ABSTRACT: Fe–polyphenols coordination polymers have emerged as a versatile theranostic nanoparticle for biological applications owing to the appealing biocompatibility of precursors from nature. Incorporating bioactive molecules with Fe–polyphenols coordination polymers greatly significant to take full advantages of their superiorities for advanced application. Herein, we show functional-protein-assisted fabrication of Fe–gallic acid (GA) nanonetworks via a mild and facile biomimeralization for photothermal therapy. Mild alkaline condition is crucial to obtain protein–Fe–GA nanonetworks with intense near-infrared absorption and their unique network structure allows reducing the leakage to the surrounding normal tissues, benefiting high photothermal therapeutic efficacy and minimal side effects. The proposed bovine serum albumin–Fe–GA nanonetworks are successfully used to eradicate tumor in vivo. In addition, this universal method can be extended to synthesize other protein-involved nanonetworks, such as human serum albumin–Fe–GA and ovalbumin–Fe–GA. More importantly, the intrinsic bioactivity of protein can be retained in the nanonetworks, and the ovalbumin–Fe–GA nanonetworks enable inducing the maturation of immune cells, showing the successful fusion of immune activity of ovalbumin into the nanonetworks. The proposed biomimeralization strategy shows a bright prospect in incorporating various functional proteins, such as enzymes and antibodies, to form protein–Fe–GA nanonetworks with good biocompatibility, favorable photothermal effect, and specific biological function.

KEYWORDS: Polyphenols, Coordination polymer, Biocompatibility, Biomineralization, Immune activation, Photothermal therapy

INTRODUCTION

Traditional cancer treatments, such as surgery excision, radiotherapy, and chemotherapy, have been widely applied for clinical cancer therapy, but these can suffer from the risk of side effects such as destroying the immune system and increasing the incidence of cancer recurrence. Photothermal therapy (PTT) with the merits of high selectivity and minimal invasiveness is emerging as an alternative method for cancer therapy in recent years. PTT utilizes near-infrared (NIR) light-absorbing agents to convert the NIR light energy into heat for thermal ablation of malignant tumors, and spatially controlled light illumination and localized accumulation of photothermal agents endow PTT with high selectivity and minimal invasiveness.1,2 Besides, PTT not only owns the ability to directly “burn” tumors but also can assist the efficacy of chemotherapy, radiotherapy, and immunotherapy, effectively inhibiting tumor relapse and metastasis.3–5 Up to now, various photothermal agents such as carbon nanostructures,6,7 noble metal nanomaterials,8,9 metal chalcogenides and oxide10–17 semimetal nanoparticles,18 black-phosphorus nanomaterials,19,20 photosensitizer-containing polymers and lipo-some,21–26 organic nanomaterials,27–29 and dyes30,31 have been extensively explored for PTT. Despite outstanding therapeutic efficacy, great concerns regarding the long-term safety of current photothermal agents seriously hinder their clinical applications.

