Mitochondria-Targeted Bovine Serum Albumin@Copper Sulfide Nanocomposites Conjugated with Rhodamine-110 Dye for an Enhanced Efficacy of Cancer Photothermal Therapy

Haibei Tong, Yan Gao, Jialiang Li, Jiachen Li, Di Huang, Jisen Shi,* Hélder A. Santos,* and Bing Xia*

1. Introduction

Mitochondria are essential subcellular organelles of eukaryotic cells, which can generate most cellular energy via oxidative phosphorylation and regulate their apoptotic pathway. The defects or dysfunctions of mitochondria can lead to cancer occurrence, metastasis or recurrence, [1–3] thus specific mitochondria-targeted therapeutic approaches have been developed to improve the efficacy of cancer therapy.[4–8] In the past decades, with the modification of mitochondria-targeted ligands (e.g., lipophilic cations, peptides, or aptamers, etc.), multifunctional nanoplatforms have been constructed for cancer diagnosis or therapy.[9–12] In addition, heat stress caused by hyperthermia can heavily disturb the cellular functions of mitochondria with high thermal sensitivity or directly destruct them, resulting in the activation of the apoptosis or necrosis pathways in cancer cells.[13,14] Compared with conventional hyperthermia approaches, photothermal nanoagents can efficiently convert near-infrared (NIR) light irradiation localized on tumor to heat energy, and thermally...
ablative tumor, minimizing the damage of the surrounding healthy tissue.[35,36] Especially, when photothermal nanoagents were designed to precisely control their specific accumulation in mitochondria, they could sharply elevate the localized temperature upon the illumination of NIR light to destroy mitochondria, which could induce the death of cancer cells with higher photothermal therapeutic efficacy.[17–25] Moreover, these mitochondria, which could induce the death of cancer cells with higher temperature upon the illumination of NIR light to destroy mitochondria, could sharply elevate the localized temperature upon the illumination of NIR light to destroy mitochondria, which could also sharply elevate the localized temperature upon the illumination of NIR light to destroy mitochondria. This indicates that photothermal nanoagents could efficiently fight against cancer in future.

Bovine serum albumin (BSA) derived from plasma exhibited wide biomedical applications due to their good biocompatibility and low cost. And they can be also used as templates to in situ synthesize versatile metal-related nanoparticles for tumor imaging and cancer therapy, attributed to their abundant reactive amino, carboxyl, and mercapto groups.[28,29] For example, copper sulfide (CuS) nanoparticles with strong absorbance in NIR spectral range were in situ formed in BSA templates, to fabricate photothermal BSA@CuS nanocomposites for cancer therapy.[30–34] Notably, it was reported that lipophilic and cationic rhodamine-110 molecules could efficiently cross the plasma and mitochondria membranes with negative charges of cancer cells, resulting in their specific accumulation in mitochondria.[13] Thus, rhodamine-110 dyes were used to be covalently grafted on the surfaces of nanoparticles to endow their mitochondria-targeting and fluorescent imaging capability for cancer therapy.[36] In our experiments, rhodamine-110 dyes were also chosen to be conjugated with BSA@CuS nanocomposites via the esterification of their carboxylic groups to construct mitochondria-targeted and photothermal R-BSA@CuS nanocomposites. Moreover, the size, morphology, elemental components, crystalline structures, fluorescence, and photothermal performances of BSA-based nanocomposites were characterized and analyzed. Finally, 2D monolayer cellular and 3D multicellular tumor spheroid experiments composed of model human breast cancer (MCF-7) cells were carried out to evaluate their subcellular distribution and anticancer efficiency of photothermal therapy.

2. Results and Discussion

2.1. Synthesis and Characterization of R-BSA@CuS Nanocomposites

BSA was chosen as templates to in situ synthesize CuS nanoparticles, because of strong coordination interactions between their reactive –NH$_2$, –COOH, and –SH moieties and Cu$^{2+}$ ions. In addition, alkaline reaction conditions used in our experiments can unfold the configuration of BSA to capture more Cu$^{2+}$ ions, and continuous heating conditions at 90 °C can improve the crystalline formation of CuS nanoparticles, improving their NIR absorbance.[30] On this basis, rhodamine-110 molecules were covalently modified with BSA@CuS nanoparticles through the activation of N-hydroxyl succinimide (NHS) and N-(3-dimethylamino)propyl)-N-ethyl-carbodiimide hydrochloride (EDC), to prepare R-BSA@CuS nanocomposites. These R-BSA@CuS nanocomposites were also stepwisely characterized by UV–Vis–NIR absorption spectra, photoluminescence (PL) spectra, X-ray photoelectron spectra (XPS), transmission electron microscopy (TEM), and dynamic-light-scattering (DLS) measurements. From Figure 1a, UV–vis–NIR spectra of BSA@CuS and R-BSA@CuS solutions both showed a NIR absorbance peak in the spectra range of 600–1200 nm, assigned to d-d transition of copper ions.[31] Compared with BSA@CuS samples, a new absorbance peak at 513 nm appeared in the spectra of R-BSA@CuS solutions, due to the modification of rhodamine-110 dyes. PL spectra in Figure 1b also showed a strong PL emission peak at 526 nm of R-BSA@CuS samples, confirming rhodamine-110 dyes’ attachment.

