

Chapter 2

Fullerene and Derivatives

2.1 Introduction

Since the discoveries of fullerene C₆₀ in 1985 [1], carbon nanotubes (CNTs) in 1991 [2], and graphene in 2004 [3], carbon-based nanomaterials have generated a great deal of interest in various biomedical applications [4, 5], such as gene delivery [6], optical imaging [7], and nanotherapeutics [8–12] due to their excellent mechanical, optical, and electrical properties [13–15]. These widespread applications of carbon-based nanomaterials are also accompanied by increasing concern regarding their interactions with tissues, cells, and biomolecules [16], and how they might affect the integrity of cells [17, 18], as well as the structure and function of proteins and nucleic acids [19, 20]. A detailed understanding at the molecular level of the interaction between nanomaterial and biomolecules is essential to the safe design and usage of nanoparticle-based biomedical technologies [21–27]. Recent experimental and theoretical studies on the interactions between proteins, nucleic acids (such as DNA), and cell membranes with the zero-dimensional (0D) fullerenes, one-dimensional (1D) carbon nanotubes (CNTs), and two-dimensional (2D) graphenes have shown that these interactions can affect both the structures and functions of biological systems, resulting in serious cytotoxicity and biosafety concerns [28–31]. For example, fullerene and its derivatives have been shown to inhibit the functions of HIV protease [32] and other important protein domains involved in protein–protein interaction (ppI), such as WW domains and SH3 domains [29, 33]. As will be detailed in the following chapters, single-walled carbon nanotubes (SWCNTs) have also been shown to disrupt the active sites of WW domains and compete with native ligands (proline-rich motifs) of SH3 domains. Given the ubiquity and importance of such proteins in signaling and regulatory pathways [34–36], interactions between carbon-based nanomaterials such as fullerene and derivatives with WW and SH3 domains are particularly disconcerting. Graphene nanosheets possess the strongest observed capacities for disrupting protein structure among these carbon-based nanomaterials with different

topologies [37] (more discussions in Chap. 4). Various strategies have been proposed to mitigate their cytotoxicity to normal cells [38], and meanwhile to target their specific cytotoxicity toward tumor and bacteria cells [39] as novel nanomedicine.

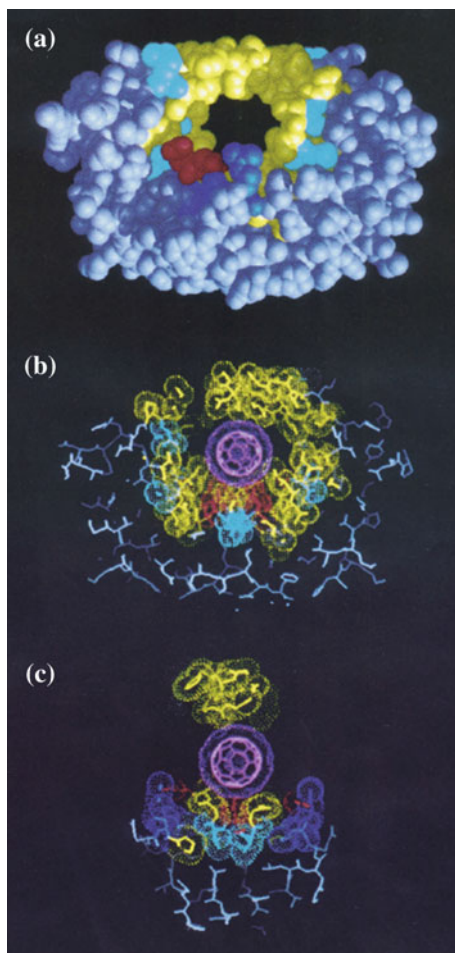
Among all carbon-based nanomaterials, fullerenes, and their derivatives are probably the most extensively studied nanoparticles for these biomedical applications mentioned above [40–42]. Besides demonstrating promises in *de novo* therapeutics, appropriate surface modifications of these nanoparticles also showed reduced potential cytotoxicity. For example, hydroxyl functionalized C_{60} protects mitochondria from superoxide, hydroxyl, and lipid radicals [43], and enhances mitochondrial enzyme activities of superoxide dismutase and glutathione peroxidase by effectively reducing oxidative stress [44]. Water-soluble immunoconjugate C_{60} effectively targets the gp240 antigen, showing great promise of applying fullerene immunotherapeutics for inhibiting cancer proliferation [45]. Even though there have been enormous progresses with the current state-of-the-art experimental techniques, applying these approaches to reveal the underlying molecular mechanisms with atomic detail has remained difficult, due to the intrinsic limitations in the temporal and spatial resolutions of these techniques, particularly for complex biological systems with a highly inhomogeneous environment. Alternatively, computational approaches can complement existing experimental techniques to investigate the nanoparticle–biomolecule interactions with atomic resolution [46].

In this chapter, we will go over some recent advances of *in silico* modeling for the interactions between fullerene and derivatives with proteins in more detail that have captivated an array of researchers from different perspectives. We start the next sections with fullerene inhibition of HIV protease, fullerene induced antibody, and related interactions, and in particular, a novel fullerene derivative, metallofullerenol $Gd@C_{82}(OH)_{22}$, and its interactions with various proteins. As for the novel metallofullerenol $Gd@C_{82}(OH)_{22}$, we will first introduce its exciting nanomedicinal potential in cancer therapeutics through inhibiting the matrix-metalloproteinase (MMP)-9 and stabilizing the collagen complex in tumor extracellular matrix (ECM). Then we discuss the potential nanotoxicity of $Gd@C_{82}(OH)_{22}$ through its inhibition of WW and SH3 domains, two representative proteins in signal transduction pathways. Finally, we also describe in detail the force field development of $Gd@C_{82}(OH)_{22}$ as an example for this important step in all nanotoxicity molecular simulations.

2.2 Fullerene Inhibition of HIV Protease

The protease specific to the human immunodeficiency virus 1 (HIVP) has been shown to be a viable target for antiviral therapy [47]. Roughly speaking, the active site of this enzyme is like an open-ended cylinder assembled almost exclusively by hydrophobic amino acids (Fig. 2.1a), except for the two key catalytic aspartic acid residues (Asp 25, Asp 125) which catalyze the protease function on the scissile

Fig. 2.1 **a** “Front” view of the HIV-1 protease. Color coding is as follows. *Yellow* Leu, Ile, Phe, Tyr, Val, Trp, Pro, Gly, Ala. *Blue* Lys, Arg. *Red* Asp, Glu. *Cyan* Thr, Ser, Gln, Asn, Cys, Met, His. *Gray* regions greater than 10 Å from the center of the active site. **b** Same view as (a) with the top scoring C60 orientation shown. The C60 is colored *magenta*, and the van der Waals surface of the active site and ligand are shown. **c** Same complex as (b) seen at a 90° cross section. (Reprinted from Ref. [48]. Copyright 1993, J. Am. Chem. Soc.)



peptide bond of the substrate. Since a fullerene, i.e., C60, molecule has approximately the same radius as the cylinder that describes the active site of the HIVP and more importantly, C60 is also primarily hydrophobic, Keyon and coworkers [48], thus conjectured the existence of a strong hydrophobic interaction between the C60 derivative and active site surfaces. This interaction should make C60 and derivatives potential inhibitors of the HIVP—a positive outcome of C60 nanotoxicity to HIV.

In their pioneering work, Keyon and coworkers [48] modeled the complexes of C60 binding with HIVP via a molecular docking program DOCK3 [49]. To test the hypothesis regarding the complementarity of the C60 with the HIVP active site (PDB 3hvp.pdb), a model of C60 was created and minimized using the SYBYL package (Version 5.4, Tripos Associates, Inc.). Atomic point charges were calculated using the Gasteiger-Huckel method. Docking to the active site of the studied

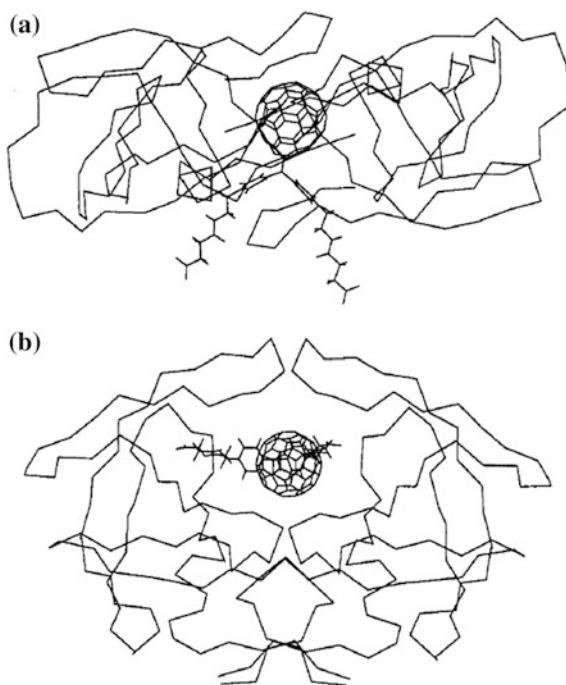
protein was done using the program DOCK3 [49]. Grids required by DOCK3 were generated against the protein HIVP using the standard AMBER united atom charges and van der Waals parameters [48]. DOCK3 finds optimal orientations of a ligand with its receptor, scoring on the basis of van der Waals contacts and complementary electrostatics. This procedure produced complexes with the C60 centered squarely at the active site, forming good van der Waals contacts with the active site surface, thereby reinforcing the hypothesis. Figure 2.1b, c display the highest scoring complex of C60 binding with HIVP in the “front” and “side” views, which show the contacts of the van der Waals surfaces.