Coordination polymer nanostructures, also known as nanoscale metal–organic frameworks, are formed by the self-assembly between metal ions and organic polydentate bridging ligands, and show great potential as a versatile nanoplatform for diverse biomedical applications due to their structural and chemical diversity and high loading capacity.32–38 Nevertheless, the majority of coordination polymers inevitably suffer from tedious synthesis process and use of toxic metal elements and organic ligands.29 The rational choice of metal center and ligands with special function is the key to developing high-performance polymers for biological applications. Fe is an essential component of hemoglobin, a substance in red blood
cells that carries oxygen around the body, and plays a crucial role in extensive biological processes. The definite biological function and biosafety of Fe element make it an excellent metal ion candidate for building coordination polymers. In light of organic ligands, some precursors derived from natural resources have attracted increasing attention and have been applied to fabricate coordination polymers instead of toxic ones such as imidazole and terephthalic acid. Gallic acid (GA) is a type of organic tea polyphenol with low-molecular weight derived from plant sources and has been extensively used in the food and medicine industries. The strong bidentate coordination interaction between Fe and the two phenolic hydroxyl groups in ortho position in GA makes it possible for the fabrication of Fe–GA coordination polymer. Moreover, the Fe–GA complex possesses intense NIR absorption derived from the strong delocalization in the π-electron structure and has been employed as an excellent PTT agent with favorable biocompatibility. Several hydrophilic polymers, such as poly(vinyl alcohol) (PVA), polyethylene glycol (PEG), and poly(vinylpyrrolidone) (PVP) have been employed as surface stabilizers for Fe–GA coordination polymers. However, previous surface modifications merely employed inactive molecules to enhance water solubility, so it is greatly significant but challenging to incorporate bioactive molecules to take full advantages of the superiorities of Fe–GA coordination polymer for advanced applications. Proteins, such as antibodies, enzymes, structural proteins, signaling proteins, and transport proteins, fulfill many functions as one type of most significant biomolecules in biology. Proteins are rich in functional groups such as amino, carboxyl, and thiol groups, thus exhibiting strong coordination capacities with transition metal ions like Fe. Moreover, the unique spatial structure and molecular chain flexibility make protein an expansive hollow nanoreactor suitable for biomineralization. Up until now, plenty of bioactive proteins, such as bovine serum albumin (BSA), human serum albumin (HSA), ovalbumin (OVA), catalase, lactoferrin, transferrin, ferritin, pepsin, trypsin, papain, horseradish peroxidase (HRP), ribonuclease, lysozyme, and insulin, have been employed for the fabrication of diverse nanostructures with fascinating properties for biological applications. For instance, noble nanoclusters (Pt, Au, Cu), metal sulfide (PbS, Bi2S3, CuS, FeS, ZnS:Mn), and metal oxide (Gd2O3, Au/Gd2O3, and MnO2) have been synthesized via biomineralization for theranostics of various diseases. Therefore, bioactive proteins as excellent templates show great potential in the fusion of diverse biological functions with the unique merits of Fe–GA coordination polymer. Herein, we showed functional-protein-assisted fabrication of Fe–GA nanonetworks via a mild and facile biomineralization way for localized photothermal therapy. Mild alkaline condition permitted the formation of Fe–GA nanonetworks with favorable NIR absorbance and impressive photothermal performance. The BSA–Fe–GA nanonetworks exhibited a unique network structure with an appropriate size of 193.5 nm, facilitating their retention in the tumor site and minimizing the leakage to surrounding normal tissues after localized administration. The BSA–Fe–GA nanonetworks with outstanding biocompatibility and excellent photothermal performance were successfully applied in localized photothermal therapy in vitro and in vivo against tumors. To testify the universality of the protein-assisted fabrication strategy of coordination polymers, other proteins like HSA and OVA were also employed to construct Fe–GA coordination polymers. Both HSA and OVA enabled the formation of protein–Fe–GA coordination polymers with similar network structure and excellent photothermal effect to those of BSA–Fe–GA nanonetworks (Scheme 1). Moreover, the proposed OVA–Fe–GA nanonetworks successfully induced the maturation of dendritic cells (DC2.4) and macrophage cells (RAW264.7). The immune activity of OVA in OVA–Fe–GA nanonetworks was retained efficiently via the mild synthesis approach, showing great potential in the development of multifunctional nanovaccine. The proposed biomineralization strategy shows a bright prospect in the incorporation of functional proteins, such as enzymes and antibodies, with Fe–GA coordination polymers for various biological applications.

■ RESULTS AND DISCUSSION

Synthesis and Characterization of BSA–Fe–GA, OVA–Fe–GA, and HSA–Fe–GA Nanonetworks. BSA–Fe–GA nanonetworks were prepared through a facile and one-step biomineralization approach. Briefly, 60 mg of BSA and 80

Scheme 1. Schematic Illustration of Functional-Protein-Assisted Fabrication of Fe–GA Coordination Polymer Nanonetworks for Localized Photothermal Therapy
mg of FeCl₃·6H₂O were dissolved in 15 mL of water with magnetically stirring. Then, 1 mL of NaOH solution (1 M) was rapidly added to the mixture liquid with pH changing from 3 to 9. GA aqueous solution (4 mL, 10 mg/mL) was then introduced into the orange complex mixture, immediately giving rise to a dark solution due to rapidly self-cross-linking of Fe³⁺ and GA along with the existence of protein in water. Alkaline aqueous environment induced loose and unfolded structure of protein, contributing a stable network of BSA−Fe−GA coordination polymer. Moreover, a mild basic condition is crucial to obtain BSA−Fe−GA nanonetworks with maximum NIR absorption, since the slightly high pH can strengthen the bidentate coordination interaction between Fe³⁺ and the two phenolic OH groups in ortho position in GA (Figure S1 and Table S1). Besides, mild synthesis condition also ensured the minimal impairment to the function of protein. The resulting BSA−Fe−GA nanonetworks were purified by dialysis against deionized water for 24 h and stored at 4 °C for further use. OVA−Fe−GA and HSA−Fe−GA nanonetworks were also synthesized under optimal synthetic conditions for BSA−Fe−GA nanonetworks.

