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Synthesis of a poly(Gd(III)-DOTA)–PNA conjugate as a potential MRI contrast agent *via* post-synthetic click chemistry functionalization†

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Herein we present a method for the simultaneous, multiple conjugation of alkynyl-(Gd(III)-DOTA) moieties to a PNA oligomer possessing a domain of azide residues. This is achieved in a single Huisgen type copper-catalyzed azide alkyne cycloaddition (CuAAC) reaction after the oligomer assembly is completed. A triplex formed between the labelled PNA and riboadenylic acid, [(Gd(III)-DOTA)₄–PNA]₂:poly(rA), displayed higher relaxivities at any field strength compared to the single-stranded probe at the same concentration of Gd³⁺ ions as determined by nuclear magnetic resonance dispersion (NMRD) studies.

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Over the past two decades, molecular imaging has been pursued utilizing various modalities including magnetic resonance imaging (MRI), single photon emission tomography (SPECT), positron emission tomography (PET), fluorescence and bioluminescence imaging. Although SPECT/PET techniques are very sensitive, the requirement for radioactive agents and the attendant risks limits their use to justifiably serious applications and not routine screening/diagnoses. Fluorescence and bioluminescence imaging have been widely employed, but are limited by fundamental drawbacks, such as low efficiency of light transmission through tissues, difficulty in quantification, and signal interference from light absorption by haemoglobin and other biomolecules.¹ Therefore, by comparison, MRI has the advantage of being a non-invasive imaging technique that provides high spatial resolution, deep tissue penetration, an unsurpassed ability to distinguish soft tissues and the ability to image subjects repeatedly. With these aforementioned advantages, MRI has proved highly useful in clinical diagnoses; however, the need for development of new contrast agents (CAs) to improve specificity and enhance sensitivity remains.^{2,3}

To date, small molecular Gd³⁺ chelates are routinely used for clinical imaging to enhance image contrast by shortening the relaxation times of proximal water protons. Modifications of Gd³⁺-based CAs have been made in attempts to improve the low molar relaxivity (r_1) of chelated gadolinium. For example, clinically used small molecule contrast agents have been conjugated to high molecular weight macromolecules including

proteins,⁴ polymers,⁵ self-assembled peptide amphiphile nanofibers⁶ and oligonucleotides.⁷ These efforts have resulted in the significant enhancement of r_1 . Additionally, the development of targeted MR contrast agents is of increasing importance, fuelled by the continual discovery of novel molecular targets such as DNA or RNA sequences, or proteins/enzymes involved in specific pathologies.

We were interested to investigate the possibility of producing a generically targeted probe that could report on global mRNA anabolism. Since the 3'-ends of all fully processed eukaryotic mRNAs, with the exception of most histone genes, have a polyadenylic acid [poly(rA)] tail, this was envisioned as a target that could serve as a scaffold for assembling a relatively high density of labels *via* triplex formation. The poly(rA) of mRNA consists of 200–250 adenosines initially in the nucleus, and is shortened to *ca.* 50 adenosines in the cytosol. The tail plays a significant role in mRNA stability, translation and transport.^{8–12} The polyadenylation of mRNA is catalysed by the enzyme poly(rA) polymerase (PAP) and by neo-PAP, a recently identified human poly(rA) polymerase, that is significantly over-expressed in human cancer cells.^{13,14} This suggests the polyadenylated RNA as a potential malignancy selective target for the development of hybridization probes.¹⁵ Some small molecules, like the protoberberine alkaloids, have been found to bind to poly(rA) with high affinity leading to self-structure formation which has been proposed as a method to interfere with the normal biological functioning of mRNA.^{15–17}

Using peptide nucleic acid (PNA) as a targeting agent offers the advantages of high affinity, specificity, biological stability. There are a limited number of examples of PNA–DOTA chelator conjugates for use in imaging by targeting mRNA.^{18–21} While gene specific agents have appeared,¹⁹ a poly(rA) targeted agent has not. Thus, in this work, a PNA hybridization probe