The hydrodynamic size and zeta potential of BSA-based samples were analyzed by DLS measurements, as recorded in Figure 1c and Table S1, Supporting Information. The results indicated that all samples had good dispersibility in aqueous solution, resulted from their strong surface negative charge in the range from −23.5 to −33.9 mV. Besides, during the formation of CuS nanoparticles in BSA templates, the protein denaturalization induced by heating or alkaline conditions led to a slight aggregation of BSA@CuS nanocomposites, with their size increased from 4.1 to 6.9 nm. For R-BSA@CuS nanocomposites, their size further reached 15.9 nm with the crosslinking of BSA templates induced by EDC/NHS reaction. And their zeta potential increased to −278 mV, because of the positive charges of rhodamine 110 molecules. Moreover, the stability of R-BSA@CuS nanocomposites in aqueous solution was also monitored, as shown in Figure S1, Supporting Information. After 1 day, their size increased from 15.9 to 28.9 nm, attributed to the swelling of BSA. And during the next 6 days, their size slightly changed in the range of less than 5 nm, with no obvious turbidity and precipitation. These results showed that R-BSA@CuS nanocomposites have stable aqueous dispersibility for their next biological application.

XPS was adopted to measure the elemental components of these samples, recorded in Figure 1d–f and Table S1, Supporting Information. BSA composed of amino acids has rich carbon, nitrogen, oxygen, sulfur, but no copper element, thus indicating that all samples had good dispersibility in aqueous solution, resulted from their strong surface negative charge in the range from −23.5 to −33.9 mV. Besides, during the formation of CuS nanoparticles in BSA templates, the protein denaturalization induced by heating or alkaline conditions led to a slight aggregation of BSA@CuS nanocomposites, with their size increased from 4.1 to 6.9 nm. For R-BSA@CuS nanocomposites, their size further reached 15.9 nm with the crosslinking of BSA templates induced by EDC/NHS reaction. And their zeta potential increased to −278 mV, because of the positive charges of rhodamine 110 molecules. Moreover, the stability of R-BSA@CuS nanocomposites in aqueous solution was also monitored, as shown in Figure S1, Supporting Information. After 1 day, their size increased from 15.9 to 28.9 nm, attributed to the swelling of BSA. And during the next 6 days, their size slightly changed in the range of less than 5 nm, with no obvious turbidity and precipitation. These results showed that R-BSA@CuS nanocomposites have stable aqueous dispersibility for their next biological application.

XPS was adopted to measure the elemental components of these samples, recorded in Figure 1d–f and Table S1, Supporting Information. BSA composed of amino acids has rich carbon, nitrogen, oxygen, sulfur, but no copper element, thus copper elemental signals in XPS were used to monitor the formation of CuS nanoparticles. In Figure 1d and Table S1, Supporting Information. BSA composed of amino acids has rich carbon, nitrogen, oxygen, sulfur, but no copper element, thus copper elemental signals in XPS were used to monitor the formation of CuS nanoparticles. In Figure 1d and Table S1, Supporting Information, the copper signal could be clearly detected in BSA@CuS (2.3%) and R-BSA@CuS (0.6%) samples, compared with 0.0% of bare BSA samples. Figure 1f also presented their high-resolution spectra with Cu (2p $^{3/2}$) and Cu (2p $^{1/2}$) signals centered at 933.1 and 953.0 eV, with typical binding energy difference (Δ = 1.9 eV). In addition, the disulfide bonds and free thiol groups of BSA were also characterized by the signals of S–H (S) at 163.9 eV in the high-resolution spectra of sulfur element, as seen in Figure 1e. However, with the disappearance of peak at 163.9 eV, two new peaks at 168.9 (S–O (= O)) and 162.6 eV (S=Cu) appeared in BSA@CuS or R-BSA@CuS samples. The result meant that S–S or S–H bonds of BSA were mostly oxidized and broken to bind Cu$^{2+}$ ions. The mass amount of oxygen element contained in BSA@CuS (22.7%) or R-BSA@CuS (21.5%) was more than that (19.8%) of BSA, confirming the occurrence of their oxidation.
Finally, TEM was also used to analyze the size, morphology, and crystalline structures of BSA-based samples, as shown in Figure 1g. In Figure 1g (1)–(3), the average size (36.5 ± 11.3 nm) of R-BSA@CuS nanocomposites was larger than that of BSA@CuS (24.7 ± 5.4 nm) or bare BSA (13.5 ± 4.6 nm), due to the aggregation of BSA induced by reaction conditions. Moreover, in Figure 1g (6), CuS nanoparticles with the size of 4.4 ± 1.8 nm (signed by red arrows) were randomly distributed in BSA templates of BSA@CuS samples. And in Figure 1g (7), similar phenomenon (3.9 ± 2.2 nm) was also observed in R-BSA@CuS samples; however, no CuS nanoparticles could be found in bare BSA samples (Figure 1g (5)). Furthermore, high-resolution TEM imaging of CuS nanoparticles was measured to analyze their crystalline structures. In Figure 1g (4) and (8), CuS (002)
and (102) lattice planes (signed by yellow lines) of BSA@CuS or R-BSA@CuS samples were clearly observed, providing direct evidence of the in situ formation of CuS nanocrystalline in BSA templates. However, according to the decreasing of copper elemental concentration in XPS data (Table S1, Supporting Information) and the sharpness of crystal lattice fringes in TEM images (Figure 1g (4),(8)), the slight decomposition of CuS nanoparticles contained in BSA templates appeared after the modification of rhodamine 110 dyes via EDC/NHS activation.

