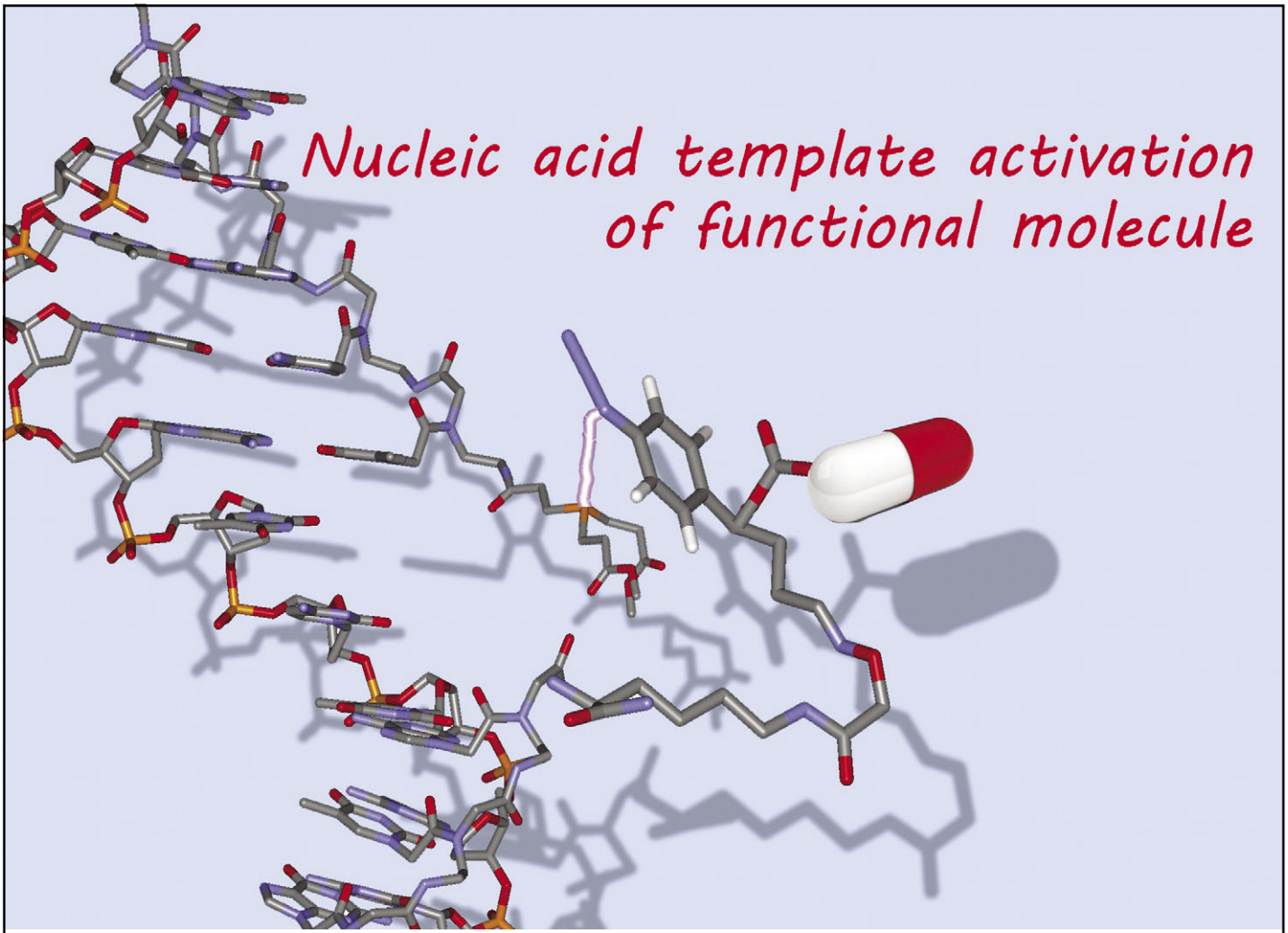


Showcasing research from Nicolas Winssinger's Laboratory, Université de Strasbourg, France

DNA-templated release of functional molecules with an azide-reduction-triggered immolative linker

Nucleic acid templated reactions have attracted significant attention for nucleic acid sensing. Herein we report a general design which extends the potential of nucleic acid templated reactions to unleash the function of a broad diversity of small molecules such as a transcription factor agonist, a cytotoxic or a fluorophore.