In order to approximate the maximum magnitude of the hydrophobic interactions, the change in solvent-exposed surface area upon binding was determined, which indicates that 298 Å² of primarily hydrophobic surface is removed from solvent exposure upon C60 binding [48]. The summation of individual surface elements according to atom type reveals that the large majority (92 %) of the desolvated surface is due to C60-carbon/HIVP-carbon atom contact. The small amount of oxygen desolvation (7 %) is due primarily to the partial blockage of the catalytic aspartates. Using the figure of 69.2 cal/(mol-Å²) recently shown to accurately describe the free energy released upon desolvation of hydrophobic molecular surface, Kenyon and coworkers estimated the resultant free energy gained upon C60 binding to be ~19 kcal/mol. However, this value has to be corrected for the free energy cost due to the loss of translational/rotational entropy that accompanies C60 binding, which has been estimated to be on the order of 7–11 kcal/mol [50]. After taking this energetic cost into account, the result is a net ΔG_{bind} of 8–12 kcal/mol. Converting this to the dissociation constant K_d values using the standard expression

$$\Delta G^0 = -RT \ln K_d \quad (2.1)$$

results in a dissociation constant on the order of 10⁻⁶–10⁻⁹ M. In order to validate these *in silico* predictions, the authors then synthesized a water-soluble C60 derivative, bis(phenethylamino-succinate) C60 (Fig. 2.2), which is relatively synthetically accessible [48]. The highest scoring DOCK3 complex of this compound with the HIVP again positions the core C60 in the center of the active site, with the charged side chains extending through the mouth of the active site into solution (Fig. 2.2). The ability of this compound to inhibit the HIVP (i.e., nanotoxic to HIVP) was assayed with an HPLC method and its K_i value was found to be 5.3 ± 0.98 μM, which agrees reasonably well with the prediction. It is of interest to note that the C60 derivative has been found to inhibit acutely and chronically HIV-1 infected human peripheral blood mononuclear cells (PBMC) with an EC₅₀ of 7 μM while showing noncytotoxicity in uninfected PBMC [48]. This introductory example demonstrates the potential for C60-based inhibitors of the HIVP. As a point of comparison, the best peptide-based inhibitors are effective in the subnanomolar range and the best nonpeptide inhibitors are effective in the high nanomolar range [51, 52].

Fig. 2.2 Top DOCK3 complex of bis(phenethylamino-succinate) C60 in **a** “front” and **b** “top” views. (Reprinted from Ref. [48]. Copyright 1993, J. Am. Chem. Soc.)



In summary, C60 fullerene and derivatives (bis(phenethylamino-succinate)C60) are found to interact with the active site of HIV-1 protease and inhibit its function (i.e., nanotoxic to HIVP). Molecular docking and simple physicochemical analysis suggest that C60 derivatives can fit snugly into the active site and remove a significant amount of primarily nonpolar surface from solvent exposure, which drives the ligand/protein association. These predictions are then validated in experimental binding assays, showing bis(phenethylamino-succinate) C60 as an effective HIVP inhibitor.

2.3 Fullerene Induced Antibody and Related Interaction

Recent biochemical and structural studies reveal the existence of certain natural proteins that can recognize specific nanoparticles [53, 54] including an antibody that was selected from the mouse immune repertoire to specifically recognize derived C60 fullerenes [53, 54], with an impressive binding affinity of 25 nM [54]. The crystal structure of the Fab fragment of this antibody has been determined [54]. Although the C60–antibody complex structure was not available, the C60 binding site was speculated to be at the interface of the antibody variable domain light and heavy chains (denoted as VL and VH in the following) lined with a cluster of shape-complementary hydrophobic amino acid residues [54]. To understand the detailed interactions

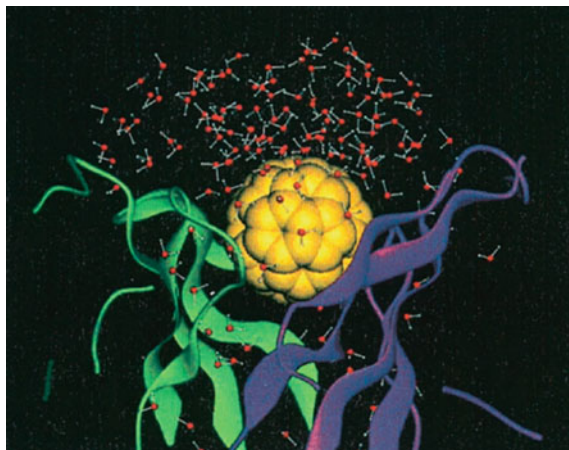


Fig. 2.3 The molecular dynamics simulation system with the stochastic boundary condition. It contains 106 protein residues (*ribbon*), the C60 (spacefilling model in *yellow*), and 166 water molecules (*ball-and-stick*). (Reprinted from Ref. [55]. Copyright 2002, Proceedings of National Academy of Sciences USA)

between the fullerene and the antibody, Ma and coworkers had studied the C60–antibody complex (Fig. 2.3) using molecular dynamics simulations [55].

The initial coordinates of the antibody were taken from the Protein Data Bank (1EMT.pdb) and the fullerene was docked into the suggested binding site [55]. In order to reduce the computer simulation resource requirement, Ma and coworkers [55] used the stochastic boundary molecular dynamics (SBMD) method, which is a more efficient method for simulating the localized interactions near the (active) site of interest in a large complex such as a protein. The CHARMM program was used for the simulation [55] with a polar-hydrogen potential function (PARAM19) and a modified TIP3P water model [56]. Atomic partial charges for the fullerene C atoms were set to neutral and the van der Waals parameters were the same as an aromatic carbon atom in the CHARMM force field [57]. The system in the SBMD simulations was typically separated into a reaction zone and a reservoir region with the reaction zone further divided into a reaction region and a buffer region [55]. In this case, the authors set the reference point for partitioning the system in SBMD as the center of C60. The reaction region around the active site was a sphere of radius r of 14 Å, a buffer region of $14 \text{ Å} < r < 16 \text{ Å}$, and a reservoir region of $r > 16 \text{ Å}$ with all atoms in the reservoir region deleted. The simulation system, shown in Fig. 2.3, consisted of 106 protein residues, a fullerene C60, and 166 water molecules. Atoms inside the reaction region were propagated by molecular dynamics, whereas atoms in the buffer region were propagated by the Langevin dynamics with position restraints [55].

During the 5-ns simulation, the authors [55] observed that a single C60 molecule can be readily accommodated in the suggested binding site of the antibody. The ball inside the binding site undergoes a small relative translational motion, but a

significant rotational motion. However, further analysis of the angular momentum reveals no favored axis of rotation. Since the ball is nearly rigid, its deformational motion is negligible. About 17 % of the surface of the ball is exposed to solvent throughout the simulation, with the antibody covering the remaining surface (Fig. 2.3) [55]. The C60 binding site is surrounded predominantly by hydrophobic amino acid side chains. Of particular interest is the presence of rich π -interactions between the fullerene and the aromatic side chains of the antibody. Phe-96 (VL), Tyr-49 (VL), and Tyr-91 (VL) residues form π - π stacking arrangements with the ball. A three-tiered π -stacking interaction is observed between the ball, Tyr-52 (VH), and Trp-33 (VH). Another interesting interaction arises from the motion of the side chains of Trp-47 (VH) and Arg-50 (VH)—a rotation of the side chain of Trp-47 (VH) results in a triple π -stacking between the ball, the guanidinium group of Arg-50 (VH), and the side chain of Trp-47 (VH) [55].

