Magnetic nanoparticle clusters for photothermal therapy with near-infrared irradiation

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In this study, the photothermal effect of magnetic nanoparticle clusters was firstly reported for the photothermal ablation of tumors both in vitro in cellular systems but also in vivo study. Compared with individual magnetic Fe3O4 nanoparticles (NPs), clustered Fe3O4 NPs can result in a significant increase in the near-infrared (NIR) absorption. Upon NIR irradiation at 808 nm, clustered Fe3O4 NPs inducing higher temperature were more cytotoxic against A549 cells than individual Fe3O4 NPs. We then performed in vivo photothermal therapy (PTT) studies and observed a promising tumor treatment. Compared with PBS and individual magnetic Fe3O4 NPs by NIR irradiation, the clustered Fe3O4 NPs treatment showed a higher therapeutic efficacy. The treatment effects of clustered Fe3O4 NPs with different time of NIR illumination were also evaluated. The result indicated that a sustained high temperature generated by NIR laser with long irradiation time was more effective in killing tumor cells. Furthermore, histological analysis of H&E staining and TUNEL immunohistological assay were further employed for antitumor efficacy assessment of PTT against A549 tumors.

1. Introduction

Nanomaterials have been extensively used in biomedical research during the past twenty years [1–4]. Especially in recent years, more and more studies focused on photo-absorbing nano-agents, and photothermal therapy (PTT) employing photoabsorbers can convert optical energy into thermal energy to kill cancer cells without affecting healthy tissues [5,6]. Compared with radiotherapy, chemotherapy and surgical management, PTT is less invasive, controllable and highly efficient. Our and other research groups have developed a large number of nanomaterials as PTT agents, such as gold-based nanomaterials [5,7–9], carbon nanotubes and graphene [10,11], all of which show strong optical absorbance in the near-infrared (NIR) tissue optical transparency window. The previous results demonstrated that photoabsorber-based therapy might be a promising approach for cancer therapy, which could effectively reduce tumor growth and enhance survival [12–15]. Even so, the potential toxicity induced by photothermal agents (especially for carbon nanotubes or graphene, and so on), is still an unresolved debate [16,17], which will inevitably limit future clinic applications of PTT. These non-degradable or slowly degradable nanoparticles easily accumulate in bodily organs, resulting in increased oxidative stress, inflammatory cytokine production and cell death [18]. Therefore, it is significant to explore a bio-safety and biodegradable photoabsorber for the photothermal ablation of cancer with NIR irradiation.

Magnetic iron oxides nanoparticles (namely Fe3O4 NPs) have received tremendous attention for their excellent magnetic, biocompatible and potentially non-toxic properties [19,20]. Fe3O4 NPs, which can be manipulated and controlled by an external magnetic field, are additional important materials and have been employed in many areas, including biology, pharmaceuticals and diagnostics. Moreover, iron is a nutrient and readily metabolized by cellular regulation using the transferrin pathway. Thus, Fe3O4 NPs are easily degradable and passes in and out of cells across the plasma membrane [21]. For the acknowledged advantages of Fe3O4 NPs, they are extremely suitable for in vivo applications. In addition,
Fe3O4 NPs are exceptional materials for hyperthermia treatment of tumors [22,23]. Under an alternating magnetic field (AMF), magnetic hyperthermia of Fe3O4 NPs is produced via dipole relaxation, which can be employed to destroy tumor cells because these cells are more sensitive to temperatures in excess of ca. 41 °C than their normal counterparts [24]. Although magnetic hyperthermia has been clinically used [25–28], the technique requires high current and voltage due to large air volume within the applied field in which energy cannot be easily focused [29].