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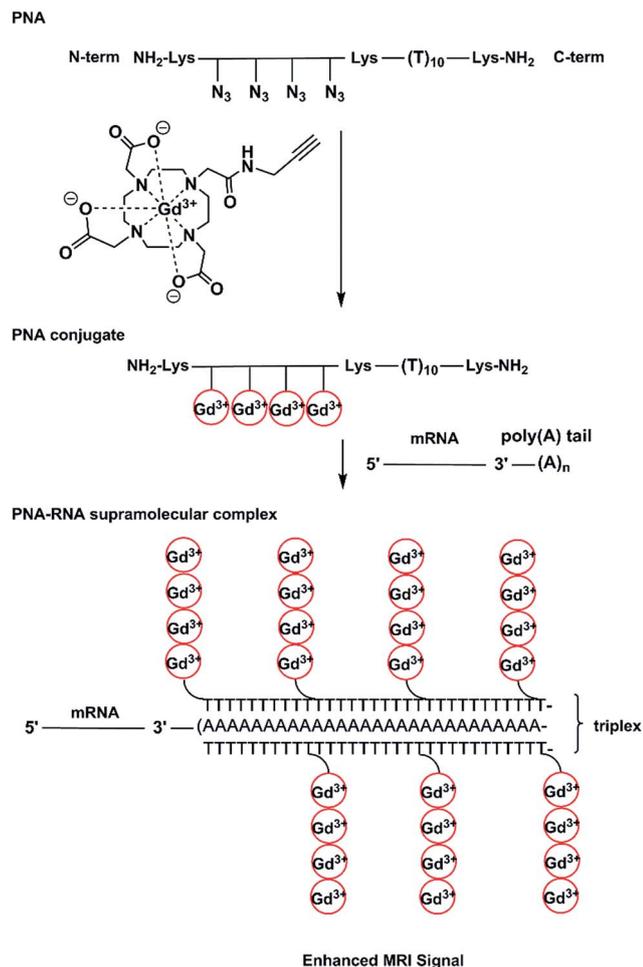


Fig. 1 Conceptual scheme for the preparation and application of (Gd(III)-DOTA)₄-PNA.

CuAAC in the presence of excess alkyne-(Gd(III)-DOTA) to yield Fmoc(Gd(III)-DOTA)₄-PNA (Fmoc-K-Gd-β-Gd-β-Gd-β-Gd-K-TTTTTT-TTTTT-K) in quantitative conjugation yield. The excess reagents, solvents, and catalyst were easily washed from the resin, whereas the workup to remove these materials using solution phase methods can be laborious, in our experience. HRMS (ESI-TOF) analysis of the Fmoc(Gd(III)-DOTA)₄-PNA conjugate showed ions with masses of 1324.0796 [M + 5H]⁵⁺ and 1103.4063 [M + 6H]⁶⁺ which are in accordance with the calculated masses of 1324.0326 [M + 5H]⁵⁺ and 1103.5285 [M + 6H]⁶⁺. After Fmoc deprotection and cleavage of the oligomer from the resin, only the usual reversed phase HPLC purification was required. Characterization of purified (Gd(III)-DOTA)₄-PNA (K-Gd-β-Gd-β-Gd-β-Gd-K-TTTTTTTTTTT-K) *via* HRMS (ESI-TOF) showed masses at 914.5258 [M + 7H]⁷⁺ and 710.6509 [M + 9H]⁹⁺ corresponding to the calculated masses of 914.2765 (M + 7H)⁷⁺ and 711.3279 [M + 9H]⁹⁺.

To determine the stoichiometry of binding of the (Gd(III)-DOTA)₄-PNA conjugate with a complementary nucleic acid, a Job plot from UV-vis data was constructed. For simplicity and accuracy of the measurement, DNA-A₁₀ was used as the target for the PNA probe. The results at two wavelengths (260 and

283 nm),⁴ indicated a binding stoichiometry of two (Gd(III)-DOTA)₄-PNA to one DNA-dA₁₀, which is consistent with triple helix formation (Fig. 2).²⁹ Since it has been shown that homothymidine PNAs form triple helices with oligoriboadenylic acid, specifically (PNA-T₈)₂:poly(rA₄₀),²⁷ we believe that (Gd(III)-DOTA)₄-PNA similarly is capable of triple helix formation with poly(rA), as illustrated in Scheme 1.