As featured in:

See Katarzyna Gorska, Alex Manicardi, Sofia Barluenga and Nicolas Winssinger, *Chem. Commun.*, 2011, **47**, 4364.

RSC Publishing

www.rsc.org/chemcomm

Registered Charity Number 207890

ChemComm

Dynamic Article Links

Cite this: *Chem. Commun.*, 2011, **47**, 4364–4366

www.rsc.org/chemcomm

COMMUNICATION

DNA-templated release of functional molecules with an azide-reduction-triggered immolative linker†

Katarzyna Gorska, Alex Manicardi, Sofia Barluenga and Nicolas Winssinger*

Received 12th January 2011, Accepted 11th February 2011
DOI: 10.1039/c1cc10222b

Nucleic acid templated reactions have attracted significant attention for nucleic acid sensing. Herein we report a general design which extends the potential of nucleic acid templated reactions to unleash the function of a broad diversity of small molecules such as a transcription factor agonist, a cytotoxic or a fluorophore.

The ability to unleash the function of a small molecule in response to a signal is of broad interest not only for its practical application in probing biological systems and as "smart therapeutics" but also from a more fundamental perspective in systems chemistry. Systems responding to nucleic acid cues (DNA or RNA) have been reported using both molecular beacons (hairpin architectures) and nucleic acid templated reactions.[1] In a pioneering study, Taylor and Ma reported the first system[2] which relied on a DNA template to trigger the hydrolysis of aryl esters catalyzed by an imidazole.[3] This principle was concurrently extended to Cu-complex-based catalysts.[4] A major focus of DNA/RNA templated reactions has been on nucleic acid sensing. The most extensively studied systems are nucleophilic ligation reactions,[5,6] templated native chemical ligations[7] and templated Staudinger reactions.[8,9–12] While nucleophilic ligation reactions and Staudinger reactions have been used to release a quencher,[6,12] these approaches have not been used to release protein ligands or therapeutics. Despite notable advances, there remains a genuine need for nucleic acid templated reactions which are broadly applicable to unleash bioactive small molecules in a bio-orthogonal fashion.[13]

Here, we present a novel and versatile design for the release of different functional molecules based on an azide-reduction triggered immolative linker (Fig. 1). Coupling the release of the functional molecule to an azide reduction was deemed most attractive by virtue of the bioorthogonality of the azide group and the well documented compatibility of the Staudinger reaction with cellular chemistry.[14] Indeed, it has been shown that nucleic acid templated reactions leveraged on a Staudinger reaction are effective in live human cells and bacteria.[10–12] While immolative properties of the *p*-aminobenzyl moiety are well known,[15] the *p*-azidobenzyl moiety has not been harnessed as an immolative linker. This linker should allow a broad variety of molecules to be coupled to the benzylic position *via* a carbonate or carbamate, which can in turn mask the function of the appended molecule. As a proof of principle, we selected a fluorophore (rhodamine) and two bioactive molecules (estradiol, a transcription factor activator and doxorubicin, a cytotoxic—see Scheme 1 for structures). For rhodamine, it is known that its fluorescence is quenched by conversion of both anilines to amides, ureas, carbamates or combinations thereof.[16] In the case of estradiol, the phenol is required for protein binding and its function can be masked by its derivatization.[17] Similarly, the cytotoxicity of doxorubicin has been dramatically reduced by conversion of the carbohydrate amino group to a carbamate.[18]

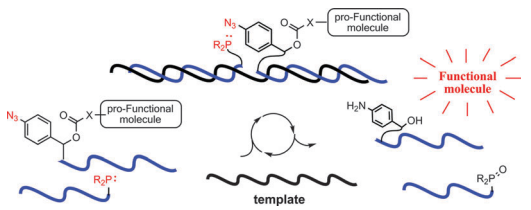
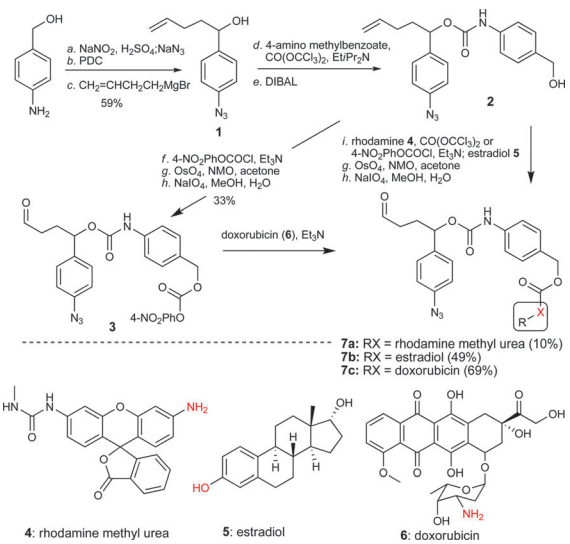


