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Incorporation of Graphene Quantum Dots, Iron, and Doxorubicin in/on Ferritin Nanocages for Bimodal Imaging and Drug Delivery

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Abstract: Graphene quantum dots (GQDs) have been emerging as the next generation
bioimaging agents because of their intrinsic strong fluorescence, photostability, aqueous
stability, biocompatibility, and facile synthesis. In this work, GQDs are encapsulated in ferritin
protein nanocages to develop multi-functional nanoplateforms towards multi-modal imaging and

cancer therapy. Encapsulation of GQDs is expected to reduce quick excretion of ultra-small GQDs from the body, which has seriously limited their bioimaging efficiency. To expand the functionality of protein nanocages as multi-modal imaging nanoprobe capable of both fluorescence and magnetic resonance imaging (MRI) for superficial and deep tissue imaging, GQDs and iron are encapsulated inside the core of AfFtn-AA (an engineered ferritin nanocage derived from the archaeon *Archaeoglobus fulgidus*). The co-encapsulation is achieved through an iron-mediated, self-assembly of ferritin dimers that results in the formation of GQD-iron complex in the ferritin nanocages ((GQDs/Fe)AA). The complex is characterized by HRTEM, XRD, SEC, DLS, and fluorescence spectroscopy. The (GQDs/Fe)AA shows high relaxivities in MRI, and pH-sensitive fluorescence with strong fluorescence at low pH values and on MDA-MB-231 cells. As an imaging agent and a drug nanocarrier, (GQDs/Fe)AA exhibits negligible cytotoxicity on the cells and a high loading capacity (35%) of doxorubicin. Taken together, the (GQDs/Fe)AA shows promising applications in cancer diagnosis and therapy as a pH-responsive fluorophore, MRI agent, and drug nanocarrier.

1. Introduction

Development of multi-functional nanomaterials has been widely explored in recent years for different biomedical applications. In particular, the integration of diagnostic and therapeutic agents with biomolecules, which induce biological functions and provide a safe interface with biological environment, is highly desirable.^[1]

The unique properties of graphene derivatives have made them attractive nanomaterials in drug delivery and biomaging.^[2-6] Among graphene derivatives, graphene quantum dots (GQDs) have minimum toxicity owing to their ultra-small size (<10 nm) and oxygen functional groups in their structure.^[7-9] The exceptional physicochemical properties of GQDs, such as extreme physiological stability and strong fluorescence, have introduced them as novel category of bioimaging agents with tunable photoluminescence for *in vivo* and *in vitro* bioimaging.^[4,10,11]

Photo stability, low expense, and facile synthesis are distinguishable characteristics of GQDs from commonly used expensive organic dyes and fluorescent proteins that are often accompanied with photobleaching. GQDs are considered as biocompatible nanomaterials in contrast to inorganic semiconductor quantum dots.^[7-9,12]

Despite the attractive properties of GQDs in bioimaging, their ultra-small size presents a drawback. A gradual decrease in fluorescence intensity of GQDs in mice tissues, with no fluorescence from any portion of the body at 24h post-injection, is explained by rapid clearance of GQDs from kidney due to their small size.^[9,13] Similar results have been reported for other carbon quantum dots, which rapidly excreted in the first hours regardless of their injection routes.^[14] Furthermore, the ultra-small size of GQDs limits their EPR effect in passive targeting.^[15] Additionally, undesired interaction of quantum dots with biomolecules leads to loss of their fluorescence, affecting their bioimaging efficiency.^[16] To address these drawbacks, encapsulation or surface coating of GQDs (e.g., with polydopamine) has been shown to prevent their quick excretion from the body and results in an efficient *in vivo* imaging.^[17,18]

The incorporation of GQDs as bioimaging agent, within protein nanocages such as ferritin, has not been reported. The core-shell structure and multi-subunit composition of ferritin nanocages provide a versatile multi-functional biomolecular platform for loading of different functional agents.^[19,20] The cage-like structure of ferritin nanocages is formed by the self-assembly of 24 subunits in the presence of divalent metal ions or at high ionic concentration.^[21-23] Encapsulation in ferritin nanocages has been shown to increase the blood circulation time of loaded agents by reducing clearance by macrophages.^[24] The protein shells also serve as protecting layers for the loaded materials, preventing their undesired interaction with other biomolecules, and preserving the fluorescence properties of loaded fluorophores. Furthermore, they are shown to be biocompatible with less side effects in cancer treatment by eliminating the undesired release of encapsulated materials and targeting of cancerous cells through their intrinsic affinity to transferrin receptors (TfR-1).^[25,26]

Previous research has reported the integration of different drug molecules and imaging agents within the internal cavity or external shell of ferritin nanocages for cancer therapy and diagnosis.^[27-35] Loading of doxorubicin (DOX) in ferritin nanocages for cancer therapy has been widely studied; however, the low efficiency of DOX-loading in ferritin poses a challenge. Enhancement in loading capacity of DOX was achieved by pH-dependent disassembly-reassembly of ferritin^[27,28] and incubation of ferritin with DOX-metal complexes.^[29] To explore the use of ferritin nanocages as contrast enhancers in biomedical imaging, iron,^[23] manganese,^[36] and gadolinium^[37,38] have been loaded onto the nanocages. The significant enhancement in relaxivities shows their potential application in magnetic resonance imaging (MRI).^[39] In addition to MRI, ferritin has also attracted applications for multi-modal imaging that includes fluorescence, photoacoustic, and positron emission tomography (PET) imaging.^[30,32,33] Among the imaging methods, simultaneous fluorescence imaging and MRI is emerging as a powerful diagnosis tool without radioactivity.^[40] Fluorescence imaging possesses excellent sensitivity, rich color choice, safety and simplicity, however, its low light penetration and strong background noise limit its main application in cellular and superficial tissue imaging. On the other hand, MRI, with high spatial resolution, can visualize deep tissues but it suffers from insensitivity.^[41] Thus, the development of dual imaging nanoprobes, which combines the advantages of MRI and fluorescence imaging is of great importance for imaging-guided surgeries and early diagnosis of diseases. Fluorescence imaging using ferritin nanocages has been reported by incorporating Cy5.5,^[32,42] fluorescein-5-maleimide,^[34,43] AlexaFluor750,^[35] green fluorescent protein (GFP),^[30] indocyanine green (ICG) dye, and fluorescein isothiocyanate,^[44] which were mainly attached to the outer surface of nanocages.

