Trivalent Gd-DOTA reagents for modification of proteins†

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The development of novel protein-targeted MRI contrast agents crucially depends on the ability to derivatise suitable targeting moieties with a high payload of relaxation enhancer (e.g., gadolinium(III)) complexes such as Gd-DOTA), without losing affinity for the target proteins. Here, we report robust synthetic procedures for the preparation of trivalent Gd-DOTA reagents with various chemical handles for site-specific modification of biomolecules. The reagents were shown to successfully label proteins through isothiocyanate ligation or through site-specific thiol–maleimide ligation and strain-promoted azide–alkyne cycloaddition.

Targeted MRI contrast agents consist of a targeting moiety and one or more gadolinium complexes. The targeting moiety is usually a small molecule or peptide. These reagents can be used to localise specific proteins – typically proteins present in the blood or extracellular matrix, or extracellular domains of membrane proteins – in vivo with high spatial resolution. Examples are MRI contrast agents targeted to serum albumin, collagen, fibrin,1–3 and various abundant receptors (e.g., progesterone, folate, dopamine glutamate). In addition, a contrast agent based on CTB (cholera toxin subunit B) has been used to successfully image neuronal connections in vivo.5

The development of protein-targeted MRI contrast agents crucially depends on the availability of suitable protein-targeting moieties that can be labelled efficiently with (multiple) gadolinium complexes while preserving: (1) affinity of the targeting moiety for its protein target; (2) strong binding of toxic Gd3+ to its chelating ligand; (3) relaxivity of the gadolinium complex; (4) rapid tissue penetration/target binding and clearance of unbound reagents; and (5) clearance of all reagent from the body before metabolism-related release of Gd3+.

For many proteins, no suitable small molecule ligands are available, and the use of biomolecules as targeting moieties would be desirable. For example, antibody- and affibody-based MRI contrast agents have been developed that enable the targeted imaging of EGFR and Her-2,6 including a two-component approach based on a biotinylated antibody and Gd-DTPA-labelled avidin to increase the gadolinium payload (with a detection limit of ca. 10⁶ receptors per cell). Affibodies and other antibody mimetics have significant advantages over antibodies as targeting moieties: they are smaller, causing rapid tissue penetration and blood clearance; they can be selected rapidly in vitro against a wide range of biomolecules; they are...
Macrocyclic ligands such as DOTA and DO3A are metabolically more stable than linear ones such as DTPA, and they bind Gd³⁺ much more tightly (pKᵢₒ = 28 for DOTA vs. 22 for DTPA),¹ minimizing potential toxicity issues. DOTA can be linked to targeting moieties through amide bond formation with one (or more) of its carboxylic acids. However, the change of a coordinating carboxylate to an amide slightly reduces the binding constant of the Gd-DOTA complex. More importantly, this substitution significantly slows the water exchange rate of the complex (a limiting factor when imaging slow-tumbling molecules).¹ ³ Our aim was to generate a toolbox of trivalent Gd-DOTA reagents that already contain Gd³⁺ and can be used to selectively label any targeting moiety (i.e. small molecules, peptides and biomolecules) in one simple step. The trivalent Gd-DOTA reagents were based on DOTA-GA in combination with a diethylenetriamine linker (Fig. 1). DOTA-GA contains a glutamic acid side chain that allows chemical modification without negatively affecting the water exchange rate of the Gd-DOTA complex. In addition, the limited rotational freedom of the short triamine linker would ensure minimal loss of reactivity through rotational motion (a limiting factor when using clinically prevalent mid-range scanners).¹ ³

One trivalent complex [Gd-DOTA]₃-X (Fig. 1, X = NCS; compound 14) has previously appeared in the patent literature.⁹ However, DOTA chemistry is notoriously sensitive to impurities and, in our experience, the details disclosed in this patent are insufficient to allow straight-forward preparation of trivalent Gd-DOTA reagents. Here, we provide detailed (optimised) procedures for the synthesis and purification of one known and two new trivalent Gd-DOTA reagents [Gd-DOTA]₃-X. In addition, we report on bioconjugation studies for the modification of proteins such as antibody mimetics.

