ACS Partner Journal

ACCOUNTS
—of materials research—

pubs.acs.org/amrcda

Article

Incorporation of Molecular Nanoparticles Inside Proteins: The Trojan Horse Approach in Theranostics

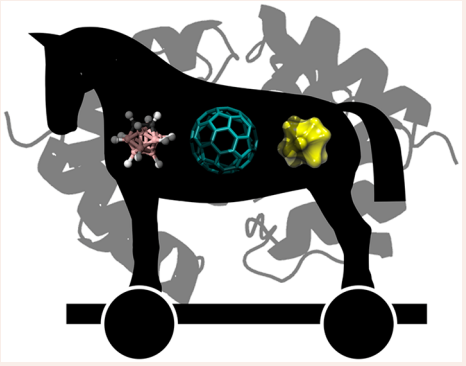
Matteo Di Giosia, Francesco Zerbetto, and Matteo Calvaresi*

Cite This: *Acc. Mater. Res.* 2021, 2, 594–605

Read Online

ACCESS | Metrics & More | Article Recommendations

CONSPECTUS: Molecular nanoparticles, MNPs, characterized by well-defined chemical formulas, structures, and sizes can interact with a variety of proteins. Fullerenes, carboranes, and gold nanoclusters well represent the diversity of MNPs properties available in nanoscience. They can have diameters smaller than 1.5 nm, be hydrophilic or hydrophobic, and can use a paraphernalia of means to establish local and global interactions with the amino acidic residues of proteins. Proteins, endowed as they are with an assortment of pockets, crevices, and gaps are natural supramolecular hosts to incorporate/hide/transport MNPs directly in water with a facile and "green" approach.



This Account identifies and discusses the rules that govern the interactions and binding between MNPs and proteins. Fullerenes are composed solely by carbon atoms arranged to form hollow polyhedra. Hydrophobic interactions occur between aliphatic residues and the fullerene surface. The amino acids most effectively interacting with fullerenes are aromatic residues that establish $\pi-\pi$ stacking interactions with the cage. Amphiphilic and charged residues produce also cation$-\pi$, anion$-\pi$, and surfactant-like interactions with the cages.

Carboranes are composed of boron, carbon, and hydrogen atoms, also arranged to form cages. They are hydrophobic with unusual properties originating from the presence of boron atoms. Hydride-like hydrogens bound to the boron atoms govern carborane chemistry. These negatively charged hydrogens do not participate in classic hydrogen bonding with water and promote hydrophobic interactions with proteins. On the contrary, the electronegativity of these hydrogens drives the formation of unconventional dihydrogen bonds with the acidic hydrogen atoms of positively charged amino acid. Carboranes also establish C$-$H$\cdots\pi$ and B$-$H$\cdots\pi$ interactions with aromatic residues.

Gold nanoclusters, AuNCs, are synthesizable with atomically precise stoichiometry. Amino acid residues with sulfur atoms or with nitrogen-containing heterocycles are the strongest Au binders. The proteins can act as supramolecular hosts but also as templates for the synthesis of AuNCs directly inside the protein core. Of the pristine amino acids, tryptophan, tyrosine, phenylalanine, and aspartic acid are the most efficient reducing groups. In a peptide sequence, the best Au-reducing moieties are obtained by nitrogen-containing residue such as glutamine, asparagine, arginine, and lysine. The investigation of the interactions between AuNCs and proteins therefore adds further complexity with respect to that of fullerenes and carboranes. The selection of the host proteins should consider that they will have to contain active sites for metal ion accumulation and ion reduction where AuNC can form and stabilize. This Account further discusses the hybridization of MNPs with proteins in view of creating innovative multifunctional theranostic platforms where the role of proteins is akin to that of "Trojan Horses" since they can (i) hide the MNPs, (ii) control their cellular uptake, (iii) drive their crossing of physiological barriers, and (iv) ultimately govern their biological fate.

1. INTRODUCTION

The investigation of the interface between proteins and nanoparticles (NPs) is a flourishing area. Proteins are ubiquitous in physiological environments while many NPs are hydrophobic and cannot operate directly in water. The interactions between biosystems and artificial NPs can create new materials and extend to water the range of applications of hydrophobic NPs. Traditionally, investigations of protein$-$NP hybrids focus on the adsorption of proteins on NP surfaces and/or the formation of protein coronas. This Account zooms in and discusses the nanobio interface of NPs incorporated inside protein pockets. In particular, we will concentrate our description on Molecular Nanoparticles (MNPs). We define MNPs as NPs characterized by a well-defined molecular

Received: March 30, 2021
Revised: May 12, 2021
Published: May 25, 2021

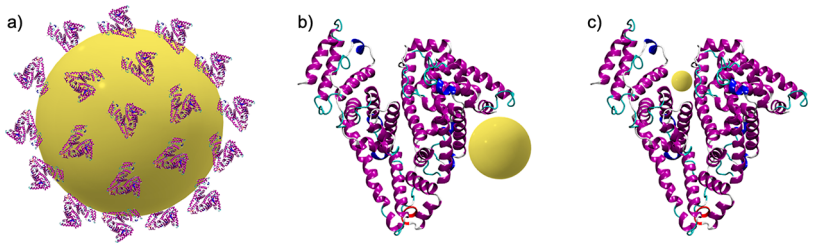


Figure 1. Interactions of proteins (HSA as an example) with (a) a nanoparticle (NP), (b) a ultrasmall nanoparticle (USNP), and (c) a molecular nanoparticle (MNP). Water not shown but implicitly present.

formula and structure. Typical examples of molecular nanoparticles are buckminsterfullerene (C_{60}), closo-carborane ($C_2B_{10}H_{12}$), and gold nanoclusters (i.e., Au_{25}). Their diameters (0.7−1.5 nm) are commensurable with many protein-binding pockets.

When exposed to biological media, all NPs interact with proteins (Figure 1). Size and shape[1,2] determine the curvature of the NP surface and are crucial parameters governing the interaction of the NPs with proteins. To date, studies have been performed identifying and characterizing the proteins interacting with "big" NPs (with diameters from 3 to 200 nm). In this case, proteins adsorb onto the NP surface, forming a stable adsorption layer, often referred to as "biomolecular corona".[3] The corona determines the biological identity of the NPs and influences the associated biological behavior. The interaction of proteins with NPs smaller than 3 nm (ultrasmall nanoparticles, USNPs) can be dramatically different with respect to the model described above. Such interactions were recently studied.[4,5] It was argued that for USNPs a hard and persistent protein corona cannot form because USNPs are of a size comparable to that of the proteins. The limited surface area of USNPs, compared to larger nanoparticles, allows the formation of either transient or stable interactions with only one or two proteins at a time.[6] A multistep mechanistic model for USNP−protein interactions was recently proposed, in analogy with the mechanism of protein−protein interaction.[7,8] The isolated USNP and the protein undergo random diffusion until they form an intermediate encounter complex.[7,8] Encounter complex formation is followed by discrete unimolecular steps of partial desolvation/ion removal and conformational rearrangement, which, together, achieve tightening of the final complex.[7,8] The case of MNPs is even more distinctive because they are smaller than the biomolecules themselves. The interaction of MNPs with proteins more closely resembles a typical biomolecular complexation (ligand−protein complex formation)[9−11] where (i) the protein maintains its tridimensional structure, (ii) the MNPs recognition is highly specific and localized in a specific protein-binding pocket, (iii) the complex is characterized by a well-defined stoichiometry. We will focus in detail on these conditions with representative MNPs in section 2.

1.1. Proteins as Supramolecular Host for MNPs

Proteins must interact with other biomolecules to perform their biological functions. Most biological processes are controlled by molecular recognition, they include the regulation of enzyme catalysis (i.e., protein−substrate binding), signaling pathways (i.e., receptor−ligand recognition), and cellular events essential to life (i.e., assembly of multiprotein complexes). Interacting biomolecules can be other proteins, nucleic acids (DNA, RNA), carbohydrates, and small molecules such as O_2 or CO_2.

Proteins are also ideal supramolecular hosts for MNPs because (i) They are endowed with an assortment of pockets, crevices and gaps able to bind MNPs of different size and shape. We refer to this concept as geometrical complementarity. (ii) The 20 different types of amino acids that form proteins feature recognition elements able to establish many different noncovalent interactions with MNPs such as hydrogen bonding, electrostatic interactions, hydrophobic interactions, van der Waals interactions, and $\pi-\pi$/cation−π/anion−π interactions. Amino acid composition and overall pocket geometry therefore govern the interaction between MNP and proteins. We refer to this concept as chemical complementarity. (iii) The high degree of preorganization of the protein host (due to its 3D structure) lowers the entropic cost for guest binding.

2. PROTEIN−MNP INTERFACE

Fullerenes, carboranes, and gold nanoclusters (AuNCs) well represent the variety of MNPs properties available in nano and materials science. Their interactions with proteins, summarized in Table 1, will be discussed in the following sections.