2.2. Photothermal Performance of R-BSA@CuS Nanocomposites

To test the photothermal effect of BSA-based nanocomposites in aqueous solution, an IR camera with thermal imaging was used to measure their temperature changes (T–t curves) under 808-nm laser irradiation. From Figure 2a, with the illumination time of 1.6 W cm\(^{-2}\) NIR laser increased from 0 to 15 min, the temperature of 0.8 mg mL\(^{-1}\) R-BSA@CuS (\(\Delta T = 25.0 \, ^\circ\text{C}\), orange), or BSA@CuS solution (\(\Delta T = 29.3 \, ^\circ\text{C}\), deep orange) sharply increased, respectively. However, DI water (\(\Delta T = 2.1 \, ^\circ\text{C}\), deep blue) or 0.8 mg mL\(^{-1}\) BSA solution (\(\Delta T = 2.3 \, ^\circ\text{C}\), light blue) showed negligible changes under the same irradiation. In Figure 2e,f, NIR light-to-heat conversion efficiency (\(\eta\)) of R-BSA@CuS or BSA@CuS nanocomposites were further calculated as 22.8% or 42.0%, respectively. The results indicated that as-prepared CuS nanoparticles formed in BSA templates had excellent photothermal effect. In contrast, the photothermal effect of R-BSA@CuS became weak, due to the slight degradation of CuS nanoparticles caused by NHS/EDC reaction. However, they were still enough for next photothermal therapy. Moreover, according to Figure 2b,c, the temperature elevation of R-BSA@CuS solution could be regulated by changing their concentration or NIR laser power. Finally, 0.8 mg mL\(^{-1}\) R-BSA@CuS solution was also illuminated by NIR laser (1.6 W cm\(^{-2}\), 15 min, Laser ON), and then cooled to room temperature by switching off NIR laser (Laser OFF), as “one cycle”, to evaluate their photothermal stability. In Figure 2d, the temperature elevation of 24.4, 24.3, 24.7, and 24.9 \(^\circ\text{C}\) was recorded after three consecutive cycles, respectively. These above results indicated that in contrast to small molecular photothermal agents, R-BSA@CuS nanocomposites had stable photothermal effect upon the repeated exposure of NIR laser, which was important for cancer photothermal therapy.

2.3. Cellular Uptake and Subcellular Distribution of R-BSA@CuS Nanocomposites

The cytotoxicity of R-BSA@CuS nanocomposites need to be assessed before their next cellular experiments. MCF-7 cells were incubated with R-BSA@CuS nanocomposites in the concentration range of 0–1600 \(\mu\text{g mL}^{-1}\), respectively. And the viability of these cell samples was further measured by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assays, as recorded in Figure S3b, Supporting Information. After 24-h or 48-h incubation, their cell viabilities in the concentration range of 0–400 \(\mu\text{g mL}^{-1}\) still retained more than 90%. However, their cell viability significantly decreased
when their concentration reached 800 or 1600 µg mL⁻¹. These results indicated that R-BSA@CuS nanocomposites with the concentration of ≤400 µg mL⁻¹ had negligible cytotoxicity for next cellular experiments.

With the conjugation of rhodamine-110 molecules, R-BSA@CuS nanocomposites showed strong fluorescence with maximum excitation wavelength at 490 nm and the maximum emission wavelength at 524 nm, as shown in Figure S2a, Supporting Information. The fluorescent signals were also observed by confocal imaging to probe their cellular internalization and subcellular distribution inside cancer cells. In our experiments, MCF-7 cells were incubated with 400 µg mL⁻¹ R-BSA@CuS nanocomposites for 0, 2, 4, 8, 12, or 24 h, respectively. And then these cell samples were washed by phosphate buffer saline (PBS) solution to remove non-internalized fluorescent nanoparticles, and then stained with 4',6-diamidino-2-phenylindole (DAPI) probes for confocal imaging. In our experiments, one channel (Ex = 488 nm, Em = 495 to 634 nm) was chosen to monitor the green fluorescent signals of rhodamine molecules, and the other channel (Ex = 405 nm, Em = 410 to 495 nm) was used for the blue fluorescence of DAPI molecules, and then these two channels were further merged together, as shown in Figure S3a, Supporting Information. Because DAPI probes were specifically localized in intracellular nuclei, the observed rhodamine fluorescence on the same slice of confocal imaging was from internalized R-BSA@CuS nanocomposites, not the physically absorbed ones outside cell membranes. According to Figure S3a, Supporting Information, the fluorescent signals of rhodamine dyes were detected in MCF-7 cells incubated with R-BSA@CuS nanocomposites after 8 h, due to their cellular internalization. Moreover, the software of Zen 2012 (blue edition, Carl Zeiss Microscopy, Gmbh, 2011) was used to calculate mean fluorescent intensity (MFI) of rhodamine channel, to semi-quantitatively analyze the amount of internalized nanocomposites, as shown in Figure S3c, Supporting Information. Because of the gradual increasing of MFI with incubation time prolonging from 0 to 12 h, the cellular internalization of R-BSA@CuS nanocomposites was time-dependent. On this basis, the incubation time of 12 h was optimized to assess their next subcellular distribution inside MCF-7 cells.

