Gene Delivery

Deutsche Ausgabe: DOI: 10.1002/ange.201508695
Internationale Ausgabe: DOI: 10.1002/anie.201508695

High DNA-Binding Affinity and Gene-Transfection Efficacy of Bioreducible Cationic Nanomicelles with a Fluorinated Core

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Abstract: *During the last two decades, cationic polymers have become one of the most promising synthetic vectors for gene transfection. However, the weak interactions formed between DNA and cationic polymers result in low transfection efficacy. Furthermore, the polyplexes formed between cationic polymers and DNA generally exhibit poor stability and toxicity because of the large excess of cationic polymer typically required for complete DNA condensation. Herein, we report the preparation of a novel class of bioreducible cationic nanomicelles by the use of disulfide bonds to connect the cationic shell to the fluorocarbon core. These bioreducible nanomicelles form strong interactions with DNA and completely condense DNA at an N/P ratio of 1. The resulting nanomicelle/DNA polyplexes exhibited high biocompatibility and performed very effectively as a gene-delivery system.*

The efficient delivery of DNA into cells is critical to the success of many biotechnology and medical applications (e.g., gene therapy).[1] Though recombinant viral gene-delivery vectors have been effective in this regard, their use in medical applications has been limited by their toxicity and immunogenicity, as well as difficulties associated with their large-scale preparation.[2] In principle, these problems could be circumvented by the use of cationic polymers, which have consequently become one of the most promising vectors for gene transfection. The growing popularity of cationic polymers in this regard can be attributed to their ease of synthesis, as well as their amenability to structural modification, which enables them to be tailored readily for specific biomedical applications.[1e,3] Cationic polymer systems deliver genes by forming complexes with DNA through electrostatic interactions. The resulting complexes protect DNA from degradation and facilitate its cellular uptake and intracellular trafficking into the nucleus. The formation of strong interactions between the DNA and polymer molecules provides a much greater degree of protection to the DNA and is therefore more effective for delivering DNA into cells. However, most of the existing cationic polymers form weak interactions with DNA, and the resulting polyplexes are generally unstable in physiological fluids, which contain serum components and salts that tend to disassociate these complexes.[4] Furthermore, transfection experiments involving cationic polymers usually require cationic polymers with a high N/P ratio, which can result in the formation of polyplexes with a highly net-positive surface charge as well as an excess of free cationic polymers within the mixture.[5] Highly positive charges on the surface of the polyplexes can interact with cellular components (e.g., cell membranes) and inhibit normal cellular processes, including clathrin-mediated endocytosis and cell-survival signaling, as well as the activity of ion channels, membrane receptors, and enzymes.[5,6] Although the presence of excess free cationic polymers can lead to enhanced gene-transfection efficiency, they can also destabilize the plasma membrane of red blood cells.[4,5,7] In light of these undesirable effects, there is an urgent need for the development of cationic-polymer vectors with a high binding affinity towards DNA.

Several novel macromolecules with high DNA-binding affinities have been described.[8] For example, Smith and co-workers prepared a series of dendrons containing multiple spermine units on their surface, and showed that these dendrons exhibited high binding affinity for DNA ($CE_{50} = 0.68$).[8d] Schmuck and co-workers optimized a short sequence of amino acids within a tweezers-like molecule capped with a tailor-made anion-binding group; the resulting system showed high affinity towards plasmid DNA.[8a,b] Even though these macromolecules with high affinity for DNA enabled the efficient shuttling of DNA into cells, their gene-transfection efficiency remained very low, most likely because their tight binding affinity prevented the efficient release of the entrapped DNA.[8a–c] An excellent gene-delivery vector must not only have a high binding affinity towards DNA, but must also have an excellent ability to release entrapped DNA after it enters the cell. To this end, we constructed a novel class of bioreducible cationic nanomicelles by using disulfide bonds to connect the cationic shell to the fluorocarbon core. We used fluorocarbon chains to form the core of the nanomicelles because of their high rigidity as compared with hydrocarbon chains. It was envisaged that the high rigidity of these chains would result in a low surface energy, which would lead to a significant increase in the stability of the resulting nanomicelles.[9] Moreover, since fluorocarbon chains are hydrophobic and lipophobic, and therefore show a high tendency towards phase separation in both polar and nonpolar environments,[10] the use of fluorocarbon cores can lead to an improvement in the ability of the resulting polyplexes to traverse the lipid bilayers of cells, as well as