Fig. 1 General concept of DNA-templated azide-triggered release of functional molecules.



Scheme 1 Synthesis of azide-based immolative linker **2** and **3** and their conjugation to functional molecules **4–6**.

Institut de Science et Ingenierie Supramoleculaires (ISIS), Université de Strasbourg—CNRS (UMR 7006), 8 allee Gaspard Monge, 67000 Strasbourg, France. E-mail: winssinger@isis.u-strasbg.fr; Fax: +33 (0)3-9024-5112; Tel: +33 (0)3 68 85 51 13

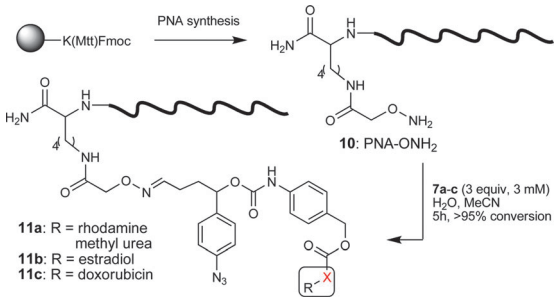
† Electronic supplementary information (ESI) available: Detailed experimental procedure. See DOI: 10.1039/c1cc10222b

Our work on nucleic acid template reactions has focused on the use of peptide nucleic acids[19] based on their resistance to biological degradation, specificity of duplex formation with short sequences and the potential to confer cellular permeability with the use of GPNA.[20] Based on the fact that a number of interesting bioactive molecules would not be compatible with the cleavage conditions of the PNA from the solid phase, we opted to conjugate the small molecule immolative linker adducts post PNA synthesis *via* a chemoselective oxime formation between a PNA bearing a hydroxyl amine and an aldehyde on the immolative linker.

The linker synthesis started from 4-aminobenzyl alcohol (Scheme 1) which was converted to 4-azidobenzaldehyde according to a known procedure[21] and treated with homoallyl magnesium bromide to obtain secondary alcohol **1** in good yield. Efforts to conjugate the different functional molecules directly to this secondary alcohol afforded poor yields presumably due to steric congestion. This led us to add an immolative linker *via* a two step sequence (carbamate formation with methyl 4-isocyanatobenzoate followed by Dibal reduction of the benzoate) to obtain the primary alcohol **2**. Compound **2** could now be coupled to the isocyante of rhodamine methyl urea **4** (prepared *in situ* with triphosgene) followed by conversion of the terminal alkene to an aldehyde (OsO_4; $NaIO_4$) to obtain rhodamine adduct **7a**. To prepare the estradiol adduct **7b**, the alcohol **2** was converted to an activated carbonate with *p*-nitrophenyl chloroformate and treated with estradiol **5** followed by conversion of the terminal alkene to an aldehyde. In the case of doxorubicin **6**, as the adduct would not be compatible with a NaIO4 treatment, alcohol **2** was first converted to the activated carbonate **3** (*p*-nitrophenyl chloroformate; OsO_4; $NaIO_4$) bearing the required aldehyde such that no further transformation would be required following its coupling to doxorubicin.

Next, we assessed the kinetics of decomposition of the immolative linker-conjugates **7a–c** upon reduction of the azide in physiologically relevant conditions. Using a large excess of phosphine (50 eq.), the pseudo-half life for the reduction of the azide was measured to be less than 6 minutes based on the consumption of **7**. Intermediate **8** could not be observed suggesting that its immolation (or that of the aza-ylide intermediate) into compounds **9** is fast. The rate of reaction for the second self-immolative linker did vary depending on the nature of the leaving group. For estradiol, intermediate **9b** could not be observed suggesting that the reaction was fast. In the case of rhodamine (**9a**) and doxorubicin (**9c**), the half life of the reaction was measured to be less than 11 and 21 minutes respectively. These differences in reaction rate are consistent with the increased pK_a of the leaving group. While there are notable differences in the rate of immolation, all the reactions proceeded with kinetics that are compatible with the time frame of biological experiments.

