SERS imaging elucidates the folate receptor-mediated photothermal/photodynamic synergistic anticancer nanodrugs-induced cell apoptosis

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Research

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Abstract

Background: Chemotherapy and radiotherapy are common methods of cancer treatment, but they are accompanied by serious side effects. Actually, many cancer cells have overexpression of folic acid (FA) receptor and by FA receptor-mediated endocytosis, anticancer drugs can be easily internalized into cancer cell, this will greatly improve the curative effect and decrease side effects. Along with the development of nanotechnology, phototherapy, owning advantages in tissue selectivity, process controlling, low toxicity and reproducible treatment, has become very promising, especially for photothermal therapy (PTT) and photodynamic therapy (PDT). Since both PTT and PDT involve the utilization of light energy, so they synergistic treatment should be a good solution by ingenious design. In this paper, based on surface enhanced Raman spectroscopy (SERS) imaging, we hope to construct a FA receptor-mediated PTT/PDT synergistic anticancer nanodrug (nanoprobe), and achieve the intracellular distribution information of the nanodrugs during cell apoptosis, and then elucidate PTT/PDT-induced cell apoptosis and synergistic efficiency.

Results: FA receptor-mediated PTT/PDT synergistic anticancer nanodrugs with tracing function are prepared by the chemical synthesis and modification of gold nanorods (GNR), involving protoporphyrin IX (PpIX), 4-mecaptobenzoic acid (MBA), and FA. Based on SERS imaging, it is found that the FA receptor-mediated endocytosis can greatly facilitate the nanodrugs internalization, in which both the number and intracellular dispersion of the PpIX-GNR-MBA-FA nanodrugs are improved relative to the GNR-MBA or PpIX-GNR-MBA compositions, and then enhance the PTT/PDT-induced cell apoptosis.

Conclusion: SERS imaging is very suitable for the phototherapy tracing due to its high sensitivity and stability. The FA receptor-mediated way can significantly facilitate nanodrugs internalization, PTT/PDT-induced cell apoptosis and synergistic efficiency. Importantly, this FA receptor-mediated SERS imaging based PTT/PDT synergistic treatment will provide a novel strategy for the design and application of anticancer phototherapy nanodrugs.

Background

To date, chemotherapy and radiotherapy are common methods of cancer treatment [1], but they are accompanied by serious side effects [2, 3]. In fact, folic acid (FA) receptor, having overexpression on the surface of various cancer cells including the brain, breast, lungs and so on, has been widely employed as tumor targeted ligand in selective drug delivery [4–6]. By FA receptor-mediated endocytosis, many anti-cancer drugs can be internalized [7, 8], this will greatly improve the curative effect and decrease side effects.

Along with rapid development of nanotechnology, phototherapy, owning obvious advantages in tissue selectivity, process controlling, low toxicity and reproducible treatment, has become very promising, especially for the photothermal therapy (PTT) and the photodynamic therapy (PDT) [9, 10]. In PTT, by using special materials with high photothermal conversion under the excitation of near infrared light

(NIR), the local temperature of cell or tissue can rapidly increase and ablate targeting tumours [11–13]. In PDT, under the light irradiation of specific wavelength, the photosensitizer can transfer the absorbed photon energy to surrounding oxygen molecules, and lead to the generation of reactive oxygen species (ROS), $^1\text{O}_2$ and other strong oxide species, and then induce cell apoptosis [14–16].

For the sake of improving treatment efficiency, the synergistic therapy of multi-methods has emerged as an efficient solution. Both PTT and PDT involve the utilization of light energy, and based on the local surface plasmon resonance (LSPR) of noble metal nanoparticles, the efficiency of PDT can be enhanced along with the photothermal effect, so PTT and PDT (PTT/PDT) synergistic treatment should achieve significant efficiency by ingenious design. Current studies have reported good efficiency of PTT/PDT synergistic therapy [17–19], but how does FA receptor-mediated endocytosis affect the amount and distribution of intracellular PTT/PDT nanodrugs are still lack.

Recently, a series of photothermal agents with high photothermal conversion efficiency, including graphene [20–22], carbon nanotubes [23], and gold nanoparticles [24, 25] are introduced into PTT. Among them, gold nanoparticles [26–28] especially gold nanorods (GNRs) have spurred extensive attentions toward biomedical materials for therapy owing to the simple preparation [29], high photothermal conversion efficiency, strong NIR absorption [30], easy modification, and good biocompatibility [31–34].