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201508695.

the endosome/lysosome membrane, thereby facilitating endosomal escape.[3c,11] Most importantly, the cationic polymers were densely packed onto the surface of the nanomicelles, thus leading to a high charge density and therefore enhancing the binding affinity towards DNA. Disulfide linkages can be reduced by intracellular glutathione, thereby facilitating the release of the entrapped DNA inside the cell. On the basis of these design features, it was envisaged that this bioreducible cationic nanomicelle system would be an excellent gene-delivery vector.

To prepare bioreducible cationic nanomicelles with a fluorocarbon core, we initially prepared a fluorocarbon chain ($-C_7F_{15}$) with an *N*-(2-(2-pyridyldithio)ethyl) terminus. The treatment of poly(ethylenimine) (PEI, $M_n = 10000$) with the Traut reagent gave the corresponding thiol-containing PEI product. This material was subsequently treated with a fluorocarbon chain bearing an *N*-(2-(2-pyridyldithio)ethyl) moiety. Thus, fluorocarbon chains were attached to PEI through a disulfide exchange reaction (Figure 1a). A variety of fluorocarbon-chain-modified PEIs were synthesized in this way, including PEI-SS-3C_7F_{15}, PEI-SS-5C_7F_{15}, and PEI-SS-10C_7F_{15}, as PEI macromolecules containing 3, 5, and 10 fluorocarbon chains, respectively. PEI-SS-5C_7F_{15} was used for the preparation of nanomicelles with sizes of 10, 30, and 50 nm (see detailed procedures in the Supporting Information).

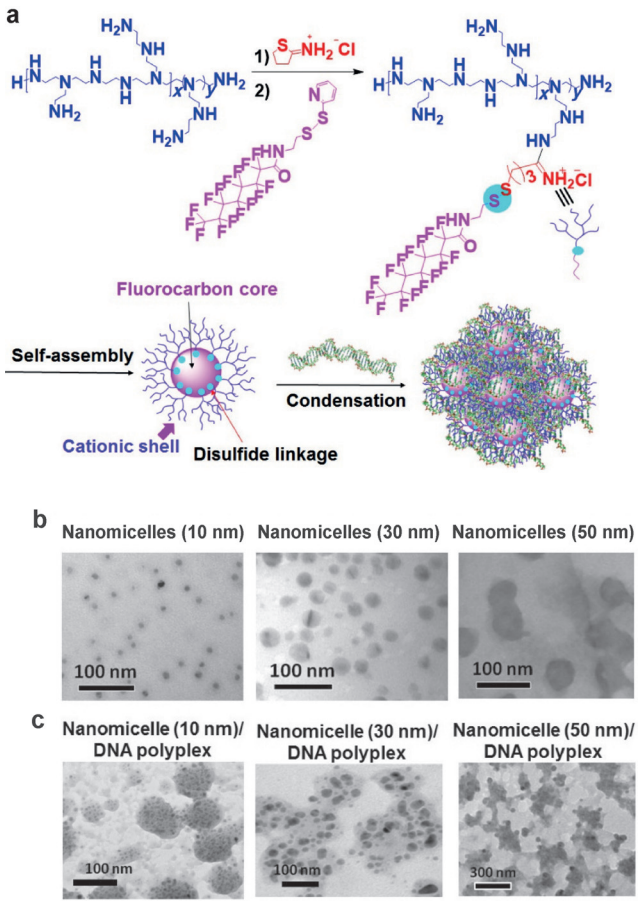

Figure 1. a) Scheme outlining the linking of fluorocarbon chains to PEI, nanomicelle formation, and the complexation of the nanomicelles with DNA. b) TEM images of the nanomicelles prepared in the current study. c) TEM images of the polyplexes formed with 10, 30, and 50 nm nanomicelles.

