Interactions between Endohedral Metallofullerenes and Proteins: The Gd@C$_{60}$−Lysozyme Model

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ABSTRACT: Endohedral metallofullerenes (EMFs) have great potential as radioisotope carriers for nuclear medicine and as contrast agents for X-ray and magnetic resonance imaging. EMFs have still important restrictions for their use due to low solubility in physiological environments, low biocompatibility, nonspecific cellular uptake, and a strong dependence of their peculiar properties on physiological parameters, such as pH and salt content. Conjugation of the EMFs with proteins can overcome many of these limitations. Here we investigated the thermodynamics of binding of a model EMF (Gd@C$_{60}$) with a protein (lysozyme) that is known to act as a host for the empty fullerene. As a rule, even if the shape of an EMF is exactly the same as that of the related fullerene, the interactions with a protein are significantly different. The estimated interaction energy (ΔG$^{\text{binding}}$) between Gd@C$_{60}$ and lysozyme is $-18.7$ kcal mol$^{-1}$, suggesting the possibility of using proteins as supramolecular carriers for EMFs. π−π stacking, hydrophobic interactions, surfactant-like interactions, and electrostatic interactions govern the formation of the hybrid between Gd@C$_{60}$ and lysozyme. The comparison of the energy contributions to the binding between C$_{60}$ or Gd@C$_{60}$ and lysozyme suggests that, although shape complementarity remains the driving force of the binding, the presence of electron transfer from the gadolinium atom to the carbon cage induces a charge distribution on the fullerene cage that strongly affects its interaction with the protein.

INTRODUCTION

Fullerenes are spherical molecules made only of carbon atoms. Atoms and molecular clusters can be encapsulated inside fullerenes to form endohedral fullerenes. The encapsulation of metal atoms creates a new class of hybrid molecules, which are called endohedral metallofullerenes (EMFs). The physicochemical properties of these hybrid materials differ from those of empty fullerenes because of the presence of electron transfer from the metal atoms to the carbon cage. The number of atoms encapsulated inside the cage defines the type of EMFs that can labeled mono-EMFs, di-EMFs, tri-EMFs, and cluster-EMFs.

EMFs can find applications in a variety of fields that span molecular electronics, photovoltaics, materials science, and nanomedicine. A further interesting potential application is the exploitation of EMFs as contrast agents (CAs) for magnetic resonance imaging (MRI) and X-ray imaging as well as radioisotope carriers in nuclear medicine. Many metal ions that have found application in medicine are toxic and cannot be administered safely in vivo. Chelating agents are often used to coordinate the metal ions and reduce their toxicity. However, in vivo, these chelate complexes are prone to metal dissociation. The release of free metal is impossible when using EMFs. EMFs therefore provide a truly unique alternative to chelating compounds because of their resistance to metabolism and their high kinetic stability. Thus, incarceration of atoms inside fullerene cages prevents direct binding of the toxic metal ions with serum components and tissues. A class of EMFs very promising for applications as MRI contrast agents (CAs) is represented by gadofullerenes. Gadofullerenes are paramagnetic compounds that entrap Gd$^{3+}$ ions. They offer fundamental advantages over conventional chelate-based MRI CAs. The aquated Gd$^{3+}$ ion is toxic, and its toxicity is usually sequestered by chelation with multidentate ligands. Both linear and macrocyclic chelating ligands were exploited, and several compounds are now available. However, even the best-designed chelates are characterized by some thermodynamic instability and the associated metabolic-ion release. A possible risk associated with the administration of these contrast agent is the acute nephrotoxicity. In particular, nephropatic patients could develop nephrogenic systemic fibrosis after exposure to gadolinium, a potentially fatal disease.