Very recently, NIR light induced photothermal effect for Fe3O4 NPs has been studied and it exhibits good photothermal converting efficiency. For example, Chu et al. applied individual Fe3O4 NPs with high concentration for the photothermal ablation of cancer by NIR laser irradiation [29]. Considering extra-high-dose magnetite might generate potential toxicity to the body, the number of usable elements should also be severely limited. Thus, Fe3O4 NPs used for PTT must be prepared, mainly involving two routes. One is to modify NIR light-absorbing materials onto the surface of magnetic NPs, another is to concentrate the magnetic NPs. Newly, it has been shown that the clustering of magnetic NPs induces a significant increase in the magnetic moment, and consequently, the clustered magnetic NPs have a much higher saturation magnetization than that of the individual NPs [30–33]. Hayashi et al. have utilized the unique merit of magnetite clusters for the magnetic hyperthermia therapy of tumors by AMF [32,33]. More significantly, previous studies have also indicated that metallic NPs clusters can induce a red-shift in the light absorption spectra [34,35], which enhances the light absorbance in the NIR region, expanding new application fields for metallic NPs clusters to be utilized as photosensitizers in the NIR PTT. Therefore, numerous papers have been reported the photothermal ablation of tumors by utilizing aggregation-induced enhanced photothermal (AIEP) of metallic NPs clusters [36,37], such as gold NPs aggregation. Until now, to the best of our knowledge, the papers on the study of photothermal effect of magnetite clusters were rarely reported, let alone for the use of magnetite clusters with NIR irradiation to destroy tumor in vivo.

Herein, we present the synthesis of magnetic colloidal nanocrystal clusters that effectively produce heat in response to harmless NIR irradiation. The biophysical parameters were not only assessed in vitro in cellular systems but also in vivo study. The results proved that the photothermal effects of clustered magnetic NPs for the treatment of A549 (Human lung adenocarcinoma epithelial cell line) tumor were better than those obtained by single crystal magnetic NPs.

2. Materials and methods

2.1. Materials

Iron (III) chloride (FeCl3·6H2O), iron (II) chloride (FeCl2·4H2O), trisodium citrate dehydrate (C3H5Na3O7·2H2O), sodium acetate anhydrous (NaOAc), ethylene glycol (EG), acetone, sodium hydroxide (NaOH), and nitric acid (HNO3) were purchased from Shanghai Chemical Reagents Company (China). MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, and other biological reagents were purchased from Invitrogen Corp. Dubecco’s modified Eagle medium (DMEM), Fetal bovine serum (FBS), Penicillin-Streptomycin solution and Trypsin-EDTA solution were purchased from Gibco (Tulsa, OK, USA). All the other chemicals were of analytical grade, and purified water was produced by a Millipore water purification system.

2.2. Synthesis of individual magnetic NPs

The individual magnetic NPs were prepared by chemical coprecipitation method [38]. In a typical recipe, FeCl3·6H2O (10.81 g, 0.04 mol) was dissolved in 50 mL deionized water. The resulting solution was transferred into a three-necked flask (250 mL), which was equipped with a mechanical stirrer, a dropping funnel, and a nitrogen inlet. Then 3.98 g of FeCl2·4H2O (0.02 mol) was also dissolved in another 50 mL deionized water, and the resulting solution was transferred into the above-mentioned three-necked flask. The as-prepared mixture was then stirred at room temperature in nitrogen atmosphere, and NaOH solution (10 wt%, 50 mL) was then slowly dropped into the flask from the dropping funnel in 1 h. After that, the mixture was continuously stirred at room temperature for 1 h, which was then stirred at 90 °C for another 2 h and cooled to room temperature under continuous stirring. The whole process was in the nitrogen atmosphere. The products were washed with deionized water three times, collected with the help of a magnet. After that, nitric acid solution (2 wt%, 100 mL) was added into the above products, which were then washed with deionized water several times and collected with the magnet, until the pH value of the supernatant is neutral. Then 100 mL (0.3 μL) of trisodium citrate was added into the resulting products, the mixture was stirred for 30 min at 90 °C in the nitrogen atmosphere. Then it was cooled to room temperature, transferred into a beaker and collected with the magnet, and 20 mL of deionized water and a lot of acetone were added into the beaker to wash the products twice. Finally, the obtained products were dispersed in 50 mL of deionized water, and stirred at 80 °C for a long time to remove acetone.

2.3. Synthesis of clustered magnetic NPs

The clustered magnetic NPs (magnetic particles) were prepared by a modified solvothermal reaction [39]. Briefly, FeCl3·6H2O (1.028 g), C8H5Na3O7·2H2O (0.24 g), and NaOAc (1.2 g) were first dissolved in 20 mL of EG under vigorous stirring for 0.5 h. The resulting solution was then transferred into a Teflon-lined stainless-steel autoclave with a capacity of 50 mL. The autoclave was sealed and heated at 200 °C for 10 h. Then it was cooled to room temperature. The as-prepared black products were washed with ethanol and deionized water several times, and collected with a magnet. The final products were dispersed in 10 mL of ethanol for the further use.