Results and discussion

Synthesis of (R)-¹Bu₄-DOTA-GA

DOTA-based building block (R)-¹Bu₄-DOTA-GA (6) was synthesised through adaptation of a literature procedure reported to give 6 in high purity and >97% enantiomeric excess (Scheme 1).¹⁰ l-Glutamic acid was converted to lactone 1 through diazotisation followed by tert-butyl ester formation, in moderate yield (over 3 steps, after crystallisation).¹ⁱ Lactone ring opening with 1 equivalent of KOH, followed by benzylation of the intermediate potassium carboxylate, gave alcohol 2, which was mesylated in good yield. Alcohol 2 was easy to store. Therefore, mesylate 3 was always prepared freshly before use.

Ley et al. synthesised benzyl-protected DOTA-GA 5 by monoalkylation of cyclen with mesylate 3 followed by triple alkylation with tert-butylbromacetate. Their procedure required 2 equivalents of cyclen (an expensive building) in the first step to avoid over-alkylation, and removal of excess cyclen after the reaction was essential to avoid problems in subsequent steps.¹² In contrast, we isolated tri-functionalised cyclen 4 as its pure HBr salt by crystallisation from chloroform/diethyl ether.¹³ Alkylation of 4 with 1.2 equivalents of 3 under basic conditions afforded 5. Benzyl ester 5 was highly sensitive to trans-esterification under basic conditions. Even after filtration of the crude reaction mixture, sufficient potassium carbonate was present to give the corresponding methyl ester upon addition of methanol.¹⁴ Therefore, 5 was purified by automated reverse phase (RP; C18) chromatography before hydrogenolysis to afford (R)-¹Bu₄-DOTA-GA (6). We also noticed that amide bond formations with 6 are easier to produce in homogeneous batches that are devoid of post-translational modifications; and they can be labelled site-specifically with suitable chemicals.

![Fig. 1](https://example.com/fig1.png)

**Fig. 1** Generic structure of trivalent Gd-DOTA reagents [Gd-DOTA]₃-X described in this study (X = reactive handle for chemoselective conjugation to biomolecules).
very sensitive to residual alcohol or water; rapid hydrolysis/methanolysis of activated esters of 6 is consistent with the sensitivity of 5 to trans-esterification. Therefore, 6 (1.35 g) was purified by automated RP (C18) chromatography and subsequent lyophilisation. The use of an acid-free eluent during this purification was crucial to avoid formation of salts, and for consistent results with subsequent amide bond formations. The ee of 6 was >97%, as determined by NMR analysis upon formation of amides with either enantiomer of z-methylbenzylamine, according to reported procedures.10

Synthesis of trivalent Gd-DOTA reagents

Trivalent linker 9 was prepared in 2 steps from 1-nitrophenylalanine methyl ester 7 according to a literature procedure (Scheme 2).14 Triamine 9 was isolated as its triple HCl salt, which is hygroscopic and needs to be stored under an inert atmosphere for consistent results in subsequent acylation with (R)-4-Bu4-DOTA-GA (6). After extensive optimisation of reaction conditions, triple acylation of triamine 9 with 6 using HATU and DIPEA afforded [(R)-4-Bu4-DOTA]3-NO2 (10) in excellent yield. In contrast to reported procedures, only a small excess of 6 (1.1 equiv. per acylation) was required. The best conversion was seen with freshly freeze-dried 6 (purified as described above) and anhydrous DIPEA. Consistent with the patent literature, purification of 10 by flash chromatography was only partially successful, and gave low yield of pure material.9 However, straight-forward size exclusion chromatography (Sephadex LH-20), followed by lyophilisation, gave 10 in high yield.

