Viral MRI contrast agents: coordination of Gd by native virions and attachment of Gd complexes by azide–alkyne cycloaddition†

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Icosahedral virus particles decorated with a Gd(DOTA) analogue by Cu-mediated azide–alkyne cycloaddition (CuAAC) and/or with Gd^{3+} ions by coordination to the viral nucleoprotein show increased T_1 relaxivity relative to free Gd(DOTA) complexes in solution.

Magnetic resonance imaging (MRI) has become a valuable technique for medical diagnostics. More than 30% of all current MRI examinations use paramagnetic Gd^{3+} complexes, such as clinically-approved Gd(DTPA) or Gd(DOTA), as contrast enhancing agents.[1] Macromolecules and nanoparticles have been explored as scaffolds for the presentation of Gd^{3+} complexes with three potential advantages in mind: (1) enhanced relaxivity per gadolinium atom by reducing the tumbling rate of the complex, (2) potential for the display of large numbers of complexes in a confined volume, providing enhanced total molar relaxivity, and (3) potential for simultaneously incorporating targeting and/or gene delivery functions.[2,3]

Virus particles and protein nanocages have been shown to be excellent scaffolds for the multivalent display of a variety of covalently-attached molecules,[4] as well as peptide epitopes incorporated into the capsid protein sequence.[5] Here we report the labelling of viral particles with Gd^{3+} in two novel ways: conjugation of a monoalkylated DOTA analogue using the Cu(I)-mediated azide–alkyne cycloaddition (CuAAC) reaction, and by a natural high affinity interaction of aqueous Gd^{3+} ions with the polynucleotide encapsulated by a virus particle. The T_1 relaxivities of the resulting particles were determined at three magnetic field strengths. This work follows three prior reports of Gd-labelled virus particles and their relaxivities. The first, from Aime and co-workers, describes the passive entrapment of a neutral Gd(DOTA) derivative in the ferritin cage, producing particles with a relaxivity largely invariant to the field strength.[6] The second contribution, from the Douglas and Young laboratories, used a natural Ca^{2+} binding site in the protein capsid of cowpea chlorotic mottle virus (CCMV) to bind Gd^{3+} and provide extraordinarily high relaxivities per metal ion.[2] Lastly, Kirshenbaum and co-workers employed a Gd(DOTA) isothiocyanate derivative to attach more than 500 such complexes to the coat protein of bacteriophage MS2.[3] The fluorocarbon lipid nanoparticles of Wickline and Lanza, bearing thousands of Gd complexes per particle, should also be noted.[7]

Our group has developed the CuAAC reaction as a useful method for bioconjugation to interesting molecular scaffolds, with a particular focus on virus particles.[8,9] The ease of installation of azide and alkyne groups, and their tolerance of diverse reaction conditions and other functionalities, makes the method well suited to practical synthesis and the use of metal complexes, such as MRI contrast agents bearing selectively reactive tails. We employed wild-type cowpea mosaic virus (CPMV)[10] and a lysine knockout mutant (K16M) of bacteriophage Qβ,[11] the latter as a virus-like particle encapsulating random cellular RNA rather than the viral genome. Both are approximately 30 nm in diameter, although with greatly different protein folds and topological structures (Fig. 1).

Each capsid was derivatized at the available amine functional groups by *N*-hydroxysuccinimide ester **1** to give the polyvalent azides **2a** and **3a** (Fig. 2). Gd(DOTA) complex **4**, containing a pendant terminal alkyne group, was prepared by a variation of a known procedure, as shown in Fig. 2. This reagent is completely stable towards hydrolysis, retains a high affinity for the bound Gd(III) ion and thus has an indefinite shelf life. Attachment of the Gd complex to the virus azides was accomplished with the aid of the Cu-bathophenanthroline complex previously described, which accelerates the reaction to allow the use of modest amounts of the azide and alkyne components.[8] Purification by sucrose gradient ultracentrifugation provided derivatized virions **2b** and **3b** in good yield (60–70% of the particles were recovered), with an average of 223 $\pm$ 20 and 153 $\pm$ 15 Gd complexes per particle, respectively, as determined by inductively coupled plasma optical emission spectrometry (ICP-OES).[12]

Inspired by both the previous observations of high affinity lanthanide interactions with calcium binding sites of CCMV[2] and

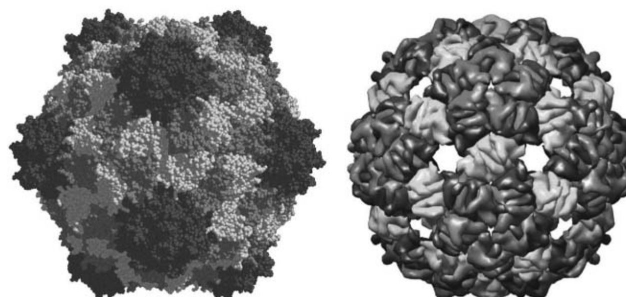

Fig. 1 Representations of the X-ray crystal structures of CPMV (left) and Qβ (right).

