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Insulin Hexamer-caged Gadolinium Ion as MRI Contrast-o-phore

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Abstract

High-relaxivity protein-complexes of Gd(III) are being pursued as MRI contrast agents in hope that they can be used at much lower doses that would eliminate toxic-side effects of Gd(III) release from traditional contrast agents. We construct here a new type of protein-based MRI contrast agent, a proteinaceous cage based on a stable insulin hexamer in which Gd(III) is captured inside a water filled cavity. The macromolecular structure and the large number of ‘free’ Gd(III) coordination sites available for water binding lead to exceptionally high relaxivities per one Gd(III) ion. The Gd(III) slowly diffuses out of this cage, but this diffusion can be prevented by addition of ligands that bind to the hexamer. The ligands that trigger structural changes in the hexamer, Cl⁻ and phenols, modulate relaxivities through an outside-in signaling that is allosterically transduced through the protein cage. Contrast-o-phores based on protein-caged metal ions have potential to become clinical contrast agents with environmentally-sensitive properties.

Graphical Abstract

Hexameric insulin with a Gd(III) ion captured in its water filled cavity is a high-relaxivity agent for magnetic resonance imaging that can change its contrast properties upon sensing ions and small molecules.

Keywords

gadolinium; protein; insulin hexamer; MRI contrast agent; environmentally-sensitive

Differences in the magnetic properties of water hydrogens in tissues can be exploited to obtain three-dimensional images in magnetic resonance imaging (MRI). The sensitivity of the method can be improved by injection of contrast agents (CAs), which increase these differences. At the moment of this writing, 30 million doses of Gd(III)-containing CAs are administered annually worldwide - approximately 1-in-3 of MRI scans.^[1]

Contrast agents are most often based on Gd(III) complexes. While these complexes are very stable *in vivo*, they need to be used at very high concentrations, which raises concerns due to potential toxicity of released free Gd(III). Thus, there is a substantial effort to optimize the properties that lead to strong contrast in order to minimize the amounts of contrast agent used. Contrast agents impact relaxation time constants, longitudinal T_1 and transverse T_2 , which reflect the rates at which proton spins in external magnetic fields return to equilibrium from excited states achieved by irradiation with radio-frequency. Dependence of these two time constants on the concentration of contrast agents is characterized by two relaxivity constants, r_1 and r_2 , which are systematically being targeted for optimization^[2-7].

Current clinical gadolinium-based CAs are small molecules with rapid tumbling times and usually only one coordinated water. These properties lead to relaxivity values that are generally below $10 \text{ mM}^{-1} \text{ s}^{-1}$ at current clinical magnetic field strengths (1.5T and 3T) and, consequently, 0.1-0.3 millimole/Kg quantities of administered doses. Hence, there is a drive to develop MRI contrast agents with high relaxivity - upwards of $100 \text{ mM}^{-1} \text{ s}^{-1}$ per Gd(III) ion, which would reduce doses. One possible way to achieve this is to use macromolecules, because large size and slow tumbling times result in dramatic increases in relaxivity.

Proteins can provide a scaffold for incorporating the Gd(III) ion into so-called ProCAs/^[8] The incorporation of the Gd(III) ion in proteinaceous structures has been achieved in several ways: 1) replacement of a protein's native metal ions by the Gd(III) ion or complexes (cf. in

apo-ferritin cage^[9];^[10] 2) bioconjugating a small molecule gadolinium complex to a protein;^[2] 3) engineering a gadolinium site into the protein or self-assembled peptides, either by modifying a native protein^[5] or by *de novo*.^[11] The modified native protein approach led not only to stable Gd(III) ion capture with exceptionally high relaxivity in MRI applications, but also to successful preclinical demonstrations of applications.^[9,12-15]

We now report an adaptation of a process of natural self-assembly of a peptide insulin to synthesize a stable hexameric cage that firmly captures one Gd(III) ion at its low-affinity, low-coordination number metal-ion binding site. This caged Gd(III) has exceptional contrast properties due to both substantial size and a large number of impacted water molecules, and represents a novel lead for creation of environment-responsive^[16] protein-based contrast agents.