The composition, size, and morphology of the as-prepared protein−Fe−GA nanonetworks were characterized by transmission electron microscopy (TEM), dynamic light scattering (DLS), Fourier transform-infrared spectroscopy (FT-IR), and inductively coupled plasma-atomic emission spectrometry (ICP-AES). The TEM images indicated BSA−Fe−GA coordination polymer owned a network structure (Figures 1a and S2a), and analogous morphologies were observed for OVA−Fe−GA and HSA−Fe−GA nanonetworks (Figure S2b,c). The hydrodynamic sizes of BSA−Fe−GA, OVA−Fe−GA, and HSA−Fe−GA nanonetworks in water were determined to be 193.5, 233.1, and 196.5 nm, respectively (Figure 1b). Protein−Fe−GA nanonetworks were dispersed in different buffer solution (10 mM, pH 4.0, 5.0, 7.0, 7.4, and 8.0) for the zeta potential test. The zeta potential values of BSA−Fe−GA, OVA−Fe−GA, and HSA−Fe−GA nanonetworks were around −20 to −30 mV in neutral and weak alkaline environment due to abundant carboxyl groups in protein and...
GA (Figures 1c and S3). The presence of BSA and GA in the prepared protein–Fe–GA nanonetworks was verified by their characteristic bands in FT-IR spectra. –CH3 symmetrical vibrations at 2932 cm⁻¹, amide I (mainly C=O stretching vibrations) at 1654 cm⁻¹, and amide II (coupling of bending vibrations of N–H and stretching vibrations of C–N) bands at 1534 cm⁻¹ all correspond to BSA in BSA–Fe–GA nanonetworks. The characteristic peak at 1265 cm⁻¹ (C–O stretch) and 1026 cm⁻¹ (O–H in-plane deformation) of GA confirmed the incorporation of GA in BSA–Fe–GA nanonetworks. Besides, the disappearance of characteristic peak at 3283 cm⁻¹ (O–H stretching) of GA indicated the coordination interaction between the phenolic groups and carboxylic groups in GA and Fe3⁺ in BSA–Fe–GA nanonetworks (Figure 1d). FT-IR spectra of GA, HSA, OVA, OVA–Fe–GA, and HSA–Fe–GA nanonetworks also proved successful incorporation of HSA and OVA into Fe–GA coordination polymers (Figure S4a,b). BSA–Fe–GA nanonetworks had a strong NIR absorbance resulting from the strong delocalization in the π-electron structure, benefiting the high photothermal efficacy under an 808 nm light irradiation.5 Even at a low concentration of 0.1 mg/mL, the absorbance of BSA–Fe–GA nanonetworks at 808 nm could reach around 0.3 (Figure 1e). The extinction coefficient of the proposed BSA–Fe–GA nanonetworks was 2.902 L·g⁻¹·cm⁻¹, which is 7.5 times larger than that of ultrasmall BSA–Fe–GA nanoparticles (0.388 L·g⁻¹·cm⁻¹) at 808 nm wavelength (Figures 1f and S5).5,67,68,72 The UV–vis–NIR absorption spectra and extinction coefficient of OVA–Fe–GA and HSA–Fe–GA nanonetworks both revealed similar NIR absorbance to that of BSA–Fe–GA nanonetworks, testifying the universality of proposed method (Figure S6a–c). The content of Fe in the proposed BSA–Fe–GA, OVA–Fe–GA, and HSA–Fe–GA nanonetworks were determined to be 16.6, 17.7, and 18.8% (w/w), respectively. The high loading of Fe–GA complex ensured the intense NIR absorption of the proposed BSA–Fe–GA nanonetworks.

Long-Term Colloidal Stability of Protein–Fe–GA Nanonetworks. The as-prepared BSA–Fe–GA nanonetworks can be easily dissolved in various media including water, PBS (10 mM, pH 7.4), normal saline, 1640 culture medium, DMEM culture medium, and fetal bovine serum (FBS), and no apparent precipitation was observed for 0.1 mg/mL BSA–Fe–GA nanonetworks for 20 days. The hydrodynamic size of BSA–Fe–GA nanonetworks exhibited no obvious change during 20 days, further confirming their long-term colloidal stability (Figure 2a,b). We further evaluated the colloidal stability of protein–Fe–GA nanonetworks in buffer solution with different pH values. Owing to these three proteins isoelectric point at around 4.8, precipitate appeared in the protein–Fe–GA solution when pH value was 4.0 or 5.0. However, no apparent precipitation and obvious hydrodynamic diameter change were observed for BSA–Fe–GA and OVA–Fe–GA nanonetworks dispersed in phosphate buffer solution (10 mM, pH 7.0, 7.4, and 8.0) for 20 days, further indicating their long-term colloidal stability in the simulated physiological environment (Figure S7a–c). HSA–Fe–GA nanonetworks showed a relatively large hydrodynamic size and poor colloidal stability, which is probably attributed to the small chemical structure difference between BSA and HSA (Figure S7d).