2.4. Characterization

Ultraviolet–visible (UV–vis) spectra were performed using a Perkin–Elmer Lambda 750 spectrophotometer. Transmission electron microscopy (TEM) images were obtained on a Tecnai G2 20 TWIN transmission electron microscope. Magnetic characterization was carried out with a vibrating sample magnetometer (VSM) on a Model 6000 physical property measurement system (Quantum, USA) at 300 K. X-ray diffraction (XRD) measurements were recorded on a X'pert PRO diffractometer to determine the composition of Fe3O4 particles. All the diffraction peaks in the XRD patterns were indexed and assigned to the typical cubic structure of Fe3O4 (JCPDS 75–1609).

2.5. Cell lines and culture conditions

A549 cells obtained from Chinese Academy of Sciences Cells Bank, Shanghai, China, were routinely cultured in RPMI-1640 cell medium supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, at 37 °C in 5% CO2 and 95% air atmosphere with >95% humidity. All experiments were performed on cells in the logarithmic phase of growth.

2.6. NIR-heating effect of magnetite NPs in solution

The individual magnetic NPs or clustering of magnetic NPs stock solution at 1 mg/mL was diluted to the different concentrations (10–80 μg/mL) and 200 μL of aliquots were deposited into wells of a 48-well cell culture plate. Wells were illuminated by an 808-nm continuous-wave NIR laser (Changchun New Industries Optoelectronics Technology, Changchun, China; fluence: 5 W/cm2, spot size: 5 mm) with the different exposure time from 60 to 180 s. Pre- and post-illumination temperatures were taken by thermocouple.

2.7. Cell viability assay

A549 cells were seeded in 96-well plates with a density of 104 cells/well, and allowed to adhere for 24 h prior to assay. The cells were exposed to the clustered magnetic NPs or individual magnetic NPs with the same concentration of 50 μg/mL, respectively. The cells were or were not irradiated by NIR laser light at a power density of 5 W/cm² with different illumination time from 60 to 180 s. After the cells were incubated at 37 °C for another 24 h, they were incubated with 0.5 mg/mL MTT in DMEM for 4 h in dark and then mixed with dimethyl sulfoxide after the supernatant was removed. The OD value at 570 nm was read using the microplate reader (Synergy TM2, BIORAD-TEK Instruments Inc. USA). Cell viability was determined by the percentage of OD value of the study group over the control group. Afterward, cells were co-stained by a mixture of Calcein AM and PI solution for 20 min. The samples were observed to observe using a ZEISS LSM710 live cell confocal laser imaging System (Carl Zeiss, Germany).

2.8. Flow cytometry analysis

To investigate the photothermal ablation of A549 cells by clustered Fe3O4 NPs without or with the 808 nm laser irradiation using flow cytometry, clustered Fe3O4 NPs dispersion (100 μg/mL in PBS solution) was added to a 12-well cell culture plate containing A549 cells with a density of 5 × 10³ cells/well in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin–streptomycin, then the A549 cells were incubated for 4 h at 37 °C in 5% CO2 and 95% air atmosphere.
with >95% humidity. Then, a group of cell solution was exposed to an 808 nm laser at a power intensity of 5 W/cm² for 180 s. After the laser irradiation treated, A549 cells were stained with Annexin-V-FITC and PI (Becton Dickinson, Mountain View, CA, USA), and analyzed by BD FACSAria I flow cytometry with excitation at 488 nm. Fluorescent emission of FITC was measured at 515–545 nm and that of DNA-PI complexes at 564–606 nm. Compensation was used wherever necessary.