Insulin spontaneously oligomerizes in solution, and in the presence of metal ions, such as Zn(II) and Co(II), this equilibrium is shifted towards hexamer at higher monomer concentrations (e.g., > 1 μM).^[17] This hexamer is not stable upon dilution, and although it can be observed by size exclusion chromatography (FPLC), it cannot be isolated without re-equilibrating back to monomer. During our research in oligonucleotide-based devices triggering insulin release,^[18] we were familiarized with a protocol for an *in situ* oxidation of Co(II) to Co(III) to form a stable $\text{Ins}_6\text{Co(III)}_2$ hexamer as an HPLC standard for quantification of less stable hexamers, e.g., $\text{Ins}_6\text{Zn(II)}_2$.^[16] The hexamer, when locked together with two Co(III) ions, has no detectable release of monomer units in buffer, with an estimated half-life for ligand exchange of two years; it releases insulin more quickly *in vivo*, with $t_{1/2}$ in hours, presumably via a metabolic reduction of Co(III) to Co(II).^[19]

In crystal structures^[20] of various insulin hexamers, metal ions hold two interdigitated *tris*- α -helical bundles via coordination with histidine side chains ($2 \times 3 \times \text{His}^{\text{B10}}$), creating a water-filled cavity inside the hexamer. The cavity is lined up by hydrophilic residues, and in its central region there is a metal ion binding site, defined by three pairs of γ -carboxylic groups of Histamates ($3 \times 2 \times \text{Glu}^{\text{B13}}$). Glutamate pairs are too far away from one another to all bind to the same metal ion at the same time; therefore, this site has relatively low affinity to metal ions, with X-ray crystallography showing binding to Cd(II), and Pb(II), and with K_D for Ca(II) and Cd(II) ions of ~ 80 and $< 10 \mu\text{M}$, respectively.^[21] The observed number of metal ions inside the cavity at this site ranges from one (Cd(II)^[22]) to three ions (Pb(II)^[22], Zn(II)^[23] and Na(I)^[24]). The site was also previously characterized via its impact on the fluorescence of lanthanides (i.e., Eu(III)) with an experimental observation that the lanthanide at that site has properties consistent with four water molecules coordinating this ion for $\text{Ins}_6\text{Zn(II)}_2\text{Eu(III)}$, and with fluorescent lifetimes indicating that up to three Eu(III) ions can be captured at the site.^[25]

Based on all these considerations, we decided to form a stable Co(III) complex in the presence of a variable number of equivalents of Gd(III), expecting that this cation, when caged within the cavity, will exhibit unique contrast properties. The hexamer formed in the presence of various amounts of GdCl_3 could be purified with chelating resin to remove excess Gd(III) ions. When adding greater than one equivalent of Gd(III) per hexamer, there is very little change in the relaxation rate before and after passing through the chelation

column, thus supporting the formation of a 1:1 complex. The product was then isolated by size exclusion chromatography (Figure S1), and, in agreement with previous reports with no added Gd(III), was stable (as a hexamer) indefinitely in HEPES buffer (time-course monitored by FPLC analysis and relaxivity; cf. Figure S2 for Mw determination).

The purified hexamer was crystallized and its X-ray structure determined at 1.8 Å resolution (Figures 1, 2, 4 and Supporting Information). Based on the B-chain configuration, it has a classical T_6 hexamer conformation.^[26-28] In it, the two Co(III) ions each bridge three insulin monomers via three His^{B10} side chains. The resulting two sets of *tris*-alpha-helical bundles are inter-digitated, defining a central cavity lined with hydrophilic residues, capped at each end by the Co(III) ions (Figure 2a).