Cytotoxicity of BSA–Fe–GA Nanonetworks. The cytotoxicity of BSA–Fe–GA nanonetworks was investigated by a standard cellular methyl thiazolyl tetrazolium (MTT) assay. Three cell lines (4T1, DC2.4 and RAW264.7) were incubated with different concentrations of BSA–Fe–GA nanonetworks ranging from 0 to 400 μg/mL. High cell viability (over 70%) was observed after the treatment of different concentrations of BSA–Fe–GA nanonetworks, demonstrating the low cytotoxicity of BSA–Fe–GA nanonetworks in vitro ensured their potential for the immune activation.

In Vitro Cellular Uptake Test. To investigate the in vitro cellular distribution of protein–Fe–GA nanonetworks, the Fe contents in cells were determined after different treatments, and the results revealed that in vitro DC2.4 and RAW264.7 cells can internalize OVA–Fe–GA and BSA–Fe–GA nanonetworks in a concentration and time dependent manner (Figure S8b,c). Such an effective phagocytosis of protein–Fe–GA nanonetworks in vitro ensured their potential for the immune activation.

Immune Activation of OVA–Fe–GA Nanonetworks in Vitro. OVA as model antigen has been widely applied to elicit immune response.73–75 To verify the function of OVA in the fabricated nanonetworks, we typically investigated the immune activation effect of OVA–Fe–GA nanonetworks via costimulatory molecules expression and cytokines secretion of DC2.4 and RAW264.7 cells.76 Before flow cytometry quantification and ELISA analysis were performed, the cytotoxicity of OVA–Fe–GA nanonetworks toward immune cells was also investigated by the standard cellular MTT assay. DC2.4 and RAW264.7 cells were incubated with different concentrations of OVA–Fe–GA nanonetworks ranging from 0 to 400 μg/mL, and the MTT results clearly showed the low cytotoxicity of OVA–Fe–GA nanonetworks (Figure S8d). The capability of OVA–Fe–GA nanonetworks to induce DCs and macrophages maturation was evaluated by upregulation of costimulatory molecules CD80 and CD83 on their membrane. CD80 can recognize and bind to B7 molecules on the T cells, providing the “second signal” for T cell activation. CD83 can modulate

Figure 2. (a) Monitoring the hydrodynamic size change of BSA–Fe–GA nanonetworks during 20 days. (b) Photos of 0.1 mg/mL BSA–Fe–GA nanonetworks dispersed in different media (from left to right: water, PBS, normal saline, 1640 culture media, DMEM culture media, and FBS) for 20 days. (c) MTT assay of 4T1 cells after incubation with different concentrations (0 to 400 μg/mL) of BSA–Fe–GA nanonetworks.
the maturity of DC and macrophage and further activate T and B cells. Taken together, increased expression of CD80 and CD83 molecules could be recognized as important markers for immune cells maturation.77 The percentages of mature DCs expressing CD80 increased from 1.37 to 18.4%, and CD83 increased from 1.36 to 13.1% after incubation with 200 μg/mL OVA−Fe−GA nanonetworks, respectively (Figures 3a,b and S9). We further carried out the same immune activation experiments on RAW264.7 cells, and the percentages of mature RAW264.7 cells with costimulatory molecules expression were determined to be 4.91% (CD80) and 11.7% (CD83) after incubation with 200 μg/mL OVA−Fe−GA nanonetworks (Figures 3c,d and S10). The CD80 and CD83 expression levels induced by OVA−Fe−GA nanonetworks in DC2.4 and RAW264.7 cells were both superior to those resulted from pure OVA at the same concentration. ELISA quantification of inflammatory cytokines (IL-6 and TNF-α) secretion of DC2.4 and RAW264.7 cells incubated with pure OVA, OVA−Fe−GA, and LPS for 24 h demonstrated that OVA−Fe−GA nanonetworks could induce higher levels of cytokines secretion compared with pure OVA (Figure 3e−h). Such evident immune responses in vitro manifested the sufficient immune activation capability of OVA−Fe−GA nanonetworks, which showed great potential in nanovaccine-based immunotherapy. In addition, BSA−Fe−GA nanonetworks also exhibited a stronger immunostimulation effect than...
free BSA, which serves as an exogenous antigen for DC2.4 and RAW264.7 cells (Figures S9–S11).78