Temperature dependent UV studies were performed at 2 μM individual strand concentration for [(Gd(III)-DOTA)₄-PNA]₂:poly(rA). Experiments were repeated thrice and the T_m values were measured from the first derivative plots (Fig. S3†). The melt curves shows a biphasic profile with T_m values of 56 °C and 85 °C, respectively, which is consistent with the presence of a triple helix, and supported by the Job plot studies.

To evaluate the (Gd(III)-DOTA)₄-PNA as an MRI probe, its NMRD profile at a series of temperatures were collected. A value of 5.6 mM⁻¹ s⁻¹ was observed at 20 MHz and 25 °C per Gd³⁺ ion (Fig. 3). The NMRD profile of the triple helix, [(Gd(III)-DOTA)₄-PNA]₂:poly(rA), at 25 °C possessed a r₁ of 6.6 mM⁻¹ s⁻¹ at 20 MHz. These findings are in good agreement with other DO₃A monoamide compounds (4–5 mM⁻¹ s⁻¹) under similar conditions.³³ Evaluation over the entire profile gives some indication of the solution structure of our probe. An increase in relaxivity is seen at ~20 MHz for both (Gd(III)-DOTA)₄-PNA and the triple helix, [(Gd(III)-DOTA)₄-PNA]₂:poly(rA) at 298 K. This increase has been attributed to the slowing of molecular rotation for the probe (Gd(III)-DOTA)₄-PNA,³⁴ and ascribed to triple helix formation when in the presence of poly(rA). This is further seen in the temperature dependent studies where the increase in relaxivity at 20 MHz is not present for higher temperatures, most likely due to destabilization of the triple-helical complex (Fig. S4†).

It is worth noting that concentrations of 20 μM or greater were easily achieved with this PNA conjugate despite the perceived poor solubility of PNAs. In this study, the solubility of the PNA probe benefits from its relatively short (10-mer) length and that it possesses a lysine on each end of this sequence as

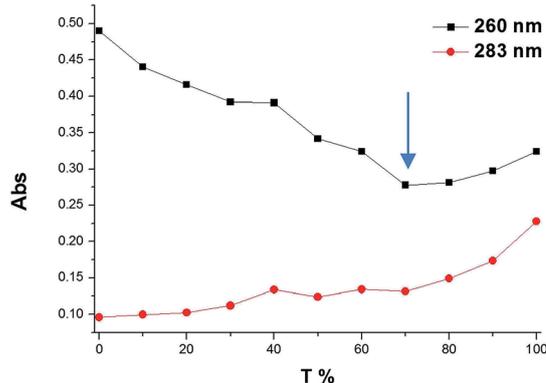


Fig. 2 Job plot of dA₁₀ (4 μM per strand) and (Gd(III)-DOTA)₄-PNA (4 μM per strand) mixtures in the molar ratios of 0 : 100, 10 : 90, 20 : 80, 30 : 70, 40 : 60, 50 : 50, 60 : 40, 70 : 30, 80 : 20, 90 : 10. Solution conditions: 100 mM NaCl, 10 mM NaH₂PO₄, 0.1 mM EDTA, pH 7, 25 °C. The arrowhead approximates the change of slope of the curves (ca. 70% T strands in complex).