We next investigated the coupling of the linker-conjugates **7a–c** to PNA. The PNA were synthesized by standard Fmoc-based solid phase chemistry with Boc protected nucleobases[22] using a Lys(Mtt)Fmoc as a first residue (Scheme 2). Prior to the cleavage of the last Fmoc, the Mtt group was cleaved (HFIP) and Boc-protected aminooxyacetic acid was introduced. Final Fmoc deprotection and cleavage from the resin (TFA) afforded the PNA **10**. Preliminary experiments for the coupling of **10** to the adduct **7a–c** in buffered conditions at pH 7 afforded



Scheme 2 Synthesis of PNA conjugates **11a–c**.

very slow coupling even in the presence of aniline as a catalyst.[23] However, efficient coupling were achieved under acidic conditions if the PNA were used directly as their TFA salts (obtained directly after cleavage or following an HPLC purification) to obtain complete conversion into conjugates **11a–c** based on MALDI analysis of the reaction.

To test the DNA templated reaction with probes **11**, we selected a sequence corresponding to microRNA21 (TAG CTT ATC AGA CTG ATG TTG A) as the template to be interrogated with the PNA probes. We had previously established that 6–8mer PNA probes were suitable for DNA template reactions and that 2–4 nucleotide distance between the PNA probes was optimal.[9,10] With these criterion, we selected a 7mer sequence for the phosphine conjugate[10] and an 8mer for the immolative linker-conjugates **11**, using a GPNA residue at every other position. In order to follow the progress of the reaction, we first focused on rhodamine release with conjugates **11a** as the reaction can be followed by continuous fluorescence monitoring. As shown in Fig. 3, in the absence of a template, the background reaction between the PNA is negligible. Using a large excess of TCEP, fluorescence increases reaching a maximum within 30 min. The sigmoidal shape of the fluorescence increase is consistent with a two step cleavage of the immolative linker (Fig. 2). In the presence of the perfect match DNA, a fast reaction is observed reaching 30% conversion with 30 min whereas a random DNA sequences or a sequence bearing two mismatches sites had significantly slower rate of reaction ($<5\%$ conversion after 30 min). Having established the viability of the present azide-triggered immolative linker in a templated reaction, we then turned our attention to the release of a bioactive small molecule. While neither estradiol nor doxorubicin have a fluorescence efficiency which enables real time monitoring of the reaction, it is possible to quantify estradiol accurately by HPLC using a fluorescence detector at nM concentration.[24] To this end, we quantified the amount of estradiol released after 30 minutes in the presence of the perfect match DNA template, a mismatched template, a random DNA template and in the absence of template. After 30 min, the reactions were quenched by the addition of H_2O_2 (to a final concentration of 10%) to oxidize the phosphine probe, and the estrogen was extracted and quantified by HPLC. As shown in Fig. 4, while the mismatched template afforded a concentration of estradiol below 20 nM, the perfect matched template afforded 180 nM concentration of estradiol which is in excess of the concentration of template (100 nM) suggesting that the template did indeed turnover. This represents an 85% yield with respect to the concentration of **11b** within 30 min of reaction. The higher conversion obtained after 30 min

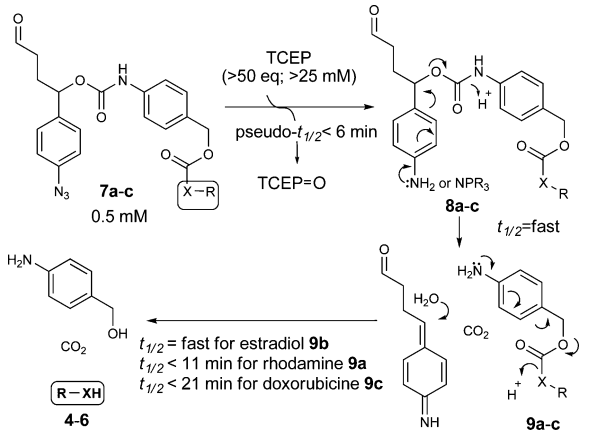


Fig. 2 Kinetic evaluation of functional molecule release upon azide reduction.