2.9. In vivo photothermal treatment

Male Balb/c mice, aging 4–5 weeks and weighing 18.8 ± 1.9 g, were supplied by the Department of Experimental Animals, Fudan University (Shanghai, China), and maintained under standard housing conditions. All animal experiments were carried out in accordance with guidelines evaluated and approved by the ethics committee of Fudan University. Through a subcutaneous injection of 2 × 10⁶ cells suspended in 100 µL PBS into the flank region, the mice bearing A549 tumor were successfully established. The longest dimension and the shortest dimension of tumors were monitored by digital calipers. Once tumors were measured ~5.0 mm in longest dimension, mice were randomized into 4 treatment groups (n = 5 per group): PBS with laser treated (Group I), individual Fe₃O₄ NPs with laser treated (Group II), clustered Fe₃O₄ NPs with laser treated (Group III and IV for different NIR illumination time). Each type of dispersion (2 mg/mL, injection volume of 25 µL) was intratumorally injected into mice. Two hours after injection, the 808-nm continuous-wave NIR laser was applied to irradiate tumors under a power intensity of 5 W/cm² for 180 s. They were sacrificed on day 1, 5 and 19 after injection, respectively. Three mice for one group were randomly selected and sacrificed at each time point and the tumors were extracted immediately after. The tumors were weighed and washed with distilled water. Afterward, the tissue was mixed with 0.5 mL of aqua regia and incubated at room temperature. Two days later, the samples were centrifuged and the upper clear solutions were collected and adjusted with distilled water. The concentrations of iron atoms in the solutions were performed by flame atomic absorption spectroscopy (Hitachi, Japan). An iron hollow cathode lamp was used as a light source. The excitation current, slit width and absorption wavelength of iron were set at 15 mA, 0.2 nm and 248.3 nm, respectively.

2.10. The retention of the magnetic NPs in the tumor sites

According to the previous literature [29], the retention of the Fe₃O₄ NPs in the tumor sites was also studied. Once tumors were measured ~5.0 mm in longest dimension, mice were randomized into 2 groups (n = 9 per group): individual Fe₃O₄ NPs with laser treated (Group I) clustered Fe₃O₄ NPs with laser treated (Group II). Each type of dispersion (2 mg/mL, injection volume of 25 µL) was intratumorally injected into mice. Two hours after injection, the 808-nm continuous-wave NIR laser was applied to irradiate tumors under a power intensity of 5 W/cm² for 180 s. They were sacrificed on day 1, 5 and 19 after injection, respectively. Three mice for one group were randomly selected and sacrificed at each time point and the tumors were extracted immediately after. The tumors were weighed and washed with distilled water. Afterward, the tissue was mixed with 0.5 mL of aqua regia and incubated at room temperature. Two days later, the samples were centrifuged and the upper clear solutions were collected and adjusted with distilled water. The concentrations of iron atoms in the solutions were performed by flame atomic absorption spectroscopy (Hitachi, Japan). An iron hollow cathode lamp was used as a light source. The excitation current, slit width and absorption wavelength of iron were set at 15 mA, 0.2 nm and 248.3 nm, respectively.

3. Results and discussion

3.1. Characterization of magnetic NPs

A TEM image shows the dimension of our obtained individual Fe₃O₄ NPs is about 15 nm (Fig. 1a), while that of the clustered Fe₃O₄
NPs has a nearly uniform size of ca. 225 nm with spherical shape (Fig. 1b). A TEM image of the clustered Fe₃O₄ NPs at higher magnification (Fig. S1) illustrates that the magnetite conglomerations are loose clusters, and the particles are composed of nanocrystals with the size of about 5–10 nm. According to the previously reported literature [44], these nanocrystals are connected with each other by amorphous matrix as a bridge. The samples of individual and clustered Fe₃O₄ NPs both show similar typical XRD patterns of magnetite (JCPDS no. 75-1609; Fig. 1c), which indicates that the nanocrystalline structure of the clustered Fe₃O₄ NPs is the same as individual Fe₃O₄ NPs. The magnetic property characterization (Fig. 1d) shows that both individual and clustered Fe₃O₄ NPs have superparamagnetic property and high magnetization with saturation value of 58.69 emu/g for the individual Fe₃O₄ NPs, and 63.70 emu/g for the clustered Fe₃O₄ NPs, respectively.