BSA@CuS nanocomposites were also conjugated with fluorescein isothiocyanate (FITC) dyes via thiourea linkage to prepare F-BSA@CuS nanocomposites as a non-targeting control. In Figure S2b, Supporting Information, F-BSA@CuS nanocomposites also exhibited their PL characteristics for confocal imaging, with maximum excitation wavelength at 490 nm and the maximum emission wavelength at 524 nm. MCF-7 cells were incubated with 400 µg mL⁻¹ R-BSA@CuS or F-BSA@CuS nanocomposites for 12 h, respectively. These cell samples were subsequently stained by lyso-tracker red or mito-tracker orange dyes for confocal imaging, respectively, as recorded in Figure 3. Apart from the above-mentioned green fluorescent channel for rhodamine, the other red fluorescent channel (Ex = 543 nm, Em = 556 to 683 nm) was for mito-tracker or lyso-tracker probes. According to the merged images shown in Figure 3a,b, for F-BSA@CuS nanocomposites, the green fluorescence from rhodamine molecules and red fluorescence from mito-tracker (or lyso-tracker) probes was obviously separated inside MCF-7 cells. However, for R-BSA@CuS nanocomposites, it was clearly observed that the red fluorescence from mito-tracker probes was mostly overlapped with the green fluorescence (Figure 3d), in contrast to the poor co-localization of lyso-tracker probes (Figure 3c). Furthermore, Pearson’s correlation between green and red fluorescence was calculated to evaluate the organelle co-localization of these nanocomposites. Pearson’s coefficient of R-BSA@CuS nanocomposites in mitochondria reached 0.69, compared with only 0.02 in lysosomes. This meant that nearly 70% of these internalized R-BSA@CuS nanocomposites went through endosomes in the cytosol, and then specifically accumulated in mitochondria, but not transferred into the lysosomes. In contrast, the subcellular distribution F-BSA@CuS nanocomposites as a non-targeting control had no significant correlation with mitochondria (Pearson’s coefficient, -0.03) or lysosomes (Pearson’s coefficient, -0.07) in MCF-7 cells, which confirmed that rhodamine-110 molecules as specific ligands enabled BSA@CuS nanocomposites to specifically accumulate in mitochondria of MCF-7 cells.

### 2.4. Enhanced Photothermal Therapeutic Efficacy of Cancer Cells

To evaluate the photothermal therapy of cancer cells based on mitochondria-targeted R-BSA@CuS nanocomposites, the viability of MCF-7 cells with different treatments was measured by MTT methods, as seen in Figure 4b. In our experiments, MCF-7 cells were incubated with 400 µg mL⁻¹ BSA@CuS or R-BSA@CuS nanocomposites for 12 h, respectively, which made nanoparticles efficiently go into cells via cellular endocytosis. Subsequently, these cell samples were repeatedly washed by fresh culture media, to eliminate the photothermal effect generated by non-internalized nanoparticles in the media. Finally, after the irradiation of NIR laser, these cell samples were cultured for 12 h for MTT analysis. Upon the exposure of NIR laser with different power intensity of 1.6, 2.4 and 3.2 W cm⁻², the temperature elevation generated by BSA@CuS nanocomposites in cell culture media was measured as 15.5, 24.4, and 32.9 °C, respectively, as shown in Figure S4, Supporting Information. According to Figure 4b, when the power intensity of NIR laser irradiation increased from 0 to 3.2 W cm⁻², the viabilities of bare MCF-7 cells group as a control were 100.0 ± 9.6%, 116.4 ± 31.4%, 91.5 ± 8.4%, and 112.2 ± 2.7%, respectively. However, for BSA@CuS group, the viabilities of MCF-7 cells incubated with BSA@CuS nanocomposites gradually decreased in the sequence of 94.2 ± 78.8% → 76.9 ± 10.7% → 70.8 ± 9.2% → 67.2 ± 3.3% under the same NIR laser irradiation. The significant differences (signed by asterisk) of the cellular viability with 1.6, 2.4, and 3.2 W cm⁻² NIR laser irradiation were statistically calculated as NS, *p < 0.05, and **p < 0.01, relative to that of MCF-7 cell without NIR irradiation. The results indicated that the cellular viability were obviously dependent on the temperature elevation generated by BSA@CuS nanocomposites under NIR laser irradiation. For R-BSA@CuS group, their cellular viabilities in sequence was 114.6 ± 31.3% → 90.8 ± 17% → 48.9 ± 2.9% → 40.4 ± 4.9%, with the significant differences of 2.4 and 3.2 W cm⁻² reaching ***p < 0.001. In particular, under 3.2 W cm⁻² NIR laser irradiation, the cellular viability (67.2 ± 3.3%) in
BSA@CuS group sharply decreased to 40.4 ± 4.9% in R-BSA@CuS group, with the significant difference of ***p < 0.001. According to these above results, non-targeting BSA@CuS nanocomposites showed their photothermal therapy of cancer cells. However, mitochondria-targeted R-BSA@CuS nanocomposites presented a significantly enhanced therapeutic efficacy, despite of the slight decreasing of their photothermal effect under the same irradiation.