The encapsulation of metals inside the fullerene cages cannot be considered as a chelation, i.e., a coordinative, dative bonding. The Gd$^{3+}$ metal ion is trapped inside the biologically stable fullerene cage, so that the toxic Gd$^{3+}$ ion is never released in vivo. This obvious advantage of gadofullerene structures is especially important for CAs that require longer retention times in vivo, such as those for blood-pool imaging. Gadofullerene species have recently been shown to exhibit $T_1$ relaxivity up to 500 times greater than those of CAs presently...
in clinical use. These new nanoscale materials are the highest performing MRI CA known to date. The carbon cage itself plays an important role in the large value of the relaxivity of the gadofullerenes. In the relaxation process, the gadofullerene can simultaneously relax the protons of many hydrogen-bonded water molecules on their ∼200 Å² paramagnetic surface. The increased contrast and stable encapsulation of Gd³⁺ potentially offer the possibility of using lower dosages of more potent and safer paramagnetic contrast agents, as compared with the performance of clinically used Gd chelates.

Although gadofullerenes overcome much of the limitations of current CAs, demonstrating superior relaxivity properties and no Gd³⁺ release, they still present important restrictions in their use:

(i) low biocompatibility,
(ii) difficulty to direct or target the gadofullerenes to specific cells or tissues,
(iii) proton relaxivities that display a remarkable pH dependency,
(iv) fullerene-based materials show aggregation phenomena that depend on the salt content of the medium and, therefore, affect their behavior in biological or medical applications,
(v) short retention times.

Conjugation of EMFs with proteins can overcome most, or all, of these limitations. The ability of fullerenes to interact with proteins was demonstrated in many systems, both experimentally and computationally. A few noteworthy advantages of protein EMF bioconjugates are

(a) a EMF-bioconjugate can be perfectly biocompatible;
(b) the relaxivity will not depend on pH or salt concentration, since the EMF-bioconjugate does not aggregate;
(c) specific tissue targeting becomes possible with binding to specific proteins or antibodies.

Crucially, conjugation of a paramagnetic gadofullerene and a protein can improve relaxivity, since the interaction with proteins can slow down the molecular rotation of the EMF and result in a relaxivity increase. In the past, molecular dynamics (MD) simulations have been carried out to investigate the interactions between gadofullerenol and proteins. The aim of this work is to investigate the possibility of conjugating pristine EMFs with proteins in a noncovalent way. Gd@C₆₀–lysozyme was chosen as a representative case because it is possible to make a comparison with the C₆₀–lysozyme system, which has been well investigated both experimentally and computationally. The analysis of the energy contributions of the binding between Gd@C₆₀ and lysozyme also supplies guidelines for understanding protein–EMF interactions.

## RESULTS AND DISCUSSION

### Density Functional Theory (DFT) Calculations on Gd@C₆₀ Molecules.

Due to the lack of crystallographic structures for Gd@C₆₀, to find the most stable state and geometry of Gd@C₆₀, the Gd ion was systematically inserted into the crystallographic structure of C₆₀ in different positions inside the cage, and a series of geometry optimization calculations were carried out, using different multiplicities. In agreement with previous calculations, it was found that the Gd ion is placed close to one of the hexagonal faces of the C₆₀ cage. The total spin multiplicity of Gd@C₆₀ is $S = 7$. ESP charges were calculated for the most stable structure of Gd@C₆₀ according to Merz–Kollman (MK) scheme (Figure 1).

![Figure 1](image1.png)

#### Docking Gd@C₆₀ to Lysozyme.

The docking procedure provides 27 privileged positions of Gd@C₆₀ interacting with lysozyme (Figure 2a). To rank the different poses, the molecular mechanics-generalized born surface area (MM-GBSA) value of the interaction between Gd@C₆₀ and lysozyme, in the minimized structures, was used as a scoring function.

![Figure 2](image2.png)
function. The position that maximizes the interaction of Gd@C₆₀ (Figure 2b) provides the initial coordinates for a 200 ns MD simulation of the adduct in an explicit water box.

To estimate the binding energy between lysozyme and Gd@C₆₀ and their components, we carried out a MM-GBSA analysis of the trajectory. This methodology has provided valuable information to estimate the interaction between proteins and carbon nanomaterials.⁶¹⁻⁶⁸

**Gd@C₆₀ Binding Pocket.** The interaction energy ($ΔG_{\text{binding}}$) between Gd@C₆₀ and lysozyme, in the Gd@C₆₀ most favorite binding pocket, was evaluated to be $-18.7$ kcal mol$^{-1}$, a value very close to the C₆₀–lysozyme interaction ($-18.5$ kcal mol$^{-1}$).⁵¹ Experimentally, lysozyme was used as a supramolecular host for C₆₀.²⁸,³⁰,⁵⁴ These results indicate the feasibility of the noncovalent bioconjugation of EMFs with proteins. Analysis of the binding components of the energy (Figure 3) shows that, even if the Gd@C₆₀ is highly charged, van der Waals interactions remain the driving force for the binding ($-41.1$ kcal mol$^{-1}$).