3.2. Photothermal effect of magnetic NPs

Fig. 2a illustrates the UV–vis spectra of the individual and clustered Fe₃O₄ NPs dispersed in deionized water. The individual Fe₃O₄ NPs have a very weak absorption in the NIR spectroscopy from 600 to 1000 nm. Surprisingly, the clustered Fe₃O₄ NPs show an obvious absorption band containing a maximum at ca. 420 nm. Moreover, compared with individual Fe₃O₄ NPs, the clustered Fe₃O₄ NPs also resulted in a significant ~3.6 fold increase in the NIR absorption at 808 nm for the aggregation of nanoparticles. Thus, the clustered Fe₃O₄ NPs that have a stronger absorption in the NIR spectroscopy may be conducive to destroying tumors in vivo as an effective thermal generator due to its in-depth tissue penetration. The photothermal effects of the PBS solutions of the individual and clustered Fe₃O₄ NPs were firstly examined and compared in vitro by NIR laser irradiation (λ = 808 nm, 5 W/cm²). As seen from Fig. 2b, the clustered Fe₃O₄ NPs were more efficient than the individual Fe₃O₄ NPs in inducing a temperature increase with the same concentration and exposure time. For example, the temperature raised by the clustered Fe₃O₄ NPs solution could reach 51.4 ± 1.2 °C with the NPs concentration of 50 µg/mL and NIR exposure time of 180 s, while that of the individual Fe₃O₄ NPs only reached 42.0 ± 1.5 °C. Compared with the previous report using individual Fe₃O₄ NPs with high concentration [29], the higher temperature was easily acquirable by clustered Fe₃O₄ NPs with lower concentration and shorter NIR irradiation time in our study, which was beneficial for the safe application in vivo. In addition, the NIR-heating effects of the individual and clustered Fe₃O₄ NPs were both time and concentration-dependent. The NPs with higher concentration performed markedly better than that of low concentration. Likewise, the longer the laser irradiation time, the higher the solution temperature is. The results indicate that optoelectronic excitation of clustered Fe₃O₄ NPs by NIR irradiation can occur rapidly, and the extra energies are efficiently transferred into molecular vibration modes to generate significant amounts of thermal energy, which could be useful as photothermal mediators.

In order to validate the feasibility of the Fe₃O₄ NPs in biomedicine, the nanomaterials of individual or clustered Fe₃O₄ NPs were administered to A549 cells in 96-well plates, respectively, and each group was subdivided into groups of with and without NIR laser exposure. The cell counting Kit-8 (CCK-8) assay was employed for the quantitative evaluation of cell viability [45]. The viability of untreated cells was assumed to be 100%. The cell viability remained about 92.63% when they were incubated with individual or clustered Fe₃O₄ NPs at higher concentration of 500 µg/mL for 24 h (Fig. S2, Supporting information), which indicated our magnetic nanomaterials had no inherent toxicity. Subsequently, the magnetic nanomaterials were applied for the photothermal ablation of cancer cells. The cytotoxic effects by NIR irradiation were shown in Fig. 3a. About 8.9%, 33.5% and 72.8% of cells were killed by clustered Fe₃O₄ NPs at the concentration of 50 µg/mL with a power density of 5 W/cm² for different illumination time of 60, 120 and 180 s, respectively. And only 0.8%, 3.5% and 14.5% of cells were killed by individual Fe₃O₄ NPs. In addition, direct irradiation of the cells remained close to 100%, exhibiting no effect on cell viability. This is mainly because the low light absorption by natural endogenous cytochromes of these cells caused minimal temperature elevation accounts for their high survival [5]. The CCK-8 experimental result indicated that clustered Fe₃O₄ NPs inducing higher temperature were more cytotoxic against A549 cells than individual Fe₃O₄ NPs by NIR irradiation. These results also proved that cancer cells could be effectively killed by clustered Fe₃O₄ NPs with lower concentration and shorter NIR irradiation time compared with previous report [29]. Fluorescence images of calcine AM (green, live cells) and PI (red, dead cells) co-stained cells after PTT were further confirmed the effectiveness of PTT using clustered Fe₃O₄ NPs (Fig. 3b). A large number of killed A549 cells were observed by
Fig. 3. (a) Relative viabilities of PBS, individual and clustered magnetic Fe$_3$O$_4$ NPs with the concentration of 50 µg/mL treated A549 cells without or with NIR laser irradiation ($\lambda = 808$ nm, 5 W/cm$^2$) for 60–180 s. (b) Confocal fluorescence images of calcein AM (green, live cells) and propidium iodide (red, dead cells) co-stained A549 cells treated by clustered magnetic Fe$_3$O$_4$ NPs at the concentration of 50 µg/mL with NIR laser irradiation ($\lambda = 808$ nm, 5 W/cm$^2$) for (1) 0 s, (2) 60 s, (3) 120 s, (4) 180 s. (c) Flow cytometry graphs of A549 cells treated by clustered magnetic Fe$_3$O$_4$ NPs without or with NIR laser irradiation ($\lambda = 808$ nm, 5 W/cm$^2$) for 180 s. The treated A549 cells were double stained by Annexin-V-FITC/PI and analyzed by flow cytometry. Quadrant I doesn't representatively correspond to live or damaged cells; Quadrant II representatively corresponds to the population of dying cells. The membranes of these cells had been compromised so PI could penetrate and then hybridize with their DNAs; Quadrant III represents the population of live cells. Since the live cells have intact cell membranes, PI could not stain their DNAs; The population of dead cells, or stained DNAs, exhibits a high PI and small forward scattering signal as shown in quadrant IV. These are free DNAs, no longer enclosed within a membrane. The analysis data reveal that cells targeted by the clustered magnetic Fe$_3$O$_4$ NPs combined with NIR lighting display irreversible damage, as shown by the larger population in quadrant IV, where the cellular membrane is broken to such an extent that the cell can no longer function nor recover from the damage [46].
clustered Fe3O4 NPs exposed to laser illumination with a power density of 5 W/cm² for 180 s. Once the irradiation time was reduced to 120 s, most of cells remained survival. In addition, cells incubated with clustered Fe3O4 NPs for irradiation time of 60 s or without NIR laser treatment both showed limited damage, which could be attributable to insufficient lethal temperature rise. The fluorescence imaging result was consistent with the CCK-8 result.