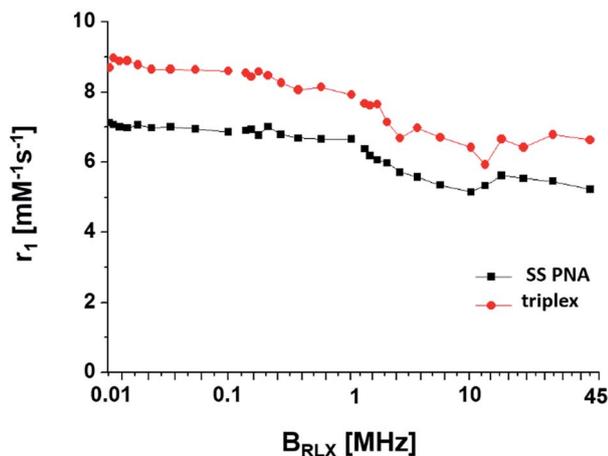


Fig. 3 Longitudinal relaxivity (r_1) of $[(\text{Gd}(\text{III})\text{-DOTA})_4\text{-PNA}]_2\text{:poly}(\text{rA})$ triplex and control linear $(\text{Gd}(\text{III})\text{-DOTA})_4\text{-PNA}$ with a constant 0.13 mM $\text{Gd}(\text{III})$ in 100 mM NaCl, 10 mM NaH_2PO_4 , 0.1 mM EDTA, pH 7, 25 °C.

well as being conjugated to the $\text{Gd}^{3+}\text{DOTA}$ complexes which add a significant polar component.

It is also noteworthy that triplex formation of this probe with an average full length poly(rA) tail of mRNA would deliver 160–200 Gd^{3+} ions to a localized microenvironment which compares very favourably with dendrimeric contrast agents that have achieved a loading of 150–200 chelators using 6th generation PAMAM dendrimers.³⁵ While there are 256 potential sites available in a G6 PAMAM dendrimer, a typical loading is closer to ~ 170 Gd^{3+} ions per dendrimer. Other dendrimers up to G10 have been synthesised with a loading of 1860 Gd^{3+} ions.³⁶ However, all these structures lack the specificity that is necessary for molecular MRI and incorporation of molecules such as polysaccharides,^{37–40} oligopeptides,^{41–44} proteins,^{44,45} antibodies⁴⁶ and oligonucleotides⁴⁷ is needed. In comparison, the $(\text{Gd}(\text{III})\text{-DOTA})_4\text{-PNA}$ probe not only consists of targeting oligomer but may deliver a high load of Gd^{3+} ions due to accumulation of probe on the repetitive sequence of the target. For actual application, another module would need to be added to the structure of the probe, that is, one that would facilitate cellular uptake and potentially nuclear localization. There are a variety of strategies already known to enhance cellular uptake of PNA⁴⁸ such as the use of cationic analogues,^{49,50} use of cell penetrating peptides,^{19,51,52} or the inclusion of specific moiety that drives receptor mediated endocytosis,⁵³ no name a few.

In summary, a poly($\text{Gd}(\text{III})\text{-DOTA}$)–PNA probe has been prepared conveniently *via* on-resin click chemistry utilizing a pre-metallated chelator. The resulting PNA conjugate recognized poly(rA) and putatively formed a stable triplex structure. The probe and its complex with poly(rA) was evaluated by NMRD studies. An increase in relaxivity was observed at ~ 20 MHz for both the $(\text{Gd}(\text{III})\text{-DOTA})_4\text{-PNA}$ and the triplex, $[(\text{Gd}(\text{III})\text{-DOTA})_4\text{-PNA}]_2\text{:poly}(\text{rA})$ at 25 °C, as compared to a monomeric chelate. By binding to poly(rA), Gd^{3+} ions would be significantly loaded to a localized microenvironment, which may improve the enhancement of contrast in MR images.

Conflicts of interest

There are no conflicts to declare.

