A supramolecular approach to multivalent target-specific MRI contrast agents for angiogenesis†

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The synthesis of a cyclic peptide–Gd(III)DTPA molecule equipped with biotin is presented, yielding a well-defined multivalent MRI contrast agent after its coupling to avidin.

Magnetic Resonance Imaging (MRI) is a powerful, non-invasive technique which plays an important role in clinical diagnosis. To enhance the contrast in T_1-weighted MR images, gadolinium(III) (Gd(III)) chelates are commonly used as MRI contrast agents.[1] Despite the efforts to improve contrast in MR images, some processes of interest, such as angiogenesis (the formation of new blood vessels), are difficult to visualize with MRI. Target-specific MRI contrast agents[2] designed to bind to proteins expressed by the cells involved in angiogenesis could improve the imaging of this process drastically due to the *in situ* accumulation of MRI contrast agent. A cyclic peptide containing the asparagine–glycine–arginine (NGR) sequence (cNGR) was identified as a specific ligand for the aminopeptidase CD13, a protein over-expressed by angiogenic endothelial cells.[3,4] By immobilizing multiple cNGR moieties and MRI labels on one single carrier, the binding of cNGR to CD13 may be improved through cooperative binding (multivalency),[5] resulting in a stronger accumulation of MRI contrast agent around regions of angiogenesis. Next to targeting, sensitivity is the other major challenge for molecular MR imaging. One way to increase the contrast is the connection of multiple Gd(III) chelates to one carrier.[1,2] High local concentrations of Gd(III) can be reached, while due to a higher molecular weight the T_1 of water molecules is more effectively reduced as compared to their monovalent low molecular weight analogues.

A strategy to synthesize such a multivalent target-specific MRI contrast agent comprises the self-assembly of monovalent target-specific MRI contrast agent units onto the periphery of a large carrier with high selectivity and high affinity. Avidin, a tetrameric protein, is capable of binding four equivalents of biotin in a strong, non cooperative fashion ($K_{ass} \approx 1.7 \times 10^{15}\ M^{-1}$)[6] and has been successfully employed as a versatile supramolecular scaffold for the synthesis of large well-defined structures.[7] In this paper we describe the synthesis of a well-defined multivalent target-specific MRI contrast agent based on the biotin–avidin system. For this, cNGR was functionalized with both the MRI label gadolinium(III) diethylene triaminepentaacetic acid (Gd(III)DTPA) and biotin (7) (Scheme 1).

† Electronic supplementary information (ESI) available: synthesis and characterization of all compounds reported in this paper and a description of the instrumentation used. See http://www.rsc.org/suppdata/cc/b5/b502347e/

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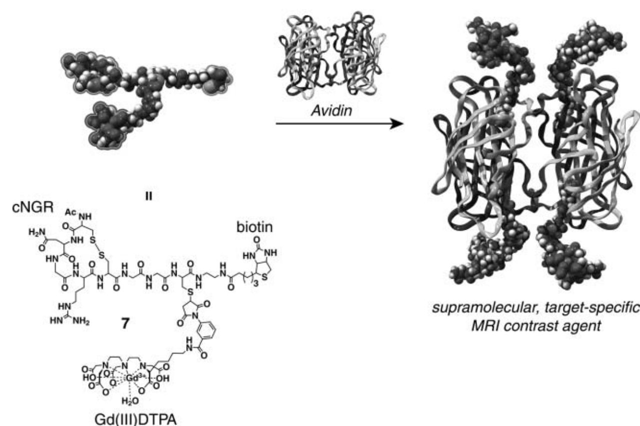

Scheme 1 Supramolecular approach for the synthesis of a multivalent target-specific contrast agent based on **7** and avidin.