Here, we present a multimodal imaging nanostructure based on engineered ferritin protein nanocages (AfFtn-AA) that encapsulate GQDs and iron ((GQDs/Fe)AA) for simultaneous fluorescence imaging and MRI. The genetically engineered ferritin was derived from the archaeon *Archaeoglobus fulgidus* and synthesized using recombinant technology. Subsequently,

GQD sheets were encapsulated in nanocages during self-assembly of dimers induced by the addition of iron (**Scheme 1**). The iron or manganese-loaded AfFtn-AA has been shown to enhance contrast in MRI.^[23,36] (GQDs/Fe)AA is introduced as a pH-responsive fluorescent probe, which exhibited strong fluorescence in cellular imaging and high relaxivities in MRI. Possibility of DOX-loading with high loading capacity in the structure of (GQDs/Fe)AA, suggests its application as a multimodal therapeutic agent for simultaneous cancer diagnosis and therapy.

2. Results and Discussion

2.1. Characterization of GQD/iron-loaded nanocages

Purified engineered ferritin (AfFtn-AA) (Figure S1) was utilized to encapsulate GQDs and iron. The dimeric subunits of AfFtn-AA self-assembled into GQDs/Fe-loaded nanocages ((GQDs/Fe)AA) upon addition of iron and GQDs. To assess the effect of GQDs on the formation of nanocages, the iron-loaded ferritin nanocages ((Fe)AA) were also prepared in the absence of GQDs. Characterizations were performed on both (Fe)AA and (GQDs/Fe)AA. The aqueous dispersions of (Fe)AA and (GQDs/Fe)AA were stable for several weeks at 4°C without any precipitation.

The iron-mediated assembly of dimers, along with the morphology of nanocages were examined by TEM of negative-stained samples. The (Fe)AA sample showed a nearly spherical core-shell structure after the addition of 500 Fe/24-merric ferritin (Fe500)AA with a narrow size distribution of the external diameter of 12.5 ± 0.7 nm (**Figure 1a**). The observation is in agreement with the typical dimension of ferritin nanocages.^[21] The clearly visible nanocages in (GQDs/Fe)AA with the same cage-like structure suggests that AfFtn-AA dimers retain the self-assembly property upon encapsulation of GQDs (Figure 1b). However, the size distribution of (GQDs/Fe)AA nanocages in Figure 1b showed a slight increase in the average external diameter of nanocages to 13.5 ± 1.0 nm. The change in the hydrodynamic diameter of nanocages after

encapsulation of GQDs was also monitored using DLS technique, which showed a shift in the hydrodynamic diameter of nanocages from 13.3 nm to 15.5 nm (Figure 1c).

Iron and high ionic concentration have been reported to mediate the self-assembly of AfFtn-AA subunits into nanocage structures.^[22,23] To investigate if iron was crucial for the loading of GQDs, experiments were performed in the absence of iron or GQDs. It was evidenced that almost 60% of the initial AfFtn-AA dimers were assembled to form (Fe)AA nanocages, while it slightly increased to 75% for (GQDs/Fe)AA. It is probably due to the interaction of GQDs with iron ions, which might facilitate iron-mediated assembly of AfFtn-AA subunits. Metal ions, such as iron ions, can easily form complexes with GQDs due to their sp²-regions and oxygen containing groups.^[45] The salt-mediated assembly of only 15% of dimers into nanocages were observed in AA+GQDs in the absence of iron, demonstrating the crucial role of iron in the high-yield formation of nanocages and encapsulation of GQDs. GQDs alone failed to induce the assembly of dimers.

2.2. Characterization of nanocores

Complete separation of free species after the self-assembly of nanocages was essential for precise characterization of loaded iron and GQDs. Thus, SEC was employed in addition to filtration to separate free GQDs, iron and dimers, and to collect protein nanocages containing loaded iron and GQDs (Figure S2).

The morphology and crystal structure of free GQDs, along with the core of nanocages in (Fe)AA and (GQDs/Fe)AA were investigated using high-resolution electron microscopy and XRD spectroscopy. The typical XRD peak of hexagonal GQDs at 22.4 degrees related to the interlayer spacing of 0.397 nm (002) is shown in **Figure 2a**. The XRD pattern of (Fe)AA also displayed four distinct peaks at 32.2, 45.9, 57.1, and 66.7 degrees equal to the D-spacing of 0.278 (110), 0.198 (113), 0.161(115), and 0.14 (106), respectively, which is in agreement with ferrihydrite XRD pattern.^[46-48] It demonstrates the ferrihydrite crystal structure of loaded iron

in the core of ferritin. The XRD pattern of (GQDs/Fe)AA revealed all diffraction peaks of ferrihydrite as well as prominent peaks of GQDs, which confirms the formation of ferrihydrite crystal along with loading of GQDs in the core of ferritin. Although no drastic variations were found in the position of ferrihydrite peaks in (GQDs/Fe)AA, the corresponding peaks of GQDs were left shifted by 2 degrees to 20.8 and 27.8 degrees. This shift was not observed in the GQDs peak of control sample containing physical mixture of (Fe)AA and GQDs. It demonstrates an increase of 0.03 nm in the interlayer spacing of encapsulated GQDs compared to free GQDs, which can be explained by incorporation of iron molecules within the layers of GQDs and formation of metal-complexes with functional groups of GQDs.^[45] Increase in the interlayer spacing of graphene layers after incorporation with magnetic nanoparticles, was also evidenced in other investigations.^[49] Additionally, a broadening in the (002) peak of GQDs was observed which might be due to the lattice distortion in the stacking order of graphene layers.^[50]

The HRTEM image of GQDs in Figure 2b confirms the high crystallinity of GQD sheets with lattice spacing of 0.33 nm and 0.275 nm, which correspond to the interlayer distance and in-plane lattice spacing of graphitic carbon, respectively. The GQDs were uniformly dispersed with an average diameter of 2.85 ± 0.75 nm, and a relatively narrow size distribution. The height profile of GQDs, obtained from AFM images showed a thickness of < 2 nm, suggesting sheet like structure of GQDs (Figure S3).

The crystal structure of nanocores of (Fe)AA and (GQDs/Fe)AA were revealed in HRTEM images using the non-stained protein samples. The HRTEM image of (Fe)AA in Figure 2c showed formation of single crystalline structure of ferrihydrites in the core of ferritin with an average diameter of 1.80 ± 1.14 nm. The measured lattice spacing of 0.2 and 0.235 are consistent with the typical ferrihydrite crystal structure.^[47,48] In contrast to (Fe)AA, HRTEM image of non-stained (GQDs/Fe)AA in Figure 2d demonstrated polycrystalline structures in the cores of nanocages attributed to both GQDs and ferrihydrite crystals. This result revealed corresponding lattice spacing of ferrihydrite (0.23, 0.24 nm), interlayer distance of GQDs (0.36, 0.37 nm), and

in-plane lattice spacing of GQDs (0.27, 0.28 nm), indicating existence of both ferrihydrite and GQDs in the core of (GQDs/Fe)AA. Additionally, incorporation of GQDs with iron nanocrystals resulted in a considerable increase in the size and size distribution of GQDs/iron nanocores, with average diameter of 4.70 ± 1.96 nm. As shown in Figure 2c and 2d, the size distribution of nanocrystals in the cores increased from ~1-4 nm in (Fe)AA to ~2-9 nm in (GQDs/Fe)AA. It also resulted in a slight increase in the external diameter of nanocages in (GQDs/Fe)AA, observed in Figure 1b and 1c.