To further explore the mechanism of above-mentioned enhanced photothermal therapy, the mitochondria damage in MCF-7 cells induced by NIR laser irradiation was assessed. Here, the depolarization of mitochondria membrane potential was detected by 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) probes. Generally, JC-1 molecules in monomer state were distributed in cytoplasm to emit green fluorescence, while the aggregation in normal mitochondria with polar membrane potential generated strong red fluorescence. However, when cells were at apoptosis or necrosis stage, JC-1 molecules difficultly accumulated in the abnormal mitochondria due to the depolarization of their membrane potential, resulting in the attenuation of their red fluorescence. In our experiments, MCF-7 cells were incubated with 400 µg mL⁻¹ BSA@CuS or R-BSA@CuS nanocomposites for 12 h, respectively. Subsequently, they were repeatedly washed by fresh culture medium, illuminated by 3.2 W cm⁻² NIR laser for 10 min, and stained by JC-1 probes for confocal imaging. Here, one channel (Ex = 543 nm, Em = 556 to 683 nm) was used to detect red fluorescent signals of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a. In bare MCF-7 cells of group 1 (as a control), strong red fluorescence of JC-1 aggregates, and the other channel (Ex = 488 nm, Em = 493 to 545 nm) was used for the green fluorescence of their monomers, and then two channels were further merged together, which was displayed in Figure 4a.
R-BSA@CuS nanocomposites. Moreover, fluorescence of these above groups was further quantitatively analyzed by flow cytometry, which was recorded in Figure 4d. In groups 1 and 2, cell percentage in Q2 quadrant reached 99.6% and 90.4%, and only tiny amount of cells distributed in Q4 quadrant (0.2% or 9.6%). In group 3, cell percentage in Q4 quadrant increased to 29.9%, with the decreasing of Q2 quadrant to 70.1%, attributed to the decreasing of their red fluorescence intensity. Especially, cell percentage of group 4 sharply increased to 76.1% in Q4 quadrant, indicating the heavy attenuation of their red fluorescence. These results showed that photothermal therapy based on non-targeting BSA@CuS nanocomposites can induce the apoptosis or necrosis of cancer cells, resulting in mitochondrial dysfunctions. In contrast, R-BSA@CuS nanocomposites mostly accumulated in the mitochondria, and generated much higher localized temperature to induce heavier mitochondrial damage under the same NIR laser irradiation. Moreover, HSP90 expression levels in MCF-7 cells with different treatments were further analyzed to evaluate their heat tolerance for NIR laser-induced hyperthermia, as shown in Figure 4c. Compared with group 2 (BSA@CuS without NIR irradiation), HSP90 expression levels of cell samples in group 3 of BSA@CuS + NIR increased by 1.4-fold. The result indicated that HSP90 overexpression was induced by photothermal therapy, which would lower the therapeutic efficacy with enhancing the heat tolerance of cancer cells. However, compared with group 4 (R-BSA@CuS without NIR irradiation), HSP90 expression levels of cell samples in group 5 of R-BSA@CuS + NIR decreased by 1.3-fold. Upon the NIR laser illumination, mitochondria-targeted R-BSA@CuS nanocomposites can directly destroy the mitochondria to inhibit the expression of HSP90 proteins, which can significantly weaken the heat tolerance of the treated cancer cells. Overall, an enhanced efficacy of photothermal therapy based on R-BSA@CuS nanocomposites was caused by their mitochondria targeting performance, which can induce much efficient damage of mitochondria, significantly suppress the heat tolerance of cancer cells, and more efficiently activate the apoptosis or necrosis pathway.
2.5. Improved Photothermal Therapy of Multicellular Tumor Spheroids

Although animal models can provide more physiologically relevant nanomedicine evaluation results, they are time-consuming, expensive, and laborious. Therefore, it is highly desirable to employ alternatives such as organoids to facilitate nanomedicine development and potentially accelerate their clinical translation.\cite{37,38} In our previous studies,\cite{39} 3D multicellular tumor spheroids exhibited attractive advantages in the in vitro simulations of tumor tissues, due to their low cost, short experimental periodicity, high-throughput screening, and convenient in situ observation. Herein, after 3-day culture of parental MCF-7 cells, multicellular tumor spheroids with the size of 100–200 µm were prepared to assess photothermal therapeutic efficacy of R-BSA@CuS nanocomposites. Firstly, as-prepared spheroids were incubated with 400 µg mL\(^{-1}\) R-BSA@CuS nanocomposites for 12 or 48 h, to mimic the accumulation of nanocomposites in tumors in vivo. By confocal imaging in z-stack mode shown in Figure 5a, green fluorescence of R-BSA@CuS nanoparticles was clearly observed, indicating that they could efficiently diffuse and accumulate in these spheroids. With the incubation time prolonging from 12 to 48 h, the depth of their fluorescent signals increased from 56.5 to 62.7 µm, with MFI amplification by ≈twofold, indicating the accumulation of more nanoparticles with longer incubation time. Moreover, these spheroids were directly stained by lyso-tracker red or mito-tracker orange dyes for confocal imaging, respectively. In Figure 5b, red fluorescence originating from mito-tracker probes and green fluorescence was mostly overlapped with Pearson's coefficient of 0.76, in contrast to the poor co-localization (0.14) of lyso-tracker probes. These results demonstrated that R-BSA@CuS nanocomposites can penetrate into tumors, enter cancer cells, and specifically accumulate in their mitochondria.

To further test photothermal therapy, these spheroids were incubated with 400 µg mL\(^{-1}\) BSA@Cu or R-BSA@CuS nanocomposites for 48 h (Day 2), respectively. The culture media of these spheroids were replaced by fresh media, and immediately irradiated by 3.2 W cm\(^{-2}\) NIR laser for 10 min.