![Energy components of $ΔG_{\text{binding}}$ of Gd@C₆₀ with lysozyme](image)

**Figure 3.** Energy components of $ΔG_{\text{binding}}$ of Gd@C₆₀ with lysozyme in the most favorite binding site.

Electrostatic interactions account for only $-3.0$ kcal mol$^{-1}$. In the Gd@C₆₀, the electron transfer due to the presence of the Gd ion inside the cage induces, at the same time, positive and negative charges on the fullerene cage, generating a plethora of stabilizing and destabilizing terms that counterbalance each other. The fact that Gd@C₆₀ maintains its hydrophobic character is proved by the negative value of the hydrophobic interactions, i.e., nonpolar solvation, that assist the binding ($-3.7$ kcal mol$^{-1}$). This value is smaller than that of the vdW interactions. Polar solvation ($12.6$ kcal mol$^{-1}$) and entropy contribution ($16.5$ kcal mol$^{-1}$) are detrimental to the binding, since their contributions are positive. The per-residue decomposition of $ΔG_{\text{binding}}$ helps in understanding the chemical origin of the various contributions.

Proteins are able to establish a large number of interactions with carbon nanoparticles that include $π-π$ stacking, hydrophobic interactions, surfactant-like interactions, and electrostatic interactions.⁶⁹⁻⁷³ Table 1 shows that a variety of interactions are present in the protein–EMF adduct. $π-π$ stacking between aromatic residues and the conjugated surface of carbon nanomaterials usually governs their interaction.⁶⁹⁻⁷⁴ For Gd@C₆₀, $π$-stacking contacts between the cage and the indolic group of Trp 123 (sandwich-like) and the phenyl group of Phe 34 (T-shaped) are important stabilizing terms (Figure 4a).

Hydrophobic interactions are established between aliphatic residues and the Gd@C₆₀ surface. Amino acids with a hydrophobic side chain tend to bind to the surface of Gd@C₆₀ reducing the interfacial energy with the water interface (hydrophobic effect).⁶⁹ Usually, the interaction energy increases with an increase in the exposed hydrophobic surface of the protein residue.⁶⁹ In the present case, the longer aliphatic chain of Val 120 interacts more strongly with the Gd@C₆₀ than Ala 122 (Figure 4b). The interactions of Cys 30 and Cys 115 with Gd@C₆₀ deserve special attention. These residues form a disulfide bond that interacts directly with Gd@C₆₀ (Figure 4b). The importance of this kind of interaction was recently highlighted by Hirano and co-workers for lysozyme and carbon nanotubes.⁷⁵,⁷⁶ Our computations evidenced surfactant-like interactions between the Thr 118 and Lys 33 residues and Gd@C₆₀ (Figure 4c).⁷⁷,⁷⁸ The hydrophobic aliphatic chains of these residues interact with Gd@C₆₀ whereas the hydrophilic groups head toward water.

In the interaction between carbon nanoparticles and proteins, electrostatic interactions are generally quantitatively smaller than those previously discussed. Positively charged residues (arginine and lysine, in particular) can interact with the carbon nanoparticle surfaces via cation–$π$ interactions. When a net charge is not present on the residue, a charge-transfer mechanism may still occur, for example, during physiosorption of aminic and amidic residues. In contrast, when we consider EMFs, where net charges exist on the fullerene cage, these interactions can have significant values.

**Table 1.** Largest Contributions ($ΔG_{\text{binding}} > 2.0$ kcal mol$^{-1}$) to $ΔG_{\text{binding}}$ of Individual Residues (kcal mol$^{-1}$) in the Most Favorite Binding Site

| Residue | $ΔG_{\text{binding}}$ (kcal mol$^{-1}$) |
|---------|----------------------------------------|
| Cys 30  | -2.7                                   |
| Lys 33  | -2.7                                   |
| Phe 34  | -2.9                                   |
| Arg 114 | -6.6                                   |
| Cys 115 | -2.4                                   |

*In bold values greater than 4 kcal mol$^{-1}$.