The synthesis of **7** comprises a sequence of highly efficient, chemoselective reaction steps, which we recently introduced as a general strategy for the double labeling of peptides.[8] Biotinylated cysteine (**2**) was reacted with the C-terminal thioester of peptide **1** containing the target-specific NGR sequence under native chemical ligation conditions (Scheme 2). This methodology was previously described by Dawson *et al.* for the synthesis of proteins of moderate size.[9,10] At this point both cysteine residues of the peptide are protected with acetamidomethyl (Acm) groups to ensure a correct "folding" of the peptide later on. The reaction was monitored employing analytical reversed phase HPLC (RP HPLC) using a C18 column for separation coupled to UV-Vis (λ_{probe} = 214 nm). Within 2 hours the thioester-functionalized peptide **1** reacted quantitatively with **2**. The ligation product was purified using preparative RP HPLC on a C18 column and subsequent lyophilization rendered **3** in 83% yield. Subsequently, the sulfhydryl group of the cysteine residue that was involved in the ligation reaction was utilized to introduce the DTPA ligand through the reaction of **3** with maleimide-functionalized DTPA (**4**) (Scheme 2). The reaction was monitored employing analytical RP HPLC coupled to UV-Vis (λ_{probe} = 214 nm). Once the reaction went to completion the reaction mixture was diluted ~30 times with 0.1 M Tris (aq, pH 6.5) after which 10 vol% of acetic acid was added. Then 1.75 equivalents of I_2 were added to remove the Acm protecting groups of **5**. The removal of the Acm groups resulted instantaneously in the correct "folding" of the peptide unit into its cyclic conformation **6** (Scheme 2). Purification using preparative RP HPLC on a C18 column and subsequent lyophilization gave **6** in 27% yield. The corresponding Gd(III)-complex **7** was obtained

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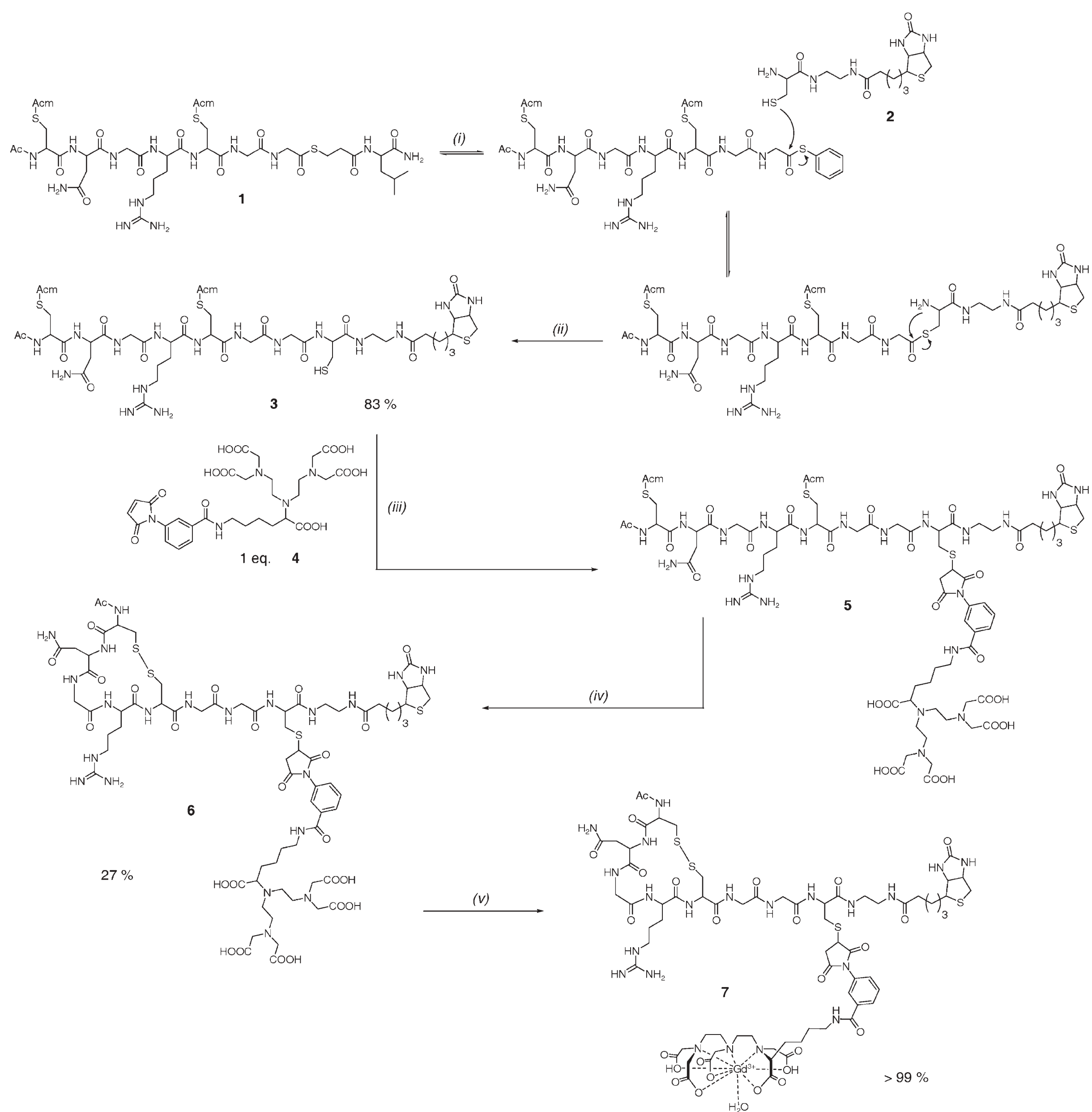