The formation of (GQDs/Fe)AA was visible from the color change from brilliant yellow in (Fe)AA to yellow-brown in (GQDs/Fe)AA upon loading of GQDs (Figure 3a (inset) and S4a).

To investigate the effect of loaded GQDs on UV-absorbance of nanocages, the spectra of (GQDs/Fe)AA were compared to those of (Fe)AA and GQDs. Free GQDs displayed two broad absorption peaks at 360 and 230 nm originating from $n - \pi^*$ transitions of C=O bonds and $\pi - \pi^*$ transitions of the aromatic C=C bonds, respectively (Figure S4b).^[51] The typical UV absorption peak of biological macromolecules at 280 nm, which is attributed to the absorbance of tyrosine and tryptophan amino acids,^[52] in addition to a sharp absorption peak at 228 nm are observed in the UV absorption of (Fe)AA and (GQDs/Fe)AA (**Figure 3a**). Although the UV-absorption peaks of GQDs were covered by the strong UV absorbance of proteins as the main phase of nanocages, an increase in the UV absorption of nanocages was observed after encapsulation of GQDs. In addition, the UV absorbance of (GQDs/Fe)AA increased by increasing feeding concentration of GQDs at the constant concentration of ferritin, indicating higher loading of GQDs by increasing R values (feeding mass ratio of GQDs to ferritin dimers).

To investigate the entrapment of GQDs and iron in nanocages, their co-elution from an SEC column was evaluated by recording chromatograms of (GQDs/Fe)AA and (Fe)AA (Figure 3b).

Prominent UV wavelength of GQDs at 230 nm, in which free GQDs showed the highest intensity of SEC peak (Figure S4c), and specific UV wavelengths of proteins at 280 nm and iron at 310 nm were used for this purpose.^[53] A considerable increase was observed in the

intensity of SEC peak of (GQDs/Fe)AA at 230 nm in elution fractions of nanocages compared to that of (Fe)AA. Therefore, the ratio of intensity of peak at 230 nm to intensity of peak at 280 nm increased by 45% in (GQDs/Fe)AA. Additionally, iron-peaks were observed in elution fractions of ferritin nanocages in (Fe)AA and (GQDs/Fe)AA (Figure 3b, inset). These results demonstrate integration of GQDs and iron within nanocages leading to their co-elution in elution fractions of nanocages. Lower intensity of iron-peak in (GQDs/Fe)AA compared with (Fe)AA is due to less amount of loaded iron in (GQDs/Fe)AA because of the presence of GQDs within the confined space of the core. This result was further confirmed in ICP analysis showing ~100% and ~90% loading of feeding iron atoms in (Fe)AA and (GQDs/Fe)AA, respectively.

Fluorescence spectroscopies of GQDs and (GQDs/Fe)AA were studied to investigate possible application of (GQDs/Fe)AA in cellular imaging. GQDs exhibited an excitation-dependent emission, which agrees well with the PL behavior of carbon quantum dots,^[54] with the highest PL at excitation wavelength of 350 nm and emission at 520 nm (Figure 3c). Strong green fluorescence of GQDs solution after exposing to UV light was easily observed by naked eyes (Figure 3c, inset). While free GQDs exhibited strong green fluorescence suggesting their potential application in cellular imaging, quench of fluorescence of GQDs after entrapment inside the cages was possible. The PL spectra of (GQDs/Fe)AA revealed significant quench in GQDs fluorescence at pH=7.4 (Figure 3d). This behavior is due to the high concentration of GQDs in the confined space of core of nanocages. The close proximity results in efficient Förster resonance energy transfer (FRET) between GQDs sheets prior to energy emissions and quench of their fluorescence.^[55,56] Additionally, π - π interaction between aromatic rings of GQDs and amino acids as well as electrostatic interactions between GQDs and Fe^{3+} ions in the cores may further enhance electron transfer leading to the quench of fluorescence.^[2,57] However, (GQDs/Fe)AA exhibited a considerable increase in the fluorescence intensity by decreasing the pH of the solution from 7.4 to 5 and 2. Ferritin nanocages have been reported to disassemble at low pH values,^[58] leading to the release of loaded GQDs and recovery of their fluorescence

property. The fluorescence of (Fe)AA remained unchanged upon pH reduction, indicating that the fluorescence recovery originates from the released GQDs from (GQDs/Fe)AA. This pH-dependent fluorescence change of (GQDs/Fe)AA is important in bioimaging of cancerous tissues because the extracellular tumor tissues, endosomes, and intracellular lysosomes are acidic, while the body fluid is neutral.

The PL spectra of (GQDs/Fe)AA was also affected by the ratio of loaded GQDs to ferritin nanocages (Figure 3e). A change in the PL spectra of (GQDs/Fe)AA from a single peak at 450 nm to a flat-top spectrum and finally to a double-peak spectrum at around 450 and 520 nm was observed by increasing R values (feeding mass ratio of GQDs to ferritin). The emission wavelengths at 520, and 450 nm correspond to GQDs and ferritin, respectively (Figure 3c and S3d). This result indicates higher loading of GQDs by increasing R values, which led to the appearance of GQDs peak at high R values. Similar change in the shape of PL spectra of mixture of GQDs and ferritin was observed by increasing R values (Figure S4e). Quantification of GQDs revealed a capacity of 8% (percentage of $W_{\text{loaded GQDs}}/W_{\text{nanocages}}$) for loading of GQDs in the cores of (GQDs/Fe)AA at R=0.5.