**Photothermal Performance of Protein–Fe–GA Nanonetworks.** To evaluate the photothermal performance of the designed nanonetworks, pure water and BSA–Fe–GA nanonetworks were both irradiated by an 808 nm laser (2 W/cm²) for 10 min. The concentration-dependent temperature elevation was observed. The 0.4 mg/mL BSA–Fe–GA nanonetworks can raise temperature by 23.6 °C, while pure water only showed a slight temperature change of 4.7 °C (Figure 4a). Besides, BSA–Fe–GA nanonetworks exhibited excellent photothermal stability, and maximum temperature increased over 20 °C during four LASER ON/OFF cycles with an 808 nm laser irradiation at power densities of 0, 0.5, 1, and 2 W/cm² (Figure 4b). The excellent photothermal heating effect of HSA–Fe–GA and OVA–Fe–GA nanonetworks were also confirmed by the fact of remarkable temperature increase, and the temperature of 0.4 mg/mL HSA–Fe–GA and OVA–Fe–GA nanonetworks can increase by 22.2 and 27.6 °C, respectively (Figure S12a,b). The infrared thermal photos further intuitively proved the outstanding photothermal effect of BSA–Fe–GA, HSA–Fe–GA, and OVA–Fe–GA nanonetworks (Figure S12c). The photothermal conversion efficiency (η) was measured according to the precious work.79 The 808 nm laser heat conversion efficiencies (η) of the as-synthesized BSA–Fe–GA, OVA–Fe–GA, and HSA–Fe–GA nanonetworks are further calculated to be 20.33, 18.76, and 14.66%, respectively (Figure S13). Therefore, the as-prepared protein–Fe–GA nanonetworks with high photothermal efficiency could serve as effective photothermal agents to ablate tumors.

**In Vitro Photothermal Therapy of BSA–Fe–GA Nanonetworks toward 4T1 Tumor Cells.** The photothermal therapy in vitro was assessed using 4T1 cells which were incubated with BSA–Fe–GA nanonetworks and irradiated by an 808 nm laser for 10 min. MTT assay revealed that the cell viability with the treatment of the nanonetworks or laser irradiation alone was similar to the negative control group. However, the cell viability showed an obvious reduction with the combined treatments of BSA–Fe–GA nanonetworks and laser irradiation. Majority of 4T1 cells were destroyed after the incubation with 400 μg/mL BSA–Fe–GA nanonetworks under an 808 nm laser irradiation at a power density of 2 W/cm² (Figure 4c). Ultimately, the temperature of 4T1 cells with different treatments after 10 min were measured by an infrared thermometer camera, indicating distinct temperature rise after PTT (Figure 4d,e). Moreover, live/dead cell dual staining was...
carried out to intuitively assess the photothermal therapy efficacy. The fluorescence images of cells costained by calcine acetoxymethyl ester (Calcine AM) and propidium iodide (PI), which differentiated the live (shown in green) and dead cells (shown in red), also proved the remarkable photothermal therapy effect of BSA−Fe−GA nanonetworks (Figure 4f).

In Vivo Photothermal Therapy of BSA−Fe−GA Nanonetworks. The BALB/c mice with xenografts 4T1 cells were randomly divided into 3 groups (n = 3) for different treatments, and BSA−Fe−GA nanonetworks or PBS were intratumorally injected to the mice. The temperature of tumor surface could increase by 37.2 °C and reach to 65.8 °C after the treatment of BSA−Fe−GA nanonetworks plus laser irradiation, which was high enough to kill tumor cells. In contrast, the tumor in mice treated with PBS (10 mM, pH 7.4) plus laser irradiation only exhibited a slight temperature increase of 8.4 °C (Figures 5a and S14). The digital photographs of 4T1 tumor-bearing mice at different time points (0, 3, 6, 21, and 30 d) after different treatments with PBS + L, BFG alone, and BFG + L L means laser irradiation (808 nm, 0.5 W/cm², 10 min). BFG: BSA−Fe−GA. TW: tumor weight; BW: body weight.
manifested the extraordinary photothermal tumor ablation effect of BSA−Fe−GA nanonetworks (Figure Sb). The largest tumors growth occurred in mice treated with BSA−Fe−GA nanonetworks alone, and the mice treated with PBS plus laser irradiation displayed insufficient thermal therapeutic effect toward tumors. Complete tumors ablation and no more tumors recurrence were observed with the treatment of BSA−Fe−GA nanonetworks plus laser irradiation. The ratio change of tumor weight to body weight of the mice in each group also indicated the remarkable photothermal therapeutic effect of BSA−Fe−GA nanonetworks (Figure 5c). These results proved the admirable photothermal therapy efficacy of BSA−Fe−GA nanonetworks in vivo.