Table 1. Main Interactions between Proteins and Selected MNPs (Fullerenes, Carboranes, and AuNCs)

	Proteins
Fullerenes	π-π stacking interactions
	van der Waals interactions
	Hydrophobic interactions
	Surfactant-like interactions
	Cation-π interactions
	Anion-π interactions
	Hydrogen bonding
	Electrostatic interactions
Carboranes	van der Waals interactions
	Hydrophobic interactions
	Dihydrogen bonding
	Hydrogen bonding
	C-H ··· π interactions
	B-H ··· π interactions
	Electrostatic interactions
AuNCs	Coordinative bonding
	Electrostatic interactions
	Hydrophobic interactions
	Hydrogen bonding
	Au ··· π interactions

https://doi.org/10.1021/accountsmr.1c00065
Acc. Mater. Res. 2021, 2, 594−605

2.1. Fullerene–Protein Interface

Fullerenes are a family of hydrophobic MNPs composed entirely of carbon atoms arranged in the form of hollow polyhedra.[12] C_{60} and C_{70} are the most common fullerenes, but higher fullerenes such as C_{76}, C_{78}, and C_{84} are also well-known.[12] Metal ions can be trapped in the cages of fullerenes, producing complexes known as endohedral fullerenes, where the metal is permanently screened from interactions with the external environment.[12] Due to the very low solubility of fullerenes in water, various fullerenes derivatives (FDs) were synthesized by chemical functionalization with hydrophilic moieties "attached" to the fullerene cage.[12]

To a first approximation, we can consider pristine fullerenes as simple hydrophobic cages. The presence of a possibly symmetric hydrophobic cavity in a protein, where a fullerene can fit, is considered a necessary prerequisite for fullerene binding.[9]

Thermodynamical analysis of the binding components of the interaction energy between C_{60} and proteins showed that van der Waals interactions play the most important role in the recognition and binding.[13] Hydrophobic interactions, i.e., nonpolar solvation, assist the binding, while polar solvation and entropy are usually detrimental to the binding.[13] The measure of the shape complementarity represents a quick way to estimate the stabilizing van der Waals and hydrophobic interactions between fullerenes and proteins (Figure 2).[9,13−18]

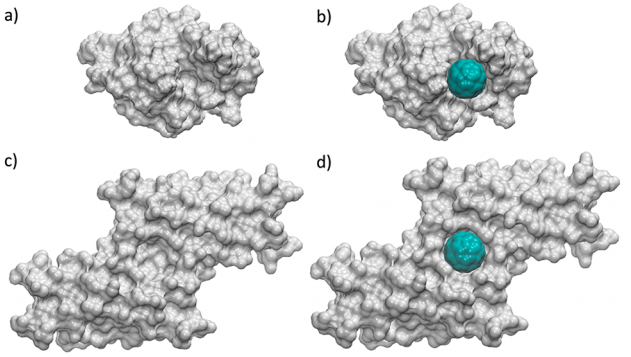


Figure 2. Surface representation of (a) lysozyme, (b) C_{60}@lysozyme, (c) COP (C_{60}-organizing peptide), and (d) C_{60}@COP showing the surface complementarity between the fullerene-binding pocket of the protein and the C_{60} cage.

With the increase of the carbon cage size, the binding strength between proteins and fullerenes increases constantly.[19,20] Then the local interactions between the amino acids and the fullerenes come into play. The amino acids most effectively interacting with fullerenes are aromatic residues (tryptophan, phenylalanine, tyrosine, and histidine) that can establish $\pi-\pi$ stacking interactions with the cage (Figure 3a,b).[9,13,14,18−24] In C_{60}@lysozyme and C_{70}@lysozyme, π-stacking interactions are sandwich-like for Trp62 and T-shape-like for Trp63 (Figure 3a).[9,13,20−22] In the design of peptides/proteins able to recognize C_{60} (i.e., COP, C_{60}-organizing peptide), the $\pi-\pi$ aromatic stacking between C_{60} and tyrosines (Figure 3b) is the driving force in the binding process.[14,25] In fact, C_{60} is wedged between two Tyr residues.

In a reductionistic approach to design a peptidic host for C_{60}, phenylalanines residues were used as recognizing moieties, able to interact with C_{60} through $\pi-\pi$ stacking, while a varying number of glycines was used as spacers, to connect the two

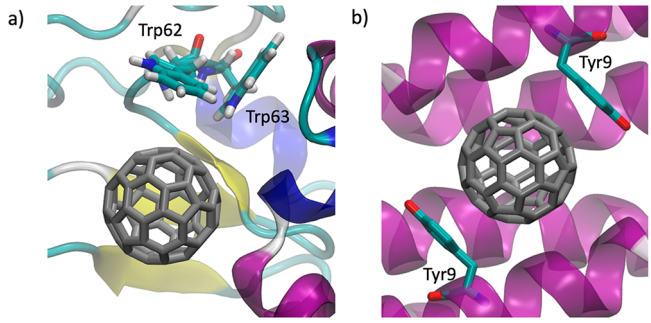


Figure 3. (a) $\pi-\pi$ stacking interactions between Trp62, Trp63, and C_{60} in C60@lysozyme. (b) $\pi-\pi$ stacking interactions between Tyr9 and C_{60} in C_{60}@COP.

terminal phenylalanines.[23] Ultimately, C_{60} was included in a lipophilic cavity and $\pi-\pi$ stacking interactions were formed. The best performance in the dispersion of C_{60} was obtained with a FGGGF peptidic nanotweezer.[23] Besides the $\pi-\pi$ interactions, fullerenes can interact also with charged amino acids via cation$-\pi$ and anion$-\pi$ interactions.[24] In drug–receptor and protein–protein complexes, cation$-\pi$ interactions contribute for 2–5 kcal mol^{-1}. If we consider the extended π-system of the fullerene, it is not surprising that lysine, arginine, asparagine, glutamine, and histidine residues constitute ∼25% of all amino acid residues of the fullerene-binding sites.[24] Of course cation$-\pi$ interactions that involve residues with a net positive charge such as arginine or lysine are much stronger than the neutral side chains of asparagine or glutamine. Electron rich regions of the fullerenes can be also involved in nonstandard hydrogen bonds with amino acid residues, acting as hydrogen bond acceptor with the hydrogen bond donor of the protein.[24] Alternatively, also anion$-\pi$ interactions can be observed in C_{60}@protein complexes. A large number of glutamic acid and aspartic acid residues are observed in fullerene protein-binding sites (∼14% of all residues).[24] The molecular electrostatic potential (MEP) surface of C_{60} (Figure 4) shows highly localized areas of positive potential. These π-holes are the favorite interaction areas for the anion$-\pi$ interactions of negatively charged residue with fullerenes.

Amphiphilic residues such as asparagine, glutamine, serine, threonine, lysine, arginine, glutamic/aspartic acid, insensitive to the sign of the charge, can stabilize the binding of the

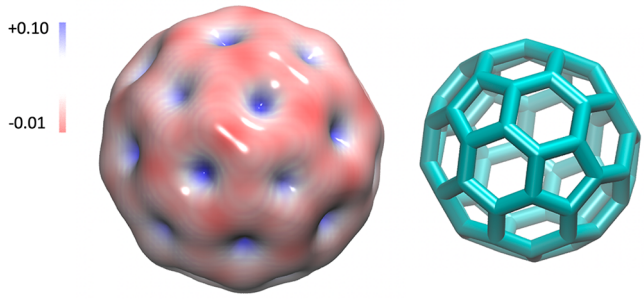


Figure 4. MEP surface of C_{60} at the M06/6-311++G** (isosurface 0.001 au). On the right, the C_{60} molecule is represented in the same orientation used to plot the MEP surface. Electron rich regions (in red) interact with protein residues via cation$-\pi$ interactions and can accept hydrogen bonds. Electron poor regions (π-holes) interact via anion$-\pi$ interactions with negatively charged residue.

https://doi.org/10.1021/accountsmr.1c00065
Acc. Mater. Res. 2021, 2, 594−605

fullerene also with a different mechanism with respect to cation−π or anion−π interactions, i.e., producing surfactant-like interactions (Figure 5a,b).[13,24] In this case, the hydrophobic part of the residue interacts with the fullerene surface, while the hydrophilic part interacts with water. In protein−fullerene complexes, several aliphatic residues usually coat the pocket and contribute to the binding (Figure 5a).[9,13,14] Hydrophobic interactions can be established with these residues, such as methionine, leucine, valine, proline, isoleucine, alanine.[9,13,14]

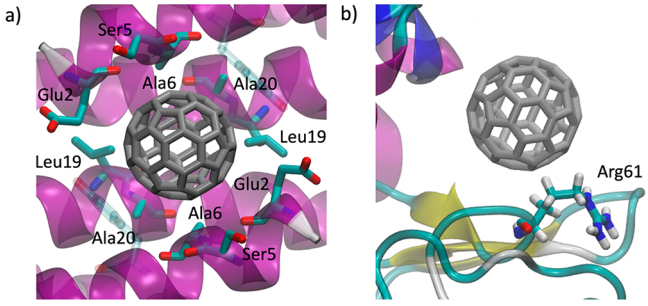


Figure 5. (a) Surfactant-like interactions between Glu2, Ser5, and C_{60} in C_{60}@COP. Aliphatic residues (Ala6, Ile19, Ala20) coat the C_{60}-binding pocket and contribute to the binding. (b) Surfactant-like interactions between Arg61 and C_{60} in C_{60}@lysozyme.

Metallohendohedral fullerenes are an important alternative. In these systems, the intrafullerene electron transfer from the encaged metal atom(s) to the fullerene cage, induces a net charge distribution on the fullerene cage that strongly affects their interaction with proteins. In this case the contribution of electrostatic interactions, which is negligible for pristine fullerenes, becomes important in the binding to a protein.[26,27]

A second alternative are fullerene derivatives (FDs). Functionalization is usually carried out to improve solubility, and upon protein binding, an energy penalty is paid for the desolvation of the hydrophilic functional groups.[19,22] A high degree of chemical functionalization generally reduces the $\pi-\pi$ interactions, while slightly increasing other interactions such as hydrogen bond, results in an overall weaker binding strength.[19,22] A systematic analysis of the interaction between FDs and proteins[18] demonstrated that proteins showing high binding interact more strongly with FDs because of hydrophobic interactions of the proteins with fullerene core. In the crystal structure of COP and fullerene derivatives, the water-solubilizing tris-acid side chain of C_{60} is not visible in the electron density map, due to its mobility, whereas the C_{60} core appears responsible for the packing arrangement. FDs with a small functional group show higher binding compared to FDs with larger (bulky) functional groups[18] that hinder sterically the interactions of the fullerene cage with the binding site.[18] The behavior of FD can be explained by the presence of three concomitant causes: (i) some proteins do not have sufficient space in the cavity to accommodate bulky functional groups attached to the fullerene surface, (ii) there is a reduction of the hydrophobic and $\pi-\pi$ interactions with the protein,[22] (iii) upon FDs binding, desolvation of the hydrophilic functional group entails an energy penalty.