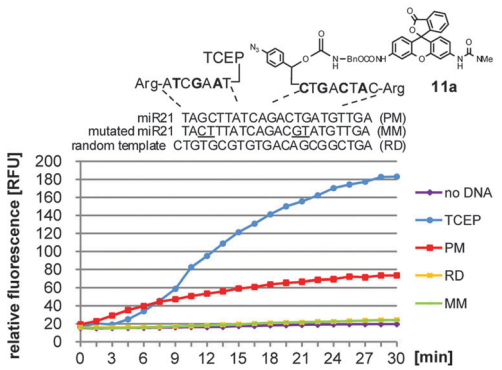


Fig. 3 Kinetics of DNA-templated release of rhodamine **4**. Conditions: 200 nM PNA rhodamine probe **11a**, 800 nM of dmTCEP–PNA probe, 100 nM DNA, in PBS, pH 7.4 with 0.1% BGG at 37 °C. Bold letters denote GPNA residues. PM: perfect match; MM: mismatched; RD: random.

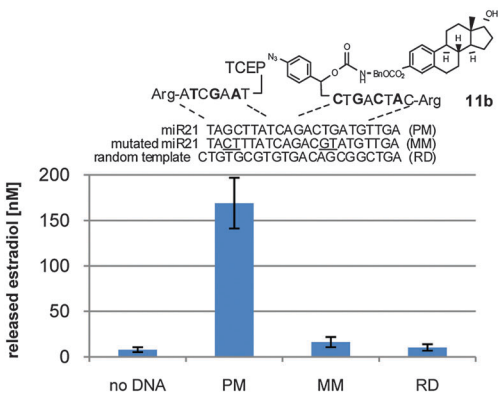


Fig. 4 Templated release of estradiol **5**. Conditions: 200 nM PNA estradiol probe **11b**, 800 nM dmTCEP–PNA probe, 100 nM DNA, in PBS, pH 7.4 with 0.1% BGG at 37 °C for 30 min. Bold letters denote GPNA residues. PM: perfect match; MM: mismatched; RD: random.

with estradiol compared to rhodamine is consistent with the faster kinetics of linker decomposition.

To the best of our knowledge, this is the first report of an azide-based immolative linker which can be triggered by Staudinger reaction to unleash the function of a broad variety of molecules. Its application to DNA-templated release of functional molecules was demonstrated with the release of a rhodamine fluorophore as well as bioactive small molecule, estradiol. The fast kinetics of the release coupled to the robustness of the chemistry should make it a useful tool in nucleic acid controlled systems.

This work was supported by a grant from the European Research Council (ERC 201749). The Institut Universitaire de France (IUF) is gratefully acknowledged for its support. A fellowship Boehringer Ingelheim Fonds (to K.G.) is gratefully acknowledged. We thank Dr Z. Pianowski for preliminary work.