The DNA-binding affinities of the cationic nanomicelles were initially evaluated by using an ethidium bromide displacement assay. This assay is a powerful comparative method, although the resulting data reflect a competition assay.[8d] The results can be presented in terms of CE_{50} values (Figure 2a), which are defined as the "charge excess" required to achieve a 50% reduction in fluorescence. A good DNA binder should give a CE_{50} value of less than 1.0.[8d] Although PEI ($M_w = 25000$) is a highly charged polymer, it forms a weak binding interaction with DNA ($CE_{50} = 1.59$). PEI-SS-5C_7F_{15} had a lower CE_{50} value of 1.39. The 30 and 50 nm micelles formed mid-strength binding interactions with DNA with a CE_{50} value of 1.05 and 1.17, respectively. This result indicated that the DNA-binding affinity can be enhanced by a self-assembly approach. Notably, the 10 nm cationic micelles exhibited a very high binding affinity towards DNA, with a CE_{50} value of 0.23.

The binding affinities of the polymers and nanomicelles towards DNA were also measured by isothermal titration calorimetry (ITC; see Figure S7 in the Supporting Information).[12–14] The binding constants (K_a) for PEI and PEI-SS-5C_7F_{15} were 8.39×10^7 and $1.03 \times 10^8\ M^{-1}$, whereas the values for the 30 and 50 nm micelles were 4.07×10^8 and $3.23 \times 10^8\ M^{-1}$, respectively. However, the K_a value for the 10 nm micelles was the highest at $1.39 \times 10^9\ M^{-1}$, which represents an increase of about two orders of the magnitude when compared with that of PEI. High binding constants are indicative of the formation of strong interactions between the micelles and DNA. These results therefore indicated that these micelles would condense DNA at a very low N/P ratio. Generally, PEI can completely condense DNA at N/P ratios above 4. However, the 10 nm micelles can completely condense DNA even at an N/P ratio of 1. On the basis of the high stability of their fluorocarbon core and their high DNA-binding affinity, the 10 nm micelle/DNA polyplexes would be stable under physiological conditions. In the aqueous NaCl (200 mM), the polyplexes formed from PEI/PEI-SS-5C_7F_{15} and DNA broke apart to release the entrapped DNA (Figure 2d). However, no DNA was released from the 10 nm micelle/DNA polyplexes under the same conditions, thus indicating that the 10 nm micelle/DNA polyplexes are highly stable.

Cellular-uptake efficiency is one of the key parameters for high gene-transfection efficacy.[15] We therefore evaluated the uptake efficiency of the different polyplexes into HeLa cells (Figure 3a). The polyplexes formed from PEI or PEI-SS-5C_7F_{15} and DNA showed similarly low levels of cellular uptake, even though they have a high surface charge (ca. 25 mV). The low cellular uptake of these polyplexes could be attributed to their poor stability. Although 10 nm micelle/DNA polyplexes formed at a N/P ratio of 2 have a slightly positive surface charge of about 9 mV (much lower than the surface charge of the PEI or PEI-SS-5C_7F_{15} polyplexes formed at an N/P ratio of 5), they showed a much higher cellular-uptake efficacy than PEI or PEI-SS-5C_7F_{15} polyplexes, probably as a result of their high stability and fluorocarbon cores.