Besides the π -stacking interactions, the complementary shape of the antibody pocket also has important impact in the recognition. The interface between the ball and the antibody binding pocket is nearly seamless and completely desolvated. Several hydrogen-bonded side chains and van der Waals contacts contribute to the formation of the complementary mostly hydrophobic pocket. For examples, Trp-103 (VH) lies at the ball-antibody interface, but is not oriented in a manner expected for π -stacking. Instead, it is hydrogen bonded to another interfacial residue, Tyr-36 (VL). This hydrogen bonding network extends to residues Asn-34 (VL) and Gln-89 (VL), both of which make contacts with the ball. Other residues that are in van der Waals contacts with the ball are hydrophobic residues Leu-46 (VL), Ala-97 (VH), and Ala-101 (VH), which further helps the binding through hydrophobic interactions.

In short, these simulation results show that the high binding affinity and specificity are achieved through complementary shape and extensive side chain interactions, including a set of rich π -stacking interactions. This finding also suggests that π -stacking is a very efficient and common mode for biological recognition of π -electron-rich carbon nanoparticles. Notably, in addition to tight binding, there is about 17 % of the surface of the fullerene persistently exposed to the solvent, leaving enough room for further manipulation of biocompatible functionalization which is an important attribute. Finally, the simulation results demonstrate that, in general, an ordinary protein binding site, such as that of an antibody, can readily bind to a carbon nanoparticle with high affinity and specificity through recognition modes commonly found in protein-ligand recognition.

2.4 Antitumor Nanomedicinal Effect of Gd@C₈₂(OH)₂₂

As mentioned above, Zhao and coworkers have recently shown that Gd@C₈₂(OH)₂₂ has antitumoral efficacy—namely Gd@C₈₂(OH)₂₂ has equivalent or better performance in inhibiting cancer growth and migration with smaller dose than conventional drugs like cyclophosphamide (CTX) and cisplatin (CDDP) [58].

Regarding its anti-cancer mechanism, subsequent studies showed that $\text{Gd@C}_{82}(\text{OH})_{22}$ is not directly involved in “killing” the cancer cells, but rather “quarantining” the cancer cells [20]. $\text{Gd@C}_{82}(\text{OH})_{22}$ is found to inhibit enzyme metalloproteinase-9 and -2 (MMP-9/MMP-2) which are needed to degrade tumor extracellular matrix (ECM) in metastasis [20]—a positive outcome of nanotoxicity $\text{Gd@C}_{82}(\text{OH})_{22}$ targeting tumor cells. Moreover, $\text{Gd@C}_{82}(\text{OH})_{22}$ was found to downregulate more than 10 different pro-angiogenic factors [59], which further supports the indirect role of $\text{Gd@C}_{82}(\text{OH})_{22}$ in anti-cancer mechanism that the metallofullereneol is able to effectively defoliate cancer tissue by restricting angiogenesis and isolating the tumor in a local environment enclosed by ECM.

In all the following subsections regarding the modeling of $\text{Gd@C}_{82}(\text{OH})_{22}$ -protein interactions, the force field parameters of $\text{Gd@C}_{82}(\text{OH})_{22}$ were developed based on the first principle quantum mechanics calculations (more details in Sect. 2.7). For the water-solvated proteins, the CHARMM22 protein force field [57, 60] with TIP3P [56] water model were used, unless otherwise explicitly stated. The long-ranged electrostatic interactions were enumerated by the particle-mesh Ewald (PME) method [61], while the relatively short-ranged van der Waals interactions were treated with a smooth cutoff of 12 Å. Periodic boundary conditions were applied for all three directions. All molecular dynamics simulations were carried out with the NAMD2 software package [62] optimized on an IBM Blue Gene supercomputer [63].

2.4.1 *Inhibitory Mechanism of $\text{Gd@C}_{82}(\text{OH})_{22}$ on MMP-9*

With human pancreatic cancer model xenografted in mice, $\text{Gd@C}_{82}(\text{OH})_{22}$ has been shown to effectively restrict tumor growth by over $\sim 50.1\%$ compared to the saline control, accompanied with a clear decrease of microvessel density on the tumor surface [20]. The anti-angiogenic activity of $\text{Gd@C}_{82}(\text{OH})_{22}$ was then investigated in more detail with in vivo cellular and in vitro biochemical assays for representative pro-angiogenic enzymes to demonstrate its capability of downregulating MMP-2/-9 in both mRNA expression and enzyme activity levels with more dramatic changes for MMP-9, while the control with $\text{C}_{60}(\text{OH})_{22}$ showing much less effect on the enzyme activity.

Under normal physiological condition with tight regulation, MMPs play a seminal role in tissue remodeling and wound healing [64]. However, once recruited in the tumorigenic environment, MMPs tend to be overexpressed, and draw a mass of blood microvessels, often resulting in rough surface morphologies by irregularly and massively formed vessels [65]. Along the cancer prognosis, MMPs are also involved in degrading the ECM layer, which is a critical step for cancer cells to escape from their local domain to migrate to other tissues. To gain a deeper understanding, Zhou and coworkers [20] then used all-atom molecular dynamics simulations to study the detailed interactions between MMP-9 and $\text{Gd@C}_{82}(\text{OH})_{22}$, $\text{C}_{82}(\text{OH})_{22}$ (control-1) or $\text{C}_{60}(\text{OH})_{22}$ (control-2).

Experiments using synchrotron radiation small angle X-ray scattering (SR-SAXS) and atomic force microscopy (AFM) [58] have demonstrated that $\text{Gd@C}_{82}(\text{OH})_{22}$ nanoparticles often aggregate to a sphere with ~ 22 -nm diameter, which is further confirmed in molecular dynamics simulations showing $\text{Gd@C}_{82}(\text{OH})_{22}$ molecules tend to cluster together before and/or after their binding on MMP-9 [20]. This indicates that even though the solubility has been significantly enhanced by the extensive hydroxylation (i.e., 22 OH groups), $\text{Gd@C}_{82}(\text{OH})_{22}$ is still largely hydrophobic. Figure 2.4 shows one representative trajectory demonstrating that $\text{Gd@C}_{82}(\text{OH})_{22}$ are indeed clustered before and after they interact with MMP-9.

Regardless of the types of nanoparticles $\text{Gd@C}_{82}(\text{OH})_{22}$, $\text{C}_{82}(\text{OH})_{22}$ or $\text{C}_{60}(\text{OH})_{22}$, the root-mean square deviation (RMSD) of MMP-9 fluctuated between 1 and 3 Å, implying there is no noticeable impact from the nanomaterial on the

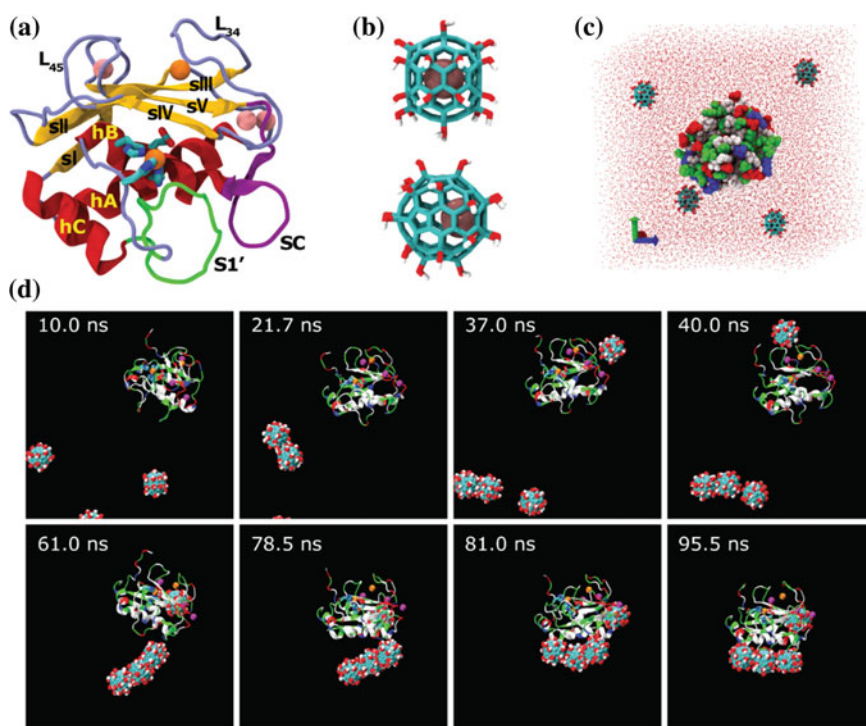


Fig. 2.4 Molecular dynamics simulations for MMP-9 and $\text{Gd@C}_{82}(\text{OH})_{22}$. **a** X-ray crystal structure of the catalytic domain of MMP-9: two Zn^{2+} and three Ca^{2+} are depicted with orange and pink balls, respectively. **b** Endohedral metallofullerenol $\text{Gd@C}_{82}(\text{OH})_{22}$, where Gd atom is marked in pink inside of $\text{C}_{82}(\text{OH})_{22}$ cage. **c** Molecular dynamics setup, where MMP-9, surrounded by four $\text{Gd@C}_{82}(\text{OH})_{22}$, is solvated with about 22,000 water molecules. **d** Temporal snapshots of $\text{Gd@C}_{82}(\text{OH})_{22}$ binding onto MMP-9. The metallofullerenol $\text{Gd@C}_{82}(\text{OH})_{22}$ is clustered before binding to MMP-9 on the hydrophobic patch near the ligand specificity loop S1'. MMP-9 is colored according to residue types: blue, red, green and white for positively charged, negatively charge, polar and nonpolar residues, respectively