![](image)

**Figure 4.** (a) $π-π$ stacking interactions, (b) hydrophobic interactions, (c) surfactant-like interactions, and (d) electrostatic interactions between lysozyme residues and Gd@C₆₀.
Analysis of the most interacting residues shows that Arg 114, Lys 33, and Asp 199 strongly interact with Gd@C_{60}. Interestingly, in the Gd@C_{60} cage, there is a dipolar distribution of charges (Figure 1b). In fact, the Gd ion is positioned close to one of the hexagonal faces of the C_{60} cage. This induces negative charges on the closest atoms via electron transfer and polarizes the Gd@C_{60} cage. In the hemisphere opposite to the Gd binding position, positive charges are induced.

On average, the fullerene cage in Gd@C_{60} is approximately half positive and half negative. This charge distribution is mirrored in the Gd@C_{60} binding pocket, where positively charged Arg 114 and Lys 33 face the negative hemisphere of the fullerene, and on the opposite side, Asp 199 faces the positive charges (Figure 4d), generating strongly stabilizing Coulombic interactions between the protein binding pocket and Gd@C_{60}.

Gd@C_{60} vs C_{60} in the C_{60} Binding Pocket. In the previous section, we observed that the presence of a Gd atom encapsulated in the C_{60} cage changes the favorite recognition pocket for the binding to lysozyme. To compare directly the differences in the protein binding between C_{60} and Gd@C_{60}, we estimated the total binding energy and their contributions, when the two fullerenes are bound in the same protein pocket (Figure 5). The C_{60} binding pocket is well characterized, and the corresponding binding thermodynamics has already been investigated both experimentally and computationally;^{28,30,31,52,54} the same binding pocket was used for Gd@C_{60}.

Thus, MD simulations were carried out for the Gd@C_{60}−lysozyme adduct, when it is bound in the C_{60} binding pocket (Figure 5a). In this case, the interaction energy ($\Delta G_{binding}$) between Gd@C_{60} and lysozyme is $-4.8$ kcal mol$^{-1}$, a lower value when compared to the interaction energy of C_{60} with lysozyme ($-18.5$ kcal mol$^{-1}$). This result confirms the predictive accuracy of the docking protocol developed for EMFs.

The analysis of the binding components of the energy (Figure 5c) of Gd@C_{60} in the C_{60} binding site provides an explanation of the different behavior of C_{60} and Gd@C_{60} in their interaction with proteins. The first difference is the presence of a new term for Gd@C_{60} corresponding to the electrostatic interaction. Whereas the highly symmetric C_{60} molecule lacks net charges on the carbon atoms, for the Gd@C_{60} cage, the presence of the electron transfer from the gadolinium atom to the carbon cage induces a charge distribution on the fullerene cage (Figure 1), generating an additional stabilizing term in the binding energy with lysozyme (Gd@C_{60} electrostatic term = $-1.8$ kcal mol$^{-1}$). The appearance of this term is extremely important because it explains qualitatively many of the differences observed with Gd@C_{60} and deserves an in-depth analysis. Even if the global effect is low, it is the result of many contributions that counterbalance each other. In a dynamical picture, one can see the effect of these stabilizing and destabilizing terms as a sequence of continuous kicks toward the Gd@C_{60} cage. This increases the mobility of the Gd@C_{60}, when compared to that of the C_{60} especially in a crevice-like binding pocket, such as the substrate binding pocket of lysozyme (Figure 6).

Figure 5. (a) Binding of Gd@C_{60} in the C_{60} binding pocket. (b) Binding of C_{60} in the C_{60} binding pocket Gd@C_{60}. (c) Total binding energy ($\Delta G_{binding}$) and energy components of $\Delta G_{binding}$ of Gd@C_{60} (in gray) and C_{60} (in black) with lysozyme, in the C_{60} binding pocket.