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Notes and references

- 1 T. F. Massoud and S. S. Gambhir, *Genes Dev.*, 2003, **17**, 545–580.
- 2 É. Tóth, L. Helm and A. E. Merbach, *Top. Curr. Chem.*, 2002, **221**, 123.
- 3 K. B. Ghaghanda, M. Ravoori, D. Sabapathy, J. Bankson, V. Kundra and A. Annapraganda, *PLoS One*, 2009, **4**, e7628.
- 4 L. S. Karfeld, S. R. Bull, N. E. Davis, T. J. Meade and A. E. Barron, *Bioconjugate Chem.*, 2007, **18**, 1697–1700.
- 5 S. Langereis, Q. G. de Lussanet, M. H. P. van Genderen, E. W. Meijer, R. G. H. Beets-Tan, A. W. Griffioen, J. M. A. van Engelshoven and W. H. Backes, *NMR Biomed.*, 2006, **19**, 133–141.
- 6 S. R. Bull, M. O. Guler, R. E. Bras, T. J. Meade and S. I. Stupp, *Nano Lett.*, 2005, **5**, 1–4.
- 7 R. Mishra, W. Su, R. Pohmann, J. Pfeuffer, M. G. Sauer and K. Ugurbil, *Bioconjugate Chem.*, 2009, **20**, 1860–1868.
- 8 J. D. Lewis, S. I. Gunderson and I. W. Mattaj, *J. Cell Sci., Suppl.*, 1995, **19**, 13–19.
- 9 A. Jacobson and S. W. Peltz, *Annu. Rev. Biochem.*, 1996, **65**, 693–739.
- 10 M. Wickens, P. Anderson and R. J. Jackson, *Curr. Opin. Genet. Dev.*, 1997, **7**, 220–232.
- 11 H. Fuke and M. Ohno, *Nucleic Acids Res.*, 2008, **36**, 1037–1049.
- 12 A. Scorilas, *Crit. Rev. Clin. Lab. Sci.*, 2002, **39**, 193–224.
- 13 S. L. Topalian, S. Kaneko, M. I. Gonzales, G. L. Bond, Y. Ward and J. L. Manley, *Mol. Cell. Biol.*, 2001, **21**, 5614–5623.
- 14 S. L. Topalian, M. I. Gonzales, Y. Ward, X. Wang and R. F. Wang, *Cancer Res.*, 2002, **62**, 5505–5509.
- 15 F. Xing, G. Song, J. Ren, J. B. Chaires and X. Qu, *FEBS Lett.*, 2005, **579**, 5035–5039.
- 16 P. Giri and G. Suresh Kumar, *Mol. Biosyst.*, 2010, **6**, 81–88.
- 17 P. Giri and G. S. Kumar, *Arch. Biochem. Biophys.*, 2008, **474**, 183–192.
- 18 N. V. Amirkhanov, I. Dimitrov, A. W. Opitz, K. Zhang, J. P. Lackey, C. A. Cardi, S. Lai, N. J. Wagner, M. L. Thakur and E. Wickstrom, *Biopolymers*, 2008, **89**, 1061–1076.
- 19 W. Su, R. Mishra, J. Pfeuffer, K.-H. Wiesmuller, K. Ugurbil and J. Engelmann, *Contrast Media Mol. Imaging*, 2007, **2**, 42–49.
- 20 M. R. Lewis, F. Jia, F. Gallazzi, Y. Wang, J. Zhang, N. Shenoy, S. Z. Lever and M. Hannink, *Bioconjugate Chem.*, 2002, **13**, 1176–1180.
- 21 X. Sun, H. Fang, X. Li, R. Rossin, M. J. Welch and J. S. Taylor, *Bioconjugate Chem.*, 2005, **16**, 294–305.
- 22 K. Westerlund, H. Honarvar, V. Tolmachev and A. Eriksson Karlström, *Bioconjugate Chem.*, 2015, **26**, 1724–1736.