global structure of MMP-9 enzyme [20]. This is in contrast with the often significant structural deformations in proteins when interacting with other carbon-based nanomaterial such as CNTs and graphene [19, 34, 37] (see the following Chaps. 3 and 4). The authors [20] then calculated the residue-specific contact probability for each fullerene derivative with the following equation,

$$p(r) = \frac{\sum_{l=1}^n \sum_{t=1}^{N_l} \sum_{m=1}^4 \delta_t^l(m, r)}{\sum_{l=1}^n \sum_{t=1}^{N_l} \sum_{m=1}^4 1}. \quad (2.2)$$

The contacting probability $p(r)$ in Eq. 2.2 of a residue r is obtained by counting the contacts $\delta_t^l(m, r)$ between the residue r and each nanoparticle m over all available simulation frames (N_l) and trajectories (n), where $\delta_t^l(m, r)$ is 1 when any heavy atom pairs between residue r and nanoparticle m is less than or equal to 5 Å at time t and trajectory l , otherwise 0. Figure 2.5 shows the residue-specific contact profiles for Gd@C₈₂(OH)₂₂, C₈₂(OH)₂₂, and C₆₀(OH)₂₂ with respective protein surfaces colored according to contact probabilities.

The distributions and frequencies of contacting residues are significantly different among these fullerene derivatives. Gd@C₈₂(OH)₂₂ is clearly distinguished from the other two fullerenols, with a strong preference in “attacking” at the lower rim of MMP-9 near the S1' loop [20] (Fig. 2.5). On the other hand, both C₈₂(OH)₂₂ and C₆₀(OH)₂₂ tend to contact with MMP-9 in broader area including the upper and lower rims of MMP-9. The S1' loop, also known as the ligand specificity loop, has long been recognized as a characteristic region in the catalytic domain due to its functional role in ligand recognition. Thus, Gd@C₈₂(OH)₂₂ could indirectly inhibit MMP-9 by allosterically modulating the ligand recognition at the ligand specificity S1' loop, in addition to partially blocking the enzymatic site of MMP-9, enhancing the target specificity in developing MMP inhibitors (MMPi's) [66] which have been recognized as critical targets for antimetastatic therapeutics [64, 67].

To gain further insight into the binding mechanism, Zhou and coworkers [20] calculated the potential of mean force (PMF) for nanoparticle binding on MMP-9 (Fig. 2.5). The binding-free energy landscape was constructed by the histogramming method [34, 68] as defined below,

$$W(x_{\text{area}}, y_{\text{dist}}) = -RT \ln p(x_{\text{area}}, y_{\text{dist}}). \quad (2.3)$$

The probability $p(x_{\text{area}}, y_{\text{dist}})$ was obtained by counting the events in a bin ($x_{\text{area}}, y_{\text{dist}}$) along with two reaction coordinates, where x_{area} and y_{dist} represent the minimum distances between the nanoparticle and catalytic Zn²⁺ ion of MMP-9, and contact area between the nanoparticle and MMP-9, respectively. The binding-free energy surface clarifies the characteristics of the binding modes found in the contact analysis. While the broadly distributed binding of C₈₂(OH)₂₂ and C₆₀(OH)₂₂ are evidenced in the multiple binding energy basins, the strong and specific binding of Gd@C₈₂(OH)₂₂ near the ligand specificity S1' loop is shown in the single and deep free energy basin, which is ~0.3–0.4 kcal/mol stronger than those of C₈₂(OH)₂₂ and C₆₀(OH)₂₂.

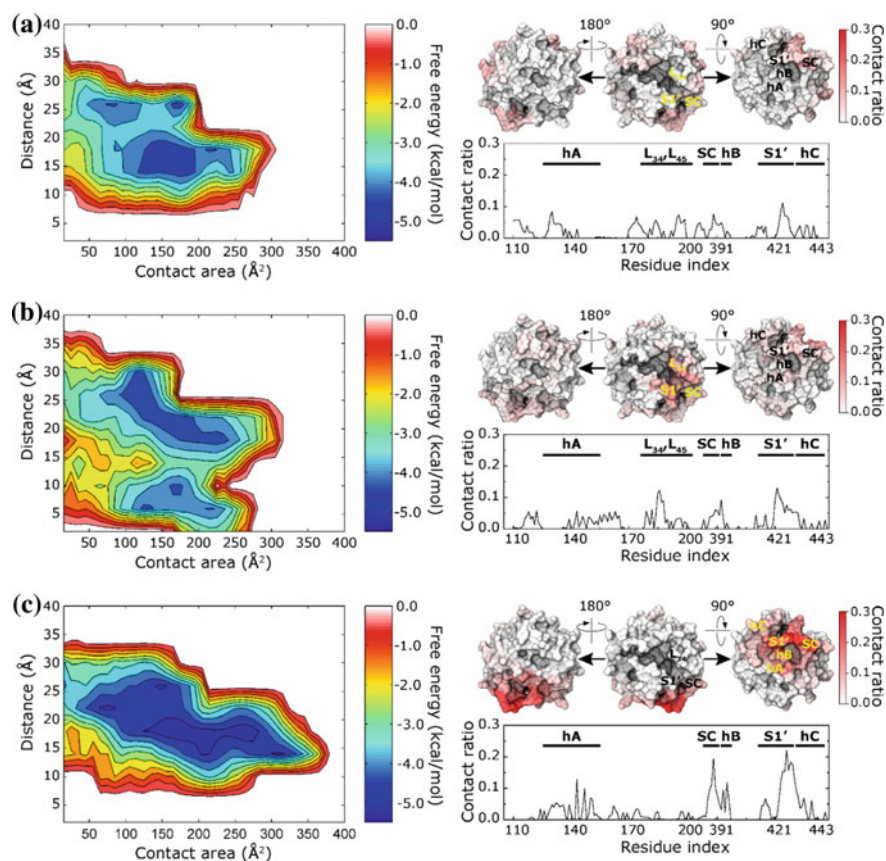


Fig. 2.5 Binding-free energy landscapes and residue-specific contacts on MMP-9 **a** and **b** Binding-free energy surfaces for fullerene $\text{C}_{60}(\text{OH})_{22}$ (**a**) and $\text{C}_{82}(\text{OH})_{22}$ (**b**) on MMP-9 shows a nonspecific binding mode (*left*) and almost all surface residues of MMP-9 contributes in contact with fullerene derivatives. **c** Metallofullerene $\text{Gd}@\text{C}_{82}(\text{OH})_{22}$ has a specific binding mode with MMP-9 (*left*) and interacts with the ligand specificity S1' loop and SC loop (*right*). Residue was assigned to be in a contact when any atom in the residue is within 5.0 Å of any atom of fullerene derivatives

$\text{Gd}@\text{C}_{82}(\text{OH})_{22}$ binding could be characterized with three distinctive phases depending on the major interactions with MMP-9 [20]: (1) nonspecific long-range electrostatic interaction, (2) nonspecific hydrophobic interaction, and (3) a more specific combined electrostatic and hydrophobic interaction, as illustrated by one typical trajectory in Fig. 2.6. Interestingly, isoelectric surface shows that a strong negative field is formed around the Zn-coordinated catalytic site of MMP-9, whereas neutral or positive fields are stretched out near the S1' loop. Given the negatively charged fullerene cage $[\text{C}_{82}(\text{OH})_{22}]^{3-}$ induced by the encaged Gd^{3+} , $\text{Gd}@\text{C}_{82}(\text{OH})_{22}$, in a long distance, tends to interact with positively charged surface residues (e.g., K433 and R440) rather than to approach the active site of the strong