![Figure 5. a) Z-stacking confocal imaging of multicellular tumor spheroids incubated with 400 µg mL\(^{-1}\) R-BSA@CuS nanocomposites for 12 or 48 h, respectively (scale bar = 100 µm). b) Confocal imaging and the corresponding Pearson's correlation analysis of multicellular tumor spheroids incubated with 400 µg mL\(^{-1}\) R-BSA@CuS nanocomposites for 48 h after the staining of lyso-tracker red dyes, and mito-tracker orange dyes, respectively (scale bar = 40 µm). c) The diameter size of multicellular tumor spheroids during the period of 4-day culture with different treatments including group 1 as a control (with no treatment), group 2 (with R-BSA@CuS, and no NIR laser irradiation), group 3 (with BSA@CuS and NIR laser irradiation), and group 4 (with R-BSA@CuS and NIR laser irradiation).](image-url)
the next period of 24 (Day 3), or 48 h culture (Day 4), these spheroids were observed by optical microscope, and their sizes were measured and statistically calculated. From Figure S4, Supporting Information, the spheroids in groups 1 or 2 still obtained their symmetrical morphology on Day 4. In contrast, the spheroids of group 3 became shrinking, and most of them in group 4 were broken into smaller fragments. During the 4-day treatment period, the sizes of these spheroids were also measured and statistically calculated in Figure 5c. The results showed that on Day 2, their sizes had negligible changes, confirming no inhibition effect of BSA-based nanocomposites on their growth. However, on Day 3, the sizes in group 3 or 4 sharply decreased to 80.5 ± 23.5 or 58.9 ± 20.9 µm with significant differences (** p < 0.001), compared with 1479 ± 37.5 µm of group 1 or 140.3 ± 37.1 µm of group 2. The significant difference between group 3 and group 4 was calculated as ** p < 0.05. It reached ** p < 0.01 after more 24 h culture on Day 4. The results demonstrated that BSA@CuS nanocomposites exhibited photothermal therapeutic effect on the growth of tumors. However, their therapeutic efficacy could be further improved by the conjugation of rhodamine-110 to endow their mitochondria-targeting capability. Accordingly, mitochondria-targeted and photothermal R-BSA@CuS nanocomposites show important potential applications on cancer photothermal therapy in future.

3. Conclusion

In summary, CuS nanoparticles were in situ synthesized in BSA templates, and then conjugated with rhodamine-110 dyes to fabricate R-BSA@CuS nanocomposites. As-prepared R-BSA@CuS nanocomposites showed excellent mitochondria-targeting and photothermal performance. Furthermore, compared with non-targeting BSA@CuS nanocomposites, mitochondria-targeted R-BSA@CuS significantly enhanced the efficacy of photothermal therapy, which was confirmed by the results of 2D monolayer cellular and 3D multicellular spheroids experiments. Therefore, we suggested that the construction strategy of mitochondria-targeted and photothermal BSA-based nanoagents opened the opportunity to develop multifunctional cancer theranostic platforms in the future, especially for combined photothermal therapy.

4. Experimental Section

Materials and Instruments: BSA and CuSO4·5H2O were obtained from Sinopharm Chemical Reagent Co. Ltd., China. NHS and EDC were obtained from Shanghai Macklin Biochemical Co., Ltd, China. Rhodamine 110 chloride and poly (2-hydroxyethyl methacrylate) (PHEMA) was purchased from Sigma-Aldrich Chemicals Reagent Co. Ltd., USA. MTT (KGA311), JC-1 (KGA601), DMEM culture medium (KGM12800S-H-500), DAPI (KGA215), Lys-Tracker red probes (KCMPP006), and mitochondrion organe probes (KCMPP003) were all obtained from KeyGen Biotechnology Co. Ltd., China. Rabbit anti-GAPDH and HRP labeled goat anti-rabbit IgG were purchased from KeyGen Biotech, China. Rabbit anti-HSP90 was obtained from Abcam, UK. Deionized (DI) water (≥18 MΩ cm resistivity, Millipore) was used in the experiments.

UV–Vis–NIR absorption spectra were measured using a Lambda 950 spectrophotometer. PL spectra were recorded by a PerkinElmer LS55 fluorescence spectrometer. XPS were performed by a Kratos AXIS Ultra DLD system with a monochromatic Al Kα X-ray beam (1486.6 eV) at 150 W in a residual vacuum of <4 × 10⁻⁶ Pa. The crystalline structures and morphology of BSA-based nanocomposites were observed by JEOL JEM-2100 UHR TEM with the accelerating voltage of 200 kV. Zeta potential and size of BSA-based nanocomposites were tested by Zetasizer NanoZS DLS measurements (Malvern Instruments, UK) at 25 °C.

Preparation of BSA-Based Nanocomposites: The synthesis procedure of BSA@CuS nanocomposites was described elsewhere. Briefly, 250 mg BSA was slowly dissolved in 7.5 mL DI water, and then 1 mL of 0.2 mol L⁻¹ CuSO₄, 0.5 mL of 1 L⁻¹ NaOH, and 2 mL of 0.2 mol L⁻¹ NaS were dropwisely added in sequence with stirring. The mixture was continuously stirred for 0.5 h at 90 °C, with its color changing from brown to deep green. And then it was repeatedly dialyzed with dialysis membrane (MWCO = 12 kDa) to prepare BSA@CuS nanocomposites.