The BSA−Fe−GA nanonetworks-based therapy did not cause notable body weight decline, showing their good biocompatibility in vivo (Figure S15). To further study the potential toxicity of the BSA−Fe−GA nanonetworks in vivo, hematoxylin and eosin staining (H&E staining) of different organs including heart, liver, spleen, and kidney and tumor of mice were carried out after diverse treatments (Figure 6). No morphological changes, inflammation, cell apoptosis, or necrosis was observed in the heart, liver, spleen, and kidney of mice in each group. No tumor tissue was found in mice treated with BSA−Fe−GA nanonetworks plus laser irradiation. On the contrary, the tumor tissue slices of mice treated with PBS plus laser irradiation and BSA−Fe−GA nanonetworks alone both illustrated a large quantity of tumor cells without obvious necrosis. These data indicated that BSA−Fe−GA nanonetworks not only possessed a powerful photothermal therapeutic effect in vivo but also exhibited good biocompatibility.

# CONCLUSIONS

In summary, we reported functional-protein-assisted fabrication of Fe−GA nanonetworks for localized photothermal therapy. The proposed BSA−Fe−GA nanonetworks not only possessed a unique network structure with an appropriate size around 200 nm minimizing the leakage to surrounding normal tissues after localized administration but also exhibited favorable NIR absorbance and impressive photothermal performance. Moreover, the proposed BSA−Fe−GA nanonetworks showed excellent biocompatibility due to intrinsic biosafety of the precursors and were successfully applied in localized photothermal therapy in vitro and in vivo. This universal design can be readily extended to fabricate coordination polymers using other functional proteins, such as HSA and OVA. The formed HSA−Fe−GA and OVA−Fe−GA nanonetworks also exhibited excellent photothermal effect due to their intense NIR absorbance. Moreover, the immune activity of OVA in OVA−Fe−GA nanonetworks was retained efficiently due to the mild synthesis condition, and the OVA−Fe−GA nanonetworks can induce the maturation of DC2.4 and RAW264.7 cells efficiently, showing great potential in the development of multifunctional nanovaccine. We believe the proposed functional protein assisted-synthesis strategy lays down a novel and universal way for the integration of functional proteins with coordination polymers for advanced applications.

# EXPERIMENTAL SECTION

Reagents and Materials. The chemical materials were all at least analysis-grade. Bovine serum albumin (BSA) and human serum albumin (HSA) were purchased from Beijing Dingguo Biotechnology Co., Ltd. (Beijing, China). Ovalbumin (OVA) was obtained from Sigma-Aldrich Corporation (Shanghai, China). FeCl3·6H2O, sodium hydroxide (NaOH), methyl thiazolyl tetrazolium (MTT), and gallic acid (GA) were provided by Aladdin Reagent Co., Ltd. (Shanghai, China). Ultrapure water (Hangzhou Wahaha Group Co., Ltd.) was used throughout the synthesis procedure. Dimethyl sulfoxide (DMSO) and formaldehyde were purchased from Concord Technology (Tianjin, China). Calcein acetoxymethyl ester (Calcein AM) and propidium iodide (PI) were obtained from dojindo laboratories (Shanghai, China). Anti-mouse CD80 FITC-conjugated and CD83 PE-conjugated antibodies were purchased from eBioscience. Mouse IL-6 and TNF-α ELISA kits were obtained from MultiSciences (Lianke) Biotech Co., Ltd. (Hangzhou, China).

Characterization. The Fe element content was quantified by inductively coupled plasma-atmospheric emission spectrometry (ICP-AES, Thermo IRIS Advantage). The size and zeta potential of protein−Fe−GA nanonetworks were determined on a Malvern Zetasizer (Nano series ZS, UK). The size and morphology of the prepared nanonetworks were characterized by a Hitachi HT7700 transmission electron microscopy (TEM) (Hitachi HT7700, Japan). Fourier transform-infrared (FT-IR) spectra (of 400−4000 cm−1) of the nanonetworks were recorded with pure KBr as the background via a Nicolet IR AVATAR-360 spectrometer (Nicolet, USA). The infrared thermal photos were taken by infrared thermometer camera (FLIR E50 series, USA). The absorption spectra of protein−Fe−GA nanonetworks were determined by a UV-3600 plus spectrophotometer (Hitachi, Japan).