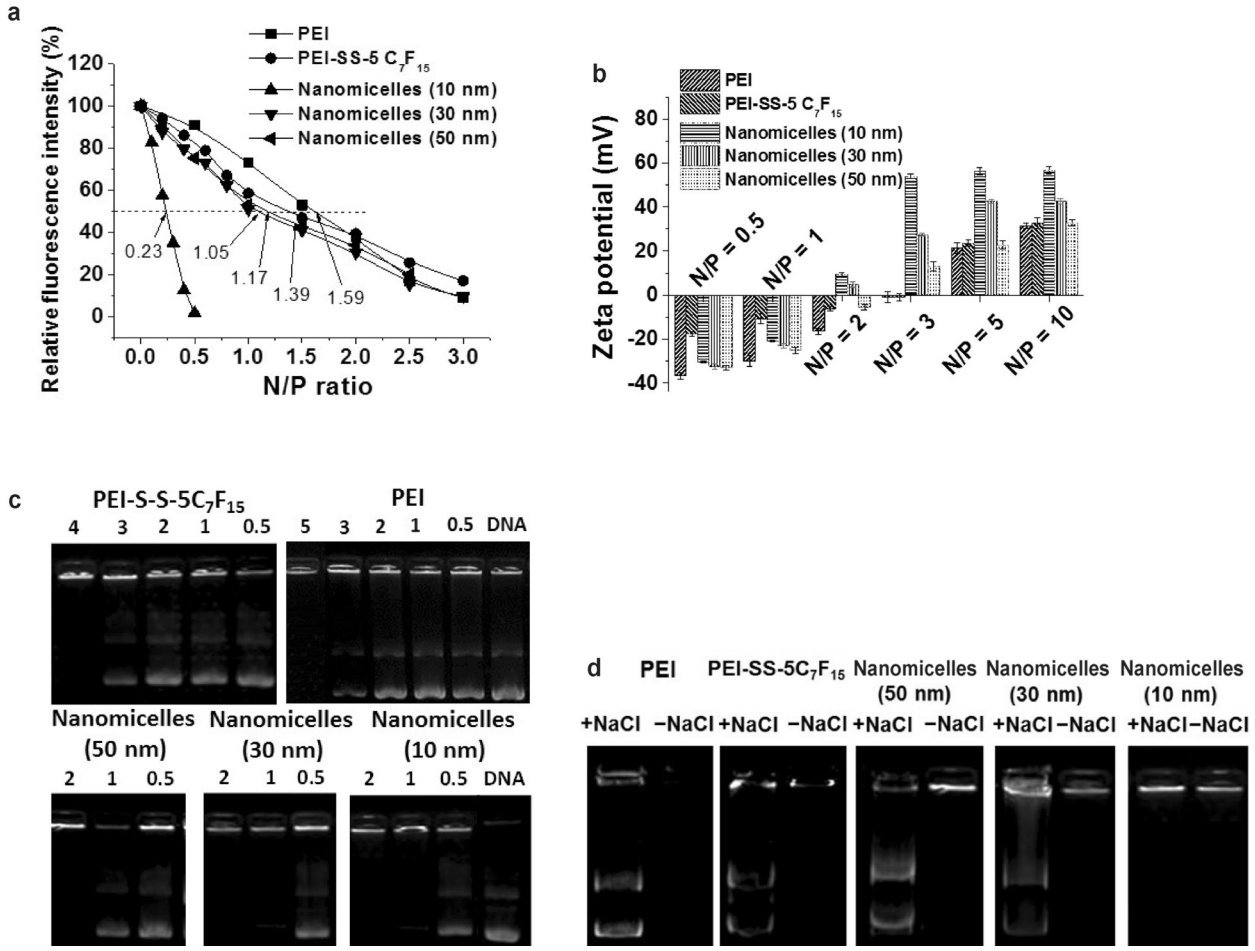

Figure 2. a) Fluorescence titration profiles for the addition of PEI, PEI-SS-5C_7F_{15}, and the micelles (10, 30, and 50 nm) to a solution of green fluorescent protein (GFP) DNA and ethidium bromide in a SHE buffer. b) Zeta potential of the polyplexes formed from the polymers or nanomicelles and DNA at different N/P ratios. c) Agarose gel electrophoresis results showing the DNA-binding capacity of the polymers and nanomicelles. d) Agarose gel electrophoresis results for the treatment of polyplexes formed from DNA and PEI (N/P = 5), PEI-SS-C_7F_{15} (N/P = 4), and nanomicelles (10, 30, and 50 nm; N/P = 2) with aqueous NaCl (200 mM).

The research groups of Vierling, Xiong, and Cheng reported increases in the transfection efficacy of cationic polymers through fluorination.[3c,10a,16] However, fluorination only led to a moderate increase in the transfection efficacy in most cases.[10a,16b] Fluorination of PEI led to a slight improvement in transfection efficacy.[16b] The self-assembly of PEI-SS-5C_7F_{15} into bioreducible cationic nanomicelles can tremendously increase the DNA-binding affinity by about two orders of magnitude. High-affinity binding to DNA provides much greater protection to the DNA molecules and is therefore more effective for delivering genetic information into cells. Moreover, the cationic shell and fluorocarbon core of the nanomicelles were connected by disulfide bonds, which were cleaved in the presence of glutathione to disassemble the nanomicelles and release the entrapped DNA (Figure 3b). It was therefore expected that the polyplexes formed from the nanomicelles would have a high gene-transfection efficacy. The 10 nm micelles exhibited a significantly higher GFP transfection efficacy than PEI in 293T cells, even at a low N/P ratio of 2 (Figure 3c). At an extremely low N/P ratio of 1, the gene-transfection efficacy of the micelles was similar to that of PEI at an N/P ratio of 10.