We recognize two halves of the cavity, defined by two B-chain trimers, **D, I, N** and **B, L, G** (Figure 2a). Asymmetrically positioned between the two Co(III) ions (Figure 2a), we observe major electron density for Gd(III) that is equally distributed (*i.e.*, each modeled with 1/3 occupancy) between three disordered carboxylates from Glu^{B13} on individual B-chains **D, I, N** (*i.e.*, DIN plane) that have more than one conformation (Figure 2a). There is a precedent for this interpretation in a Cd(II) containing hexamer (Ins₆Cd(II)₂Cd(II)).^[22]

At the nearly identical position in the other half of the cavity, defined by the **B, L, G** B-chains (*i.e.*, in BLG plane), but rotated by 44° on C3 axis) we observe a density that can be attributed either to three water molecules, or to a lower level occupancy of Gd(III). We cannot resolve this solely through crystallographic means, but the latter situation is consistent with Gd(III) distributed between six Glu^{B13} equally in solution, with the occupancy difference being a reflection of the asymmetry in the T₃ structures

In insulin, with approximately 2:1 preference for occupancy of BLG over the DIN plane, or about 0.5 kcal.mol⁻¹. This means that our X-ray structure is a superposition between six different conformations in which Gd(III) can exist, three with higher (in DIN plane) and three with lower (in BLG plane) occupancy. When Gd(III) is in one of these positions, the other five are likely occupied with water molecules, however, coordinated waters were not identified due to the partial occupancy of the Gd(III) ions.^[22] This dynamic distribution of a single Gd(III) ion over six glutamates is consistent with saturation of water proton relaxivity at one equivalent of Gd(III) ion during formation of the hexamer.

We also observe weak electron density on the 3-fold crystallographic symmetry axis within bonding distance to the Gd(III) ion close to DIN plane. This was previously assigned to a partial (*e.g.*, 1/2) occupancy by a chloride ion for Ins₆Cd(II)₂Cd(II),^[22] but we cannot exclude the possibility that it is another water molecule.

The Ins₆Co(III)₂Gd hexamer, at pH 7.0, 37 °C, and in a magnetic field of 1.4 T, had relaxivity values of $r_1 = 138 \pm 5 \text{ mM}^{-1}\text{s}^{-1}$, and $r_2 = 260 \text{ mM}^{-1}\text{s}^{-1} \pm 10 \text{ mM}^{-1}\text{s}^{-1}$, regardless if one, two, three, or four equivalents of Gd(III) were added at the assembly stage. Therefore, the ratio of one Gd(III) ion per hexamer exists in solution as well (Figure 3a, b). Changes in relaxation time (T_i) were even detected at sub-μM concentrations (Figure 3 upper inset, LoD^[29] for T_1 and T_2 of 500 and 200 nM respectively), indicating the potential

for minimization of quantities of Gd(III) administered, and potential, as well as limitations, as a targeted-MRI contrast agent.^[13,14,30,31]

The r_1 and r_2 relaxivities are amongst the highest reported per Gd(III) ion (cf., Table). In the case of the hexamer, the tumbling time is calculated at ~20 ns (50 MHz), a close match to the Larmor precession time for the water proton at 1.4T (60 MHz), which is a requirement to maximize relaxivity through an increase in molecular size (i.e. tumbling time). This effect is limited to lower fields for r_1 . Indeed, when we characterized the hexamer at higher fields (1.5T vs 3, 7, and 9.4T, all at room temperature) r_1 values decreased as magnetic field strength increased, while r_2 values remained exceptionally high (Table). Relaxivity plots are shown in Figure S3.

The relaxivity values increased with an increase above room temperature, a property that is favorable for *in vivo* use. Typically, this suggests that the system is limited by slow rates of water exchange.^[32] While the exact cause of this phenomenon awaits more precise biophysical characterization, the temperature could impact the rate of diffusion of water, conformational equilibrium of hexamer resulting in shifts towards cavities with a larger number of water molecules, and/or a change in K_D value for Gd(III). It is difficult to give a precise estimation of the K_D of the complex that is based on sterically hindered capture, as opposed to classical coordination, but its k_{off} can be approximated by experiments in which phosphate ion is added to remove generated free Gd(III) from solution, leading to the loss of contrast (see below). This k_{off} was $(2.4 \pm 0.2) \times 10^{-6} \text{ s}^{-1}$, which is slower than one that characterizes streptavidin-biotin interactions.⁴⁵