Next, 14.0 mg rhodamine 110, 8.6 mg NHS, and 7.2 mg EDC were dissolved in 1.5 mL DMF solution, which was continuously stirred for 4 h in dark. The resultant mixture was dropwisely added into 4.5 mL of 15.6 mg mL⁻¹ BSA@CuS aqueous solution, and then continuously stirred for 12 h in dark. Finally, the reaction solution was repeatedly dialyzed with dialysis membrane (MWCO = 12 kDa) to prepare R-BSA@CuS nanocomposites, which was freeze-dried and stored at 4°C for next experiments.

50 mg BSA@CuS nanocomposites was dissolved in 10 mL carbonate buffer (pH 9–9.5), and then dropwisely added with 0.25 mL of 1 mg mL⁻¹ FITC in DMSO solution to be stirred at 4°C in dark. After 24 h, the reaction solution was repeatedly dialyzed to prepare F-BSA@CuS nanocomposites, and then freeze-dried and stored at 4°C for next experiments.

Photothermal Conversion Efficiency Test: An optical-fiber-coupled power-tunable diode laser with the wavelength of 808 nm (Hi-Tech Optoelectronics, China) was adopted as NIR laser source with the irradiated area of ∼0.8 cm² and distance of ∼2.5 cm. Under NIR laser irradiation (1.6 W cm⁻², 15 min), thermal imaging and temperature elevation of 1 mL DI water, BSA, BSA@CuS, and R-BSA@CuS aqueous solution with the concentration of 0.8 mg mL⁻¹ were observed by a IR thermal camera (FLK-T32, Fluke, USA), respectively. The solutions of R-BSA@CuS nanocomposites with different concentrations (0.1, 0.2, 0.4, 0.8, and 1.6 mg mL⁻¹) and different power density (0.8, 1.6, 2.4, and 3.2 W cm⁻²) were irradiated for 15 min to evaluate their photothermal effect. To investigate the photothermal stability, 0.8 mg mL⁻¹ R-BSA@CuS solution was further irradiated by 1.6 W cm⁻² NIR laser for 15 min, followed by naturally cooling to room temperature without laser irradiation, regarded as one cycle of “Laser ON/OFF”. Finally, the efficiency of light-to-heat conversion of BSA@CuS and R-BSA@CuS was further calculated according to the method reported in our previous work [39].

Cell Culture and MTT Assays: MCF-7 cells were cultured in DMEM medium which was supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin at 37 °C under a humidified atmosphere with 5% CO₂. MTT staining was adopted to investigate their cellular viabilities. After washing three times with PBS solution, 100 µL of MTT solution at 570 nm in each well was recorded by using a filter Max F5 spectrophotometer. The optical density was calculated as cell viability (%).

Cytotoxicity Tests: To evaluate the biocompatibility of R-BSA@CuS nanocomposites, MCF-7 cells (1.5 × 10⁴ cells per mL) were dispersed within 96-well plates to a total volume of 100 µL per well containing 25, 50, 100, 200, 400, 800, and 1600 µg mL⁻¹ R-BSA@CuS for 24 or 48 h, respectively, and then their cellular viability was analyzed by MTT methods.

To evaluate photothermal therapeutic efficacy, MCF-7 cells (1.5 × 10⁵ cells per mL) were dispersed within 96-well plates to a total volume of

Part. Part. Syst. Charact. 2021, 38, 2100013 © 2021 The Authors. Particle & Particle Systems Characterization published by Wiley-VCH GmbH
100 µL per well, containing 400 µg mL⁻¹ R-BSA@CuS or BSA@CuS for 12 h, respectively. And then these cell samples were repeatedly washed by fresh culture medium, and irradiated by NIR laser with different power intensities of 0, 1.6, 2.4, and 3.2 W cm⁻² for 10 min, respectively. After next 12-h culture, their cellular viability was analyzed by MTT methods.

Conical Imaging and Flow Cytometry: To observe the cellular uptake of R-BSA@CuS nanocomposites, cells were plated into 6-well culture plates, and the number of cells per well was ~1.5 × 10⁴ cells (per sterile slide is placed in each well). After 12-h incubation, their culture medium was replaced by the medium containing 0.4 mg mL⁻¹ R-BSA@CuS nanocomposites. After 0, 2, 4, 8, and 12 h, these cell samples were washed by PBS, and stained by DAPI probes for confocal imaging (LSM710 NLO, Zeiss, Germany).

To observe the subcellular distribution of R-BSA@CuS nanocomposites, cells were plated into 6-well culture plates, and the number of cells per well was ~1.5 × 10⁴ cells. After 12-h incubation, cell culture medium was replaced by the medium containing 0.4 mg mL⁻¹ R-BSA@CuS or F-BSA@CuS nanocomposites, respectively. After 12 h, these cell samples were washed by PBS, and stained by mito-tracker orange or lyso-tracker red probes for confocal imaging, respectively.

To monitor mitochondria damage, cells were plated into 6-well culture plates, and the number of cells per well was ~1.5 × 10⁴ cells. After 12-h incubation, cell culture medium was also replaced by the medium containing 0.4 mg mL⁻¹ R-BSA@CuS or BSA@CuS nanocomposites, respectively. After another 12-h culture, these cell samples were washed by fresh culture medium, irradiated by 3.2 W cm⁻² NIR laser for 10 min, and stained with JC-1 probes for confocal imaging. And the fluorescent intensity of these above cell samples was also quantitatively analyzed by FACScan flow cytometry (BD Biosciences, USA).