2.2. Carborane−Protein Interface

Carboranes are hydrophobic MNPs composed of boron, carbon, and hydrogen atoms. Carborane structures can be complete (closo-) polyhedra or missing one (nido-), two (arachno-), or more vertices.[28−30] The icosahedral closo-carboranes are particularly stable. Another very important class of carborane derivatives are the dicarbollide anions, $[B_9C_2H_{11}]^{2-}$. These anions form sandwich compounds, with many metal ions, referred to as bis(dicarbollides).[28−30] Due to the increasing interest for carboranes in medicinal chemistry, many carborane derivatives were recently synthesized.[28−30] The nonclassical bonds of boron generate a complex overall electronic structures where carbon and boron atoms are hexacoordinated and the electrons are three-dimensionally delocalized (boron clusters can be described as 3D aromatic compounds).[28,29] The hydrogen atoms bound to boron atoms are hydride-like and have a negative charge. Carboranes show high hydrophobicity since the presence of the hydride-like hydrogens prevents the formation of classic hydrogen bonding with water.[28,29] Maximizing hydrophobic interactions with proteins is crucial for the formation of carborane−protein complexes. The carborane cage occupies a volume similar to that of adamantane and has a diameter of about 5.5 Å, which is just a little larger than that of a rotating phenyl group (ca. 4.7 Å).[28,29]

In the binding with proteins, carboranes are usually buried inside a concave hydrophobic pocket, where shape complementarity plays, as in the case of fullerenes, a crucial role (Figure 6a,b).[31−33] The possibility to exploit carboranes as building blocks to create important interactions with hydrophobic patches of proteins was pointed out already in the first paper that described the determination of the first carborane−protein complex structure (Figure 6c). In this system, cobalt bis(1,2-dicarbollide) ion bound to the hydrophobic pockets formed by side chains of HIV-protease residues (Figure 4b).[31] Direct evidence that the carborane cage binds to a hydrophobic surface was also provided by the X-ray structure of

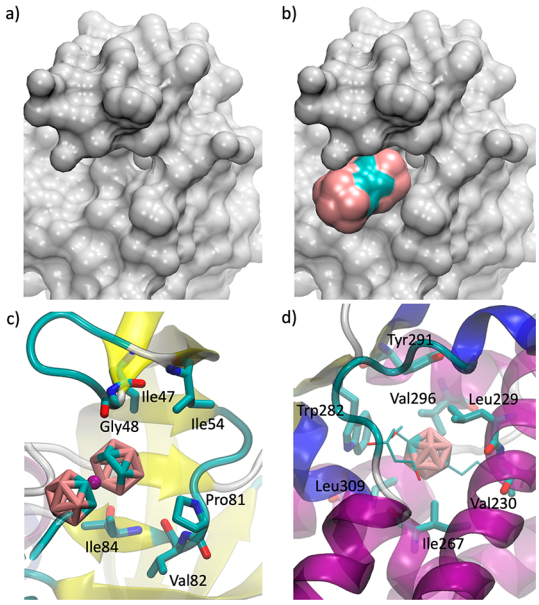


Figure 6. Surface representation of (a) HIV protease (HIV-PR) and (b) cobalt bis(1,2-dicarbollide)@HIV-PR. (c) Interactions between cobalt bis(1,2-dicarbollide) and hydrophobic residues (Ile47, Gly48, Ile54, Pro81, Val82, Ile84) of HIV-PR. (d) Interactions between a *p*-carborane derivative and hydrophobic residues (Leu229, Val230, Ile267, Tyr291, Trp282, Val296, Leur309) of vitamin D receptor ligand-binding domain.

https://doi.org/10.1021/accountsmr.1c00065
Acc. Mater. Res. 2021, 2, 594−605

vitamin D receptor ligand-binding domain complexed to a carborane-derivative agonist (Figure 6d).[32]

Favorable interactions of the carborane and metallacarborane cluster with the hydrophobic pocket formed by enzyme active sites are leitmotifs in the crystal structure of carborane derivatives with proteins.[28−33] Since carboranes are isosteric to a rotating phenyl group, multiple favorable pockets exist in proteins.[33] A crucial difference between fullerenes and carboranes is that fullerenes interact with proteins directly with their carbon atoms, while the outer sphere of carboranes consists of hydrogen atoms. In other words, the intermolecular interactions between proteins and carboranes are mediated by hydrogen atoms. In carboranes, the acidic C−H groups and the hydridic B−H groups compete or act synergically. On the one hand, the acidic C−H bonds can form "classical hydrogen bonds", for example, with the carbonyl groups of the peptide bonds or with amino acids that are acceptors of hydrogen bonds. On the other hand, the hydride-like hydrogens form unconventional dihydrogen bonds.[34] These bonds, also called proton−hydride bonds, generally occur between a positively charged hydrogen atom of a classical proton donor and a hydridic proton acceptor (Figure 7a).[34] The strength of these

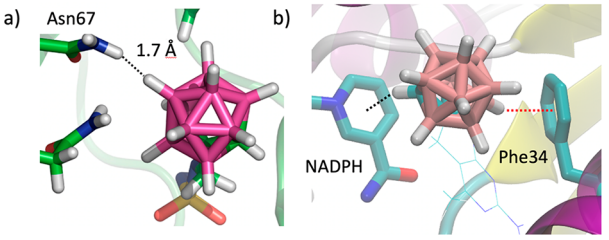


Figure 7. (a) Interaction between a monoanionic nido cage carborane derivatives with human carbonic anhydrase II (hCAII). A strong dihydrogen bond with Asn67 is formed. Adapted with permission from ref 35. Copyright 2013 American Chemical Society. (b) C−H···π (black dashed line) and B−H···π (red dashed line) interactions in the crystal structure of dihydrofolate reductase (hDHFR), in a complex with a closo-*o*-carborane derivative.

dihydrogen bonds depends on the hydridic character of the proton, which is a function of the carborane chemical formula, typology and the position of the B−H vertex.[35] For example, the neutral closo-carborane cage is bound to human carbonic anhydrase II mainly via dispersion interactions and forms only very weak dihydrogen bonds. On the contrary, the monoanionic nido cage forms short and strong dihydrogen bonds (stabilization of up to 4.2 kcal/mol; H···H distances of 1.7 Å, Figure 7a) with the polar hydrogen of protein NH_2 groups.[35] Electrostatic interactions have been proposed also to intervene in the stabilization of bound carboranes, via hydridic B−H···Na^+ Coulomb interaction.[36] Carboranes also establish C−H···π and B−H···π interactions (Figure 7b).[37] For example, the crystal structure of dihydrofolate reductase, in a complex with a closo-*o*-carborane derivative, showed the typical C−H···π and B−H···π interactions between the *o*-carborane moiety and the π-systems of NADPH and Phe34 (Figure 7b).[38]

2.3. Gold Nanocluster−Protein Interface

A number of stoichiometrically precise gold nanoclusters (AuNCs) have been synthesized (i.e., $Au_{25}(SR)_{18}$, $Au_{36}(SR)_{23}$, $Au_{38}(SR)_{24}$, $Au_{102}(SR)_{44}$, $Au_{144}(SR)_{60}$).[39] These systems have allowed in-depth studies of the properties of AuNCs. The size of AuNCs approaches the Fermi wavelength of conduction electrons, so that the continuous density of states of bulk gold breaks into discrete energy levels, resulting in size dependent optical, electronic, and chemical properties, that include HOMO−LUMO transitions, magnetism, redox properties, optical chirality, quantized charging, strong photoluminescence, and catalytic activity.[39] Bare AuNCs are unstable in solution and need to be protected by ligands (i.e., thiolates, phosphines, or polymers), which in turn strongly affect their properties.[39] Inspired by natural biomineralization processes, proteins can be used as templates for the synthesis and stabilization of AuNCs.[40,41] Inside proteins, AuNCs form by a sequential process that is triggered by gold deposition and aggregation into small clusters that act as nucleation centers for AuNCs growth.[40,41]

The study of the interaction between AuNCs and proteins adds further complexity with respect to that of fullerenes and carboranes in two aspects: (i) the proteins can be considered as supramolecular receptors that can be used to host a preformed AuNCs, and (ii) the proteins also offer the opportunity of protein-assisted synthesis of AuNCs directly in their core. In this case, it is crucial to identify a protein pocket containing sites for metal ion accumulation and reduction. Preformed AuNCs can interact with proteins through two modalities: (i) host−guest interaction between the protein and the ligand-protected AuNCs;[42−44] (ii) ligand exchange with selected amino acids of the protein, for instance, a protein residue such as cysteine can displace a protecting group of the AuNC.[45] The two modalities of interaction can act together.[45] An essential role in the binding is exerted by electrostatic and hydrophobic forces (Figure 8).[42,43] Hydrogen bonding between AuNC ligands and protein residues can be used to control the host−guest recognition process.[44]

The variety of hybrids that can be produced can have substantially different properties even upon small modifications. For instance, AuNCs with the same core and surface charge but protected by two different ligands, namely, *p*-mercaptobenzoic acid and glutathione, possess very different association rate constants with proteins.[7]

The different functional groups present in proteins, such as sulfhydryl groups, hydroxyl groups, carboxyl groups, makes them ideal scaffolds also to accumulate and reduce Au ions. The resultant AuNCs are sterically protected by the bulky protein. The synthetic methodology is straightforward. In the pioneering work by Xie et al. bovine serum albumin (BSA)-AuNCs hybrids were synthesized[40] using a facile one-pot synthesis, under mild reaction conditions. Besides BSA, different proteins, such as human serum albumin, ovalbumin, lysozyme, papain, trypsin, pepsin, transferrin, insulin, keratin, gelatin, aprotinin, apoferritin, immunoglobulin G, glucose oxidase, Y globulin, horseradish peroxidase, urease, fibrinogen, esterase, lactoferrin, zein, and β-lactoglobulin, were used to prepare protein−AuNCs hybrids.[41] Notably, the number of gold atoms in the cluster differs from protein to protein.[41] The proposed mechanism for the formation of a protein−AuNC hybrid consists of three different steps, (i) ionic binding of gold ions to specific amino acid residues of the protein, (ii) reduction of the gold ions, and (iii) growth of AuNCs. Amino acid residues in the protein have a triple role in the AuNCs synthesis providing anchoring sites for the gold ions, acting as the reducing agent, and stabilizing the AuNCs. Major efforts were made to understand the role of protein size, amino acid content, metal coordination environment in the process of