Hydrogenation of the nitro group of 10 gave aniline [(R)-4-Bu4-DOTA]3-CH2-NH2 (14) as a pure compound, which was converted into [Gd-DOTA]3-CH2-NH2 (12) in two steps. The use of methanesulfonic acid, in addition to TFA and the cation scavenger triethylsilane, was essential for the deprotection of all 12 tert-butyl esters of 11.15 The reaction worked best when kept at or just above 0 °C; significant decomposition and reduced yields were observed if the reaction mixture was allowed to warm up. Therefore, the deprotected intermediate (as its methanesulfonate salt) was separated from the deprotection cocktail by precipitation upon addition of cold diethyl ether. The resulting trivalent DOTA ligand was then directly charged with Gd3+ to give 12 by high resolution ESI-MS.15 Purified aniline 12 was prepared in batches of ca. 350–400 mg, and could be stored at −20 °C for >1 year without signs of decomposition.

Next, aniline 12 was modified to install reactive handles for the labelling of targeting moieties, including peptides and biomolecules. Modifications of 12 were performed on small scales (6–50 mg) to produce material for bioconjugation studies. Acylation with the N-hydroxysuccinimide ester of 2-maleimidoacetic acid (2-maleimidoacetic acid-OSu),16 afforded [Gd-DOTA]3-maleimide (13) suitable for selective modifications of thiols (e.g., cysteines). Treatment of 12 with thiophosgene gave [Gd-DOTA]3-[O3CCH2CSNH2] (14) for reactions with nucleophilic amines (e.g., lysines). Isothiocyanate 14 could also be converted into the azide [Gd-DOTA]3-CH2-N3 (15), suitable for, for example, copper-catalysed azide–alkyne cycloadditions (CuAAC) or strain-promoted azide–alkyne cycloadditions (SPAAC). 2-Maleimidoacetic acid-OSu and 3-azidopropylamine were separated from trivalent Gd-DOTA reagents 13 and 15, respectively, using size exclusion chromatography before bioconjugation reactions; isothiocyanate 14 did not require purification.

Bioconjugation studies with trivalent Gd-DOTA reagents

Next, the suitability of trivalent Gd-DOTA reagents 13, 14 and 15 for protein modification was investigated. Initially, bioconjugation experiments were performed with lysozyme. Incubation of lysozyme (71 µM in 0.1 M sodium carbonate) with three equivalents of 14 resulted in rapid bioconjugation (Fig. 2a and b). SDS-PAGE analysis showed mainly single and double labelling of lysozyme, and some triple labelling (Fig. 2a, lane 1). The identity of the singly- and doubly-labelled lysozyme was confirmed by ESI-MS (Fig. 2b and the ESI†).17 Preincubation of
can be selected rapidly in vitro against a range of targets using phage display protocols. Adhirons have excellent binding affinity and specificity, high thermal stability, low production costs and no disulfide bonds. Compared to antibodies, their much smaller size would facilitate more rapid tissue penetration and blood clearance, which is advantageous for in vivo imaging.

In addition, the ease of engineering of site-specific modifications, including single or multiple cysteines, makes Adhirons well-suited for site-specific chemical labelling. To test the site-specific modification of Adhirons with trivalent Gd-DOTA reagents, bioconjugation reactions were performed with Adhiron15C, which contains a single cysteine on its C-terminus (pointing away from the binding loops) (Fig. 2c–f). Adhiron15C contains a C-terminal His tag and was purified by Ni nitritolactacetic acid (NTA) affinity chromatography. Initially, labelling with maleimide 13 was performed directly in the elution buffer, at a protein concentration of 84 μM. SDS-PAGE analysis showed formation of a single labelling product even in the presence of 40 equiv. of 13 (Fig. 2c) and high resolution ESI-MS confirmed the identity of Adhiron15C-[Gd-DOTA]3 (Fig. 2d).