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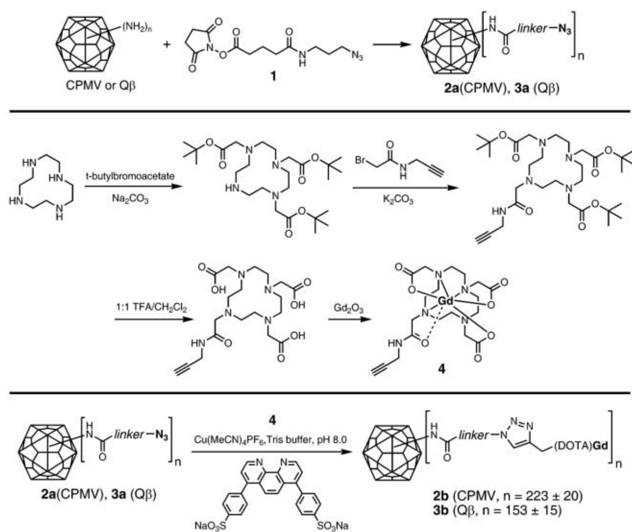

Fig. 2 Synthesis of Gd(DOTA)-derivatized virus particles.

the general affinity of lanthanides for RNA, we also explored the association of aqueous Gd^{3+} ions with CPMV.[13] Since lanthanide ions form insoluble hydroxides at pH 7 in the absence of chelating ligands, and since lanthanide phosphates are similarly insoluble, wild-type CPMV (5 mg mL^{-1}) was incubated in a solution of 50 mM $GdCl_3$ and 30 mM EDTA in 0.1 M HEPES buffer at pH 7.0, providing a molar excess of 10 000 Gd^{3+} ions per virus particle (Fig. 3).[14] A white gelatinous precipitate formed immediately; Gd-hydroxides were ruled out by a control experiment omitting the virus. The Gd-EDTA-CPMV reaction mixture was then dialyzed against a solution of EDTA (5 mM in 0.1 M HEPES, pH 7.0) for 2–3 h, during which time the precipitate dissolved to yield a clear colorless solution. Dialysis was then repeated with a fresh EDTA solution (5 mM in 0.1 M HEPES, pH 7.0, 12–24 h) and then twice against pure HEPES buffer (0.1 M, pH 7.0, 12 h) to remove excess Gd and EDTA species.

After dialysis, the virus was purified on a 10–40% sucrose gradient to further remove any virus decomposition fragments or small molecules. The sucrose band containing intact virions was pelleted and resuspended; HEPES, PBS or phosphate buffers could be used without the formation of precipitates. Gd concentrations were again determined by ICP-OES, giving reproducible values of 80 $\pm$ 20 Gd per virion. The above procedure was repeated with Tb^{3+} ions, with identical results.

The high affinity nature of the CPMV–lanthanide^{3+} interaction was apparent from the following observations: (a) lanthanide ions remained associated with CPMV throughout multiple dialysis and

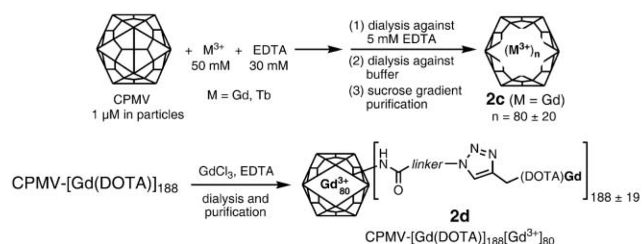

Fig. 3 High affinity binding of lanthanide ions by CPMV and synthesis of the dual Gd-labelled particles **2d**.

gradient procedures, reaching a stable loading value, (b) CPMV was able to compete with much higher concentrations of EDTA for the lanthanide ions, and (c) repeated treatment of the purified CPMV–Gd particles with EDTA-containing buffer did not extract Gd from the particle. The CPMV–Gd interaction requires packaged RNA; particles lacking genomic RNA[15] do not bind significant amounts of Gd^{3+}. Since the number of bound lanthanide ions is approximately equal to the number of protein molecules that make up each capsid, we speculate that a single RNA + protein site for each asymmetric unit (60 per particle) may be responsible for the high affinity interaction.