https://doi.org/10.1021/accountsmr.1c00065
Acc. Mater. Res. 2021, 2, 594−605

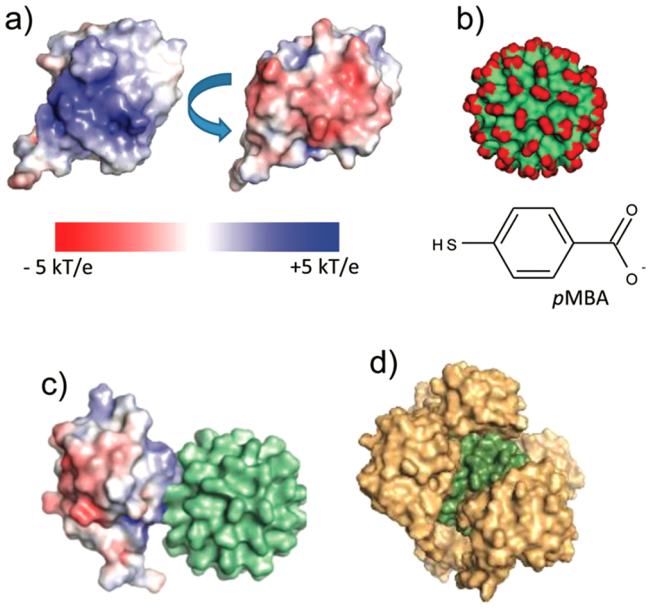


Figure 8. AuNC coated with pMBA ligands (AuMBA) and ubiquitin. (a) Electrostatic surface potential of ubiquitin (1UBQ) calculated with APBS. (b) AuMBA (red, −COO groups) and structure of *p*-mercaptobenzoic acid (pMBA) passivating ligand. (c) Schematic representation of an AuMBA particle (in green) bound to ubiquitin through the single positive surface patch on the protein surface. (d) Schematic illustration of an AuMBA particle partially coated by interacting ubiquitin molecules. Adapted with permission from ref 8. Copyright 2020 The Royal Society of Chemistry.

formation, and the properties of the AuNCs.[46] All 20 amino acids were tested to determine their reducing power and binding capability to HAuCl4.[47] Amino acid residues containing nitrogen heterocycles (histidine and tryptophan) or sulfur (cysteine, methionine) in their side chains are the strongest Au binders.[47] The analysis of the interaction distances of Au−protein residue side chains in the Protein Data Bank (Figure 9) indicated that the favorite residues for Au binding are Cys and His.[48] Au ions interact also with other amino acids such as Lys, Arg, Gln, Met, Glu, Asp, Asn, Thr, and Ser and both the N-terminal and C-terminal regions of the proteins.[48]

The lone pairs of sulfur, nitrogen, and oxygen usually interact with the Au ions, but Au···π interactions are also

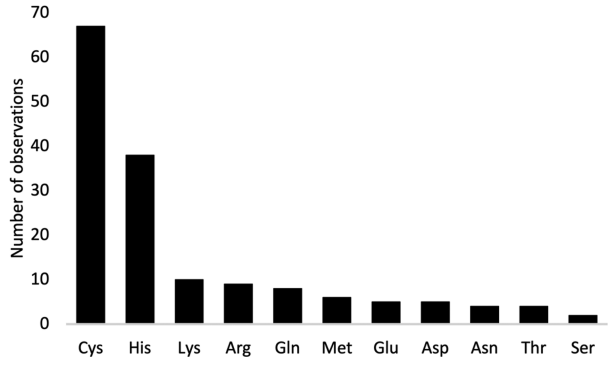


Figure 9. Column chart of most frequently bound residues to Au atoms in the structures of gold/protein adducts reported in the PDB. Adapted with permission from ref 48. Copyright 2020 Elsevier. Data kindly provided by the authors.

observed.[48] Au ions can bind to a single side chain, completing its coordination sphere with (i) solvent ligands, (ii) the ligands already present in the structure of the initial Au salt, or (iii) one or more protein residues. For instance, dyads such as His/His, His/Lys, His/Cys, His/Gln, Cys/Cys, Cys/Asp, and Cys/Asn are able to trap Au atoms between their side chains.[48] Importantly, the binding of Au ions to proteins generally does not affect their overall conformation.[48] The interaction of proteins with Au compounds mainly occurs via ligand displacement and Au ions bind residue side chains through relatively strong coordinative bonds.[48] Two mechanisms can be operative: (1) Au(III) compounds break down before binding; i.e., the Au(III) ion is reduced to Au(I), which then binds to the protein. (2) An initial binding of Au(III) to the protein is followed by ligand detachment/metal reduction or by metal reduction/ligand detachment.[48] The formation of AuNCs involves the reduction of Au(III) to Au(I), followed by dismutation of Au(I) to Au(0) and Au(III). It can be activated by external reducing agents (i.e., $NaBH_4$)[49] or directly by the protein amino acid residues.[40] Tryptophan, tyrosine, phenylalanine, and aspartic acid are the most efficient reducing groups as pristine amino acids, while in a peptide sequence the residues with the best performances contain nitrogen such as glutamine, asparagine, arginine, and lysine.[47] Also disulfide bridges play a crucial role in AuNC formation, in particular during the AuNCs growth. After the disulfide bond breakage, the cysteine residues can form Au−S bonds able to stabilize the AuNC core by thiolate linkages. The conformation of the protein is also involved in the regulation of AuNCs. For instance, modulating BSA conformation yields Au_4, Au_8, Au_{10}, Au_{13}, and Au_{25}.[50]

A crystalline protein cage built by cross-linking treatment of a single crystal of apo-ferritin was used as a model to characterize structurally the process of gold ion binding inside the protein scaffold and in the initial formation of the core of the gold nanocluster.[49] Wild-type ferritin has two specific metal-binding regions, centered at Cys48 and surrounded by His49, Glu45, and Arg52. Since Au ions have high affinity for sulfur, additional Cys residues were added, replacing the Glu45 and Arg52 residues by Cys (apo-E45C/R52C-rHLFr).[49] Three different Au-binding sites were identified in the crystal structure of the mutated ferritin cage (Figure 10a−e), involving Cys48, His49, Cys52, Met96, Cys126, His114, and His147. Reduction with sodium borohydride ($NaBH_4$) induced a rearrangement of the Au ions and amino acids (Figure 10f) and the formation of a AuNC, which, once formed, is stabilized by His114, Glu 130 and Cys126 (Figure 10g).

3. IDENTIFICATION OF MNPS CARRIER PROTEIN

The characterization of protein−MNPs hybrids follows a robust procedure: in fact, methodologies used to study protein−molecule interactions such as X-ray diffraction crystallography,[14,31,32,49] NMR,[9,20,23] mass spectrometry,[11,50] and optical spectroscopies[9−11,14,31,32,49,50] can be analogously used to investigate the protein−MNP interface. It is the identification of new proteins able to bind or template the synthesis of a MNP that represents a real needle-in-a haystack-problem. To date, relatively few proteins are known to interact with MNPs. The total number of proteins found in terran biological organisms is likely to exceed ten million. As of March 2021, 176 000 protein structures had been deposited in the Protein Data Bank (PDB). Within this "space" there must

https://doi.org/10.1021/accountsmr.1c00065
Acc. Mater. Res. 2021, 2, 594−605

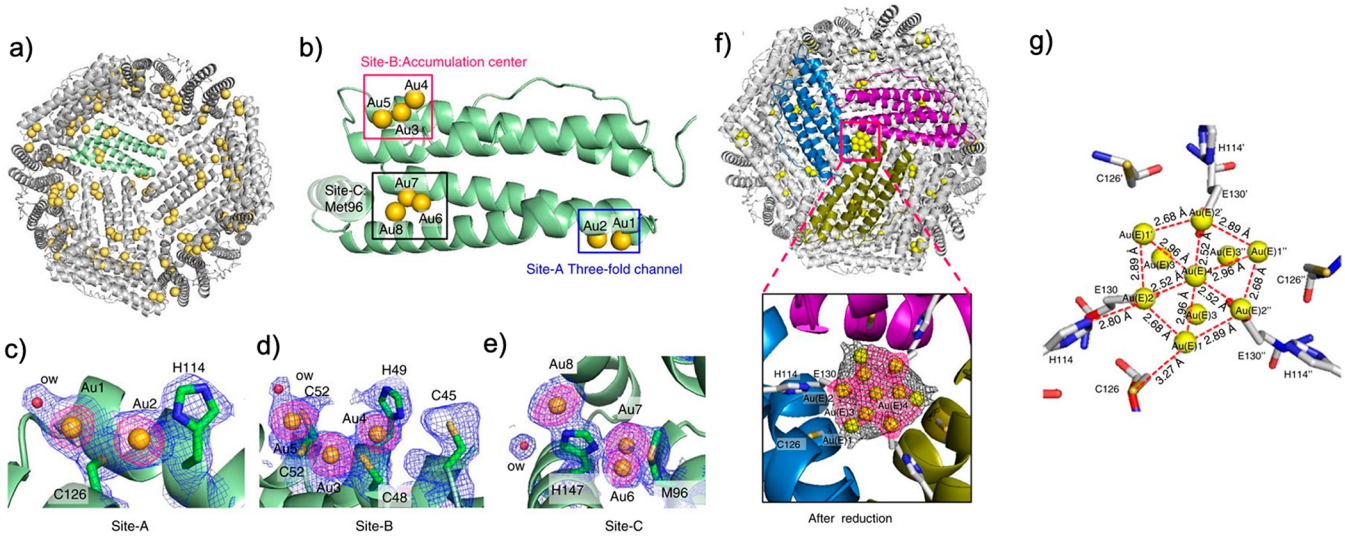


Figure 10. Crystal structure of Au·CL-apo-E45C/R52C-rHLFr. (a) Entire structure showing the positions of Au ions immobilized on the interior surface. (b) Monomeric subunit showing the binding sites of Au ions. (c)−(e) Coordination structures of Au at (c) Site-A, (d) Site-B, and (e) Site-C. Au atoms in yellow. (f) Crystal structure of Au^0(E)·CL-apo-E45C/R52C-rHLFr showing the formation of Au subnanocluster after the reduction of preorganized Au atoms in Au·CL-apo-E45C/R52C-rHLFr with $NaBH_4$. (g) Binding site of the Au subnanocluster formed in Au^0(E)·CL-apo-E45C/R52C-rHLFr with selected bond distances. Adapted with permission from ref 49. Copyright 2017 Springer Nature.