While the nature of nanocage cores is hydrophilic, the encapsulation of hydrophilic GQDs inside the cores during the iron-mediated assembly of ferritin is expected. However, their interaction with outer shell of nanocages would be also possible. To ensure that GQDs are loaded inside the cores, the PL spectra of (Fe)AA+GQDs were compared with free GQDs and (GQDs/Fe)AA at the same concentration of GQDs and ferritin before separation of unbounded GQDs at R=0.3 and 0.15, (Figure S4f and S4g). Possible interaction of GQDs with outer surface of (Fe)AA should result in a quench in fluorescence of GQDs. However, addition of GQDs to (Fe)AA did not cause any quench in fluorescence intensity of GQDs, while a reduction in emission intensity of (GQDs/Fe)AA was observed compared to free GQDs. After separation of free GQDs, (Fe)AA+GQDs only exhibited the single emission peak of ferritin at 450 nm, which revealed complete separation of GQDs and the absence of loaded GQDs in the mixture. The

absence of considerable decrease in fluorescence intensity of GQDs in (Fe)AA+GQDs along with its PL behavior after separation of unbounded GQDs indicate that any external interaction between GQDs and outer shell of the nanocages is unlikely.

2.3. DOX loading

The potential application of (GQDs/Fe)AA as a multifunctional carrier for drug delivery, and imaging was evaluated by loading of DOX. Since loading of DOX in the core of nanocages during self-assembly of dimers was expected to reduce amount of loaded GQDs and Fe, another procedure was employed for DOX loading. DOX was simply loaded on nanocages during mixing of drug molecules and (GQDs/Fe)AA nanocages, followed by separation of free DOX (Figure S5a).

A high loading efficiency of 83 % (percentage of $W_{\text{loaded DOX}}/W_{\text{feed DOX}}$) and loading capacity of 35% (percentage of $W_{\text{loaded DOX}}/W_{\text{nanocages}}$) were achieved. To investigate successful loading of DOX on (GQDs/Fe)AA, fluorescence spectra of (GQDs/Fe)AA-DOX and free DOX at the same DOX concentration was studied (**Figure 4**). Significant fluorescence quenching was observed for DOX after loading on (GQDs/Fe)AA, suggesting close proximity of DOX to (GQDs/Fe)AA, verifying its interaction with nanocages. A color change from pink to brownish-pink was also visible during the loading process (Figure 4, inset).

To investigate DOX loading mechanism, the same experiment was repeated for mixture of dimers of ferritin and DOX, which exhibited no change in fluorescence intensity of mixed DOX compared to free DOX (Figure S5b). PL spectra of mixture after separation of free DOX showed negligible fluorescence, demonstrating separation of all DOX molecules and no interactions between DOX and ferritin dimers. This result confirms the attachment of drug molecules to nanocages due to their morphology and specific structure after the assembly of dimers and formation of the nanocage. The size of DOX molecules, along with preloading of cores with GQDs and iron, may limit their penetration through the channels and internalization

inside the cores. Thus, DOX might be mostly attached to the exterior surface and channels of nanocages. Ferritin has 14 major channels including 8 channels at the 3-fold symmetries and 6 channels at the 4-fold symmetries. Studies showed that DOX molecules can form complexes with iron ions that exist in the channels of nanocages, which may significantly contribute to their high loading onto ferritin nanocage^[29,59]. Previous reports suggest that particles <2 nm can easily penetrate through the channels due to highly dynamic and flexible feature of channels.^[57] Considering 1.5 nm as maximum possible molecular size of DOX,^[60] it is not impossible that a fraction of the 35% DOX is loaded inside the ferritin shell. Taken together, the loading on the exterior surface and the channels may contribute to the high loading of DOX onto our ferritin nanocage.

Encapsulation of DOX in the core of nanocages using pH-dependent disassembly-reassembly of ferritin is the most common procedure reported in literatures.^[27-29,31] The reported loading capacity ranges from 2.8%,^[28] 4%,^[27] 10.87%,^[31] to 21.82%^[29]. The disassembly-reassembly process is not fully reversible and affects the rearrangement of protein conformation as well as protein loss during loading.^[58] In this work, high loading capacity of 35% was achieved through simple incubation of DOX with (GQDs/Fe)AA with reduced protein loss. While various efforts have been reported to address the challenge of low loading efficiency of DOX in ferritin, such as by incubation of ferritin with DOX-metal complexes^[29] and by chemical or genetic modification of ferritin nanocages,^[29,31] the AfFtn-AA has been found to show superior ease of handling and better loading efficiency.

2.4. Fluorescence (Cellular) and Magnetic Resonance Imaging

Leveraging on pH-dependent fluorescence of (GQDs/Fe)AA and strong fluorescence of GQDs, potential application of (GQDs/Fe)AA in cellular imaging was examined. MDA-MB-231 cells were incubated with GQDs, (Fe)AA and (GQDs/Fe)AA, and the cells were observed with confocal microscope. The strong green fluorescence from GQDs in **Figure 5a** indicated the

translocation of nanomaterials through the cell-membrane and their localization in the nucleus and cytoplasm of the cells after 1 hr, which was desirable for imaging application of GQDs. Although fluorescence of GQDs quenched after encapsulation inside the cores of nanocages, a considerable green fluorescence was emitted from the cells incubated with (GQDs/Fe)AA (Figure 5b). Additionally, the cells incubated with (Fe)AA did not exhibit any noticeable fluorescence (Figure 5c), indicating that this green fluorescence originates from GQDs. The observation can be explained by the disassembly or degradation of nanocages and release of encapsulated GQDs along with iron in the acidic environment of intracellular lysosomes. The recovery of fluorescence of released GQDs after disassembly or degradation of nanocages at low pH values was demonstrated in Figure 3d. Although (GQDs/Fe)AA showed a clearly visible intracellular fluorescence, its fluorescence intensity was relatively lower than GQDs, which is also confirmed in their PL spectra in Figure 3d. It can be explained by incomplete or prolonged degradation of nanocages especially at pH 5, resulting in less amount of released GQDs and lower fluorescence intensity. Although the release mechanism of iron from ferritin inside the cells has not been fully understood, several investigations demonstrated that ferritin has to be degraded to release iron.^[61-63] The studies on different cell lines also reported that degradation of ferritin and release of iron occur in lysosomes by lysosomal proteases and they showed that acidic environment of lysosomes is crucial for this degradation.^[62,63] Therefore, the free GQDs released from degraded nanocages in lysosomes emitted the noticeable green fluorescence suggesting the potential use of (GQDs/Fe)AA for intra-cellular imaging.