Notes and references

1 A. P. Silverman and E. T. Kool, *Chem. Rev.*, 2006, **106**, 3775; M. F. Jacobsen, E. Clo, A. Mokhir and K. V. Gothelf, *ChemMedChem*, 2007, **2**, 793; S. Tyagi, *Nat. Methods*, 2009, **6**, 331.
2 Z. Ma and J. S. Taylor, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 11159.
3 Z. Ma and J. S. Taylor, *Bioorg. Med. Chem.*, 2001, **9**, 2501; Z. Ma and J. S. Taylor, *Bioconjugate Chem.*, 2003, **14**, 679; J. Cai, X. Li and J. S. Taylor, *Org. Lett.*, 2005, **7**, 751.
4 J. Brunner, A. Mokhir and R. Kraemer, *J. Am. Chem. Soc.*, 2003, **125**, 12410; F. H. Zelder, J. Brunner and R. Kramer, *Chem. Commun.*, 2004, 902.
5 Y. Z. Xu, N. B. Karalkar and E. T. Kool, *Nat. Biotechnol.*, 2001, **19**, 148; S. Sando and E. T. Kool, *J. Am. Chem. Soc.*, 2002, **124**, 9686; H. Abe and E. T. Kool, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 263; D. J. Kleinbaum and E. T. Kool, *Chem. Commun.*, 2010, **46**, 8154.
6 S. Sando and E. T. Kool, *J. Am. Chem. Soc.*, 2002, **124**, 2096.
7 T. N. Grossmann and O. Seitz, *J. Am. Chem. Soc.*, 2006, **128**, 15596; T. N. Grossmann, A. Strohbach and O. Seitz, *ChemBioChem*, 2008, **9**, 2185.
8 J. Cai, X. Li, X. Yue and J. S. Taylor, *J. Am. Chem. Soc.*, 2004, **126**, 16324; R. M. Franzini and E. T. Kool, *ChemBioChem*, 2008, **9**, 2981; H. Abe, J. Wang, K. Furukawa, K. Oki, M. Uda, S. Tsuneda and Y. Ito, *Bioconjugate Chem.*, 2008, **19**, 1219.
9 Z. L. Pianowski and N. Winssinger, *Chem. Commun.*, 2007, 3820.
10 Z. Pianowski, K. Gorska, L. Oswald, C. A. Merten and N. Winssinger, *J. Am. Chem. Soc.*, 2009, **131**, 6492.
11 K. Furukawa, H. Abe, K. Hibino, Y. Sako, S. Tsuneda and Y. Ito, *Bioconjugate Chem.*, 2009, **20**, 1026.
12 R. M. Franzini and E. T. Kool, *J. Am. Chem. Soc.*, 2009, **131**, 16021.
13 It has recently been demonstrated that singlet oxygen could be generated in live cells in response to a nucleic acid cue: D. Arian, E. Clo, K. V. Gothelf and A. Mokhir, *Chem.–Eur. J.*, 2010, **16**, 288.
14 E. M. Sletten and C. R. Bertozzi, *Angew. Chem., Int. Ed.*, 2009, **48**, 6974.
15 P. L. Carl, P. K. Chakravarty and J. A. Katzenellenbogen, *J. Med. Chem.*, 1981, **24**, 479.
16 L. D. Lavis, T. Y. Chao and R. T. Raines, *ACS Chem. Biol.*, 2006, **1**, 252; S. P. Leytus, L. L. Melhado and W. F. Mangel, *Biochem. J.*, 1983, **209**, 299.
17 F. G. Cruz, J. T. Koh and K. H. Link, *J. Am. Chem. Soc.*, 2000, **122**, 8777; H. Li, J. M. Hah and D. S. Lawrence, *J. Am. Chem. Soc.*, 2008, **130**, 10474.
18 T. W. Hudyma, K. Bush, K. L. Colson, R. A. Firestone and H. D. King, *Bioorg. Med. Chem. Lett.*, 1993, **3**, 323; I. Niculescu-Duvaz, D. Niculescu-Duvaz, F. Friedlos, R. Spooner, J. Martin, R. Marais and C. J. Springer, *J. Am. Chem. Soc.*, 1999, **42**, 2485.
19 M. Egholm, O. Buchardt, L. Christensen, C. Behrens, S. M. Freier, D. A. Driver, R. H. Berg, S. K. Kim, B. Norden and P. E. Nielsen, *Nature*, 1993, **365**, 566; P. E. Nielsen, *Mol. Biotechnol.*, 2004, **26**, 233; Z. L. Pianowski and N. Winssinger, *Chem. Soc. Rev.*, 2008, **37**, 1330.
20 P. Zhou, M. Wang, L. Du, G. W. Fisher, A. Waggoner and D. H. Ly, *J. Am. Chem. Soc.*, 2003, **125**, 6878; A. Dragulescu-Andrasi, S. Rapireddy, G. He, B. Bhattacharya, J. J. Hyldig-Nielsen, G. Zon and D. H. Ly, *J. Am. Chem. Soc.*, 2006, **128**, 16104.
21 J. T. Spletstoser, P. T. Flaherty, R. H. Himes and G. I. Georg, *J. Med. Chem.*, 2004, **47**, 6459.
22 S. Pothukanuri, Z. Pianowski and N. Winssinger, *Eur. J. Org. Chem.*, 2008, 3141.
23 A. Dirksen, S. Dirksen, T. M. Hackeng and P. E. Dawson, *J. Am. Chem. Soc.*, 2006, **128**, 15602.
24 Y. Yoon, P. Westerhoff, S. A. Snyder and M. Esparza, *Water Res.*, 2003, **37**, 3530.