Table 2. Application in Nanomedicine of Fullerenes, Carboranes, and AuNCs in Therapy and Imaging[a]

		Fullerenes	Carboranes	Gold NCs
Therapy	Photodynamic and sonodynamic (PDT, SDT)	✓✓✓	✓	✓✓
	Photothermal therapy (PTT)	✓		✓✓✓
	Microwave, Radiowave and Magnetic Induced Hyperthermia (MilH, RIH, MaIH)	✓✓		✓✓✓
	Neutron Capture Therapy (NCT)	✓	✓✓✓	
	Radiotherapy (RT)	✓✓	✓✓	✓✓✓
	Chemotherapy (CT)	✓✓✓	✓✓✓	✓
Imaging	Fluorescence Imaging (FI)	✓✓	✓	✓✓✓
	Raman Imaging (RI)	✓✓	✓✓	✓✓✓
	Photoacoustic Imaging (PAI)	✓✓✓		✓✓✓
	X-ray Computed Tomography (X-CT)	✓	✓	✓✓
	Magnetic Resonance Imaging (MRI)	✓✓✓	✓✓	✓
	Positron Emission Tomography (PET)/ Single-Photon Emission Computed Tomography (SPECT)	✓✓	✓✓	✓

[a]The number of ticks in the table reflects the potential of the MNP in the different application fields, from a critical analysis of the scientific literature.

likely be the right protein able to bind/template the synthesis of a selected MNPs, but its identification is a profound challenge that involves significant trial-and-error work. High-throughput virtual screening and *in silico* rational design approaches are trying to accelerate this process that can be considered at the forefront of materials discovery.

3.1. Virtual Screening

In drug discovery high-throughput virtual screening is routinely used to verify the ability of proteins to recognize target molecules. It is used to search libraries of small molecules in order to identify those structures most likely to bind to a drug target, typically a protein receptor or enzyme. The process can be reversed: a protein database can be screened to select proteins able to recognize and bind an individual molecule. Docking protocols build geometries of interaction and quantify them, giving as a result a rank of the possible binders. The combination of these protocols (high throughput virtual screening and docking) identify the "best" proteins for a selected MNP.[18,51,52]

3.2. Protein Engineering

Rational design and protein engineering are successful methodologies to develop new functional systems with technological applications in several fields such as imaging, sensing, conductive materials, logic circuits, and catalysis.[53] It is possible to introduce in a protein a binding pocket for a specific MNP[14,25] or to design a coordination site in well-

https://doi.org/10.1021/accountsmr.1c00065
Acc. Mater. Res. 2021, 2, 594−605

defined cavities of a protein for the synthesis and stabilization of AuNCs.[53,54] Existing proteins or de novo designed peptide/protein sequences can be used.

4. PROTEINS AS "TROJAN HORSES"—APPLICATION OF PROTEIN−MNP HYBRIDS IN THERANOSTICS

The MNPs discussed in this Account (fullerenes, carboranes, gold nanoclusters) are among the most promising theranostic agents for applications in nanomedicine (Table 2).[12,28−30,41,55] Due to their peculiar chemical−physical properties (fullerenes, carboranes, AuNCs) or to the possibility to entrap/bound d-block and f-block elements (metalloendohedral fullerenes and metallocarboranes), these MNPs can be used (i) in the generation of reactive oxygen species upon light and ultrasound irradiation (photodynamic and sonodynamic therapy),[20,23,56] (ii) in the conversion of electromagnetic radiation in heat (thermotherapies) following stimulation by light, microwave, radiowave, or magnetic fields stimulation,[41,55] (iii) in neutron capture therapy,[28] (iv) in radiotherapy,[39,55] or (v) as chemotherapeutic agents.[28−30,51,55] At the same time MNPs have intrinsic multimodal imaging capabilities because they are fluorescent[39,41,55] and are characterized by photoacoustic effects,[20] give a strong Raman signal,[39,55] and can be used as contrast agents in X-ray computed tomography,[55] magnetic resonance imaging,[55] positron emission tomography,[55] and single photon emission computed tomography.[55]

In many instances, many MNPs have demonstrated superior properties with respect to approved therapies and imaging techniques. They still present important restrictions for their use due to one or more of the following problems: (i) poor water solubility and/or low biocompatibility, (ii) dependency of their therapeutic efficacy/imaging properties/toxicity on the features of the physiological environment (salt content and local pH), (iii) aggregation phenomena, (iv) nonspecificity of cellular uptake, and (v) poor pharmacokinetic/pharmacodynamic properties. Many of these difficulties can be overcome by the integration MNPs with the specific characteristics of proteins to create innovative multifunctional theranostic platforms.

4.1. MNPs Dispersion in Physiological Environments by Proteins

Incorporation of MNPs in proteins allows dispersion of the MNPs in water with a "green" and facile approach, circumventing the insolubility of some MNPs in physiological environments and their tendency to aggregates, which induces toxicity.[9,17,57] Due to poor solubility in aqueous solution, some MNPs, such as C_{60}, are usually not in single "molecular" form in physiological environments, while in C_{60}@protein hybrids the fullerene is monomolecularly dispersed.[9,17,57] Proteins provide steric protection to the MNPs endowing them with high stability also in conditions that may otherwise prove extreme. It is the isoelectric point of the protein that governs the stability of the hybrid and not the intrinsic chemistry of the MNP.[17] The biocompatibility and stability of these hybrids in a wide range of pH values and ionic forces makes them ideal for biological applications.[9,17,56,57] The use of proteins, as natural supramolecular hosts, avoids complicated synthetic procedures, usage of artificial hosts that can be toxic, or the need of noxious organic solvents for MNP dispersion. Proteins can be easily extracted and purified from common renewable resources such as wheat, rice, maize, milk, and egg; these "cheap" and "sustainable" biosystems can be turned into nanotechnological objects of high practical interest. The capability to maintain the biological conformation of the protein upon formation of the hybrid is crucial for their administration into biological systems since conformational changes that modify the folding of a protein may trigger an immune response leading to toxicity.

4.2. Cellular Uptake and Biodistribution of Protein−MNP Hybrids

The protein-protected MNPs maintain the biological identity of the carrier protein, without intermediate complications arising from the formation of protein coronas.[58] Bioconjugation also controls the uptake of the MNPs, their cellular trafficking, and their ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) properties.

Cellular Uptake. The dispersion of MNPs with self-targeting proteins can address MNPs toward specific cells.[59] For example, the high levels of expression of transferrin and ferritin receptors in cancer cells or the selective accumulation of human serum albumin in cancer tissues make the use of these proteins attractive to target cancer cells.[59]

As a representative example, we can consider the case of transferrin−AuNCs hybrids, where transferrin has the dual function to act as a reducing, templating, and stabilizing agent for AuNCs and as a ligand to target transferrin receptors, overexpressed in cancer cells.[60] Lysozyme or lectins[61] can be used to recognize the external membrane of bacteria. MNPs bound/synthesized inside antibody cavities, maintain the intrinsic recognition/targeting ability of the carrier protein.[62] The platform of chemical functionalities present on the protein surface can be also used for further modifications with targeting tags, such as antibodies, vitamins or homing peptides (Figure 11). Functionalization of BSA-stabilized AuNCs by

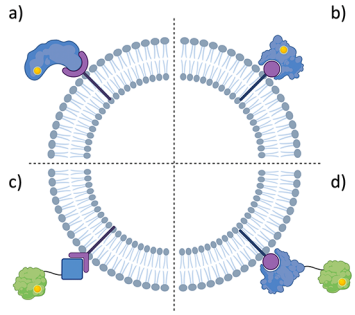


Figure 11. Cellular uptake of the MNP−protein hybrids induced by (a) proteins targeting specific receptors, (b) antibody recognition, tags as (c) vitamins or homing peptides, and (d) antibodies, chemically conjugated to the proteins. Created with BioRender.com.

folic acid enhanced the uptake by HeLa tumor cells that express high levels of folic acid receptors. The opposite occurs with A549 tumor cells that express low levels of folic acid receptors.[63]

Cellular Trafficking. The intracellular protein trafficking ensures that proteins are delivered to the correct subcellular compartment in the cell and is a prerequisite for correct cell function. The selection of a specific protein for the dispersion of a MNP allows to regulate its local accumulation in subcellular compartments. Such accumulation enhances both imaging and therapeutic performances of MNPs. As an example, the conservation of the "biological identity" of the carrier protein induce a lysosomal accumulation of C_{70}@