To evaluate the application of (GQDs/Fe)AA as MRI contrast agent, MRI images of samples containing various concentrations of (GQDs/Fe)AA nanocages at different echo (T_E) and inversion (T_I) times were recorded using a 3.0 T whole-body MRI scanner. A noticeable darkening of T_2 -weighted images and brightening of T_1 -weighted images was observed by increasing concentration of nanocages and loaded iron, showing the dual contrast enhancement caused by (GQDs/Fe)AA (Figure 5d). For better evaluation of efficiency of this magnetic–

ferritin nanocomposite in enhancing contrast of MR images, the longitudinal (r_1) and transverse (r_2) relaxivities were obtained by measuring signal intensities of the MR images at various concentrations of (GQDs/Fe)AA. The intensity values were used to quantify the transverse (T_2) and longitudinal (T_1) relaxation times, followed by determining r_1 and r_2 as the gradients of the fitted linear line by plotting the inverse of T_1 and T_2 against loaded iron concentration (Figure 5e). The T_1 and T_2 relaxation times decreased sharply by increasing concentration of iron and the calculated r_1 and r_2 values for each protein nanocages are $727 \text{ mM}^{-1}\text{s}^{-1}$ and $1607 \text{ mM}^{-1}\text{s}^{-1}$, respectively. The r_1 value is comparable to that of Gd-loaded ferritin with 10 Gd/cage ($800 \text{ mM}^{-1}\text{s}^{-1}$).^[38] Additionally, the obtained relaxivities demonstrated a two orders of magnitude enhancement in relaxivities of iron-loaded modified ferritin compared to iron-loaded human ferritin with same amount of loaded iron ($r_1=2.3 \text{ mM}^{-1}\text{s}^{-1}$, $r_2=11 \text{ mM}^{-1}\text{s}^{-1}$).^[23,64] The higher relaxivity is likely due to more diffusion of water molecules through their channels.^[38] Free GQDs did not show noticeable decrease in relaxation times by increasing GQD concentration, and revealed very low r values (Table S1). The relaxivities of (Fe)AA and (GQDs/Fe)AA were comparable (Figure S6 and Table S1), indicating that the loaded iron was the main contrasting agent in (GQDs/Fe)AA nanocomposite.

2.5. Cytotoxicity

GQDs are known as biocompatible nanomaterials with low *in vivo* and *in vitro* toxicity on a series of cell lines.^[7-9] Ferritin is a natural protein found in most living systems suggesting their biocompatibility and biodegradability. As a result, an inconsiderable toxicity was expected to be observed for (GQDs/Fe)AA on majority of cell lines. Although ferritin has not shown any obvious cytotoxicity against several cell lines, such as MDA-MB-231, MCF-7, T47D, SKBR3, and HeLa,^[63,65] its significant cytotoxicity was reported on mouse embryonic fibroblast cells (MEFs).^[63] Additionally, protective effect of ferritin on proliferation of breast cancer cells restricted its biomedical application in treatment of breast cancer.^[65-67] Thus, in order to

1 evaluate any potential toxic effect of (GQDs/Fe)AA and its possible application in cancer
2 therapy, MTT assay was carried out on normal 3T3 MEF, and MDA-MB-231 breast cancer
3 cells at nanocage concentrations of 0.13-1.04 μM which is equivalent to 5-40 $\mu\text{g.mL}^{-1}$ of loaded
4 GQDs. It is a suitable range of concentration for cellular imaging, MRI and drug delivery. For
5 better understanding of the cytotoxic effect of (GQDs/Fe)AA, the cytotoxicity of control
6 samples including GQDs and (Fe)AA at the same ferritin and GQDs concentrations were
7 examined.
8

9 GQDs showed high cell viability (more than 85%) on the MDA-MB-231 cells at concentrations
10 of lower than 100 $\mu\text{g.mL}^{-1}$, while they exhibited similar cell viability on 3T3 cells at
11 concentrations of lower than 50 $\mu\text{g.mL}^{-1}$ (Figure S7a and S7b). It is in agreement with other
12 investigations, which studied cytotoxicity of GQDs on MDA-MB231 and 3T3 cell lines.^[6,7,68]
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14 Moreover, **Figure 6a** revealed the proliferative effect of (Fe)AA on MDA-MB-231 cells which
15 significantly decreased ($P < 0.05$) by increasing concentration of (Fe)AA. This observation is
16 consistent with several investigations which evidenced that ferritin stimulates proliferation of
17 breast cancer cells such as MDA-MB-321 cells.^[65-67] Although (Fe)AA did not show any
18 cytotoxicity on MDA-M231, it exhibited a low cytotoxicity with cell viability of 88% at ferritin
19 concentration of 1.04 μM on 3T3 cells. The cytotoxicity of (Fe)AA on 3T3 cells is because of
20 the release of iron from nanocages which generates damaging ROS. Our *in vitro* results for
21 (Fe)AA are consistent with previous findings which reported high resistance of cancerous cells
22 to iron toxicity, while MEFs exhibited considerably lower resistance and higher cytotoxicity.^[63]
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24 In an experiment reported by Asano *et al.*, the degradation of ferritin was observed in both iron-
25 replete and depleted conditions in non-cancerous MEFs, while ferritin only degrades in iron-
26 depleted conditions in cancerous cells.^[63] Therefore, it is expected that when 3T3 and MDA-
27 MB-231 cells are incubated with the same amount of nanocages, the ferritin starts to degrade
28 inside lysosomes. The increase of iron level inside the cells caused the degradation of ferritin
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to stop in MDA-MB-231 cells but continue in 3T3 cells as it is not cancerous. Hence, leading to higher iron release in 3T3 and higher toxicity.

As shown in Figure 6a and 6b, the cytotoxicity of (GQDs/Fe)AA is cumulative cytotoxicities of GQDs and (Fe)AA. It revealed degradation of nanocages and release of iron and GQDs in acidic environment of lysosomes, which was monitored by fluorescence of released GQDs in cellular imaging (Figure 5b). As a result, similar to GQDs and (Fe)AA, (GQDs/Fe)AA exhibited no significant toxicity on MDA-MB-231, and low cytotoxicity with cell viability of higher than 80% on 3T3 cells at ferritin concentration of lower than 1.04 μ M. Additionally, in contrast to (Fe)AA, (GQDs/Fe)AA did not stimulate proliferation of breast cancer MDA-MB-231 cells, making it superior than (Fe)AA for drug delivery and imaging in breast cancer therapy. For precise determination of the cytotoxicity of (GQDs/Fe)AA, *in vivo* experiments are imperative.

3. Conclusion

GQDs and iron were successfully loaded in the cores of modified ferritin nanocages with a polycrystalline structure attributed to GQDs and ferrihydrite crystals. A slight increase in the size of nanocages, along with an increase in the size of encapsulated nanocrystals were observed after encapsulation of GQDs. The (GQDs/Fe)AA showed quenching in fluorescence upon loading of GQDs in ferritin nanocage. The fluorescence was recovered after the release of GQDs from disassembled nanocages in acidic environment of the lysosome enabling the imaging of MDA-MB-231 breast cancer cells. (GQDs/Fe)AA shows high relaxivities in MRI, originated from loaded iron, which was comparable with the reported relaxivity of Gd-loaded ferritin. The high loading capacity of (GQDs/Fe)AA for DOX indicates its potential application in cancer therapy, along with MRI and pH-sensitive fluorescence imaging. (GQDs/Fe)AA as an imaging agent and a drug nanocarrier exhibited negligible cytotoxicity on MDA-MB-231 and 3T3 cells. It also eliminated the commonly observed proliferation effect of ferritin

nanocages on breast cancer MDA-MB-231 cells. The pH-sensitive fluorescence, high MRI relaxivities, high DOX-loading capacity and low cytotoxicity of (GQDs/Fe)AA demonstrate its promising application as a bimodal imaging agent in cancer diagnosis and therapy.