Scheme 2 The synthesis of the biotinylated target-specific contrast agent **7**. (i) 2 vol% thiophenol, 2 vol% benzylmercaptan, 6 M guanidine in 0.1 M Tris, 1 h, pH $\approx$ 7, 37 °C; (ii) spontaneous rearrangement; (iii) 0.1 M Tris, 1 h, pH 6.5, RT; (iv) 1.75 equiv. I_2, 10 vol% acetic acid, 1 h, RT; (v) 1 equiv. $GdCl_3$, H_2O, pH 6.5–7; (Acm = $–CH_2CONHCH_3$).

in quantitative yield (> 99%) through the addition of 1 equivalent of $GdCl_3$ in H_2O (Scheme 2). The formation of **7** was confirmed with ESI-MS.

To verify the binding stoichiometry between **7** and avidin, the HABA (4′-hydroxyazobenzene-2-carboxylic acid) assay was performed probing the UV-Vis absorption at 500 nm.[11] Upon addition of **7** to a solution of avidin in PBS buffer containing 5 equivalents of HABA per binding site, HABA is expelled from the biotin binding pocket of avidin, resulting in a decrease in the absorption at 500 nm of HABA bound to avidin (Fig. 1). After the addition of 3.8 equivalents of **7** the absorption spectrum at 500 nm does not change any further, indicating that all binding sites of avidin are occupied by **7**.

The longitudinal relaxivity r_1, which is an intrinsic property of the MRI contrast agent, was determined from concentration dependent measurements of the longitudinal relaxation time (T_1) of **7** at 1.5 T at 20 °C. The data gave a good linear fit to the equation $(1/T_1)_{observed} = (1/T_1)_{diamagnetic} + r_1[Gd(III)]$ ($R^2 > 0.999$) and an r_1 of 8.1 $mM^{-1}s^{-1}$ was calculated. This value is higher than the r_1 of parent Gd(III)DTPA (r_1 = 4.2 $mM^{-1}s^{-1}$ at 1.5 T and 20 °C), which can be explained in terms of molecular weight (1.9 kDa for **7** *versus* 0.5 kDa for Gd(III)DTPA).

To gain insight into the effect of binding of **7** to avidin on the r_1 of **7** an E-titration[12] was performed using MR (at 1.5 T and 20 °C), adding a 0.12 mM solution of avidin in PBS buffer (pH 7.4) to a 0.08 mM solution of **7** in PBS buffer (pH 7.4). This resulted in a

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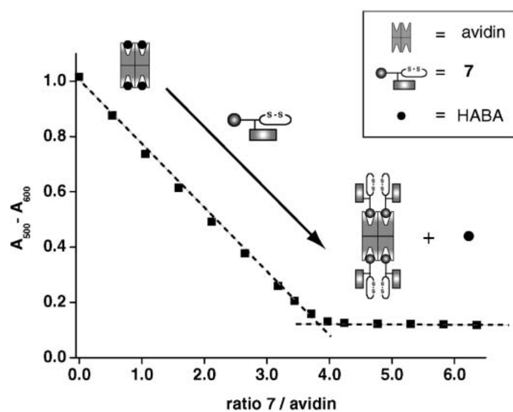