Figure 6. Trajectories in the C_{60} binding site during the 200 ns MD simulation of the (a) Gd@C_{60} cage (Gd ion is removed for clarity) and (b) the C_{60} cage.
In the case of C$_{60}$ (Figure 6b), protein residues stick to its surface to maximize vdW interactions ($C_{60}$ vdW contribution = $-45.1$ kcal mol$^{-1}$) and become glued to the fullerene cage ($C_{60}$ entropic term = $18.1$ kcal mol$^{-1}$). In contrast, the binding of the Gd@C$_{60}$ to the C$_{60}$ binding pocket is characterized by higher mobility (Figure 6a), due to the charges that are present in the Gd@C$_{60}$ cage. The enhanced mobility of both Gd@C$_{60}$ and protein residues interacting with the fullerene cage reduces the entropic penalty of Gd@C$_{60}$ upon lysozyme binding (Gd@C$_{60}$ entropic term = $13.8$ kcal mol$^{-1}$), but at the same time decreases the van der Waals interactions, which are the driving force for the interaction between Gd@C$_{60}$ and lysozyme (Gd@C$_{60}$ vdW contribution = $-26.2$ kcal mol$^{-1}$).

Hydrophobic interactions, i.e., nonpolar solvation, assist the binding, even if the corresponding value for Gd@C$_{60}$ (Gd@C$_{60}$ nonpolar solvation term = $-2.8$ kcal mol$^{-1}$) is smaller than that for the empty hydrophobic C$_{60}$ cage (C$_{60}$ nonpolar solvation term = $-4.3$ kcal mol$^{-1}$). The polar solvation term is detrimental for the binding for both C$_{60}$ and Gd@C$_{60}$, but the penalty is smaller for Gd@C$_{60}$ than C$_{60}$. The binding of the fullerene cage occurs in the substrate binding pocket of lysozyme, that is a region exposed to water and where amino acids with polar side groups are located. The hydrophilic parts of these residues, upon formation of the complex with fullerenes, are forcefully desolvated, causing a destabilization of the system. For the hydrophobic C$_{60}$ cage (C$_{60}$ polar solvation term = $12.8$ kcal mol$^{-1}$), this destabilization is greater when compared to that of the charged cage of Gd@C$_{60}$ (Gd@C$_{60}$ polar solvation term = $12.1$ kcal mol$^{-1}$).

To understand the differences between C$_{60}$ and Gd@C$_{60}$ upon binding, it is interesting to re-analyze the solvation binding terms, from the point of view of C$_{60}$ and Gd@C$_{60}$ (Figure 7).

![Figure 7. Total solvation energy and polar and nonpolar energy components of total solvation energy for Gd@C$_{60}$ (in gray) and C$_{60}$ (in black) with lysozyme, in the C$_{60}$ binding site.](image)

It is clear that the difference is due to the presence of charges in the Gd@C$_{60}$ cage. The binding between C$_{60}$ and a protein is always favored because C$_{60}$ is a purely hydrophobic sphere. When it binds to a protein binding pocket, there is always a stabilizing nonpolar term, whereas the polar solvation is null (net charges on the cage are zero). The nonpolar solvation term takes also into account the entropy increase due to the water molecules in the first hydration shell that are tightly bound to the protein and are set free upon noncovalent interactions with the fullerene (hydrophobic effect).

On the opposite, for Gd@C$_{60}$ even if a stabilizing nonpolar term is still present, the polar solvation term tends to overcome it because of the net charges present in the Gd@C$_{60}$ cage. Only if there is an ideal pocket able to accommodate the Gd@C$_{60}$ cage can the binding with protein take place.