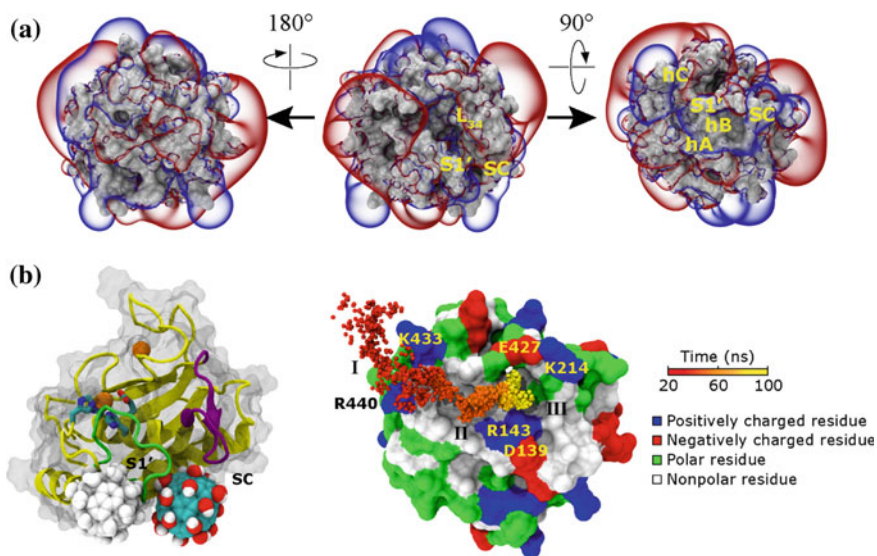


Fig. 2.6 Electrostatic potential map and representative inhibitory pathway. **a** While a large negative potentials prevails around the active site of MMP-9 (*center*), a hydrophobic patch with positive hot spots is stretched out over the area encompassing the ligand specificity S1' and SC loops (*right*). The electrostatic potential was computed by using APBS 1.3 tool [69]. *Blue* and *red* lobes represent isoelectric potential surfaces of +0.5 and -0.5 kT, respectively. **b** *Left* A representative binding mode shows that Gd@C₈₂(OH)₂₂ (a *solid ball*) binds between the S1' ligand specificity loop (*green ribbon*) and the SC loop (*purple ribbon*) leading to the ligand binding groove. Alternatively, Gd@C₈₂(OH)₂₂ (a *gray ball*) can bind at the back entrance of the S1' cavity leading into the active site (ball-and-stick for active sites and orange ball for the catalytic Zn²⁺). *Right* A possible binding pathway: depending on major driving forces, Gd@C₈₂(OH)₂₂ binds on MMP-9 along with three different phases. Phase-I a diffusion-controlled nonspecific electrostatic interaction, Phase-II a transient nonspecific hydrophobic interaction and Phase-III a specific hydrophobic and hydrogen-bonded stable binding

negative electrostatic shield. However, when Gd@C₈₂(OH)₂₂ is driven near the surface, it readily moves to a nearby hydrophobic channel with mostly short-ranged nonspecific hydrophobic interactions albeit transient [20]. As mentioned above, because Gd@C₈₂(OH)₂₂ is largely hydrophobic, the interaction with hydrophobic residues would be energetically beneficial due to the hydrophobic interaction. Once the hydrophobic channel is crossed, Gd@C₈₂(OH)₂₂ ends up in between the S1' loop and SC loop with a large contact area (Fig. 2.6b), resulting in a position more ideal and well-balanced among the hydrophobic interactions (e.g., L212, F396, L397, P429, and P430), π -cation interactions (e.g., R143 and K214), and hydrogen bonds (e.g., T426, E427). Thus, both the thermodynamics (free energy landscape) and kinetics analyses confirm that Gd@C₈₂(OH)₂₂ can form a more specific and energetically more favorable binding at the ligand specificity S1' loop.

2.4.2 Mechanical Impact of $\text{Gd@C}_{82}(\text{OH})_{22}$ on Collagen Complexes

In this subsection, we will turn our focus to the extracellular matrix (ECM), and illustrate how the nanoparticle could affect the structural integrity of collagen complex, a major constituent of the extracellular matrix [70, 71] (as well as the proteolytic target for MMP-2/MMP-9). In vivo tests with mice showed that $\text{Gd@C}_{82}(\text{OH})_{22}$ treatment thickens the fibrous ECM layer [58, 72]. The collagen content is known to decrease as the tumor advances [73]. Hence, the phenomenal antimetastatic activity with $\text{Gd@C}_{82}(\text{OH})_{22}$ is believed to be closely related to the enhanced resistance to ECM degradation. Although the ECM degradation is mainly lowered by $\text{Gd@C}_{82}(\text{OH})_{22}$ inhibition to MMP, recent studies also show that $\text{Gd@C}_{82}(\text{OH})_{22}$ can directly interact with collagen as well [72], where the mechanical properties (e.g., density and stiffness) are remarkably changed. Thus, it may provide another aspect for better understanding of how $\text{Gd@C}_{82}(\text{OH})_{22}$ plays as an antimetastatic nanomedicine. Li and coworkers approached this collagen- $\text{Gd@C}_{82}(\text{OH})_{22}$ interaction problem with two levels of structural complexity: (1) a single collagen triplex and (2) a tetramer of collagen triplexes.

With regard to a single collagen triplex, the simulation was initiated with 14 $\text{Gd@C}_{82}(\text{OH})_{22}$ molecules distributed over water solvent without contacting the collagen. Overall, the $\text{Gd@C}_{82}(\text{OH})_{22}$ molecules were frequently interacting with the collagen triplex at various different sites, as accompanied with intermolecular aggregation among themselves (Fig. 2.7). A more detailed analysis revealed that $\text{Gd@C}_{82}(\text{OH})_{22}$ has preferential bindings from N-terminus to the middle, followed by C-terminus (i.e., on average up to 4 and 2 in the N-terminus and the middle, respectively, while only 1 in the C-terminus). The nanoparticle adsorption contributed structural integrity of collagen triplex. Compared to the control without any nanoparticle, the residue-specific analysis shows that (1) $\text{Gd@C}_{82}(\text{OH})_{22}$ can sustain the triplex closer to its crystal structure especially at the N- and C-termini and (2) also suppresses thermal fluctuations in both ends, with more prominent effect on the C-terminus.

The site-preference of adsorption observed here is due to the multiple different energy sources between the collagen triplex and $\text{Gd@C}_{82}(\text{OH})_{22}$. The long-range electrostatic interaction may play a major role in determining the initial contact rate along the axis of collagen triplex. That is, the N-terminal contact can be intensified by favorable long-range electrostatic interaction between the positively charged N-termini from the three chains and the negative fullerene cage of $\text{Gd@C}_{82}(\text{OH})_{22}$. However, once $\text{Gd@C}_{82}(\text{OH})_{22}$ begins to contact with the surface residues in close distance, it can afford to utilize diverse short-ranged interactions such as hydrophobic interaction and hydrogen bonds. As such, even though the number of $\text{Gd@C}_{82}(\text{OH})_{22}$ adsorbed at the C-terminus is relatively small, this nanoparticle may act as an effective structural stabilizer through stable short-range interactions once attached.

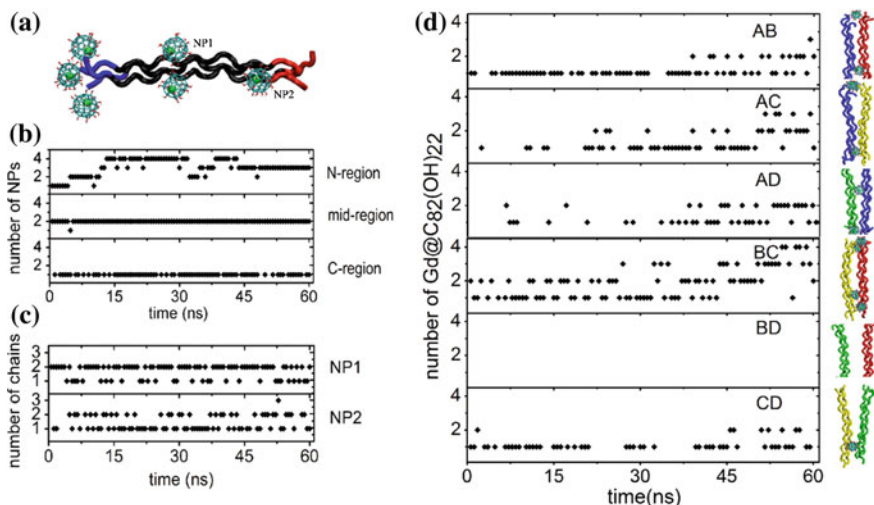


Fig. 2.7 Gd@C₈₂(OH)₂₂ binding dynamics on collagen complex. **a** Representative snapshot of collagen triplex bound by Gd@C₈₂(OH)₂₂ ($t = 50$ ns). The N-terminal, middle and C-terminal regions of collagen triplex is colored in blue, black and red, respectively. **b** Number of adsorbed Gd@C₈₂(OH)₂₂ molecules in each region of a collagen triplex. **c** Number of collagen peptide chains with which the representative Gd@C₈₂(OH)₂₂ molecule is interact. The adsorbed Gd@C₈₂(OH)₂₂ can simultaneously interact with two peptide chains, acting as the “nanoparticle-mediated bridge” between two chains. **d** The number of Gd@C₈₂(OH)₂₂ simultaneously contact with collagen triplex pairs: A–B, A–C, A–D, B–C, B–D, and C–D; together with corresponding snapshots at $t = 60$ ns (on the right side)