https://doi.org/10.1021/accountsmr.1c00065
Acc. Mater. Res. 2021, 2, 594−605

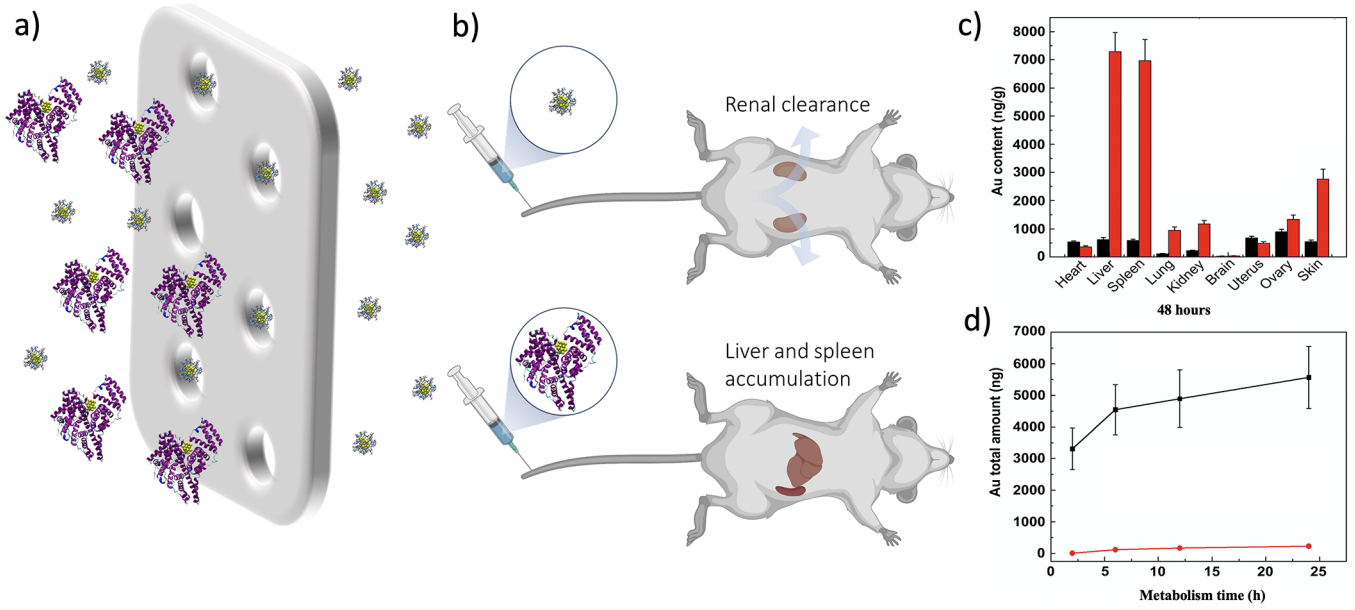


Figure 12. (a) Cartoon representation of the renal filtration barrier. The size threshold for kidney filtration is ~6 nm. GSH-Au_{25}NCs, with a size smaller than 6 nm can pass through the glomerular filtration membrane readily, while BSA-Au_{25}NCs (8−10 nm) are blocked by the barrier. (b) Biodistribution of GSH-Au_{25}NCs and BSA-Au_{25}NCs in treated mice. GSH-Au_{25}NCs showed very efficient renal clearance and are excreted into urine. In contrast, BSA-Au_{25}NCs were retained in the body, with elevated concentrations in the liver and spleen. Created with BioRender.com. (c) Biodistribution of the GSH- and BSA-Au_{25}NCs treated mice at 48 h. (d) Time-dependent Au content (total metabolism) in 24 h. GSH-Au_{25}NCs (black) and BSA-Au_{25}NCs (red). Panels c and d adapted with permission from ref 65. Copyright 2012 Elsevier.

lysozyme in HeLa cells.[20] Analogous experiments carried out with C_{70}@BSA showed that C_{70}@BSA undergoes neither endocytic uptake nor trafficking to the lysosomes.[20]

Crossing Physiological Barriers. In the human body, physiological barriers allow separation between different compartments of the body and/or with the outer environment. Sometimes proteins can be used as molecular Trojan horses to ferry MNPs through these physiological barriers.[64] The blood brain barrier (BBB) is a physical and metabolic barrier between the central nervous system (CNS) and the peripheral circulation that protects the brain from pathogens and other toxins but makes CNS diseases hard to treat with conventional drugs. The BBB is selectively permeable to specific proteins, such as transferrin, insulin, or apolipoproteins because they can cross the barrier through receptor-mediated trafficking. Binding of a MNP with one of these proteins can allow the passage of the BBB.

Alternatively, binding with proteins can stop the elimination through physiological barriers.[65] The glomerular filtration barrier (GFB) functions is a highly organized, semipermeable membrane that in kidneys filters excess fluid and waste products out of the blood into the urine, eliminating them from body. GFB also prevents the passage of the majority of proteins (serum albumin in particular) into urine retaining them in the circulation. The glomerular filtration barrier is a sort of "size cutoff" slit, which retains objects (such as proteins or nanoparticles) larger than 6−8 nm in the body and rapidly excretes smaller ones.[65] Incorporation of a MNP in proteins, such as albumins, blocks the filtration of small MNPs from the kidney (Figure 12), modifying their clearance and blood half-life.

ADMET Properties. Use of protein also allows the *in vivo* regulation of pharmacokinetic and pharmacodynamic properties of the MNPs. Some proteins, such as hormones, have short elimination half-life, a property crucial for close regulation of endogenous levels and for ADMET. In contrast, proteins with transport function (such as albumin) and long-term immune function (such as immunoglobulins) are characterized by half-lives of several days. The pharmacokinetics and pharmacodynamics of the MNP−protein hybrids are governed by the host protein and can increase the retention time of the MNPs for effective applications. Preservation of the biological identify of the protein allows the hybrid to escape clearance by reticuloendothelial cells and to have a long blood circulation time. AuNCs of the same size, stabilized by a small peptide, such as glutathione (GSH) or synthesized inside bovine serum albumin (BSA), showed strongly different pharmacokinetics and biodistribution.[65] AuNCs protected with GSH showed very efficient renal clearance with 36% of the AuNCs excreted into urine in 24 h and only 6% remaining in the mouse after 28 days (Figure 12). In contrast, more than 95% of AuBSA were retained for 28 days, with elevated concentrations in the liver and spleen (Figure 12).[65] This difference is attributed to the size of the protein, in fact the GSH-protected AuNCs can be taken up by the kidneys, but not the AuNCs incorporated in BSA.

5. CONCLUSIONS AND PERSPECTIVES

The understanding of MNP−protein interactions at the molecular level represents a foundational keystone in nanoscience. In this account, we identified and discussed the rules that govern the nanobio interface between proteins and representative MNPs (fullerenes, carboranes, and gold nanoclusters). MNPs are characterized by a well-defined molecular formula and structure and their diameters (0.7−1.5 nm) are commensurable with many protein pockets. Proteins behave as supramolecular hosts able to recognize MNPs directly in water, resembling a typical biomolecular complexation (ligand−protein complex formation). Proteins can establish many different noncovalent interactions with MNPs such as

https://doi.org/10.1021/accountsmr.1c00065
Acc. Mater. Res. 2021, 2, 594−605

hydrogen or dihydrogen bonding, electrostatic interactions, hydrophobic interactions, van der Waals interactions, $\pi-\pi$/cation$-\pi$/anion$-\pi$ interactions, depending on the surface chemistry of the MNP. It is fair to say that to date understanding of protein−nanoparticle interactions has only been partly achieved and much remains to be done when it comes to design. The reason mainly lies in the fact that we know relatively few proteins that interact with MNPs. Every new discovery was mainly guided by chemical intuition, scientific curiosity, and ultimately, serendipity. A crucial role will be carried out by computational methodologies, as virtual screening and protein engineering, that will accelerate the design/discovery of MNP-binding proteins. Finally, the application of the MNP−protein hybrids, as theranostic platforms, in nanomedicine was discussed. The integration of the chemical-physics properties of MNPs with the specific characteristics of proteins creates innovative multifunctional theranostic platforms. The biocompatible incorporation of a MNPs inside a protein "hides" MNPs, controlling (i) the absorption mechanism of the MNPs, (ii) the crossing of physiological barriers, and (iii) their biological fate, and ultimately makes MNPs "go where no MNP has been before". Other MNPs such as polyoxometalates (POM),[66] polyhedral oligomeric silsesquioxane (POSS),[67] metal (i.e., silver[68] or copper[69]), and metal oxide molecular nanoparticles can benefit from the proposed approach. Last, but not least, hybrid MNP−protein systems have great potential for the technological exploitation of MNPs and the possibility to engineer a protein to tune MNP properties via noncovalent interactions will represent a sound basis also for many scientific applications such as the development of new sensors, catalysts, and bioelectronic devices.[70]

AUTHOR INFORMATION

Corresponding Author

Matteo Calvaresi − *Dipartimento di Chimica "G. Ciamician", Alma Mater Studiorum - Università di Bologna, 40126 Bologna, Italy;* orcid.org/0000-0002-9583-2146; Email: matteo.calvaresi3@unibo.it

Authors

Matteo Di Giosia − *Dipartimento di Chimica "G. Ciamician", Alma Mater Studiorum - Università di Bologna, 40126 Bologna, Italy;* orcid.org/0000-0003-3494-298X

Francesco Zerbetto − *Dipartimento di Chimica "G. Ciamician", Alma Mater Studiorum - Università di Bologna, 40126 Bologna, Italy;* orcid.org/0000-0002-2419-057X

Complete contact information is available at: https://pubs.acs.org/10.1021/accountsmr.1c00065

Notes

The authors declare no competing financial interest.

Biographies

Matteo Di Giosia received his Ph.D. in Chemistry from University of Bologna in 2017. He spent research periods as a visiting fellow at Centre for BioNano Interactions at University College Dublin and Technion Israel Institute of Technology. He is currently a postdoctoral research fellow at the University of Bologna. His research activity is focused on the development of biohybrid materials based on proteins and carbon nanostructures (fullerenes, nanotubes, graphene, and nanodiamonds) for applications in nanomedicine.

Francesco Zerbetto was born in 1959 and received his Ph.D. in 1986 from the University of Bologna. He was then Research Associate at NRC, Ottawa, until 1990 when he was appointed Researcher at the University of Bologna. He has been Professor of Physical Chemistry since 2001. His research, in computational and theoretical chemistry, has covered vibrationally resolved electronic spectroscopies, tunneling effect, nonlinear optics, carbon nanostructures, chirality, interlocked molecules, surface science, self-assembly, and more recently signal processing.

Matteo Calvaresi obtained his Ph.D. from the University of Bologna in 2008. In 2011, he became Assistant Professor of Organic Chemistry and was promoted to Associate Professor in 2018. He was a Visiting Researcher at Peking University and Technion Israel Institute of Technology and Visiting Professor at University of Pune. Currently, he leads the NanoBio Interface Lab. His research interests are focused on the interactions between proteins and nanomaterials, both experimentally and computationally. He has been working at the cutting edge between nanotechnology and biology, creating photo-theranostic platforms based on the integration of proteins/phages with nanoparticles/photosensitizers to detect and kill via photodynamic and photothermal therapies cancer cells/bacteria.

ACKNOWLEDGMENTS

M.D.G. was supported by a FIRC-AIRC fellowship for Italy (id. 22318). The research leading to these results has received funding from AIRC under MFAG 2019-ID. 22894 project - P.I. M.C.

REFERENCES

(1) Calvaresi, M. The Route towards Nanoparticle Shape Metrology. *Nat. Nanotechnol.* **2020**, *15*, 512−513.

(2) Boselli, L.; Lopez, H.; Zhang, W.; Cai, Q.; Giannone, V. A.; Li, J.; Moura, A.; Araujo De, J. M.; Cookman, J.; Castagnola, V.; et al. Classification and Biological Identity of Complex Nano Shapes. *Commun. Mater.* **2020**, *1*, 35.