Combining the two labelling procedures provided CPMV particles carrying both types of Gd species (Fig. 3). Thus, treatment of a covalently-labelled CPMV particle bearing 188 Gd(DOTA) species per virion (analogous to **2b**) with Gd^{3+}/EDTA provided the hybrid particle **2d**, with a total of 268 $\pm$ 30 Gd atoms per particle, as revealed by ICP-OES. Therefore, the presence of attached Gd(DOTA) did not affect the internal binding of Gd^{3+}.

The T_1 relaxivities of the resulting particles were determined at proton Larmor frequencies of 64, 200 and 500 MHz (Table 1). All of the particles yielded similar values at 64 MHz, ranging from 1230 (CPMV–Gd) to 4150 (dual labelled particle) $mM^{-1}s^{-1}$, and from 11.9 {Qβ–[Gd(DOTA)]} to 15.5 (dual labelled **2d**) $mM^{-1}s^{-1}$ on a per-Gd basis. The latter values represent enhancements of 2 to 3 times per Gd relative to Magnevist (the first clinically approved intravenous MRI contrast agent, with a relaxivity of 5.2 $mM^{-1}s^{-1}$ at 64 MHz). These values are comparable to those of Kirshenbaum and co-workers for Gd(DTPA) complexes attached to bacteriophage MS2 (14.0 $mM^{-1}s^{-1}$ at 64 MHz),[3] but far lower than those reported by Douglas and co-workers for Gd ions attached to the calcium binding site of CCMV (202 $mM^{-1}s^{-1}$ at 61 MHz).[2] As expected, T_1 relaxivities were found to be diminished at higher field strengths.

There was no substantial difference in relaxivity exhibited by the two types of Gd centers attached to CPMV. We had anticipated that Gd(DOTA) species would experience a relatively modest enhancement with respect to free Gd(DOTA) upon attachment to the viral carrier, since these complexes (as well as the similar DTPA chelates) exhibit slow water exchange kinetics. They would therefore be expected to benefit only slightly from the great increase in rotational and translational correlation time provided by their immobilization on the large viral cage. The relaxivity results suggest that the nucleoprotein-bound Gd centers present in **2c** and **2d** are also likely to be well shielded from solvent water molecules. This is plausible since the lanthanide ions are associated with the RNA encapsulated by the viral capsid,[16] and are much more tightly bound than the high relaxivity centers engineered on CCMV.[2]

Table 1 T_1 relaxivities for derivatized virus particles at multiple proton Larmor frequencies[a]

Sample	Relaxivity per Gd (and per particle)/$mM^{-1}s^{-1}$		
	64 MHz	200 MHz	500 MHz
2b	12.8 (2850)	10.8 (2410)	3.0 (670)
2c	15.4 (1230)	11.0 (880)	1.5 (120)
2d	15.5 (4150)	9.4 (2520)	4.4 (1180)
3b	11.9 (1820)	5.1 (780)	3.5 (540)

[a] All values have an estimated error of 15%.

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We have demonstrated the use of the versatile copper(I)-catalyzed azide–alkyne cycloaddition reaction to decorate virus particles with suitably derivatized Gd chelates. The alkyne form of Gd(DOTA) described here has found other uses in our laboratory that will be described separately. We have also described the observation of a high affinity natural RNA-associated lanthanide binding property of wild-type CPMV.

While the two- to three-fold enhancement in Gd relaxivity of the reported particles is encouraging, more needs to be done to improve the efficiency of signal generation at target sites if the nanoparticle approach is to bear practical fruit. Ignoring differences in instrumentation and diagnostic methods, a simple consideration of molecular weights provides an idea of the nature of the problem; the clinically-used Gd(DOTA) molecule is approximately 560 Da, and 5 g (9 mmol) of the agent is administered in an average MRI exam. Particles **2d** and **3b** mass approximately 20 000 Da per Gd center, and thus must either provide 35-times (20 000/560) better relaxivity per metal, or be targeted to a desired location (tissue or tumor) 35-times as efficiently as Gd(DOTA). The properties and tailorability of nanoparticle platforms bring both of these goals well within reach.

We acknowledge the National Institutes of Health (R01-CA112075) for their support of this work.

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