Till now, a series of photosensitizer agents, including phthalocyanine green (ICG) [35], dihydroporphyrin (Ce6) [36], hematoporphyrin (HP) [37], pheophytin [38], protoporphyrin IX (PpIX) [39–41], have been developed. However, the traditional PDT treatment suffers from several drawbacks such as high oxygen dependent, poor water solubility and poor targeting of photosensitizer. Moreover, the generated ROS has very short half-life and limited diffusion distance, so the efficiency of PDT is greatly restricted [42, 43]. Due to the advantages in easy modification, strong photodynamic effect, cellular respiration promotion, PpIX is becoming a better candidate of photosensitizer [44, 45]. Furthermore, it is found that PpIX is temporarily quenched after conjugation with GNRs, and released when the probe is internalized into cancer cell as a result of avoiding the inconvenience in the dark treatment [18].

For nanodrugs design and application, by the internalized number and distribution information, both fixed-point irradiation and selective treatment can be easily achieved, thereby improving efficiency and saving energy. So far, many methods, including fluorescence imaging [31, 32], photoacoustic imaging [46] and magnetic resonance imaging (MRI) [47], have been employed to implement targeted and precise cancer treatment. However, these methods have disadvantages in low stability or low sensitivity. Surface enhanced Raman scattering (SERS) imaging, a developing solution with high stability and sensitivity, high specificity and in situ detection, is becoming a good solution for phototherapy application [48–52].

In view of the above facts, we hope to construct a FA receptor-mediated PTT/PDT synergistic anti-cancer nanodrug with the tracing function based on SERS imaging. Figure 1 shows the synthesis illustration, and the final structure of the nanodrug is named as PpIX-GNR-MBA-FA (Fig. 1C), in which the GNR realize photothermal conversion, but also enhance the efficiency of photodynamic. In order to achieve the

conjugation between GNR and PpIX, the thiolation of PpIX with cysteamine hydrochloride is used (Fig. 1A). Raman reports molecules 4-Mercaptobenzoic Acid (MBA) is selected as the bridge between GNR and FA, so not only the FA receptor mediated path is achieved, but also the nanodrug is equipped with SERS signal (Fig. 1B). After that, by using the prepared PpIX-GNR-MBA-FA SERS nanoprobe to image receptor-mediated internalization, we can achieve intracellular distribution information of PTT/PDT nanodrugs during cell apoptosis, and elucidate the FA receptor mediated PTT/PDT synergistic anti-cancer nanodrugs-induced cell apoptosis.

Results And Discussion

Synthesis and characterization of PTT/PDT nanodrugs

In order to achieve the FA receptor mediated PpIX-GNR-MBA-FA nanoprobe, we first need to prepare PpIX-SH and FA-MBA compositions, respectively. Figure 2A and 2B show the corresponding FTIR spectra. In Fig. 2A, it is observed that two new peaks at 1564 and 1622 cm^{-1} , attributed to the amide bond, appear in the PpIX-SH compared with the PpIX, and the characteristic peak of the PpIX-SH at 1988 cm^{-1} , corresponding to the stretching vibration of -SH in cysteamine hydrochloride [51], is also observed. Clearly, this result demonstrates that a thiol group has been introduced into PpIX by the conjugation between the carboxyl group of PpIX and the amino group of cysteamine hydrochloride. In addition, a significant blue shift appears in the fluorescence spectrum of PpIX-SH compared with PpIX (Fig. S1), indicating the strong electronegativity of sulfhydryl group in the cysteine [18].

While, in Fig. 2B, the peak at 1602 cm^{-1} , attributed to the vibration of the benzene ring skeleton, is observed in above three compositions. Differently, two peaks at 1678 and 3285 cm^{-1} , respectively attributed to the carbon oxygen acid bond of the amide bond and -NH- in amide bond, appeared in MBA-FA, indicating that MBA and FA have been successfully conjugated by amide bonds.

Subsequently, the PpIX-SH and MBA-FA compositions are conjugated to GNRs, respectively. Figure 2C presents the corresponding UV-Vis absorption spectra, in which an absorption peak of longitudinal surface plasmon resonance (SPR) near 785 nm is observed in all compositions, including GNR, GNR-MBA, GNR-PpIX, GNR-MBA-FA, PpIX-GNR-MBA, and PpIX-GNR-MBA-FA. Compared with the GNR UV-Vis spectrum, a blue shift 12 nm appears in the GNR-MBA-FA, reflecting the strong electronegativity of the exposed carboxyl group due to the amino terminus of FA is conjugated to the carboxyl terminus of MBA, and the sulfhydryl terminus of MBA is conjugated to GNR, as shown in Fig. S2. Moreover, a red shift 10 nm is observed in the characteristic peak at 780 nm of PpIX-GNR-MBA and PpIX-GNR-MBA-FA due to the strong conjugation of the ring structure between PpIX and MBA. In addition, it is found that a characteristic peak at 280 nm corresponding to FA and the characteristic peak of PpIX near 400 nm , appear in the UV-Vis spectrum of PpIX-GNR-MBA-FA.