Although the labelling of Adhiron15C with maleimide 13 was successful, a large excess of this precious reagent was needed. To check if inefficient labelling was caused by imidazole and/or tris(2-carboxyethyl)phosphine (TCEP) present in the Adhiron elution buffer, ligation reactions were also attempted after purification of Adhiron15C by dialysis into labelling buffer (PBS containing 10% glycerol and 0.05% Tween-20; pH 7.4) and/or gel filtration. However, this did not significantly improve the labelling reaction, and neither did the use of polymer-supported TCEP (instead of TCEP solution) to reduce disulfide bonds of Adhiron dimers before labelling. Therefore, a more efficient 2-step protocol for Adhiron labelling based on SPAAC was developed. Treatment of Adhiron15C (50 μM in labelling buffer) with the commercially available linker dibenzocycloocty maleimide (DBCO-Mal; see the ESI† for structure; 20 equiv.) gave Adhiron15C-DBCO (Fig. 2f).

After buffer exchange (PD-10 column) and concentration, Adhiron15C-DBCO (89 μM in labelling buffer) was treated with azide 15 (2 equiv.), leading to rapid formation of Adhiron15C-DBCO-[Gd-DOTA]3 by SPAAC (Fig. 2e and f). Although conversion was still not complete (most likely because of the sluggish maleimide ligation to form Adhiron15C-DBCO), this procedure gave similar conversion to direct ligation with maleimide 13, but is significantly more efficient in terms of the required amount of trivalent Gd-DOTA reagent.

Conclusions

Trivalent Gd-DOTA reagents have been developed for the site-specific functionalisation of (bio)molecules bearing amines, thiols, and/or (strained) alkynes. Detailed synthetic procedures have been reported, including recommendations regarding purification and storage to assure high-yielding reactions with sensitive intermediates. Procedures for the (site-specific) modification of different proteins were developed. Our reagents allow a range of chemoselective ligation reactions with targeting moieties, including biomolecules. Therefore, they may contribute to the
development of novel MRI contrast agents targeted to proteins for which no suitable and/or easy-to-functionalise small molecule- or peptide-based binders are available, but for which phage display protocol can deliver antibody mimetics with high affinity and selectivity. In addition, our reagents could triple the gadolinium payload of previously described CTB-based neuronal tracers. We are currently using our reagents to optimise the gadolinium payload on Adhiron-based contrast agents, for example through multiple labelling or the inclusion of a polyvalent scaffold.  

### Experimental

#### Synthetic procedures

The following compounds were prepared using literature methods and full reaction details can be found in the ESI:

1. 194 (m, 1H), 1.44
2. 3.34 (dd, H2O, 10 : 90 : 0.1 to 85 : 10 : 0.1). The eluate was concentrated in vacuo and redissolved in MeOH, then purified by size exclusion chromatography (Sephadex LH-20; elution with MeOH). The purified product was concentrated in vacuo. The residue was taken up in H2O, then lyophilised to give the title compound as a colourless amorphous solid (650 mg, 88%). δH (500 MHz; CDCl3) 8.06–8.12 (m, 2H), 7.49–7.60 (m, 2H), 4.34–4.58 (m, 1H), 2.80–3.92 (m, 77H), 2.15–2.61 (m, 6H), 1.90–2.13 (m, 6H), 1.37–1.51 (m, 108H);

2. [R]-Bu4-DOTA-GA benzyl ester 5. A solution of benzyl 5-(tert-butoxy)-4-(methanesulfonyloxy)-5-oxopentanoate (1.2 mL, 30 mmol) in MeCN (20 mL) was added dropwise to a suspension of trialkylated cyclen (aspirator) and back-filled and stirred at 40 °C until complete according to LCMS (16 h). The reaction mixture was cooled to room temperature, filtered over Celite, and concentrated in vacuo. The crude product was purified on silica gel (C18 chromatography using gradient elution (MeCN : H2O)) to give the title compound as a colourless amorphous solid (380 mg, 93%).