Another known contributor to high relaxivity is the number of water molecules bound to the metal ion.^[33-35] Typically, Gd(III) has a coordination number of 8 or 9, but in the hexamer reported here, it is only bound, in any of the major conformations, to at most just one of the glutamates and a chloride ion as non-water ligands, with six or seven other coordination sites available for water molecules (Figure 2b). In a fluorescence study of Eu(III) in a Zn(II)-insulin hexamer, the number of water molecules bound was estimated as four.^[25] We could identify twelve water molecules in the cavity of our crystal structure forming two planes with six waters in each. These are primarily hydrogen-bonded to the side chains of Ser^{B9}, His^{B10} and Glu^{B13} on the B-chain alpha helices. Another five water molecules could be present based on the assumption that when Gd(III) is fixed at one position, the other five positions are occupied by water molecules. Overall, the Gd(III) ion is caged within a cavity with as much as seventeen water molecules, and with a low number of its coordination sites filled by non-water ligands; which is a unique situation for a gadolinium-MRI contrast agent.

In order for the hexamer to be useful as a contrast agent, the high relaxivity must be maintained *in vivo*. Stability also prevents toxicity of free Gd(III) and of insulin monomer components, with the latter readily addressed by inactive insulin analogs. Stability of the hexamer itself is measured by FPLC, but this method would not detect that Gd(III) left the cavity in the absence of concomitant degradation. In contrast, because $1/T$ is directly proportional to the concentration of Gd(III) in the central cavity, the relaxivity monitoring captures all changes in the cations molecular environment.

Purified $\text{Ins}_6\text{Co(III)}_2\text{Gd(III)}$ hexamer is stable indefinitely by FPLC and relaxation measurements at pH 7.0 at room temperature and at a concentration less than $200\mu\text{M}$, which is consistent with exchange experiments with radio-labeled insulin that measured half-lives of over two years.^[19] At higher concentrations a precipitate is observed, in both concentration- and temperature-dependent manner (Supporting Information). We observed a decrease in relaxation rate ($1/T_2$) of $<3\%$ per day for $\text{Ins}_6\text{Co(III)}_2\text{Gd(III)}$ at 37°C , without any decomposition of hexamer by FPLC even after several weeks of monitoring. In the presence of 150mM Cl^- ions, the behavior was similar. In contrast, the phosphate anion, known to form an insoluble complex with Gd(III) , removes it from the cavity at a higher rate (Figure 4a), while also not degrading the hexamer (Figure S4). Ca(II) ions at 10mM can also interfere with the Gd(III) - likely via transmetalation (Figure 4a).

These results can be rationalized by the presence of six major tunnels in T_θ -hexamers that lead from the protein surface to the cavity hosting Gd(III) . Based on the previous studies measuring the escape of Cd(II) ,^[21] the channels represent pathways for a potential escape of a metal ion from the cavity (Figure 4c, and Figure S5), and as an entrance of other agents to reach it. In a control experiments, we monitored relaxivity of a preformed ' $\text{Ins}_6\text{Co(III)}_2$ ' in the presence of free Gd(III) , observing a slow rate of increase with an estimated $t_{1/2}$ of ~ 40 hours (Figure S6). This rate is significantly faster than the reported rate of exchange of monomeric units,^[19] and is consistent with the potential for equilibration between cavity and environment through these channels.

In vitro stability of the Gd(III) hexamer was also tested in the presence of ascorbic acid (Figure 4a and Figure S4), confirming that Co(III) hexamer is degraded to monomers by reducing agents (cf., release *in vivo* of insulin with $t_{1/2}$ in hours^[19]). Phenolic ligands, used for stabilization of hexamers in formulations, such as resorcinol (and tylenol), also degrade the hexamer (Figure S4), as expected, based on their redox activity.