Similar results were obtained for a luciferase reporter gene assay (Figure 4a). The high transfection efficacy of the 10 nm micelles was further confirmed in HeLa cells (Figure 4b), A549 cells, HepG2 cells, and MCF-7 cells (see Figures S9–S11). Although a variety of polymers with very high DNA-binding affinities have been reported, the gene-transfection efficacies of these materials are typically low,[8a,c] most likely because of difficulties in releasing the entrapped DNA into cells. In this study, we used disulfide bonds to connect the cationic shells to the fluorocarbon cores, which not only led to stronger binding to the DNA but also enabled the facile release of the bound DNA, thus leading to high gene-transfection efficacy. Viral systems are by far the most effective means of DNA delivery (usually >90% efficiency).[3b] The flow cytometry results showed that the gene-transfection efficacy of the 10 nm micelles was around 95%, even at an N/P ratio of 2 (Figure 4d), and thus reached the level of viral vectors.[17]

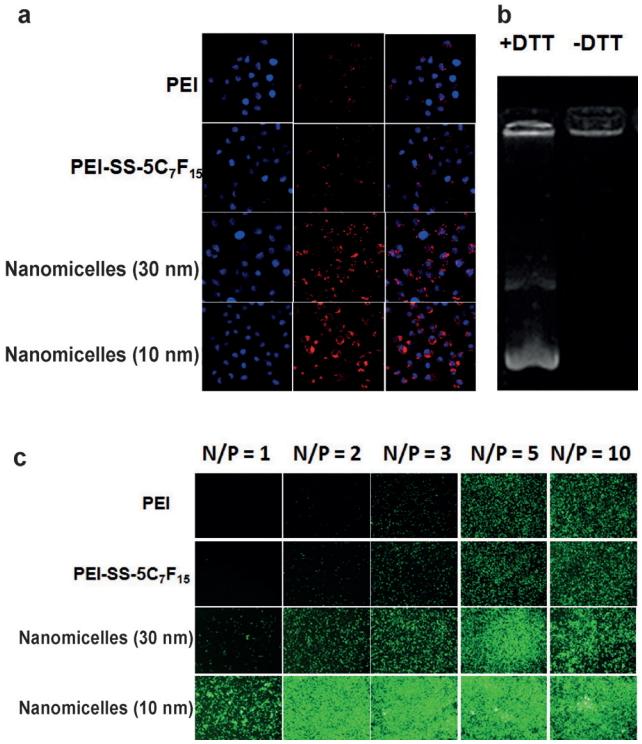

Figure 3. a) Confocal images of HeLa cells treated with PEI/DNA complexes at an N/P ratio of 5, PEI-SS-5C_7F_{15}/DNA polyplexes at an N/P ratio of 5, 30 nm micelle/DNA polyplexes at an N/P ratio of 5, and 10 nm micelle/DNA polyplexes at an N/P ratio of 2 for 2 h (DNA was labeled with Cy5, and the cell nuclei were stained with 4′,6-diamidino-2-phenylindole). b) Agarose gel electrophoresis results following the treatment of the polyplexes formed from 10 nm micelles/DNA with dithiothreitol (DTT; 10 mM). c) GFP expression in the 293T cells.

In general, the polyplexes formed between cationic polymers and DNA in the presence of an excess of the cationic polymer are highly dynamic complexes, in which the polymers retain a degree of mobility, whereas the DNA is relatively restrained.[18,19] In the presence of serum, the polyplexes and the free polymers could potentially bind with the serum proteins, which would break the dynamic equilibrium and result in the cleavage/aggregation of the polyplexes. However, the 10 nm micelle/DNA polyplexes have a slight positive charge on their surfaces (ca. 9 mV), which would reduce their ability to bind with the serum. Furthermore, as shown above, the 10 nm micelles bind very tightly to the DNA, and the resulting polyplexes would be difficult to break apart. Gene-transfection experiments were conducted in 293T cells with a medium containing 0, 10, 30, or 50% FBS. Notably, a high level of gene transfection was observed for the 10 nm micelles at a low N/P ratio of 2, even in the medium containing 50% FBS (Figure 4c). The FBS therefore had very little effect on gene transfection, whereas gene transfection by the PEI polyplexes significantly decreased in the presence of FBS (Figure 4c).