4. Experimental Section

Materials: Chloramphenicol, ampicillin, isopropyl β -D-1-thiogalactopyranoside (IPTG), 4',6-diamidino-2-phenylindole (DAPI), uranyl acetate, DOX, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) powder and paraformaldehyde were purchased from Sigma. Dimethylsulfoxide (DMSO), HNO₃ 68%, ammonium sulfate, ferrous sulfate, and Amicon ultra centrifugal filter units (10 kDa, 100 kDa MWCO) were purchased from Merck Millipore, and the Vulcan CX-72 carbon black was obtained from Cabot Corporation. The Dulbecco's Modified Eagle's Media (DMEM), penicillin streptomycin (Pen Strep), fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) and LB broth media were from Thermo Fisher Scientific. The plasmids containing engineered *A. fulgidus* ferritin gene that results in closed-pore structure were generous gifts from Professor Imke Schröder at University of California, Los Angeles. The MDA-MB-231 breast cancer and 3T3 mouse embryonic fibroblast (MEFs) cell lines were kind gifts from Professor Kathy Luo. A water purification system (Milli-Q Gradient) was utilized to provide ultrapure water.

Synthesis of GQDs: GQDs were synthesized by oxidation of CX-72 carbon black in presence of nitric acid.^[11] Briefly, 0.22 g CX-72 carbon black was mixed with 50 mL of 6 M nitric acid, followed by overnight refluxing of mixture at 135°C. The mixture was then cooled to room temperature, and remaining solid precipitate was separated using qualitative filter papers. The acquired liquid was warmed up to evaporate the nitric acid, and water was added to the obtained solid to prepare reddish aqueous dispersion of GQDs. The GQDs with lateral size of below 10

kDa were separated by ultrafiltration using 10 kDa MWCO Amicon ultra-centrifugal filter devices at 15,000×g for 30 min. The prepared GQDs were dried and stored for further experiments.

Synthesis of ferritin (AfFtn-AA): Engineered *A. fulgidus* ferritin (AfFtn-AA) was prepared following previously published methods.^[22] Briefly, *A. fulgidus* ferritin (AfFtn) gene was cloned into a pET-11a vector and amino acids of lysine250 and arginine151 were replaced by alanines by site directed mutagenesis. The *E. coli* transformed with the plasmid containing AfFtn-AA gene was grown on LB medium containing ampicillin and chloramphenicol as selection pressure for 16 hr. The overnight culture was then added into LB medium (1 L) containing the same antibiotics in a 250-rpm rotating chamber at 37°C. The gene expression was induced by addition of IPTG (1 mM) at optical density of ~0.6 at wavelength of 600 nm. After 4 hr of induction, the cells were centrifuged at 4000×g. The obtained cell pellet was resuspended in Buffer A (50 mM NaCl, 25 mM HEPES, pH 7.5) and incubated on ice for 30 min. The suspension was then sonicated for 20 min followed by heating at 85°C for 10 min to denature the *E. coli* proteins. The insoluble fractions containing cell debris and denatured proteins were separated by ultra-centrifugation at 274,000×g at 4°C for one hr. The ferritin in the supernatant was purified in a Fast Protein Liquid Chromatography (FPLC, AKTA, GE Healthcare) system with a 20 mL HiPrep Phenyl FF column (GE Healthcare). Column binding and elution of protein were done using Buffer B (buffer A+500 mM ammonium sulfate) and buffer A, respectively. The protein was analyzed using bicinchoninic acid (BCA) assay (BCA Kits, Thermo Fisher Scientific, Waltham, USA) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The purified protein was kept in buffer A at 4 °C.

Preparation of AfFtn-AA nanocages containing GQDs/iron nanocores ((GQDs/Fe)AA): Freshly prepared AfFtn-AA ferritin solution was exchanged with 1 M HEPES buffer at pH 8 and was concentrated to 1 μM using 10 kDa MWCO Amicon ultra centrifugal filter. The

aqueous GQDs solution (0.25 mg mL^{-1}) was added to the ferritin solution, followed by incubation at room temperature for 2 hr. Subsequently, a fresh ferrous sulfate solution in 0.1% HCl was added slowly in three steps to the mixture up to 1 mM Fe^{2+} . The mixture was incubated for 2 hr at room temperature, and for 14 hr at 4°C . The unbounded iron, GQDs and ferritin dimers were removed by filtration using 100 kDa MWCO Amicon ultra centrifugal filter at $4200\times g$ for 0.5 hr. To ensure complete separation of the unbounded materials, the mixture was treated with Superdex 200 10/300 GL column (a size exclusion chromatography, GE Healthcare), with Buffer A as the mobile phase. The collected purified (GQDs/Fe)AA nanocages were sent for characterizations. The same experiment was repeated for different control samples in the absence of GQDs and/or iron. Amount of loaded iron in nanocages was measured using inductively coupled plasma (ICP) analysis in an ICP-MS instrument (Perkin Elmer, ELAN DRC-e). The loaded GQDs were quantified by measuring the concentration of unbounded GQDs using their maximum UV-vis absorbance at 230 nm.

DOX-Loading: preparation of (GQDs/Fe)AA-DOX: DOX ($230 \text{ }\mu\text{M}$) was added to the (GQDs/Fe)AA solution ($0.7 \text{ }\mu\text{M}$) in HEPES buffer, followed by incubation at room temperature for 4 hr and at 4°C for 14 hr. The unbounded DOX molecules were separated by repeated ultrafiltration at $4200\times g$ for 30 min using 100 kDa MWCO Amicon ultra centrifugal filter. Loaded DOX concentration was measured by determining the amount of unbounded DOX, which was subtracted from the initial amount of DOX. A calibration curve generated at series concentrations of DOX ($0.01\text{--}10 \text{ }\mu\text{M}$) using their fluorescence intensities at excitation and emission wavelengths of 458 and 595 nm, respectively, was used for quantification of DOX.