3. Enolate was concentrated in vacuo to give an aqueous solution (5 m, 2H), 2.51–2.59 (m, 1H), 2.39–2.48 (m, 1H), 1.98–2.06 (m, 1H), 1.87–1.96 (m, 1H), 1.40–1.48 (m, 3H, 63H); δC (125 MHz; CDCl3) 176.1, 171.4, 170.8, 170.3, 81.6, 81.3, 63.6, 56.1, 55.7, 52.0, 51.7, 49.4, 33.1, 28.3, 28.2, 27.93, 27.87, 25.3; m/z HRMS (ESI) calcd for C14H25N2O10: 701.4695 [M + H]+, found 701.4702.

4. [R]-Bu4-DOTA-3a-NO2 10. DIPEA (526 μL, 3.20 mmol) was added to a cooled (0 °C), stirred solution of HATU (546 mg, 1.41 mmol), 6 (400 mg, 1.14 mmol), and 2-amino-3-(4-nitrophenyl) propyl (2-aminoethyl)amine trihydrochloride 9 (55 mg, 0.32 mmol) in DMSO (6 mL). The reaction mixture was stirred at 0 °C for 30 minutes, then allowed to warm to room temperature and stirred overnight. Once the reaction was complete according to LCMS, the reaction mixture was concentrated in vacuo and redissolved in MeOH, then purified by size exclusion chromatography (Sephadex LH-20; elution with MeOH). The purified product was concentrated in vacuo. The residue was taken up in H2O, then lyophilised to give the title compound as a colourless amorphous solid (650 mg, 88%). δH (500 MHz; CDCl3) 8.06–8.12 (m, 2H), 7.49–7.60 (m, 2H), 4.34–4.58 (m, 1H), 2.80–3.92 (m, 77H), 2.15–2.61 (m, 6H), 1.90–2.13 (m, 6H), 1.37–1.51 (m, 108H); m/z HRMS (ESI) calcd for C116H207N16O29: 763.1744 [M + 3H]+, found 763.1739.

5. [R]-Bu4-DOTA-3-NH2 11. 10% Pd/C (180 mg) suspended in MeOH (1 mL) was added to a solution of 10 (410 mg, 0.18 mmol) in MeOH (5 mL) under an atmosphere of nitrogen. The reaction was then placed under an atmosphere of hydrogen (balloon; three cycles of evacuation (aspirator) and back-filling) and stirred at 40 °C until complete according to LCMS (16 h). The reaction mixture was cooled to room temperature, filtered over Celite, and concentrated in vacuo to give the title compound as a colourless amorphous solid (380 mg, 93%). δH (500 MHz; CDCl3) 6.91–7.02 (m, 2H), 6.53–6.62 (m, 2H), 4.31–4.21 (m, 1H), 1.63–3.92 (m, 89H), 1.39–1.50 (m, 108H); m/z (LC-ESI-MS) calcd for C14H25N2O10: 752.8 [M + 3H]+, found 753.3 (pattern for sequential deprotection of Bu groups observed).

6. [Gd-DOTA-3]-NH2 12. Pre-cooled (0 °C) TFA (3.5 mL) and TES (1.2 mL, 30 mmol) were added to solid 11 (500 mg, 0.22 mmol), after which vigorous bubbling was observed. The reaction temperature was maintained at 0 °C for 20 minutes. Then, MeOH (227 μL) was added and the reaction mixture was stirred for 4 h at 0 °C. Once the reaction was complete according to LCMS, the reaction mixture was poured into cold ether (40 mL) in a falcon tube. The resulting precipitate was stored in the fridge for 1 hour then centrifuged (1800 × g, 5 min). The supernatant was decanted off and the residual dissolved in water, then lyophilised to give a colourless amorphous solid (510 mg). This solid was dissolved in H2O (4 mL) and the pH adjusted to 6.5 with 2 N NaOH. To this solution was added GdCl3.6H2O (3 × 90 mg, 0.73 mmol) successively in 3 portions; after each addition the pH was monitored until no further change was observed (ca. 20 min) and the pH readjusted to 6.5 with 2 N NaOH before the next addition. After the final addition, the pH was again adjusted to 6.5 and the cloudy pink solution was heated to 50 °C overnight. Once complete according to LCMS the mixture was filtered over Celite and lyophilised. The crude product was purified by size exclusion chromatography.
(Sephadex LH-20; elution with H2O). The purified product was lyophilised to give the title compound as a pale pink amorphous solid (360 mg, 75% over 2 steps). m/z HRMS (ESI, negative mode) calculated for C69H96Gd3N16O27S: 695.1369 M+1, found 695.1369 (complex pattern due to Gd isotopes; the predicted and observed isotopic distributions were identical, see ESI†).