Generally, the formation of polyplexes between a cationic polymer and DNA typically requires a large excess of the cationic polymer, which consequently leads to an excess of the free polymer within the system.[20,21] For PEI to attain good gene transfection, it has to be complexed with DNA at an N/P ratio of 10, which leaves an excess of seven equivalents in the mixture.[20,21] Unfortunately, the presence of free PEI can destabilize the plasma membranes of red blood cells and induce the aggregation of erythrocytes.[15] We performed an erythrocyte aggregation test (see Figure S12a,b for microscopy and SEM images of the mouse erythrocytes after their treatment with a buffer, the 10 nm micelles, and a solution of PEI). The results revealed that the 10 nm micelles did not induce aggregation of the erythrocytes. In contrast, the PEI solution did lead to significant aggregation. The cytotoxicity of the bioreducible nanomicelles was evaluated in an MTT assay. The nanomicelles showed much better biocompatibility than PEI. This enhanced biocompatibility is related to the introduction of the fluorocarbon chain.

In conclusion, the self-assembly of polycationic polymers bearing a fluorocarbon core gave bioreducible cationic nanomicelles with a high DNA-binding affinity. The bioreducible cationic nanomicelles exhibited excellent DNA-condensation properties, even at an N/P ratio of 1. Moreover, the polyplexes formed by the condensation of these nanomicelles with DNA were very stable under physiological conditions, but were disrupted by intracellular glutathione, which led to the release of the entrapped DNA and high levels of gene-transfection efficacy (ca. 95% in 293T cells). These results therefore show that the construction of bioreducible cationic nanomicelles is an attractive strategy for producing effective gene vectors.

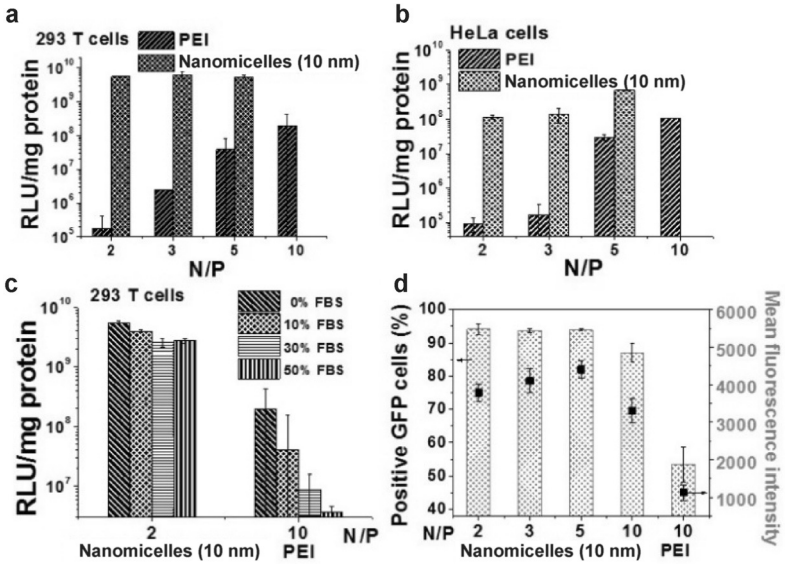

Figure 4. a) Luciferase transfection efficiency of 10 nm micelles and PEI in 293T cells. b) Luciferase transfection efficiency of 10 nm micelles and PEI in HeLa cells. c) Luciferase transfection efficiency of 10 nm micelles and PEI in 293T cells with 0, 10, 30, and 50% FBS. d) Proportion of GFP-positive cells and the mean fluorescence intensity for GFP transfection with 10 nm micelles and PEI in 293T cells.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (51273187, 21374107, 21474097) and the Fundamental Research Funds of the Central Universities (WK2060200012).

Keywords: biotechnology · DNA-binding affinity · gene delivery · nanomicelles · nanostructures

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 755–759
Angew. Chem. **2016**, *128*, 765–769

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Received: September 16, 2015
Published online: November 20, 2015