### CONCLUSIONS

The calculated interaction energy between Gd@C$_{60}$ and lysozyme is evaluated to be $-18.7$ kcal mol$^{-1}$, suggesting the possibility of using proteins as supramolecular carriers for EMFs. π–π stacking (with Trp 123, Phe 34), hydrophobic interactions (with Val 120, Ala 122, Cys 30, Cys 115), surfactant-like interactions (with Thr 118, Lys 33), and electrostatic interactions (with Arg 114, Lys 33, Asp 199) govern the global interaction between lysozyme and Gd@C$_{60}$.

vdW interactions and shape complementarity remain the driving forces for the binding, even if the presence of the electron transfer from the gadolinium atom to the carbon cage induces a charge distribution on the fullerene cage that strongly affects its interaction with lysozyme. In fact, a different protein binding pocket, is identified for Gd@C$_{60}$ with respect to C$_{60}$. Although C$_{60}$ has a natural tendency to bind to proteins, Gd@C$_{60}$ prefers to remain in water because, upon binding, a positive polar solvation term (which is null for C$_{60}$) exists and represents an energy penalty. In a dynamical picture, the presence of many stabilizing and destabilizing electrostatic terms that are located closely to each other causes continuous kicks to the Gd@C$_{60}$ cage, increasing its mobility. In conclusion, only the interaction with a well-suited binding pocket, showing charge complementarity to the EMF and a well-defined complementary shape, allows EMF binding to the protein. The presence of net charges on the cage of EMF increases the constraints to make a protein binding pocket suitable for EMF and makes its identification more complicated.

### COMPUTATIONAL DETAILS

**QM Calculation on Gd@C$_{60}$ Molecules.** The electronic structure and geometry of the Gd@C$_{60}$ adduct were investigated by means of DFT calculations using the Gaussian 09 series of programs.$^{79}$ Full geometric optimizations for Gd@C$_{60}$ were carried out. A benchmark to evaluate the performance of different DFT functionals and basis sets to reproduce experimental data on gadofullerenes was recently performed by Zhou and co-workers.$^{80}$ Following their results, the pure generalized gradient approximation functional Perdew–Burke–Ernzerhof$^{81}$ was used for DFT calculations. To take into account the scalar relativistic effects, the effective core potential triple split basis set (CEP-121G)$^{82}$ was used to describe the Gd atom, whereas for the carbon atoms, we used the common medium-sized double-split 6-31G* basis set.$^{79}$ When pure functionals are used, the common medium-sized double-split 6-31G* is good enough to calculate reasonable septet–nonet gap and Gd–C distance.$^{80}$ Frequency calculations were also performed after geometry optimization to ensure that the obtained structures are real minima on the potential energy surface.

**Parameterization of the Gd@C$_{60}$ Molecule for Molecular Mechanics.** The fullerene cage was modeled using the CA atom type (sp$^3$ Aromatic Carbon parameter). ESP charges were calculated for the most stable structure of Gd@C$_{60}$ according to the Merz–Kollman (MK) scheme, a protocol used previously to study Gd–DOTA complexes.$^{83}$ van der Waals parameters for the Gd atom were taken from the same study.$^{83}$
Docking Gd@C_{60}. A docking protocol recently validated for the study of interactions between proteins and nano-objects, was used to generate the coordinates of the adduct between lysozyme and Gd@C_{60}. This methodology is based on a two-step protocol of docking. The shape complementarity algorithm of PatchDock was used to generate initial docking models of lysozyme and Gd@C_{60}. The docking poses were then refined with FireDock. Sidechain flexibility is modeled by rotamers and Monte Carlo minimization. Following the rearrangement of the side chains, the relative position of the docking partners is refined by Monte Carlo minimization. All poses were fully minimized by AMBER. The f12SB force field was used to model the proteins. The minimization was carried out with the Sander program, using the Generalized Born model for the solvation and no cut-off for van der Waals and electrostatic interactions was adopted.

**MD Production and MM-GBSA Analysis.** The same protocol of equilibration, MD production, and molecular mechanics-generalized born surface area (MM-GBSA) recently used for C_{60}—lysozyme complexes was used in this work. The overall sampling time was 200 ns. The calculation and decomposition of binding free energy, $\Delta G_{\text{bind}}$, between fullerenes and lysozyme were performed using the MM-GBSA scheme. To obtain an estimate of the binding entropy, the translational and rotational entropies (standard statistical mechanical formulas), and the vibrational entropy contribution (normal-mode calculations, nmode program in AMBER) for the complex, receptor, and ligand are calculated during the MD trajectory. The results are averaged using the PTRAJ program via MMPBSA.py.

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The authors declare no competing financial interest.

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