Preparation of Protein−Fe−GA Nanonetworks. First, 0.1 mg/mL protein (BSA, OVA, or HSA) and 80 mg of FeCl3·6H2O were dispersed in 15 mL of ultrapure water under gentle magnetic stirring for 3 min, and then 1 mL of NaOH (1 M) was added to adjust pH to 9. After stirring for 0.5 h, GA aqueous solution (4 mL, 10 mg/mL) was introduced to the mixture and reacted for another 10 h. The obtained protein−Fe−GA nanonetworks were purified by dialysis (molecular weight cutoff is 8−14 kDa). Then, the purified nanonetworks were stored at 4 °C for following use.

Long-Term Colloidal Stability. First, 0.1 mg/mL BSA−Fe−GA nanonetworks were dispersed in ultrapure water, PBS (10 mM, pH 7.4), normal saline, Gibco 1640 culture medium, Gibco DMEM culture medium, and fetal bovine serum (FBS), and their photos were then taken for 20 days. The hydrodynamic sizes of BSA−Fe−GA nanonetworks dissolved in water at different time points were also recorded.

To further investigate the influence of pH on the colloidal stability of different protein−Fe−GA nanonetworks, 0.1 mg/mL BSA−Fe−GA, OVA−Fe−GA, and HSA−Fe−GA nanonetworks were dispersed in different buffer solution including sodium acetate−acetic acid buffer solution (10 mM, pH 4.0 and 5.0) and phosphate buffer solution (10 mM, pH 7.0, 7.4, and 8.0), and their photos were then taken during 20 days. The hydrodynamic sizes of BSA−Fe−GA, OVA−Fe−GA, and HSA−Fe−GA nanonetworks in different buffer solution at different time points were also recorded.

Measurement of the Extinction Coefficient at 808 nm of Protein−Fe−GA Nanonetworks. The NIR absorbance in protein−Fe−GA nanonetworks with different concentrations were determined by a UV-3600 plus spectrophotometer. The extinction coefficient at 808 nm was then calculated according to the Lambert−Beer law (A/L = εc, where A is the absorbance intensity, L is the length of the cuvette, C is the concentration, and ε is the extinction coefficient).

Assessment of Photothermal Performance. Different concentrations (0.1, 0.2, and 0.4 mg/mL) of BSA−Fe−GA nanonetworks and ultrapure water were irradiated with an 808 nm laser irradiation (2 W/cm2) for 10 min. HSA−Fe−GA and OVA−Fe−GA nanonetworks solution were also irradiated with the same parameters. The temperature increase of pure water and protein−Fe−GA nanonetworks (0.1, 0.2, 0.4 mg/mL) was recorded by an infrared thermometer camera during the photothermal heating process. Magnetic stirring was necessary to ensure the homogeneity of heat distribution.

DOI: 10.1021/acssuschemeng.8b04656
To calculate the photothermal conversion efficiency, protein–Fe–GA solutions (0.4 mg/mL) were irradiated with an 808 nm laser (2 W/cm²) until the solution temperature reached a steady state. Then, the solution naturally cooled down to the ambient temperature after the stoppage of laser irradiation. The photothermal conversion efficiency (\(\eta\)) of protein–Fe–GA nanonetworks were calculated as follow equation:

\[
\eta = \frac{h(s(T_{\text{max}} - T_{\text{f}}))}{Q_s(1 - 10^{-5})}
\]

where \(h\) is the heat transfer coefficient and \(s\) is the surface area of the container. \((T_{\text{max}} - T_{\text{f}}))\) is the temperature change of the protein–Fe–GA solution at the maximum steady-ambient temperature. \(Q_s\) represents heat dissipated from light absorbed by the solvent and the container. \(I\) is the power density of the laser, and \(A\) is the absorbance of protein–Fe–GA at 808 nm.

**In Vitro Cytotoxicity Assay.** The cytotoxicities of BSA–Fe–GA and OVA–Fe–GA nanonetworks were evaluated by 4T1 cells and immune cells (DC2.4 and RAW264.7 cells), respectively. 4T1 cells were cultured in Gibco DMEM with 10% FBS and 1% streptomycin–penicillin in an atmosphere of 5% CO₂ at 37 °C. DC2.4 and RAW264.7 cells were cultured in Gibco DMEM with 10% FBS, 1% streptomycin–penicillin, and 1% L-glutamine in an atmosphere of 5% CO₂ at 37 °C. The cells were seeded in a 96-well plate at a density of \(1 \times 10^5\) per well. After 24 h, the cells were washed by PBS (10 mM, pH 7.4) to remove dead cells and incubated with fresh media containing BSA–Fe–GA and OVA–Fe–GA nanonetworks with different concentrations for another 24-h incubation. After being washed with PBS again, the cells were treated with fresh medium containing 10% FBS, 5 mg/mL, and then incubated for 4 h in an atmosphere of 5% CO₂ at 37 °C. Then, 120 μL of DMSO was respectively added into each well to replace the media and dissolved the purple formazan crystals. After 10 min of gentle shaking, the absorbance of each well at 490 nm was recorded by a microplate reader (Biotek, USA). The cell viabilities were calculated according to the following formula.