To make clear the cell death mode after photothermal treatment, an Annexin-V-FITC/PI method was conducted by flow cytometry. Fig. 3c showed the flow cytometry graphs of the cells by clustered Fe3O4 NPs with and without laser irradiation. Annexin-V-FITC emission signal was plotted on the x-axis, while PI emission signal was plotted on the y-axis. The graphs quadrants were divided into four quadrants. The quantities of living cells, apoptotic cells and necrotic cells were determined by the percentage of Annexin V+/PI-, Annexin V+/PI+ and Annexin V-/PI-. Almost no apoptosis or necrosis cells were observed in the group of no NIR treatment. After laser irradiation, the apoptosis rate of cells reached 67.7%, while the necrosis rate was only 6.4%. The flow cytometry data revealed that cells treated by clustered Fe3O4 NPs displayed irreversible damage upon photothermal treatment, and their cellular membranes were broken to such an extent that the cells could no longer function nor recover from the damage. Therefore, the mechanism of in vitro PTT is mainly triggered by cell apoptosis, not necrosis.

3.3. Photothermal ablation of tumor in vivo

Encouraged by the effective photothermal outcome in vitro, we next performed a pilot in vivo photothermal treatment study. In this work, A549 tumor-bearing mice were established by subcutaneous injection of 2 × 10⁶ A549 cells suspended in RMPI-1640 medium into the flank region. Once the tumors were measured ca. 5.0 mm in longest dimension, mice were randomized into four treatment groups (n = 5 per group) mentioned in the experimental section. There was no statistical difference among group mean tumor volumes at the onset of treatment (P > 0.05). Twenty-four hours before irradiation, mice were then injected intratumorally with 25 μL of treatment solution containing 50 μg of individual or clustered Fe3O4 NPs in Group II, III and IV, leaving light gray scars on the original tumor sites. The tumors were illuminated with an 808-nm NIR laser (5 W/cm²; spot size, 5 mm) for 120 s in Group III and for 180 s in Group II, IV. No mice died during the course of therapy.