[Gd-DOTA]3-maleimide 13. To a solution of 12 (50 mg, 0.024 mmol) in HEPES buffer (300 mM, pH 7.3, 300 μL) and DMSO (200 μL), a solution of 2-maleimidoacetic acid-OSu (18 mg, 0.072 mmol) in DMSO (100 μL) was added. Because a precipitate formed, the reaction mixture was centrifuged (17 000 × g, 1 min), and the supernatant was removed and retained before the pellet was redissolved in DMSO (100 μL) and added back to the supernatant. The reaction was spun (Stuart rotator) at room temperature for 4 h before a further aliquot of 2-maleimidoacetic acid-OSu (18 mg, 0.072 mmol) in DMSO (200 μL) was added. The reaction mixture was spun for a further 2 h, then loaded onto a size exclusion chromatography (LH20) column, and the product was eluted with H2O. The eluted product was lyophilised to give the title compound as an off-white solid (20 mg, 40%, some starting material 12 present according to LCMS), which was used immediately for bioconjugation reactions. m/z HRMS (ESI, negative mode) calculated for C74H101Gd3N17O30: 726.4889 M+1, found 726.4918 (complex pattern due to Gd isotopes; the predicted and observed isotopic distributions were identical, see ESI†).

[Gd-DOTA]3-ITC 14. Thiophosgene (3.3 μL, 0.013 mmol) was added to a biphasic solution of 12 (50 mg, 0.024 mmol) in chloroform–H2O (50:50, 1 mL), and the reaction mixture was stirred vigorously overnight. Once the reaction was complete according to LCMS, the reaction mixture was diluted with water (3 mL) and the aqueous layer was extracted with chloroform (2 × 3 mL). The organic fractions were discarded and the remaining aqueous solution was lyophilised to give the title compound as a pale red powder (169 mg, 75% over 2 steps). Further aliquots of 2-maleimidoacetic acid-OSu (18 mg, 0.072 mmol) in DMSO (200 μL) were added. Because a precipitate formed, the reaction mixture was centrifuged (17 000 × g, 1 min), and the supernatant was removed and retained before the pellet was redissolved in DMSO (100 μL) and added back to the supernatant. The reaction was spun (Stuart rotator) at room temperature for 4 h before a further aliquot of 2-maleimidoacetic acid-OSu (18 mg, 0.072 mmol) in DMSO (200 μL) was added. The reaction mixture was spun for a further 2 h, then loaded onto a size exclusion chromatography (LH20) column, and the product was eluted with H2O. The eluted product was lyophilised to give the title compound as an off-white solid (5 mg, 76%). m/z HRMS (ESI, negative mode) calculated for C69H96Gd3N16O27S: 728.4955 M+1, found 728.4918 (complex pattern due to Gd isotopes; the predicted and observed isotopic distributions were identical, see ESI†).

Bioconjugation reactions

Labelling of lysozyme with [Gd-DOTA]3-ITC 14. Hen egg white lysozyme at 71 μM in 0.1 M Na2CO3 (pH was not adjusted) was incubated at 25 °C with a 3-fold molar excess of [Gd-DOTA]3-ITC 14 for 18 h. For SDS-PAGE analysis, reactions were quenched by addition of one volume of 3 × SDS-PAGE loading buffer (containing Tris buffer) and one volume of 150 mM DTT prior to heating to 100 °C for 5 minutes and analysis by SDS-PAGE and HRMS.