- 23 X. Wang and R. H. E. Hudson, *ChemBioChem*, 2015, **16**, 2156–2161.
- 24 V. Castro, H. Rodríguez and F. Albericio, *ACS Comb. Sci.*, 2016, **18**, 1–14.
- 25 A. H. St Amant, C. Engbers and R. H. E. Hudson, *Artif. DNA PNA XNA*, 2013, **4**, 4–10.
- 26 N. V. Amirkhanov and E. Wickstrom, *Nucleosides, Nucleotides Nucleic Acids*, 2005, **24**, 423–426.
- 27 A. Gourishankar and K. N. Ganesh, *Artif. DNA PNA XNA*, 2012, **3**, 5–13.
- 28 O. Almarsson and T. C. Bruice, *Proc. Natl. Acad. Sci. U. S. A.*, 1993, **90**, 9542–9546.
- 29 S. K. Kim, P. E. Nielsen, M. Egholm, O. Buchardt, R. H. Berg and B. N. V. F., *J. Am. Chem. Soc.*, 1993, **115**, 6477–6481.
- 30 R. F. H. Viguier and A. N. Hulme, *J. Am. Chem. Soc.*, 2006, **128**, 11370–11371.
- 31 D. E. Prasuhn, R. M. Yeh, A. Obenaus, M. Manchester and M. G. Finn, *Chem. Commun.*, 2007, 1269–1271.
- 32 F. Wojciechowski, A. Groß, I. T. Holder, L. Knörr, M. Drescher and J. S. Hartig, *Chem. Commun.*, 2015, 13850–13853.
- 33 S. Aime, P. L. Anelli, M. Botta, F. Fedeli, M. Grandi, P. Paoli and F. Uggeri, *Inorg. Chem.*, 1992, **31**, 2421–2428.
- 34 S. Aime, M. Botta and E. Terreno, *Adv. Inorg. Chem.*, 2005, **57**, 173–232.
- 35 E. C. Wiener, M. W. Brechbiel, H. Brothers, R. L. Magin, O. A. Gansow, D. A. Tomalia and P. C. Lauterbur, *Magn. Reson. Med.*, 1994, **31**, 1–8.
- 36 L. H. Bryant, M. W. Brechbiel, C. Wu, J. W. M. Bulte, V. Herynek and J. A. Frank, *J. Magn. Reson. Imag.*, 1999, **9**, 348–352.
- 37 E. K. Woller, E. D. Walter, J. R. Morgan, D. J. Singel and M. J. Cloninger, *J. Am. Chem. Soc.*, 2003, **125**, 8820–8826.
- 38 D. Zanini and S. Pamam, *J. Org. Chem.*, 1998, **63**, 3486–3491.
- 39 M. L. Wolfenden and M. J. Cloninger, *Bioconjugate Chem.*, 2006, **17**, 958–966.
- 40 D. Zanini and R. Roy, *J. Am. Chem. Soc.*, 1997, **119**, 2088–2095.
- 41 K. Sadler and J. P. Tam, *Rev. Mol. Biotechnol.*, 2002, **90**, 195–229.
- 42 L. Crespo, G. Sanclimens, M. Pons, E. Giralt, M. Royo and F. Albericio, *Chem. Rev.*, 2005, **105**, 1663–1681.
- 43 D. T. S. Rijkers, G. W. van Esse, R. Merckx, A. J. Brouwer, H. J. F. Jacobs, R. J. Pieters and R. M. J. Liskamp, *Chem. Commun.*, 2005, 4581–4583.
- 44 I. van Baal, H. Malda, S. A. Synowsky, J. L. J. van Dongen, T. M. Hackeng, M. Merckx and E. W. Meijer, *Angew. Chem., Int. Ed. Engl.*, 2005, **44**, 5052–5057.
- 45 R. Kluger and J. Zhang, *J. Am. Chem. Soc.*, 2003, **125**, 6070–6071.
- 46 C. Wu, M. W. Brechbiel, R. W. Kozak and O. A. Gansow, *Bioorg. Med. Chem. Lett.*, 1994, **4**, 449–454.
- 47 Y. Choi, A. Mecke, B. G. Orr, M. M. Banaszak Holl and J. R. Baker, *Nano Lett.*, 2004, **4**, 391–397.
- 48 U. Koppelhus and P. E. Nielsen, *Adv. Drug Delivery Rev.*, 2003, **55**, 267–280.
- 49 P. Zhou, M. Wang, L. Du, G. W. Fisher, A. Waggoner and D. H. Ly, *J. Am. Chem. Soc.*, 2003, **125**, 6878–6879.
- 50 R. Mitra and K. N. Ganesh, *J. Org. Chem.*, 2012, **77**, 5696–5704.
- 51 F. Gallazzi, Y. Wang, F. Jia, N. Shenoy, L. A. Landon, M. Hannink, S. Z. Lever and M. R. Lewis, *Bioconjugate Chem.*, 2003, **14**, 1083–1095.
- 52 K. Kaihatsu, K. E. Huffman and D. R. Corey, *Biochemistry*, 2004, **43**, 14340–14347.
- 53 M. Równicki, M. Wojciechowska, A. J. Wierzba, J. Czarnecki, D. Bartosik, D. Gryko and J. Trylska, *Sci. Rep.*, 2017, **7**, 7644.