Fig. 1 HABA assay showing the decrease in UV-Vis absorption at 500 nm upon the addition of **7** (0.26 mM in PBS buffer, pH 7.4) to a 0.5 mL solution of avidin (10 μM in PBS buffer, pH 7.4) containing 5 equiv. of HABA per binding site (the dashed lines through the data points serve to guide the eye).

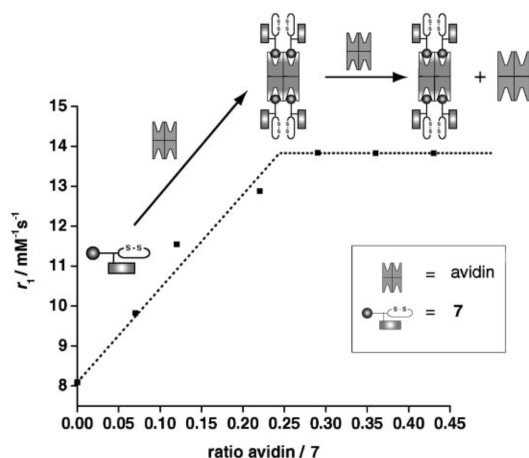

Fig. 2 E-titration[12] at 1.5 T and 20 °C showing the r_1 of **7** (0.08 mM in PBS buffer, pH 7.4) in the presence of increasing amounts of avidin (dotted line: fitted data with $r_{1,\mathrm{free}}$ = 8.1 $mM^{-1}s^{-1}$, yielding $r_{1,\mathrm{bound}}$ = 13.8 ± 0.5 $mM^{-1}s^{-1}$ and N = 4.1 ± 0.3).

linear increase in the r_1 of **7** (Fig. 2). The addition of more than 0.24 equivalents of avidin did not result in any further changes in the r_1 of **7** (Fig. 2). This behavior rules out non-specific binding between **7** and avidin.

In a previous study we found that the r_1 of biotinylated Gd(III)DTPA increases by a factor of 3 from $r_{1,\mathrm{free}}$ = 6.1 $mM^{-1}s^{-1}$ to $r_{1,\mathrm{bound}}$ = 17.5 $mM^{-1}s^{-1}$ upon binding to avidin.[13] This result was assigned to a strong reduction in the molecular tumbling rate of the Gd(III)DTPA moiety upon binding of the biotinylated Gd(III)DTPA contrast agent to the large protein avidin (molecular weight of 64 kDa). By fitting the data of the E-titration to a mathematical model describing the binding of multiple substrates to a multivalent protein with N identical, independent binding sites,[13] and by taking into account that $K_{\mathrm{ass}} = 1.7 \times 10^{15}$ M^{-1} and $r_{1,\mathrm{free}}$ = 8.1 $mM^{-1}s^{-1}$, an $r_{1,\mathrm{bound}}$ of 13.8 ± 0.5 $mM^{-1}s^{-1}$ and an N of 4.1 ± 0.3 were calculated. Remarkably, the increase in r_1 of **7** by a factor of 1.7 is not as pronounced as in the case of biotinylated Gd(III)DTPA, where we found a 3-fold increase. This may be attributed to the longer spacer between the Gd(III)DTPA moiety and the biotin unit in the case of **7**, which maintains a higher degree of flexibility for the Gd(III)DTPA moiety.

In conclusion, by exploiting the strong and specific binding of biotin to avidin, a well-defined multivalent target-specific MRI contrast agent based on avidin and **7** was synthesized through self-assembly. The availability of nanoparticles coated with avidin[14] allows an even higher loading of Gd(III)DTPA and cNGR peptide units per carrier. The profits of a high loading of Gd(III)DTPA moieties as well as target-specific peptides will be investigated *in vivo* using a murine model to assess its efficacy as a multivalent target-specific MRI contrast agent.

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