Characterization of ferritin structures upon loading: Transmission electron microscopy (TEM, Tecnai T12, FEI), high resolution TEM (HRTEM, Philips CM300), X-Ray diffraction (XRD, Bruker D8 Advance) and nanosizer (Zetasizer, Malvern, UK) were employed to confirm successful encapsulation of iron and GQDs, along with their size and crystalline structure inside the cavities. To prepare TEM samples, suspensions of samples were placed on the copper grids

and allowed to dry at room temperature. In order to observe the structure of protein nanocages, the samples containing nanocages were negatively stained by 1.5% uranyl acetate, and the grids were dried under ambient condition. The encapsulated iron and GQDs inside the cores were observed using unstained sample. Size distribution and average particle size, were quantified from TEM images using the ImageJ software (U. S. National Institutes of Health, Bethesda, MA, USA). Fluorescence and UV spectra of nanocomplexes were recorded using a UV-vis spectroscopy (Shimadzu UV 2450, Kyoto, Japan) and a spectrophotometer with multiple detection modes (SpectraMax M5 Multi-Mode Microplate Reader).

MRI: MRI samples including (Fe)AA and (GQDs/Fe)AA loaded with 1000 Fe/cage (protein concentration range of 0-10 μM and GQDs at concentration range of 0-1 mg mL^{-1}) were embedded in 0.8% agarose gel in 24-well tissue culture plates. The samples in each well were imaged by a 3.0 T whole-body scanner (Magnetom Verio, Siemens) using a dedicated knee coil. Different inversion (TI) and echo times (TE) with slice thickness = 3.0 mm, matrix = 512×512 , field of view = 27 cm, and number of averages = 1 were applied for taking T1 and T2-weighted images. The T1-weighted images were obtained at inversion times of 30, 50, 100, 200, 400, 500, 600, 800, 100, 1200, 1800, 2000, and 2500 ms at TR (repetition time) = 2800 ms, TE = 8.5 ms. The T2-weighted images were obtained at echo times of 10, 15, 20, 40, 60, 80, 100, 150, and 200 ms at TR = 1000 ms. Finally, the longitudinal and transverse relaxation times (T1 and T2*) and relaxivities (r1 and r2) were calculated by measuring the signal intensity of MR images obtained for each sample at different TI and TE values (Supporting Information).

Cell culture: MDA-MB-231 breast cancer and 3T3 mouse embryonic fibroblast (MEFs) cell lines were used to examine cellular toxicity and imaging of the cells incubated with (GQDs/Fe)AA, (Fe)AA, and GQDs. The cells were seeded in the flasks containing DMEM medium, supplemented with 10% (v/v) FBS and 1% (v/v) Pen Strep, followed by incubation in a humid atmosphere at 37 °C and 5% CO₂.

In vitro cellular imaging: Cellular imaging experiments were carried out on MDA-MB-231 cells using confocal laser microscopy to study bioimaging application of (GQDs/Fe)AA and to visualize its uptake by the cells. The cells were cultured in eight-well confocal chambers (15,000 cells/well) and incubated for 24 hr to reach 80% confluency. The cell medium in each well was then replenished with fresh medium containing free GQDs (40 $\mu\text{g mL}^{-1}$), (GQDs/Fe)AA (1 μM), and (Fe)AA (1 μM). After 1 hr of incubation, the cells were washed twice with fresh medium followed by addition of DAPI to stain the nucleus. The cells were then incubated for 15 min, and washed twice with fresh medium and phosphate-buffered saline (PBS). Fixation of cells was carried out by their incubation with 100 μL of 4% paraformaldehyde for 10 min. Cells were subsequently washed with PBS and stored at 4°C in PBS. Finally, the fixed cells were imaged using a confocal laser scanning microscope (Carl Zeiss, LSM 510. Meta Laser). The experiment was repeated thrice.

In vitro cellular toxicity assay: Toxicity of (GQDs/Fe)AA, GQDs and (Fe)AA on MDA-MB-231 and 3T3 cells was assessed using MTT assay. The MDA-MB-231 and 3T3 cells were seeded on 96-well plates at density of 6,000 cells/well and 4,500 cells/well, respectively. After 24 hr of incubation, fresh media containing samples were replenished with previous media and the cells were incubated for another 48 hr. Samples included (Fe)AA and (GQDs/Fe)AA at nanocages concentrations of 0.13, 0.26, 0.52, 1.04, 2.08, and 2.6 μM , and free GQDs at 5, 10, 20, 40, 80, 100, 150, and 200 $\mu\text{g mL}^{-1}$. Each sample was added to more than three wells. Subsequently, 20 μL of MTT solution (5 mg mL^{-1}) was added to each well and the cells were incubated for 3 hr. After removing the cell medium, 150 μL DMSO was added to the wells to dissolve the formed formazan crystals. The relative cell viabilities were calculated after measuring the UV absorbance of each well at 570 nm using a microplate reader (SpectraMax M5 Multi-Mode Microplate Reader). The experiment was repeated thrice.

Statistical Analysis. Data are presented as the mean $\pm$ standard deviation from three independent experiments. Student's T-test was employed to analyze the statistical differences for two group

data sets by use of SPSS software. P values of less than 0.05 were considered as statistically significant differences. All cell viability results were normalized to the untreated group of cells.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Scheme 1. Schematic illustration of Steps for preparation of (GQDs/Fe)AA-DOX

Figure 1. TEM image and size distribution of (a) (Fe)AA, (b) (GQDs/Fe)AA. (c) Size distribution of (Fe)AA and (GQDs/Fe)AA measured by DLS.

Figure 2. (a) XRD patterns of GQDs, (Fe)AA, (GQDs/Fe)AA and (Fe)AA+GQDs. (b,c,d) HRTEM images and size distribution of (b) GQDs, (c) iron nanocrystals inside cores of (Fe)AA. (d) GQDs/Fe nanocrystals inside the cores of (GQDs/Fe)AA.

Figure 3. (a) UV-vis absorption spectra of (Fe)AA and (GQDs/Fe)AA at R=0.3 and 0.15 (feeding mass ratio of GQDs to ferritin dimers). Inset: photographs of aqueous dispersion of (Fe)AA (left) and (GQDs/Fe)AA (right). (b) SEC chromatogram of (Fe)AA and (GQDs/Fe)AA at 230, 280 and 310 nm. Inset: a zoom on 310 nm at A4-A6 fraction. (c) PL spectra of GQDs at different excitation wavelengths. Inset: pictures of aqueous dispersion of GQDs under visible light (left) and UV light at 365 nm (right). (d) PL spectra of (GQDs/Fe)AA and (Fe)AA compared to same concentration of GQDs, before and after disassembly of nanocages by reducing pH from 7.4 to 2 and 5. (e) PL spectra of (Fe)AA and (GQDs/Fe)AA at R=0.05-0.5, after separation of unbound materials.