(3) Cedervall, T.; Lynch, I.; Lindman, S.; Berggård, T.; Thulin, E.; Nilsson, H.; Dawson, K. A.; Linse, S. Understanding the Nanoparticle - Protein Corona Using Methods to Quantify Exchange Rates and Affinities of Proteins for Nanoparticles. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104* (7), 2050−2055.

(4) Zarschler, K.; Rocks, L.; Licciardello, N.; Boselli, L.; Polo, E.; Garcia, K. P.; De Cola, L.; Stephan, H.; Dawson, K. A. Ultrasmall Inorganic Nanoparticles: State-of-the-Art and Perspectives for Biomedical Applications. *Nanomedicine* **2016**, *12* (6), 1663−1701.

(5) Boselli, L.; Polo, E.; Castagnola, V.; Dawson, K. A. Regimes of Biomolecular Ultrasmall Nanoparticle Interactions. *Angew. Chem.* **2017**, *129* (15), 4279−4282.

(6) Muraca, F.; Boselli, L.; Castagnola, V.; Dawson, K. A. Ultrasmall Gold Nanoparticle Cellular Uptake: Influence of Transient Bionano Interactions. *ACS Appl. Bio Mater.* **2020**, *3* (6), 3800−3808.

(7) Lira, A. L.; Ferreira, R. S.; Torquato, R. J. S.; Zhao, H.; Oliva, M. L. V; Hassan, S. A.; Schuck, P.; Sousa, A. A. Binding Kinetics of Ultrasmall Gold Nanoparticles with Proteins. *Nanoscale* **2018**, *10* (7), 3235−3244.

(8) Ferreira, R. S.; Lira, A. L.; Sousa, A. A. Quantitative Mechanistic Model for Ultrasmall Nanoparticle-Protein Interactions. *Nanoscale* **2020**, *12* (37), 19230−19240.

(9) Calvaresi, M.; Arnesano, F.; Bonacchi, S.; Bottoni, A.; Calò, V.; Conte, S.; Falini, G.; Fermani, S.; Losacco, M.; Montalti, M.; et al. C60@Lysozyme: Direct Observation by Nuclear Magnetic Resonance of a 1:1 Fullerene Protein Adduct. *ACS Nano* **2014**, *8* (2), 1871−1877.

(10) Yin, M. M.; Chen, W. Q.; Lu, Y. Q.; Han, J. Y.; Liu, Y.; Jiang, F. L. A Model beyond Protein Corona: Thermodynamics and Binding

Stoichiometries of the Interactions between Ultrasmall Gold Nanoclusters and Proteins. *Nanoscale* **2020**, *12* (7), 4573−4585.
(11) Goszczyński, T. M.; Fink, K.; Kowalski, K.; Leśnikowski, Z. J.; Boratyński, J. Interactions of Boron Clusters and Their Derivatives with Serum Albumin. *Sci. Rep.* **2017**, *7* (1), 1−12.
(12) Goodarzi, S.; Da Ros, T.; Conde, J.; Sefat, F.; Mozafari, M. Fullerene: Biomedical Engineers Get to Revisit an Old Friend. *Mater. Today* **2017**, *20* (8), 460−480.
(13) Calvaresi, M.; Bottoni, A.; Zerbetto, F. Thermodynamics of Binding between Proteins and Carbon Nanoparticles: The Case of C60@Lysozyme. *J. Phys. Chem. C* **2015**, *119* (50), 28077−28082.
(14) Kim, K.-H.; Ko, D.-K.; Kim, Y.-T.; Kim, N. H.; Paul, J.; Zhang, S.-Q.; Murray, C. B.; Acharya, R.; DeGrado, W. F.; Kim, Y. H.; et al. Protein-Directed Self-Assembly of a Fullerene Crystal. *Nat. Commun.* **2016**, *7*, 11429.
(15) Calvaresi, M.; Furini, S.; Domene, C.; Bottoni, A.; Zerbetto, F. Blocking the Passage: C_{60} Geometrically Clogs K^+ Channels. *ACS Nano* **2015**, *9* (5), 4827−4834.
(16) Trozzi, F.; Marforio, T. D.; Bottoni, A.; Zerbetto, F.; Calvaresi, M. Engineering the Fullerene-Protein Interface by Computational Design: The Sum Is More than Its Parts. *Isr. J. Chem.* **2017**, *57* (6), 547−552.
(17) di Giosia, M.; Valle, F.; Cantelli, A.; Bottoni, A.; Zerbetto, F.; Calvaresi, M. C60 Bioconjugation with Proteins: Towards a Palette of Carriers for All PH Ranges. *Materials* **2018**, *11* (5), 691.
(18) Ahmed, L.; Rasulev, B.; Kar, S.; Krupa, P.; Mozolewska, M. A.; Leszczynski, J. Inhibitors or Toxins? Large Library Target-Specific Screening of Fullerene-Based Nanoparticles for Drug Design Purpose. *Nanoscale* **2017**, *9* (29), 10263−10276.
(19) Wu, X.; Yang, S.-T.; Wang, H.; Wang, L.; Hu, W.; Cao, A.; Liu, Y. Influences of the Size and Hydroxyl Number of Fullerenes/Fullerenols on Their Interactions with Proteins. *J. Nanosci. Nanotechnol.* **2010**, *10* (10), 6298−6304.
(20) Di Giosia, M.; Soldà, A.; Seeger, M.; Cantelli, A.; Arnesano, F.; Nardella, M. I.; Mangini, V.; Valle, F.; Montalti, M.; Zerbetto, F. A Bio-Conjugated Fullerene as a Subcellular-Targeted and Multifaceted Phototheranostic Agent. *Adv. Funct. Mater.* **2021**, *31*, 2101527.
(21) Yang, S. T.; Wang, H.; Guo, L.; Gao, Y.; Liu, Y.; Cao, A. Interaction of Fullerenol with Lysozyme Investigated by Experimental and Computational Approaches. *Nanotechnology* **2008**, *19* (39), 395101.
(22) Bai, Y.; Wu, X.; Ouyang, P.; Shi, M.; Li, Q.; Maimaiti, T.; Lan, S.; Yang, S. T.; Chang, X. L. Surface Modification Mediates the Interaction between Fullerene and Lysozyme: Protein Structure and Antibacterial Activity. *Environ. Sci.: Nano* **2021**, *8* (1), 76−85.
(23) Di Giosia, M.; Nicolini, F.; Ferrazzano, L.; Soldà, A.; Valle, F.; Cantelli, A.; Marforio, T. D.; Bottoni, A.; Zerbetto, F.; Montalti, M.; et al. Stable and Biocompatible Monodispersion of C 60 in Water by Peptides. *Bioconjugate Chem.* **2019**, *30* (3), 808−814.
(24) Giełdoń, A.; Witt, M. M.; Gajewicz, A.; Puzyn, T. Rapid Insight into C60 Influence on Biological Functions of Proteins. *Struct. Chem.* **2017**, *28* (6), 1775−1788.
(25) Liutkus, M.; López-Andarias, A.; Mejías, S. H.; López-Andarias, J.; Gil-Carton, D.; Feixas, F.; Osuna, S.; Matsuda, W.; Sakurai, T.; Seki, S.; et al. Protein-Directed Crystalline 2D Fullerene Assemblies. *Nanoscale* **2020**, *12* (6), 3614−3622.
(26) Ma, D.; Meng, X. Y.; Bell, D. R.; Liu, S.; Zhou, R. Inhibition of CYP2C8 by Metallofullerenol Gd@C82(OH)22through Blocking Substrate Channels and Substrate Recognition Sites. *Carbon* **2018**, *127*, 667−675.
(27) Bologna, F.; Mattioli, E. J.; Bottoni, A.; Zerbetto, F.; Calvaresi, M. Interactions between Endohedral Metallofullerenes and Proteins: The Gd@C 60 -Lysozyme Model. *ACS Omega* **2018**, *3* (10), 13782−13789.
(28) Scholz, M.; Hey-Hawkins, E. Carbaboranes as Pharmacophores: Properties, Synthesis, and Application Strategies. *Chem. Rev.* **2011**, *111*, 7035−7062.
(29) Leśnikowski, Z. J. Challenges and Opportunities for the Application of Boron Clusters in Drug Design. *J. Med. Chem.* **2016**, *59* (17), 7738−7758.
(30) Issa, F.; Kassiou, M.; Rendina, L. M. Boron in Drug Discovery: Carboranes as Unique Pharmacophores in Biologically Active Compounds. *Chem. Rev.* **2011**, *111* (9), 5701−5722.
(31) Cigler, P.; et al. From Nonpeptide toward Noncarbon Protease Inhibitors: Metallacarboranes as Specific and Potent Inhibitors of HIV Protease. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102* (43), 15394−15399.
(32) Fujii, S.; Masuno, H.; Taoda, Y.; Kano, A.; Wongmayura, A.; Nakabayashi, M.; Ito, N.; Shimizu, M.; Kawachi, E.; Hirano, T.; et al. Boron Cluster-Based Development of Potent Nonsecosteroidal Vitamin D Receptor Ligands: Direct Observation of Hydrophobic Interaction between Protein Surface and Carborane. *J. Am. Chem. Soc.* **2011**, *133*, 20933−20941.
(33) Calvaresi, M.; Zerbetto, F. In Silico Carborane Docking to Proteins and Potential Drug Targets. *J. Chem. Inf. Model.* **2011**, *51* (8), 1882−1896.
(34) Fanfrlik, J.; Lepsik, M.; Horinek, D.; Havlas, Z.; Hobza, P. Interaction of Carboranes with Biomolecules: Formation of Dihydrogen Bonds. *ChemPhysChem* **2006**, *7*, 1100−1105.
(35) Pecina, A.; Lepšik, M.; Rezac, J.; Brynda, J.; Mader, P.; Řezáčová, P.; Hobza, P.; Fanfrlík, J. QM/MM Calculations Reveal the Different Nature of the Interaction of Two Carborane-Based Sulfamide Inhibitors of Human Carbonic Anhydrase II. *J. Phys. Chem. B* **2013**, *117* (50), 16096−16104.
(36) Fanfrlik, J.; Brynda, J.; Rezac, J.; Hobza, P.; Lepsik, M. Interpretation of Protein/Ligand Crystal Structure Using QM/MM Calculations: Case of HIV-1 Protease/Metallacarborane Complex. *J. Phys. Chem. B* **2008**, *112* (47), 15094−15102.
(37) Frontera, A.; Bauza, A. Closo-Carboranes as Dual CH···p and BH···p Donors: Theoretical Study and Biological Significance. *Phys. Chem. Chem. Phys.* **2019**, *21*, 19944−19950.
(38) Reynolds, R. C.; Campbell, S. R.; Fairchild, R. G.; Kisliuk, R. L.; Micca, P. L.; Queener, S. F.; Riordan, J. M.; Sedwick, W. D.; Waud, W. R.; Leung, A. K. W.; et al. Novel Boron-Containing, Nonclassical Antifolates: Synthesis and Preliminary Biological and Structural Evaluation. *J. Med. Chem.* **2007**, *50* (14), 3283−3289.
(39) Jin, R.; Zeng, C.; Zhou, M.; Chen, Y. Atomically Precise Colloidal Metal Nanoclusters and Nanoparticles: Fundamentals and Opportunities. *Chem. Rev.* **2016**, *116* (18), 10346−10413.
(40) Xie, J.; Zheng, Y.; Ying, J. Y. Protein-Directed Synthesis of Highly Fluorescent Gold Nanoclusters. *J. Am. Chem. Soc.* **2009**, *131* (3), 888−889.
(41) El-Sayed, N.; Schneider, M. Advances in Biomedical and Pharmaceutical Applications of Protein-Stabilized Gold Nanoclusters. *J. Mater. Chem. B* **2020**, *8* (39), 8952−8971.
(42) Shang, L.; Brandholt, S.; Stockmar, F.; Trouillet, V.; Bruns, M.; Nienhaus, G. U. Effect of Protein Adsorption on the Fluorescence of Ultrasmall Gold Nanoclusters. *Small* **2012**, *8* (5), 661−665.
(43) Yin, M.-M.; Dong, P.; Chen, W.-Q.; Xu, S.-P.; Yang, L.-Y.; Jiang, F.-L.; Liu, Y. Thermodynamics and Mechanisms of the Interactions between Ultrasmall Fluorescent Gold Nanoclusters and Human Serum Albumin, γ - Globulins, and Transferrin: A Spectroscopic Approach. *Langmuir* **2017**, *33*, 5108−5116.
(44) Zhu, H.; Li, J.; Wang, J.; Wang, E. Lighting Up the Gold Nanoclusters via Host - Guest Recognition for High-Efficiency Antibacterial Performance and Imaging. *ACS Appl. Mater. Interfaces* **2019**, *11*, 36831−36838.
(45) Paramanik, B.; Kundu, A.; Chattopadhyay, K.; Patra, A. Study of Binding Interactions between MPT63 Protein and Au Nanocluster. *RSC Adv.* **2014**, *4*, 35059−35066.
(46) Xu, Y.; Sherwood, J.; Qin, Y.; Crowley, D.; Bonizzoni, M.; Bao, Y. The Role of Protein Characteristics in the Formation and Fluorescence of Au Nanoclusters. *Nanoscale* **2014**, *6* (3), 1515−1524.
(47) Tan, Y. N.; Lee, J. Y.; Wang, D. I. C. Uncovering the Design Rules for Peptide Synthesis of Metal Nanoparticles. *J. Am. Chem. Soc.* **2010**, *132* (16), 5677−5686.