Insulin hexamers are known to undergo gross structural rearrangements of the cavity in the presence of various ligands,^[36] and we hoped to take advantage of these to achieve sensing. For example, when a T_θ -hexamer is converted to a R_θ -hexamer form, the channels leading from the bulk solvent to the central cavity collapse (cf., Figure 4c with 4d). Because Co(III) is not expected to fully switch in both positions to a tetrahedral ligand field arrangement (as observed in R_θ -hexamers), we decided to study $\text{Ins}_6\text{Zn(II)}_2\text{Gd(III)}$ formed *in situ*, which is expected to have full flexibility. This formulation is stable at $10\mu\text{M}$ concentrations of monomeric insulin, and has similar and stable relaxivity, comparable to that of $\text{Ins}_6\text{Co(III)}_2\text{Gd(III)}$, which makes it suitable to measure relaxivities of otherwise unstable hexamers. Indeed, in the presence of SCN^- , and also Cl^- (Figure 4b, and Figure S7, respectively), we observed a $1/T_2$ increase of over 20% , consistent with the change in the structure that was previously proposed to occur at the high concentrations of this anion.^[37] At the same time, we observe complete protection from the degradation by ascorbic acid, indicating that the complex does not equilibrate any more to release dimers and Zn(II) . Interestingly, addition of resorcinol, caused a downward shift by approximately 15% (Figure 4b), while still protecting fully from ascorbic acid (i.e., indicating conformational change, rather than re-equilibration). These are very large changes in absolute numbers, indicating outstanding potential to use allosteric effects in proteins to achieve 'smart' behaviors of

ProCAs (cf. Figure 4c and d). Further, we observed that resorcinol and thiocyanate provided substantial protection from complexation agents such as ascorbic acid.

In conclusion, we report synthesis, structure, and properties of a Gd(III)-containing peptide-cage based on insulin. This cage has several unique properties: It contains Gd(III) captured in a cavity with a large number of water molecules, while coordinated to only one-to-two non-water ligands. Second, both high molecular mass and high-water occupancy contribute to exceptionally high relaxivity values. Third, the stability of the hexamer is extraordinary, with the exception of reducing conditions. And, fourth, the hexamer provided us with the first example of an allosteric change in a protein impacting contrast properties.^[43, 44] The hexamer system is in this respect similar to GFP, a barrel protein with chromophore centrally positioned, but which can be sensitized to the outside environment. Thus, while this specific hexamer, as described in our work, is not likely to be directly used in clinics due to bioactivity of insulin monomers, it represents the first-in-class contrast agent on which new targeted and environmentally sensitive systems can be built.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Assembly of the 'Ins₆Co(III)₂Gd' hexamer. Schematic showing Ins₆Co(III)₂Gd hexamer self-assembly from six insulin monomers one Gd(III) ion, and two cobalt(II) ions, followed by peroxide oxidation of the cobalt to 'lock-in' the structure. Both monomer and hexamer pictures are generated from our X-ray crystallographic structure (cf. Figure 2). Gd(III) ion locations in the structure are shown colored aqua (in monomer Glu^{B13}). Each Co(III) ion (pink) is shown bound to His^{B10} on one of two 'tris- α -helix bundles' (one colored blue and one colored red) which are interdigitated with one another, thus forming a large cavity inbetween.

Figure 2.

X-ray crystal structure. a) Results showing the amino acids that line the cavity containing the Gd ion that is disordered over six sites: three Gd_D sites (major) have 66% of the total electron density (at 1/3 of this each); and three Gd_B sites (minor) equally share the remaining electron density. The Cl ion site is 1/2 occupied, and the Co(III) ion sites are fully occupied. Located water molecules in the central cavity are depicted by red colored spheres. Inset view looks down the C3 axis at the atoms in the cavity. b) Structure depicting only one gadolinium ion-binding site with a Gd ion. The remaining Gd-binding sites are depicted occupied by superimposed waters (transparent red spheres) to depict plausible locations²⁶ for additional Gd-coordinated water molecules to illustrate the Gd ion is likely to be coordinated to several water molecules within the cavity. Upper inset view looks down the

C3 axis at the atoms in the cavity. Lower inset shows an X-ray crystal structure (pdb 1trz)²⁴ with sodium in analogous positions to the gadolinium. The closest Glu^{B13} residue to the Na(I) ion is orientated in an equatorial position for binding.

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Figure 3.

