers

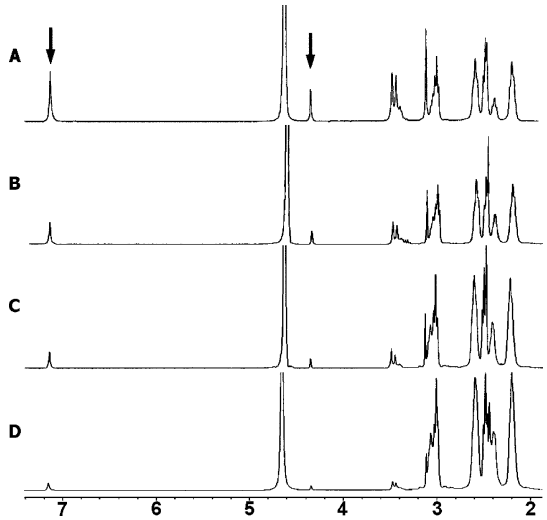

Fig. 5 ^{1}H-NMR of amine-terminating dendrimers from generation one to generation four. (A) G_1–NH_2 (B) G_2–NH_2 (C) G_3–NH_2 (D) G_4–NH_2. Arrows indicate the signals for the benzyl moiety.

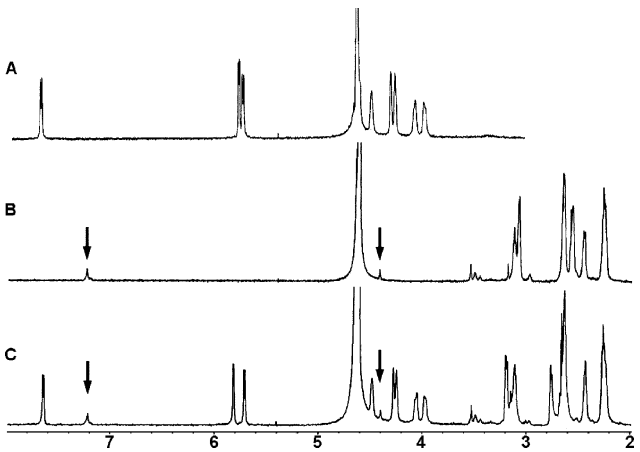

Fig. 6 ^{1}H-NMR analysis of (A) poly(rU) (B) G_3–NH_2 (C) poly(rU)/G_3–NH_2 at N/P = 1 : 1. Arrows indicate the signals for the benzyl moiety.

and poly(rU). The fact that the chemical shifts of the benzyl group in the dendritic molecules did not change when complexed with poly(rU) (Fig. 6), suggests that this benzyl group at this terminal of tri(ethylene glycol) chain may not have a strong interaction with the RNA molecules and is therefore available for receptor-mediated targeting purposes.

Moreover, the tri(ethylene glycol) derived dendritic compounds G_n showed comparable inhibitory effects on the *Candida* ribozymes to those observed with triethanolamine derived dendrimers.[11] This indicates that the presence of the tri(ethylene glycol) unit does not adversely affect the RNA binding properties of these dendritic constructs. Moreover, the flexible and hydrophilic feature of the tri(ethylene glycol) moiety may provide the dendrimer with some plasticity when interacting with RNA molecules, facilitate the adaptive interplay between complementary molecular shapes and conformation, and thus enhance the

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interactions between the dendritic molecules and structured RNA molecules.

Conclusion

In conclusion, tri(ethylene glycol) derived dendritic compounds with various amine end groups were synthesized and their properties of binding and assembly with RNA were assessed using the *Candida* ribozyme as a model RNA molecule. The amine terminating dendrimers bind strongly to the ribozyme and efficiently inhibit the activity of the ribozyme, as shown by their effects on both electrophoretic mobility in agarose gel and the catalytic activity of the ribozymes. These dendrimers exert their inhibitory effect by affecting the *Candida* ribozyme folding through competing with Mg^{2+} *via* electrostatic interactions, as evidenced by Mg^{2+} displacement experiments and CD spectroscopic studies. In addition, the RNA/dendrimer complexes form stable, well-defined, spherical nanoscale particles, which reflects further the strong electrostatic interactions occurring between RNA and dendrimers, and the cooperative effect as well as the self-assembly processes at work. Moreover, the NMR studies showed that the presence of a tri(ethylene glycol) linker did not adversely affect the RNA binding properties of the dendritic molecules and that the other end of the tri(ethylene glycol) linker is accessible for targeting. Therefore, the short tri(ethylene glycol) unit could act as a linker to connect a ligand to a dendrimer for specific RNA targeting or RNA delivery. Recently, ligand conjugated PAMAM dendrimers using poly(ethylene glycol) as a linker have been found to be safe and efficient vectors for specific DNA delivery.[13] Taken all together, we expect that the PAMAM dendrimer systems as described here may also find promising uses for specific RNA targeting and RNA delivery. We are working actively in this direction.

Acknowledgements

We are grateful to Bao Penghui and Chen Zhiqiang for ribozyme preparation, Dr. Liu Minghua for CD analysis. Dr Laurence Charles thanks Spectropôle, the Analytical Facility of Aix-Marseille Universities, for technical support in this study by allowing special access to the instruments. Financial support from the Ministry of Science and Technology of China (N°2003CB114400, 2003CB514102, 2003AA2Z3506), National Science Foundation of China (N°20572081, 30330170, 30170213), Wuhan University, Cheung Kong Scholar Foundation, AFM and CNRS is gratefully acknowledged.

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