https://doi.org/10.1021/accountsmr.1c00065
Acc. Mater. Res. 2021, 2, 594−605

(48) Giorgio, A.; Merlino, A. Gold Metalation of Proteins: Structural Studies. *Coord. Chem. Rev.* **2020**, *407*, 213175.
(49) Maity, B.; Abe, S.; Ueno, T. Observation of Gold Sub-Nanocluster Nucleation within a Crystalline Protein Cage. *Nat. Commun.* **2017**, *8*, 14820.
(50) Yu, Y.; Luo, Z.; Teo, C. S.; Tan, Y. N.; Xie, J. Tailoring the Protein Conformation to Synthesize Different-Sized Gold Nanoclusters. *Chem. Commun.* **2013**, *49*, 9740−9742.
(51) Calvaresi, M.; Zerbetto, F. Baiting Proteins with C60. *ACS Nano* **2010**, *4* (4), 2283−2299.
(52) Calvaresi, M.; Zerbetto, F. Fullerene Sorting Proteins. *Nanoscale* **2011**, *3* (7), 2873.
(53) Beloqui, A.; Cortajarena, A. L. Protein-Based Functional Hybrid Bionanomaterials by Bottom-up Approaches. *Curr. Opin. Struct. Biol.* **2020**, *63*, 74−81.
(54) Aires, A.; Llarena, I.; Moller, M.; Castro-Smirnov, J.; Cabanillas-Gonzalez, J.; Cortajarena, A. L. A Simple Approach to Design Proteins for the Sustainable Synthesis of Metal Nanoclusters. *Angew. Chem., Int. Ed.* **2019**, *58*, 6214−6219.
(55) Chen, Z.; Ma, L.; Liu, Y.; Chen, C. Applications of Functionalized Fullerenes in Tumor Theranostics. *Theranostics* **2012**, *2*, 238−250.
(56) Soldà, A.; Cantelli, A.; Di Giosia, M.; Montalti, M.; Zerbetto, F.; Rapino, S.; Calvaresi, M. C60@lysozyme: A New Photosensitizing Agent for Photodynamic Therapy. *J. Mater. Chem. B* **2017**, *5* (32), 6608−6615.
(57) Di Giosia, M.; Bomans, P. H. H.; Bottoni, A.; Cantelli, A.; Falini, G.; Franchi, P.; Guarracino, G.; Friedrich, H.; Lucarini, M.; Paolucci, F.; et al. Proteins as Supramolecular Hosts for C60: A True Solution of C60 in Water. *Nanoscale* **2018**, *10* (21), 9908−9916.
(58) Fleischer, C. C.; Payne, C. K. Nanoparticle-Cell Interactions: Molecular Structure of the Protein Corona and Cellular Outcomes. *Acc. Chem. Res.* **2014**, *47* (8), 2651−2659.
(59) Zhang, N.; Mei, K.; Guan, P.; Hu, X.; Zhao, Y. Protein-Based Artificial Nanosystems in Cancer Therapy. *Small* **2020**, *16* (23), 1907256.
(60) Guevel, X. L.; Daum, N.; Schneider, M. Synthesis and Characterization of Human Transferrin-Stabilized Gold Nanoclusters. *Nanotechnology* **2011**, *22* (27), 275103.
(61) Cantelli, A.; Piro, F.; Pecchini, P.; Di Giosia, M.; Danielli, A.; Calvaresi, M. Concanavalin A-Rose Bengal Bioconjugate for Targeted Gram-Negative Antimicrobial Photodynamic Therapy. *J. Photochem. Photobiol., B* **2020**, *206*, 111852.
(62) Zhang, X.; Chen, M.; Zhang, Y.; Hou, Y.; Wu, Y.; Yao, M.; Li, L.; Shi, L.; Liu, T.; Hu, B.; et al. Monoclonal-Antibody-Templated Gold Nanoclusters for HER2 Receptors Targeted Fluorescence Imaging. *ACS Appl. Bio Mater.* **2020**, *3* (10), 7061−7066.
(63) Zhang, P.; Yang, X. X.; Wang, Y.; Zhao, N. W.; Xiong, Z. H.; Huang, C. Z. Rapid Synthesis of Highly Luminescent and Stable Au20 Nanoclusters for Active Tumor-Targeted Imaging in Vitro and in Vivo. *Nanoscale* **2014**, *6* (4), 2261−2269.
(64) Wiley, D. T.; Webster, P.; Gale, A.; Davis, M. E. Transcytosis and Brain Uptake of Transferrin-Containing Nanoparticles by Tuning Avidity to Transferrin Receptor. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110* (21), 8662−8667.
(65) Zhang, X. D.; Wu, D.; Shen, X.; Liu, P. X.; Fan, F. Y.; Fan, S. J. In Vivo Renal Clearance, Biodistribution, Toxicity of Gold Nanoclusters. *Biomaterials* **2012**, *33* (18), 4628−4638.
(66) Zhang, C.; Bu, W.; Ni, D.; Zuo, C.; Cheng, C.; Li, Q.; Zhang, L.; Wang, Z.; Shi, J. A Polyoxometalate Cluster Paradigm with Self-Adaptive Electronic Structure for Acidity/Reducibility-Specific Photothermal Conversion. *J. Am. Chem. Soc.* **2016**, *138* (26), 8156−8164.
(67) Zhang, W.-B.; Yu, X.; Wang, C.-L.; Sun, H.-J.; Hsieh, I-F.; Li, Y.; Dong, X.-H.; Yue, K.; Van Horn, R.; Cheng, S. Z. D. Molecular Nanoparticles Are Unique Elements for Macromolecular Science: From "Nanoatoms" to Giant Molecules. *Macromolecules* **2014**, *47* (4), 1221−1239.
(68) Abdulhalim, L. G.; Kothalawala, N.; Sinatra, L.; Dass, A.; Bakr, O. M. Neat and Complete: Thiolate-Ligand Exchange on a Silver Molecular Nanoparticle. *J. Am. Chem. Soc.* **2014**, *136* (45), 15865−15868.
(69) Ma, B.; Wang, S.; Liu, F.; Zhang, S.; Duan, J.; Li, Z.; Kong, Y.; Sang, Y.; Liu, H.; Bu, W.; et al. Self-Assembled Copper-Amino Acid Nanoparticles for in Situ Glutathione "aND" H 2 O 2 Sequentially Triggered Chemodynamic Therapy. *J. Am. Chem. Soc.* **2019**, *141* (2), 849−857.
(70) Aires, A.; Maestro, D.; Ruiz del Rio, J.; Palanca, A. R.; Lopez-Martinez, E.; Llarena, I.; Geraki, K.; Sanchez-Cano, C.; Villar, A. V.; Cortajarena, A. L. Engineering Multifunctional Metal/Protein Hybrid Nanomaterials as Tools for Therapeutic Intervention and High-Sensitivity Detection. *Chem. Sci.* **2021**, *12*, 2480−2487.

https://doi.org/10.1021/accountsmr.1c00065
Acc. Mater. Res. 2021, 2, 594−605