Structurally, Gd@C₈₂(OH)₂₂ was shown to enhance the interchain hydrogen bonding probabilities, especially at the both terminal regions. For example, an average of 0.8 and 1.4 hydrogen bonds exist at the N- and C-terminal regions of the collagen triplex, respectively, in the absence of Gd@C₈₂(OH)₂₂, while this number increases to 0.9 and 1.4, respectively, in the presence of Gd@C₈₂(OH)₂₂. This implies that the reduced thermal fluctuations with Gd@C₈₂(OH)₂₂ might facilitate the hydrogen bonding formation. In addition, simulations often showed that Gd@C₈₂(OH)₂₂ not only interacts with each individual chain of the triplex, but also with multiple chains simultaneously (Fig. 2.7). The interchain hydrogen bonds are well-known for retaining the collagen triplex structure [74–79]. In this regard, a so called “nanoparticle-mediated bridge” was proposed as a plausible mechanism to explain how Gd@C₈₂(OH)₂₂ contributes to the structural stability of the collagen complex (Fig. 2.7). Indeed, Gd@C₈₂(OH)₂₂ mediates the interchain interactions of collagen triplex by binding in between the collagen monomer chains and holding them tightly through hydrogen bonds.

Gd@C₈₂(OH)₂₂ can also interact with a bundle of collagen triplexes (Fig. 2.7d). In simulation with a complex of four collagen triplexes, the Gd@C₈₂(OH)₂₂

molecules prefer to bind on at least one of the collagen triplexes with high probability, where 19 out of a total of 20 $\text{Gd@C}_{82}(\text{OH})_{22}$ were found to be adsorbed. The nanoparticle adsorption highly enhanced the bundle stability (i.e., $\text{RMSD} \approx 4 \text{ \AA}$), compared to the control simulations with no nanoparticles (i.e., $\text{RMSD} \approx 12 \text{ \AA}$). Similar to the case of a single triplex, $\text{Gd@C}_{82}(\text{OH})_{22}$ were shown to effectively mediate the interactions between the collagen triplexes themselves. Up to four $\text{Gd@C}_{82}(\text{OH})_{22}$ were found in between pairs of collagen triplexes, forming stable hydrogen bonds. This contributes not only to the conformational stabilization of individual triplex, but also to the organized orientation among the collagen triplexes. Interestingly, the ordered arrangement has been validated in CD spectra of the collagen solution. The triple helical structures are characterized with a positive and negative peak at 220 and 197 nm, respectively [80], where both peaks increase with the presence of $\text{Gd@C}_{82}(\text{OH})_{22}$, indicating that $\text{Gd@C}_{82}(\text{OH})_{22}$ enhances a short-ranged oligomer or microfibril formation.

2.5 $\text{Gd@C}_{82}(\text{OH})_{22}$ Inhibition of Protein–Protein Interaction

Two representative protein domains, the WW-domain and SH3 domain, ubiquitous in molecular signaling transduction pathways, are examined in detail in this subsection [29, 33] to illustrate the potential side effect of $\text{Gd@C}_{82}(\text{OH})_{22}$ in interfering protein–protein interactions (ppi) [81–83].

2.5.1 *Direct Inhibition of WW-Domain*

WW-domain exists in regulatory pathways as mediating signal transduction by recognizing the proline-rich motifs (PRMs). Due to its involvement in the epithelial sodium channel function, it has been speculated that WW-domain could be involved in several human diseases such as Alzheimer’s disease [84, 85].

The simulation results of Zhou and coworkers [29] indicate that WW-domain is highly liable to the $\text{Gd@C}_{82}(\text{OH})_{22}$ molecules. $\text{Gd@C}_{82}(\text{OH})_{22}$ not only interacts with the WW-domain, but also directly blocks the putative binding site for the native ligand PRM (Fig. 2.8). It interacts directly with various critical residues, including Y28, K30, H32, Q35, T37, and W39, which are responsible for recognizing the PRM ligand [86–88]. The potential of mean force (PMF) indicated that the binding is quite stable (i.e., $\Delta G = -5.44 \text{ kcal/mol}$, Fig. 2.8c), where the PMF was calculated with Eq. 2.3 using two reaction coordinates: the minimum distance between $\text{Gd@C}_{82}(\text{OH})_{22}$ and two signature residues Y28 and W39, and the contact

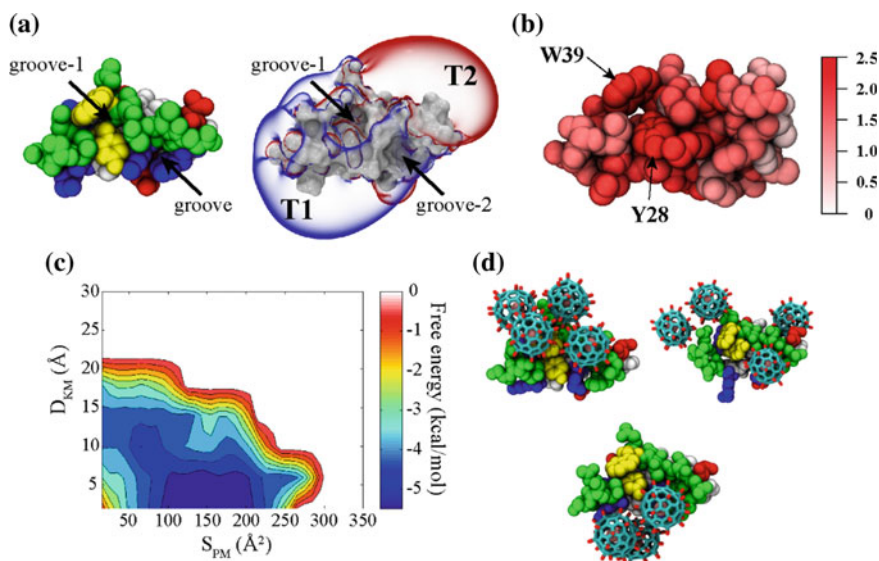


Fig. 2.8 Residue-specific contacts and binding-free energy surface from the binary system of Gd@C₈₂(OH)₂₂ and WW domain. **a** WW-domain is colored according to residue types with Y28 and W38 highlighted in yellow negatively charged, positively charged, polar, and nonpolar residues are colored in red, blue, green and white, respectively. **b** Site-specific contact ratio of WW domain, where residues in PRM binding site have high contact ratio to Gd@C₈₂(OH)₂₂. **c** The binding-free energy surface, where D_{KM} is the minimum distance between Gd@C₈₂(OH)₂₂ and the signature residues (Y28 and W39) of the WW-domain and S_{PM} is the contacting surface area. **d** Representative binding modes found in the global minimum. Yellow key residues, white hydrophobic, green non-charged polar, red negatively charged, and blue positively charged residues

area between Gd@C₈₂(OH)₂₂ and the WW-domain. The representative binding modes (Fig. 2.8d) also demonstrate that the Gd@C₈₂(OH)₂₂ molecules are clustered on the WW-domain, directly blocking the PRM binding grooves. Gd@C₈₂(OH)₂₂ is shown to favorably occupy the PRM binding site of the WW-domain via multiple different interactions, including π - π stacking with the aromatic residues Y28 and W39, π -cation interaction with the positively charged K30, and hydrogen bonds with various nearby protein residues through its surface -OH groups.

To further understand the potential disturbance to the native PRM ligand binding, Zhou and coworkers [29] also studied a ternary binding system containing the WW-domain, Gd@C₈₂(OH)₂₂ and the PRM ligand (i.e., G₁TPPPPYTVG₁₀). The simulation shows that Gd@C₈₂(OH)₂₂ generally wins over the native ligand in almost all highly contacting sites of the WW-domain. Even the PRM itself, especially around the central (P₃)PPPY₇ motif, shows a higher preference to bind to Gd@C₈₂(OH)₂₂, indicating that the native binding between the WW-domain and PRM is very likely disturbed in the presence of this nanoparticle.