Western Blot Assays: MCF-7 cells with different treatments were washed with PBS solution and digested with Trypsin at 37 °C. Then they were lysed in Lysis Buffer with 1% of Protease Inhibitor Cocktail Kit (KeyGen Biotech, China) on ice. Bicinchoninic acid protein assay kit (KeyGen Biotech, China) was employed to measure total protein concentration of each sample. Glyceraldheyde-3-phosphate dehydrogenase (GAPDH) was set as the loading control. Equal amount of protein of each sample was loaded on an SDS-PAGE gel. After electrophoresis, the resulting gel was transferred to a nitrocellulose (NC) filter membrane. Then, the blotted NC membrane was blocked in Tris buffered saline Tween-20 (TBST) containing 5% of skim milk and shaken for 2 h, then washed with TBST three times. After that, the NC membrane was incubated with a rabbit monoclonal anti-HSP90 as the primary antibody overnight at 4 °C with shaking and washed with TBST for three times. The NC membrane was then incubated with secondary antibody HRP labeled goat anti-rabbit IgG at room temperature for 2 h followed by three times of washing with TBST. After incubation with the enhanced chemiluminescence reagent, HSPs and GAPDH were visualized using a ChemiDoc MP Imaging System (SYNGENE G: BOXChemIXR5, UK). The grey shades of each band of the blotted NC membrane were analyzed using Gel-Pro32 software.

3D Multicellular Tumor Spheroid Tests: According to the culture protocol reported in the previous reference,[39] 450 µg PHEMA was firstly incubated in 10 mL 95% ethanol solution with stirring at 37 °C for 36 h. 0.3 mL of polymer solution were dropped into each well of 96-well plates for 12 h with UV curing. To prepare multicellular tumor spheroids, ~6 × 10⁵ MCF-7 cells in 0.3 mL DMEM medium were plated into these low-attached wells, and then cultured for ~3 days at 37 °C under a humidified atmosphere with 5% CO₂, to form multicellular tumor spheroids. These spheroids were re-suspended, and filtered with simple precipitation to obtain their uniform size in the range of 100–200 µm. After the dispersion with the intensity of ~20 per well in the 96-well plate, they were incubated with 0.4 mg mL⁻¹ R-BSA@CuS nanocomposites for 24 or 48 h, and then washed by PBS solution for confocal imaging in Z-stack mode, respectively. The above-mentioned spheroids samples were also stained with mito-tracker orange or lyso-tracker red probes for confocal imaging. Moreover, incubated with 400 µg mL⁻¹ R-BSA@CuS or BSA@CuS nanocomposites, these spheroids were cultured for 48 h, respectively washed with PBS solution, irradiated with 3.2 W cm⁻² NIR laser for 10 min, and then continued to be cultured for 2 days. During the experimental periods, the morphology of these spheroids was observed by inverted fluorescence microscope (DMIL LED Fluo, Leica, Germany), and their measured size were statistically analyzed.

Statistical Analysis: SPSS statistics software was employed in the statistical analysis. All data were reported as mean ± standard deviation unless specifically noted otherwise. Statistically significant differences (**p < 0.01, *p < 0.05) were determined by ANOVA with Tukey’s post-test method. Mass amount of elements, hydrodynamic size, zeta potential of BSA-based nanocomposites, PL excitation and emission spectra of R-BSA@CuS and F-BSA@CuS nanocomposites. Confocal imaging of cellular internalization of R-BSA@CuS nanocomposites and their corresponding MFI. MTT results of MCF-7 cells incubated with R-BSA@CuS nanocomposites with the concentration in the range of 0–1600 µg mL⁻¹. T–t curves of 400 µg mL⁻¹ R-BSA@CuS nanocomposites under NIR laser irradiation with different power intensity. Optical microscopy imaging of spheroids with different treatments.

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

Acknowledgements
H.T. and Y.G. contributed equally to this work. H.A.S. acknowledges financial support from the HiLIFE Research Funds, the Sigrid Jusélius Foundation, and the European Research Council Proof-of-Concept Grant (Grant No. 825020). B.X. thanks financial support from National Natural Science Foundation of China (Nos. 30930077 and 31000164), Natural Science Foundation of Jiangsu Province (No. BK20130964), and Bilateral Chinese-Croatian Scientific Project (No. 6-5).

Conflict of Interest
The authors declare no conflict of interest.

Data Availability Statement
Research data are not shared.

Keywords
bovine serum albumin, copper sulfide nanoparticles, heat tolerance, mitochondria-targeted, photothermal therapy

Received: January 15, 2021
Revised: February 2, 2021
Published online: March 7, 2021

[1] P. E. Porporato, V. L. Payen, J. Pérez-Escuredo, C. J. De Saedeleer, P. Danhier, T. Copetti, S. Dhup, M. Tardy, T. Vazeille, C. Bouzin, O. Feron, C. Michiels, B. Gallez, P. Sonveaux, Cell Rep. 2014, 8, 754.
[2] K. Ishikawa, K. Takenaga, M. Akimoto, N. Koshikawa, A. Yamaguchi, H. Imanishi, K. Nakada, Y. Honma, J.-I. Hayashi, Science 2008, 320, 661.