Site-specific labelling of Adhiron15C with [Gd-DOTA]3-maleimide 13. The following stock solutions were prepared: [Gd-DOTA]3-maleimide 13 (10 mM in H2O); Adhiron15C (110 μM in elution buffer: 50 mM NaH2PO4, 500 mM NaCl, 300 mM imidazole, 10% glycerol, pH 7.4); tris[2-carboxyethyl]phosphine (TCEP; 50 mM in water). A solution of Adhiron15C (22.8 μL, 2.5 nmol), 13 (6 μL, 60 nmol) and TCEP (1.2 μL, 60 nmol) were mixed together and incubated at room temperature. After 3 h, a further aliquot of 13 solution (4 μL, 40 nmol) was added and the incubation continued for another 3 h. The reaction mixture was analysed by SDS-PAGE and HRMS.

Two-step site-specific labelling of Adhiron15C with [Gd-DOTA]3-azide 15. A sample of Adhiron15C in elution buffer was dialysed (2 × 1: 1 000) into labelling buffer (PBS containing 20% glycerol and 0.05% Tween-20; pH 7.4) to give a protein solution of 57 μM. 6.1 mL of this solution (0.35 μmol) was treated with TCEP in H2O (50 mM; 350 μL; 17.5 μmol), labelling buffer (185 μL) and DBCO-Mal in DMSO (20 mM; 350 μL, 7 μmol) to give a final protein concentration of 50 μM. The reaction was rocked for 6 h and monitored by mass spectrometry. Upon completion, the material was passed through a buffer exchange column (PD-10, GE Healthcare) according to manufacturer’s instructions, eluting 0.5 mL fractions with labelling buffer. Fractions containing protein were identified by BioRad colourimetric assay and pooled. The protein was then concentrated to 89 μM by spin concentrator (3 kDa cut-off), analysed by HRMS, and used immediately in the next step (or flash frozen and stored at −80 °C if required).

2 mL of this Adhiron15C-DBCO solution (0.17 μmol) was treated with [Gd-DOTA]3-azide 15 (2 mM in H2O; 175 μL; 0.35 μmol) and the solution was rocked for 6 h. Upon completion, the material was passed through a buffer exchange column (PD-10, GE Healthcare) according to manufacturer’s instructions, eluting 0.5 mL fractions with labelling buffer. Fractions containing protein were identified by BioRad colourimetric assay and pooled. Adhiron15C-DBCO-[Gd-DOTA] was concentrated to 323 μM by spin concentrator (3 kDa cut-off), analysed by SDS-PAGE and HRMS, flash frozen and stored at −80 °C.

Acknowledgements

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Notes and references

4 Mn(II) and iron oxide-based contrast agents have been discontinued, see ref. 2.


7 D. Artemov, N. Mori, R. Ravi and Z. M. Bhujwalla, Cancer Res., 2003, 63, 2723; on average, the constructs contained 12.5 Gd-DTPA moieties per avidin.


11 In the first step of this sequence, a syringe pump was used to add aqueous sodium nitrite solution to the L-glutamate solution; this approach gave significantly higher yields than portion-wise addition of solid sodium nitrite.


13 Unfortunately we did not manage to exploit this reactivity to shorten the synthesis by hydrolysing the ester during workup.


15 To avoid chelation of metal ions other than Gd$^{3+}$ by the deprotected DOTA derivative, glassware was cleaned with concentrated sulfuric acid before use, HPLC-grade solvents, TFA and MsOH were used for the conversion of 11 $\rightarrow$ 12. No products incorporating metal ions other than Gd$^{3+}$ were detected by HRMS.


17 The intensity of ESI-MS peaks resulting from lysozyme-[(Gd-DOTA)$_3$]$_2$ was weak (see the ESI†), and minor amounts of lysozyme-[(Gd-DOTA)$_3$] could not be detected by ESI-MS analysis of reaction mixtures. We suggest that SDS-PAGE analysis better reflects the abundance of different labelling species than ESI-MS.