\[
\text{Cell viability} = \frac{\text{OD}_{\text{exp}}}{\text{OD}_{\text{con}}} \times 100\%
\]

where \(\text{OD}_{\text{exp}}\) and \(\text{OD}_{\text{con}}\) are the optical density (OD) for cells treated with different concentrations of nanonetworks and cells in control group, respectively.

**In Vitro Cellular Uptake Test.** The cellular uptake of protein–Fe–GA by DC2.4 and RAW264.7 cells was investigated by ICP-AES measurement of Fe content in cells. Briefly, DC2.4 and RAW264.7 cells were seeded in 12-well plates at a density of \(5 \times 10^4\) cells per well. After 24 h, the cells were washed by PBS (10 mM, pH 7.4) to remove dead cells and incubated with fresh media containing BSA–Fe–GA and OVA–Fe–GA nanonetworks with different concentrations (0, 100, 200, and 400 mg/L) for different incubation time (8, 12, and 24 h). After that, each group of cells was washed three times with PBS. The cells in each well were processed with aqua regia for 2 days and then diluted with water to 4 mL for ICP-AES test.

**In Vitro Activation and Maturation of Immune Cells Induced by OVA–Fe–GA and BSA–Fe–GA Nanonetworks.** Immature immune cells including DC2.4 and RAW264.7 were seeded in a 12-well plate at a density of \(5 \times 10^4\) per well and cultured for 24 h. Then, the cells were washed with PBS and treated with fresh culture media containing OVA, BSA, OVA–Fe–GA, BSA–Fe–GA, and LPS for another 24 h, respectively. The cell culture supernatants were collected together and then purified by centrifugation for 3 min at 1000 rpm. The obtained supernatants were stored at \(-80\) °C for following analysis. The amounts of pro-inflammatory cytokines IL-6 and TNF-α released by cells after different treatments were analyzed by ELISA according to the manufacturer’s directions. Then, cells were subsequently washed with PBS and stained for 1 h at 4 °C with FITC-conjugated anti-CD80 and PE-conjugated anti-CD83 antibodies. After again washing 3 times with PBS and dispersing with 200 μL of PBS, these labeled cells were then collected for flow cytometry.

**In Vivo Photothermal Therapy of 4T1 Cells with BSA–Fe–GA Nanonetworks.** 4T1 cells were seeded in a 96-well plate at a density of \(1 \times 10^4\) per well and cultured in an atmosphere of 5% CO₂ at 37 °C for 24 h. Then, the cells were cultured with different concentrations of BSA–Fe–GA nanonetworks (0, 200, and 400 μg/mL) for 1 h, following by an 808 nm laser irradiation at a power density of 0, 0.5, 1, and 2 W/cm² for 10 min. The infrared thermal photos of cells with different treatments were recorded by an infrared thermometer camera. The cell viabilities were investigated by a standard MTT assay as aforementioned. Calcein acetoxymethyl ester (Calcein AM) and propidium iodide (PI) were used to stain live and dead cells after different treatments. After incubation for 15 min, each well was washed twice with PBS carefully to remove dyes completely. The fluorescence images were obtained on an inverted fluorescence microscope.

**Animal Model.** The tumor models were established by the subcutaneous planting of 4T1 cells onto the back of the BALB/c mice (15–18 g, HFK Bioscience Co., Ltd., Beijing, China). When the tumor size reached 5–7 mm in diameter, in vivo photothermal therapy was carried out on the mice.

**Statistical Analysis.** The data are presented as the means ± standard deviation (SD). The comparison between groups was analyzed with two-tailed t tests or one-way ANOVA. Differences were considered statistically significant when the \(p\) values were less than 0.05 (> 0.05).

**Acknowledgments**

The authors declare no competing financial interest.

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**Notes**

Y.Q.W. and J.Z. contributed equally to this work. This work was supported by the financial support from the National...
Natural Science Foundation of China (Grant Nos. 21435001 and 21874101) and Natural Science Foundation of Tianjin City (No. 18JCYBJC20800).

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