Figure 2D presents the SERS spectra of GNR, GNR-MBA, GNR-MBA-FA, PpIX-GNR-MBA, PpIX-GNR-MBA-FA, respectively. We can see that two strong characteristic peaks at 1077 and 1583 cm^{-1} , respectively

attributed to the sulfhydryl group and the in-plane deformation of the hydrocarbon bond of benzene ring of MBA, appear in all compositions except GNR, and the corresponding intensity of SERS signal is decreased with the number increasing of the conjugated function molecules, the possible reason is the crowding each other of GNR surface molecules. Specially, there is little change in characteristic peaks after the covalent attachment of the FA and MBA compared to the GNR-MBA, indicating the mutual exclusion of the sulfhydryl group with stronger binding force. By the way, the peak of 640 cm^{-1} is a characteristic peak of DMSO solvent. Though another characteristic peak around 1585 cm^{-1} also appears in GNR-PpIX (Fig. S3), but it is not suitable for Raman probe due to the low signal intensity and big peak width.

Also, from the TEM imaging results of GNR and PpIX-GNR-MBA-FA (Fig. 2E and 2F), we can see that the rod-like GNRs with mean length of $39 \pm 2\text{ nm}$ and width of $10 \pm 2\text{ nm}$ are observed, in which a gray shell of $2 \sim 6\text{ nm}$ around the GNR surface appears, further indicating that the conjugation among MBA, FA and PpIX is successful. Considering dispersion is an important parameter for nanodrugs application, we also measure the corresponding hydrodynamic size distribution by the dynamic light scattering (DLS) method, as shown in Fig. S4. Although some aggregation occurs, the properties of PTT and PDT properties will not be affected [51].

Cell internalization assays

Photothermal nanodrugs first need to be internalized into cancer cell, and the internalized number is closely related to its efficacy. Using inductively coupled plasma-mass spectrometry (ICP-MS) method [53], Fig. 3 shows the average number of the internalized GNR-MBA, PpIX-GNR-MBA, and PpIX-GNR-MBA-FA by HeLa cells were 7.57×10^3 , 5.70×10^4 , and 3.37×10^5 , respectively. This result demonstrates that the FA receptor-mediated mode can greatly facilitate the GNRs internalization for 50-fold increasing.

Also, Raman characterization of the internalized PpIX-GNR-MBA-FA is performed. Figure 4B presents the Raman spectrum of HeLa cells co-cultured with the prepared PpIX-GNR-MBA-FA nanoprobe for 2 h, in which the peaks at 1003 , 1450 , and 1660 cm^{-1} represent the characteristics peaks of HeLa cell, two peaks at 1077 and 1583 cm^{-1} corresponding to MBA are observed. Along with the co-cultured time is increased to 4 h, the intensity of two peaks at 1077 and 1583 cm^{-1} is significantly improved (Fig. 4C). In contrast, two peaks at 1077 and 1583 cm^{-1} are not found in control group (Fig. 4A), indicating that the PpIX-GNR-MBA-FA nanoprobe has been internalized into HeLa cells.

Following, SERS imaging is used to assess the internalized number and distribution of the PpIX-GNR-MBA-FA nanoprobe. Figure 5 shows the bright field image and Raman spectral imaging result of HeLa cells incubated with the GNR-MBA, PpIX-GNR-MBA, PpIX-GNR-MBA-FA, respectively. We can see that all three compositions are internalized into HeLa cell: the GNR-MBA composition is mainly distributed in a small area around the nucleus (Fig. 5b₁-b₅) while no peaks appear in control group (Fig. 5a₁-a₅). Specially, along with the internalized number increasing, the distribution of the PpIX-GNR-MBA composition becomes dispersed (Fig. 5c₁-c₅) and the PpIX-GNR-MBA-FA nanoprobe is dispersed

throughout whole cell, further indicating that FA receptor can greatly facilitate GNRs internalization and dispersion. That is to say, both the internalized number and dispersion of the PpIX-GNR-MBA-FA nanoprobe are good relative to other compositions while its signal intensity is not particularly strong due to (1) PpIX and FA occupy most of the hot spots of GNR surface, so the amount of the conjugated MBA is decreased, and the corresponding spectral imaging quality becomes bad due to the weak intensity of Raman signal; (2) Raman signal intensity is decreased due to the nanoprobe dispersion. Clearly, above Raman spectral imaging result of two characteristic peaks at 1077 and 1583 cm^{-1} reflects that the more assembling of nanoprobe, the stronger intensity of Raman signal. Of course, the intensity change inconsistent of two characteristic peaks might result from the intracellular interference.