2.5.2 Indirect Inhibition of SH3-Domain

Next, the authors [33] explored the interaction between Gd@C₈₂(OH)₂₂ and SH3 domain (Src homology 3), another highly promiscuous protein–protein interaction mediator involved in signaling and regulatory pathways. Similar to WW-domain, Zhou and coworkers [33] investigated the PRM inhibitory action of Gd@C₈₂(OH)₂₂ by configuring a binary binding system containing the SH3 domain and Gd@C₈₂(OH)₂₂, and a ternary complex among the SH3 domain, Gd@C₈₂(OH)₂₂, and a native PRM ligand (P₁PPVPPRR₈).

In the binary system, simulation results again indicate that Gd@C₈₂(OH)₂₂ prefers to interact with the SH3 domain especially around the PRM binding site. Although this is similar to WW-domain, much larger area from the SH3 domain is involved in contacting with Gd@C₈₂(OH)₂₂, including the PRM binding site as well as the RT and n-Src loops (Fig. 2.9). The binding-free energy surface reflects the fact that two binding modes (i.e., on-site and off-site bindings) are populated almost equally in the SH3 domain with the off-site mode being slightly more stable than the on-site one by $\Delta\Delta G \approx -0.04$ kcal/mol (Fig. 2.9c). The PRM binding site of SH3 domain is characterized by linear arrangement of the aromatic and hydrophobic residues (i.e., F141, W169, P183, and Y186) for the PxxP motif of the PRM in the PPII helix. Moreover, the RT loop, composed of a cluster of negatively

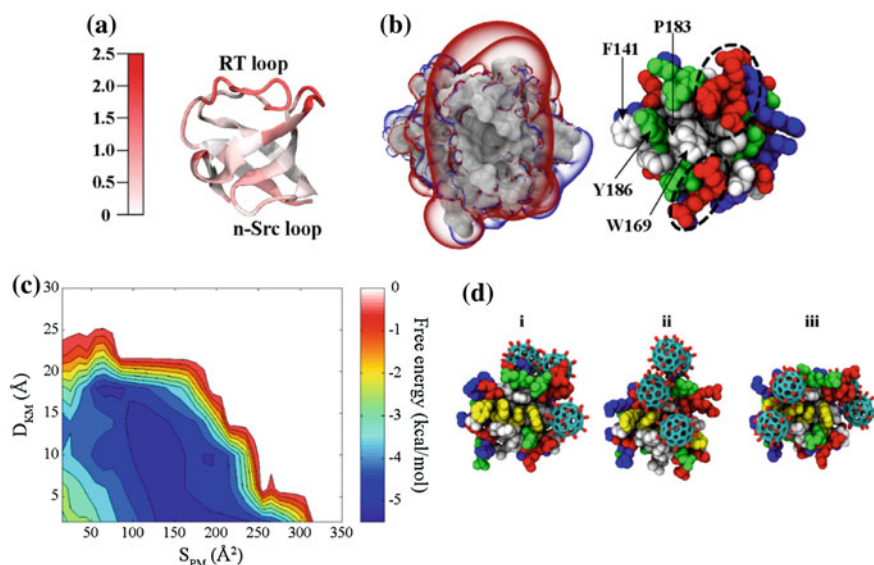


Fig. 2.9 Residue-specific contacts and binding-free energy surface from the binary system of Gd@C₈₂(OH)₂₂ and SH3 domain. **a** Residue-specific contacts of Gd@C₈₂(OH)₂₂ on SH3 domain. **b** Electrostatic potential surface of SH3 domain, where a large negative electrostatic field is formed around the PRM binding site by negative residue clusters at n-Src and RT loops. **c** Binding-free energy landscape in the binary system and **d** Representative structures in the low energy basin

charged residues including D147, E148, E149, and D150, is known to recognize PRM and guide its binding orientation through long-range electrostatic interaction with the C-terminal arginine of PRM [89].

The representative binding modes found in the two energy basins illustrate how the $\text{Gd@C}_{82}(\text{OH})_{22}$ nanoparticles block the PRM binding site directly, or interact with the RT loop thus interfering the binding indirectly. Different from WW-domain, the PRM binding site of the SH3 domain is being shielded with a large negative electrostatic field generated by the acidic residues of the RT and n-Src loops, which makes $\text{Gd@C}_{82}(\text{OH})_{22}$ binding more diffused over the area including the binding site and its surrounding regions. Again, these results indicate that $\text{Gd@C}_{82}(\text{OH})_{22}$ may also interfere the protein–protein mediation by the SH3 domain, even though it could be less serious than the WW-domain, as shown in the dual and more diffused binding modes (Fig. 2.9).

The $\text{Gd@C}_{82}(\text{OH})_{22}$ binding, equilibrated between the on- and off-site bindings in the binary system, has been dramatically shifted toward the off-site mode in the presence of PRM. The representative binding mode found in the energy minima of $\text{Gd@C}_{82}(\text{OH})_{22}$ and the SH3 domain shows that PRM is always being distracted from the putative binding site, indicating a more favored indirect interference in this case [33]. Therefore, $\text{Gd@C}_{82}(\text{OH})_{22}$ may interfere the SH3 domain more by indirectly distracting PRM from the off-sites, rather than directly blocking the native binding site as in the WW-domain case. Overall, both cases implicate that the protein–protein interaction mediated by either WW or SH3 domain might be impaired by the exposure of $\text{Gd@C}_{82}(\text{OH})_{22}$.

2.6 Metallofullerenol $\text{Gd@C}_{82}(\text{OH})_{22}$ Force Field Development

In this subsection, we will describe in detail the force field development of a fullerene derivative, metallofullerenol $\text{Gd@C}_{82}(\text{OH})_{22}$, as an example for this important parametrization step in nanotoxicity modeling. Fullerene derivatives are one of the mostly investigated carbon allotropes for biomedical applications [40, 42]. Their large carbonaceous surfaces are ready for modification with various chemicals to enable the fullerenes as effective nanocarriers for diagnostics and therapeutics [41, 90]. In particular, endohedral metallofullerenes have attracted special attentions for their potential applications as bioimaging agents, where the hollow fullerene cages serve as a rigid template for magnetosensitive heavy metal(s) while facilitating the exterior surface for further chemical modification. Among these heavy metals, gadolinium (Gd) is one of the most extensively studied metals, mainly as contrasting agent for magnetic resonance imaging (MRI) due to its strong paramagnetism at room temperature [91]. Recent studies have shown that Gd^{3+} ion encaged in fullerene with multiple exterior hydroxyl groups (i.e., $\text{Gd@C}_{82}(\text{OH})_x$) could remarkably enhance the proton relaxivity by more than 20 times better than the commercial organometallic Gd-complexes [92]. More interestingly, Zhao and coworkers have

recently shown that $\text{Gd@C}_{82}(\text{OH})_{22}$ also has antitumoral efficacy [58]. It is shown that $\text{Gd@C}_{82}(\text{OH})_{22}$ has equivalent or better performance in inhibiting cancer growth and migration even with much smaller dose than conventional drugs like cyclophosphamide (CTX) and cisplatin (CDDP) [58]. Thus, it is of great interest to model this novel metallofullerenol and its interactions with proteins, which requires a reliable force field.

2.6.1 Force Field Parameterization from Quantum Mechanics

The classical force field of $\text{Gd@C}_{82}(\text{OH})_{22}$ was parameterized by Zhou and coworkers [20] based on first-principal density functional theory (DFT) with the spin-polarized generalized-gradient approximation (GGA) using the Perdew–Burke–Ernzerhof (PBE) exchange–correlation functional [93]. The Slater basis sets were constructed at the level of triple- ζ plus one polarization function. The heavy atoms were treated with the frozen-core approximation (i.e., for $[1\text{S}^2]$ of O and C, and for $[1\text{S}^2-4\text{d}^{10}]$ of Gd, respectively) [94]. The zero-order regular approximation (ZORA) was applied for the scalar relativistic effect [95]. All calculations were performed with the Amsterdam Density Functional (ADF 2010.01) program [96, 97]. Geometry optimization was first performed for $\text{Gd}^{3+}@\text{[C}_{82}(\text{OH})_{22}]^{3-}$ and $\text{C}_{82}(\text{OH})_{22}$ with septet and singlet spin multiplicities for their respective electronic ground states. The geometries were confirmed to be in the energy minima by calculating the vibrational frequencies. The atomic point charges were finally derived by simulating multiple moments (e.g., monopole, dipole, and quadrupole) of corresponding systems in order to avoid strong basis set dependence as found in Mullikan analysis. Dispersion terms for fullerenol and Gd were adapted from corresponding atom types in CHARMM force field, and Ref. [98].