Fig. 4 showed the infrared thermal images of tumor surface in Group I, II and IV mice. In Group IV, the maximum temperature of the tumor surface rapidly increased 216.1 °C to reach 55.9 °C attributing to the clustered Fe3O4 NPs with a good NIR-heating property. In addition, as can be seen from the figure, the increase in NIR irradiation time from 120 s to 180 s could not significantly increase the temperature of tumor surface, showing the temperature reached a balance after two minutes of NIR irradiation. At that time, the NIR laser didn’t stop working, and kept irradiating for one minute to maintain a high temperature in the tumor site to effectively kill A549 cells. In Group II, the maximum temperature of the tumor surface reached 50.3 °C, which was inferior to Group IV due to the weak photothermal effects. In contrast, less temperature changed in Group I by PBS with NIR treated, and increased only 4.2 °C. Furthermore, in order to investigate the effect of PTT using clustered Fe3O4 NPs with different NIR irradiation time, the 808-nm continuous-wave NIR laser was employed to irradiate tumors under a power intensity of 5 W/cm² for 120 s in Group III. Compared with Group IV, the duration time of high temperature in Group III reduced one minute. As we know, high temperature can lead to cell apoptosis, even instantaneous coagulative necrosis and irreversible cell death [47]. Tumor sizes after diverse treatments were measured every two days (Fig. 5a). The tumors of Group I, II and III grew rapidly. There was no statistically significant difference in final tumor size (P > 0.05), indicating that tumor growth was not affected by PBS or individual Fe3O4 NPs with NIR irradiation for 180 s, and clustered Fe3O4 NPs with NIR irradiation time of 120 s. On the contrary, a statistically significant of tumor growth was observed in mice treated with clustered Fe3O4 NPs plus NIR laser for illumination time of 180 s (P < 0.05). Effects were most pronounced in the Group IV, where mean tumor volume at 19 days was reduced from 955.3 mm³ in controls to 222.8 mm³ (P = 0.0139). At 19th days, mice were euthanized, tumors were excised and weighted (Fig. 5). The weights of tumors for Group I, II, III, IV and V were 0.5311 ± 0.0599 g, 0.4369 ± 0.0452 g, 0.4072 ± 0.0479 g and 0.2297 ± 0.0333 g, respectively (Fig. 5d). Obviously, the antitumor efficiency of Group IV was particularly prominent and was superior to all the other groups (P < 0.01), showing an inhibition rate of 56.75%, 47.43% and 43.59% compared with Group I, II and III. However, there was no statistically significant difference in Group III compared with Group I, II, indicating that a lack of sustained high temperature would hardly effectively kill the tumor cells.
Accordingly, histological analysis of H&E staining illustrated that the tumors in test groups exhibited a scarlike structure containing numerous collagen bundles (Fig. S3), especially for Group IV. However, a lot of tumor cells were observed in Group I, II and III. The antitumor efficacy of PTT against A549 tumor was also evaluated by the TUNEL immunohistological assay (Fig. S4), which exclusively detected apoptosis in tumor tissues. TUNEL signals (brown fluorescence) were observed in Group II, III and IV. Tumor tissues treated with clustered Fe3O4 NPs plus laser irradiation for 180 s showed more TUNEL signals than did tumors treated with PBS and individual Fe3O4 NPs plus laser irradiation for 180 s, and clustered Fe3O4 NPs plus laser irradiation for 120 s, indicating that photothermal ablation of clustered Fe3O4 NPs with long time NIR treatment was more efficient for tumor therapy. Last but not the least, to investigate the metabolism of the Fe3O4 NPs after therapy, the iron atoms captured in the tumors were determined by flame atomic absorption spectroscopy. The analytical results exhibited that 98.7%, 77.8%, 53.5% of individual Fe3O4 NPs or 99.1%, 72.3%, 46.7% of clustered Fe3O4 NPs in the tumors for 1, 5 and 19 days post-injection with NIR irradiation with 180 s (Fig. S5). The analytical result showed that our Fe3O4 NPs could be slowly cleared from the tumors after therapy. Besides, the high Fe3O4 NPs uptake in the first few days is particularly important for photothermal cancer treatment if repeated NIR irradiation therapy was needed.

4. Conclusions

The individual and clustered Fe3O4 NPs with the same nanocrystalline structure were successfully synthesized. The clustered Fe3O4 NPs were more efficient than the individual Fe3O4 NPs in inducing a temperature increase. Significantly greater cell killing was detected when A549 cells incubated with clustered Fe3O4 NPs were irradiated with NIR illumination. The flow cytometry analysis demonstrated that the mechanism of in vitro PTT was mainly triggered by cell apoptosis, not necrosis. Using an A549 tumor model, a higher therapeutic efficacy was obtained by the NIR-induced hyperthermia of the clustered Fe3O4 NPs. We believe that our study has the potential in the future clinical application of cancer therapies for the clustered Fe3O4 NPs with good photothermal effect and excellent biological safety.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2014.10.064.

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