Figure 4. (a) PL spectra of (GQDs/Fe)AA-DOX and DOX at the same concentration of drug at excitation wavelength of 458 nm. Inset: photographs of mixture of (GQDs/Fe)AA and **DOX** after incubation times of 0, 30 and 60 minutes.

Figure 5. Fluorescence pictures of MDA-MB-231 cells incubated with (a) 40 $\mu\text{g/mL}$ GQDs, (b) 1.04 μM (GQDs/Fe)AA and (c) 1.04 μM (Fe)AA for 1 hr. (Right: Overlay fluorescence image of nucleus stained with blue DAPI and green GQDs), Left: green fluorescence of GQDs). Scale bars are 50 μm . (d) T_1 and T_2 -weighted images of (GQDs/Fe)AA embedded in a 0.8% agarose gel at different concentrations and at $T_I=1800$ ms and $T_E=200$ ms. (e) Effect of concentration of loaded iron on T_1 and T_2 relaxation times of (GQDs/Fe)AA, to determine its r_1 and r_2 values.

Figure 6. Relative viability of (a) MDA-MB-231 (b) 3T3 cells, incubated with GQDs, (Fe)AA, and (GQDs/Fe)AA at different concentrations of nanocages and GQDs for 48 h. Data are shown as the mean $\pm$ standard deviation. Symbols indicate $P < 0.05$ versus (+) (GQDs/Fe)AA group at the same nanocage concentration, (*) same group at nanocage concentration of 1.04 μM .

Loading of graphene quantum dots, iron, and doxorubicin into ferritin protein nanocages show promising applications as multimodal carrier for pH-responsive fluorescence and magnetic resonance imaging as well as drug delivery for cancer diagnosis and therapy.

Multifunctional Ferritin Nanocages

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Incorporation of Graphene Quantum Dots, Iron, and Doxorubicin in/on Ferritin Nanocages for Bimodal Imaging and Drug Delivery

Supporting Information

Incorporation of Graphene Quantum Dots, Iron, and Doxorubicin for Expanded Multifunctionality of Ferritin Nanocages

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1. Experimental Section

Preparation of AfFtn-AA nanocages containing iron/GQDs nanocores ((GQDs/Fe)AA): Complete Separation of free species after self-assembly of nanocages was essential for precise characterization of loaded iron and GQDs. Thus, in addition to filtering, a chromatography method was employed to particularly separate free GQDs. Small molecules like free iron could be easily separated in a Hiprep 26/10 desalting column, however, ferritin nanocages and GQDs were eluted at the same fractions, and separation of free GQDs was not possible using this column (Figure S2a). Thus, the Superdex 200 SEC column was employed, which eluted protein nanocages, dimers and GQDs in elution volume of 8-14 mL (fractions of A₄, A₅, A₆), 14-18 mL (fractions of A₇, A₈) and 18-24 mL (fractions of A₉, A₁₀, A₁₁), respectively (Figure S2b). After SEC of (GQDs/Fe)AA, the protein nanocages containing loaded iron and GQDs in fractions of A₄-A₆ were collected for characterization of nanocores.

Magnetic resonance Imaging: To determine the relaxivities of nanocomposite, the intensities of MR images, which were obtained at different echo (T_E) and inversion (T_I) times at various concentrations of (GQDs/Fe)AA, (Fe)AA and GQDs, were measured using Imagej. Subsequently, the following correlations were fitted to intensity values (I_{T_1} , I_{T_2}) versus T_I and T_E times using MATLAB to calculate the longitudinal (T₁) and transverse (T₂) relaxation times for each sample, respectively.

$$I_{T_1} = A - Be^{-T_I C}, \quad C = 1/T_1$$

$$I_{T_2} = Ae^{-T_E B} + C, \quad B = 1/T_2$$

The $1/T_1$ and $1/T_2$ data were plotted against iron concentrations, and the slope of the best fitted linear lines determined the longitudinal (r_1) and transverse (r_2) relaxivities.

2. Results

2.1. Characterization of nanocages

Figure S1. FPLC chromatogram of AfFtn-AA dimers solution containing impurities at 280 nm

2.2. Characterization of nanocores

Figure S2. Chromatograms of GQDs at 230 nm, and (Fe)AA nanocages containing dimers at 280 nm, using (a) Desalting column, and (b) SEC column

Figure S3. AFM image of GQDs and corresponding AFM height profile

Figure S4. (a) Photographs of freeze dried AfFtnAA dimers, (Fe)AA and (GQDs/Fe)AA. (b) UV-vis absorption spectra of GQDs. Inset: photograph of synthesized concentrated GQDs (c) SEC chromatogram of GQDs at 230 and 280 nm. (d) PL spectra of ferritin dimers at different excitation wavelengths. (e) PL spectra of GQDs and ferritin+GQDs at different R values (feeding ratio of GQDs to ferritin dimers) at constant concentration of GQDs by increasing ferritin concentration (f) PL spectra of GQDs, (GQDs/Fe)AA and (Fe)AA+GQDs at R=0.3 before separation of free GQDs and after separation of free GQDs (dashed lines). (g) PL spectra of GQDs, (GQDs/Fe)AA and (Fe)AA+GQDs at R=0.15 before separation of free GQDs.

3.3. DOX loading

Figure S5. (a) Standard calibration curve of DOX, based on its fluorescence intensity at excitation and emission wavelengths of 458 and 595 nm, respectively. (b) PL spectra of DOX, and ferritin dimers+DOX at the same concentration of drug, before and after separation of free drug at excitation wavelength of 458 nm.

3.4. Fluorescence (cellular) and Magnetic Resonance Imaging

Figure S6. Effect of concentration of loaded iron on T_1 and T_2 relaxation times of (Fe)AA, to determine its r_1 and r_2 relaxivities.

Table S1. Calculated r_1 and r_2 values for (GQDs/Fe)AA, (Fe)AA for each protein cages and for GQDs.

Components	r_1	r_2
(GQDs/Fe)AA	$727 \text{ mM}^{-1}\text{s}^{-1}$	$1607 \text{ mM}^{-1}\text{s}^{-1}$
(Fe)AA	$691 \text{ mM}^{-1}\text{s}^{-1}$	$1698 \text{ mM}^{-1}\text{s}^{-1}$
GQDs	$1.2 \text{ mM}^{-1}\text{s}^{-1}$	$0.2 \text{ mM}^{-1}\text{s}^{-1}$

3.5. Cytotoxicity

Figure S7. Relative viability of (a) MDA-MB-231 (b) 3T3 cells, after incubation with GQDs for 48 and 72 h.