2.6.2 Validation of $\text{Gd@C}_{82}(\text{OH})_{22}$ Force Field Parameters

Although some of the experimental results are available for the electronic properties of Gd@C_{82} [99, 100] or $\text{Gd@C}_{82}(\text{OH})_{22}$ [101, 102] in solid state, no experimental data on multipole moments, such as dipole and quadrupole, of metallofullerenol $\text{Gd@C}_{82}(\text{OH})_{22}$ have been determined yet due to difficulties related to their aggregation in gas phase or aqueous solution phase. The good news is that, for the case of Gd@C_{82} , recent studies have shown that DFT-level quantum mechanical calculations agree well with experimental observations such as the ground state spin multiplicity and optimized geometry of Gd atom in the fullerene cage [103]. Gd atom has been observed in the vicinity of a C–C double bond on the C_2 molecular axis of C_{82} , in contrast to the other Group 3 metallofullerenes M@C_{82} (i.e., $M = \text{La}, \text{Sc}$, etc.) where La and Sc atoms are located near a six-membered ring in the

opposite side of the C–C double bond [100]. This geometry has been successfully predicted in the DFT calculations [103]. This made it possible to accurately predict the ground state spin multiplicity (i.e., $M = 7$) as determined from magnetic studies [104, 105]. Interestingly, Zhou et al. [20] also found in their DFT calculations that Gd atom is stabilized near the C–C double bond rather than the opposite six-membered ring.

In addition to the multiple moment prediction, the atomic partial charges were further evaluated in order to test if the empirical parameters provide accurate energetics. Similar to the protocols used in standard force field developments, such as CHARMM [57, 60], Zhou et al. [20] computed the interaction energy profiles between $\text{Gd@C}_{82}(\text{OH})_{22}$ and a probe water molecule as a function of intermolecular distance using both quantum mechanics (QM) and molecular mechanics (MM). Figure 2.10 shows one representative energy profile for the probe water molecule approaching toward $\text{Gd@C}_{82}(\text{OH})_{22}$ along an arbitrary direction ($\text{Gd@C}_{82}(\text{OH})_{22}$ has a roughly isotropic spherical structure with the 22 OH groups distributed uniformly on the sphere). The direct comparison demonstrates that the MM partial charges for the metallofullerenol $\text{Gd@C}_{82}(\text{OH})_{22}$ do seem to reasonably reproduce the QM energy profile, in particular, the minimum energy value (about -14 kcal/mol) and its position (about 7.1 Å). Profiles along other directions show similar behavior. The authors attribute this good agreement in QM and MM energy profiles to the exceptionally well-delocalized electron density over the fullerenol cage $\text{C}_{82}(\text{OH})_{22}$.

The delocalization of electron density on the fullerenol cage was further validated by re-parameterizing the atomic partial charges with a “wrong” Gd position.

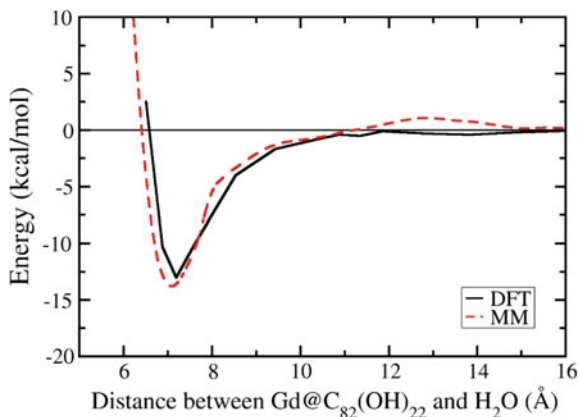


Fig. 2.10 Potential energy profiles between quantum mechanics (DFT) and molecular mechanics (MM) for $\text{Gd@C}_{82}(\text{OH})_{22}$ and a probe water (TIP3P) as a function of intermolecular separation. Our developed empirical force field parameters (i.e., atomic partial charges) for $\text{Gd@C}_{82}(\text{OH})_{22}$ reasonably reproduce the quantum mechanics DFT-based energy profile, in particular, the minimum energy value (about -14 kcal/mol) and its position (about 7.1 Å). (Reprinted from Ref. [20]. Copyright 2012, Proceedings of National Academy of Sciences USA)

Table 2.1 Atomic charge comparison between charge fits parameterized at two different Gd positions of Gd@C₈₂(OH)₂₂

Gd at C–C double bond (C=C)				Gd at six-membered ring (C ₆)				Charge difference	
C=C side		C ₆ side		C=C side		C ₆ side		C=C side	C ₆ side
Atom	$q_{C=C}$	Atom	q_{C_6}	Atom	$q_{C=C}$	Atom	q_{C_6}	$\Delta q_{C=C}$	Δq_{C_6}
C37	−0.193	C29	−0.036	C37	−0.149	C29	−0.098	−0.044	0.062
C46	−0.134	C30	−0.035	C46	−0.082	C30	−0.092	−0.051	0.057
C55	0.367	C53	−0.113	C55	0.350	C53	−0.137	0.017	0.025
C56	0.361	C54	−0.085	C56	0.342	C54	−0.137	0.020	0.052
C27	0.345	C40	0.331	C27	0.348	C40	0.396	−0.003	−0.065
C28	0.352	C41	0.250	C28	0.354	C41	0.400	−0.002	−0.150
Average	0.183		0.052		0.194		0.055	−0.011	−0.003

As mentioned above, the other Group 3 metallofullerenes La@C₈₂ or Sc@C₈₂ have their La/Sc atoms located near a six-membered ring, i.e., in the opposite side of the C–C double bond where Gd sits [100]. Zhou et al. [20] thus located the Gd atom in the opposite of its correct position to be at La/Sc's location, and then redid the charge fitting and compared how the nearby carbon atom charges change with the Gd position. Table 2.1 summarized the partial charges of the six nearest carbon atoms at either the C–C double bond or the six-membered ring site in both cases. A careful comparison shows that the average charge differences (Δq) are only −0.011e and −0.003e at the C–C double bond side and six-membered ring site, respectively. This implies that the adsorbed electrons from the endohedral Gd are fairly well distributed (or delocalized) over the entire fullerene cage C₈₂(OH)₂₂, which fortunately makes the partial charges (force field parameters) relatively insensitive to the motion of the encaged Gd atom as well as external molecules such as waters.

Overall, the MM energy profile seems to reproduce the QM energy profiles reasonably well, which implies additional scaling factors are not necessary for our current empirical atomic partial charges. Subsequent further tests on charge delocalization confirm the reliability of the empirical force field in a slightly perturbed environment such as internal motion of Gd or external approaching water molecules.

2.7 Summary and Future Perspectives

In this chapter, we discussed the interaction between fullerene and its derivatives with various proteins from both nanotoxicology and nanomedicine (i.e., positive outcomes of nanotoxicity targeting HIV or cancer) perspectives. Fullerene C₆₀ and derivatives can be utilized as inhibitors to HIV-1 protease, while they may cause antibody neutralization at the same time. Of particular interest, an endohedral metallofullerenol Gd@C₈₂(OH)₂₂ is found to be effective against tumor metastasis

through inhibiting of MMP-9, an enzyme necessary for the tumor ECM degradation and angiogenesis.

Large-scale molecular dynamics simulations show that $\text{Gd@C}_{82}(\text{OH})_{22}$ displays a strong and specific binding at the ligand specificity S1' loop of MMP-9, thus disabling its function (i.e., nanotoxic to MMP-9). In addition, $\text{Gd@C}_{82}(\text{OH})_{22}$ is also shown to influence the mechanical properties of the collagen complex, a major constituent of ECM layer, where $\text{Gd@C}_{82}(\text{OH})_{22}$ can stabilize a single collagen triplex as well as a bundle of triplexes, effectively mediating their interchain interactions. Both effects contribute to its nanomedicinal roles. On the other hand, from the nanotoxicology perspective, theoretical studies also show that $\text{Gd@C}_{82}(\text{OH})_{22}$ might interact with the ubiquitous WW and SH3 domains, both critical in signal transduction via protein-protein mediation. Despite the differences in detailed mechanisms for the two domains, $\text{Gd@C}_{82}(\text{OH})_{22}$ appears to effectively inhibit their functions in binding with the native PRM peptides.

These findings might have provided new insights and directions in designing novel therapeutic nanomaterials as direct “nanodrugs,” instead of just “nanocarriers” carrying conventional small molecule drugs, in fighting with fatal diseases such as HIV/AIDS and cancer. Further investigations on these fullerene-based novel nanodrugs are highly desired with combined *in silico*, *in vitro* and *in vivo* approaches. Meanwhile, additional studies on their potential nanotoxicity to other proteins, DNA, cell membranes, as well as cells, tissues, and animal models are a must in order for a deeper understanding of the toxicity with underlying molecular mechanisms. We also envision more development efforts on applying fullerene and its derivatives in broader biomedical applications. These studies on fullerene and its derivatives' nanotoxicity should also stimulate and facilitate the cytotoxicity studies of other nanomaterials in this emerging field.

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