1.4T relaxation measurements at 37°C and MRI. a) r_1 relaxivities. b) r_2 relaxivities. ($r_{1,2} = 1/T_{1,2} - 1/T_{1,2}^{\text{without contrast}}$), where $1/T_{1,2} = 1/T_{1,2}^{\text{with contrast}}$, with contrast $- 1/T_{1,2}^{\text{without contrast}}$ obtained from slopes of linear fits for 1 (blue), 2 (red), 3 (green), 4 (purple) equivalents of Gd(III) per hexamer. Slopes are virtually identical, showing that the central cavity of the hexamer is capable of binding to no more than one equivalent of Gd(III). Lower insets show slope comparison to the commercial MRI contrast agent Magnevist (orange) r_1 (3.2 mM⁻¹ s⁻¹) and r_2 (3.7 mM⁻¹ s⁻¹) at much higher concentrations. Upper insets show measurements used to calculate a Limit of Detection (LoD) of 500nM (T1) and 200 nM (T2). c) 3T clinical MRI image of phantom samples of $\text{Ins}_6\text{Co(III)}_2\text{Gd}$ at various concentrations compared to Magnevist (cross-section of samples in plastic tubes surrounded by a waterbath).

Figure 4.

Stability of gadolinium-hexamers exposed thiocyanate, and to the physiological additives: calcium, phosphate, and ascorbate, over 24 hours in 100mM HEPES at pH 7.4 and 37°C. Y-axis is $1/T_2$, with contrast and is directly proportional to the gadolinium-hexamer concentration. a) Stability of the hexamer Ins₆Co(III)₂Gd. b) Stability of hexamer formulations of Ins₆Zn(II)₂Gd. Arrows indicate the significant increase, then drop, in relaxivity when first thiocyanate (150mM) is added to Ins₆Zn(II)₂Gd, then resorcinol (100mM), demonstrating that the relaxivity can be modulated allosterically. Addition of thiocyanate and/or resorcinol to Ins₆Zn(II)₂Gd affords various levels of protection against hexamer degradation by ascorbic acid. Structure of the *T₆*-insulin hexamer Ins₆Co(III)₂Gd. Entrances to the major tunnels, affording a connection between the Gd(III) ion and the bulk solvent, are indicated by the green arrows. Atoms colored crimson are amino acids 1-9 of the B-chain, and these are extended in *T₆* (Octahedral Co(III) ion is colored pink). d) *R₆* insulin hexamer Ins₆Zn(II)/resorcinol from X-ray structure (1evr).^[42] shows amino acids 1-9 of the B-chain are coiled when the hexamer binds resorcinol, resulting in collapse of the major tunnels. (Cl⁻ ion coordinated to tetrahedral Zn(II) ion is colored green). Note: concentrations of additives are higher than their typical physiological levels. 100mM resorcinol; “Ca²⁺” is 10mM calcium chloride; “PO₄³⁻” is 10mM sodium phosphate; 200μM ascorbic acid. Hexamers are at 10μM. Results for the chloride ion are given in the Supporting Information (Figure S7).

Relaxivities (per Gd(III) ion) of selected 'high relaxivity' Protein-Gd(III) complexes (including $\text{Ins}_9\text{Co(III)}_2\text{Gd}$ at various magnetic field strengths).

Table:

Protein	r_1 /mM ⁻¹ s ⁻¹	r_2 /mM ⁻¹ s ⁻¹	K_d /μM	Mag. Field /T	Temp. /°C	q	MW /kDa	Type	Reference
BSA	~60	-	130	1.4	37	-	69	-	38
IgG	103	207	6	1.4	19	-	150	-	39
Glut. Syn.	172	-	-	1.2	25	4	50×12	Replacement of Mn(II) &/or Mg(II)	40
(BSA)(GdEDTA) _n	36	20	-	0.5	37	-	-	Bioconjugated	41
ProCAI	117	129	0.000001	1.5	25	2	12	Engineered binding site	5
MB1-1	10	89	5	7.0	20	3	11.7	Engineered <i>de novo</i>	11
$\text{Ins}_6\text{Co(III)}_2\text{Gd}$	138	261	-	1.4	37	-	35	Replacement of Ca(II)	This study
"	98	222	-	1.5	room	"	"	"	"
"	44	253	-	3.0	room	"	"	"	"
"	37	229	-	3.0	room	"	"	"	"
"	40	203	-	3.0	room	-	-	-	-
"	12	265	-	7.0	27	"	"	"	"
"	10	223	-	9.4	room	"	"	"	"