Collectively, the highly internalized number and good dispersion of the PpIX-GNR-MBA-FA nanoprobe relative to the GNR-MBA or PpIX-GNR-MBA compositions can greatly facilitate nanodrugs internalization.

Cytotoxicity and phototoxicity assays

In order to present the efficacy of the PpIX-GNR-MBA-FA nanoprobe, we first employ CCK-8 to assess the bio-compatibility. As shown in Fig. 6A, in the case of no laser irradiation, even if the concentration of PpIX-GNR-MBA-FA nanoprobe reaches 10 $\mu\text{g/mL}$, the cell viability still maintains more than 85% after co-incubation for 24 h, indicating the low chemical-toxicity of the PpIX-GNR-MBA-FA nanoprobe.

Moreover, to assess the anti-cancer activity of the PpIX-GNR-MBA-FA nanoprobe, the photo-toxicity is tested by the survival rate. Since the photosensitizer PpIX works at wavelength 633 nm and the GNR perform photothermal at wavelength 785 nm, HeLa cells are irradiated with the 633 nm laser (6.54 mW/cm^2 for 20 min) for PDT, the 785 nm laser (177 mW/cm^2 for 10 min) for PTT and the PTT/PDT synergetic treatment with the 785 nm laser then the 633 nm laser. As shown in Fig. 6B, it is found that the viabilities of PTT, PDT and PTT/PDT are decreased with the GNRs concentration increasing, and the high photo-toxicity of the PpIX-GNR-MBA-FA nanoprobe is obtained at 10 $\mu\text{g/mL}$. Compared with the cell viability of 31% for PTT and 25% for PDT, the cell viability is reduced to 8% for PTT/PDT treatment. Clearly, this result illustrated the excellent efficiency of PTT/PDT synergistic treatment relative to the individual PTT or PDT. In contrast, the cell viability maintains more than 96% in control group (0 $\mu\text{g/mL}$), indicating that the side effects of laser irradiation can be neglected.

Figure 6C shows the corresponding cell morphological changes during laser irradiation by an optical microscope. For PTT treatment, HeLa cells are co-cultured with the PpIX-GNR-MBA-FA nanoprobe, after 785 nm laser irradiation (10 mW for 10 s), the cell membrane becomes blurry, and some small bubbles appear due to the local thermal effect of GNRs leads to the local temperature increasing around the probe. In PDT treatment, it is observed that after laser irradiation, the cell membrane became blurry, some small bubbles appear, and then gradually become large. The possible reason is that the PpIX irradiated by 633 nm laser would lead to the generation of ROS, SO and other strong oxide species, and then rapid oxidation of intracellular macro-molecules, as a result of O_2 production and bubble formation. Actually, the speed of bubble formation is slow due to the PDT process is complicated. Compared with the result

of PDT, the speed of bubbles formation in PTT is improved due to photo-effect and heat-effect will cause the rapid increasing of local temperature while the bubble size in PDT is larger than PTT, indicating that the efficiency of PDT is better than PTT. Clearly, this result is consistent with the above cell viability assay. For PTT/PDT synergistic treatment, HeLa cells are co-cultured with the PpIX-GNR-MBA-FA and then irradiated with 785 nm laser (10 mW, 10 s) and 633 nm laser (0.37 mW, 10 s) in turn, we can see that both the number and size of the bubbles formation are larger than individual PTT or PDT. Accordingly, the efficiency of PTT/PDT synergistic treatment was significantly better than individual PTT or PDT.

Conclusions:

In summary, based on SERS imaging, we design and prepare a FA receptor-mediated PTT/PDT synergistic anti-cancer nanodrug PpIX-GNR-MBA-FA with the tracing function by modifying photosensitizer PpIX, Raman reporter molecule MBA, and the mediated molecule FA to the GNR surface. It is found that the FA receptor-mediated mode can facilitate nanodrugs internalization, in which both the internalized number and intracellular dispersion of the nanodrugs are improved relative to the GNR-MBA or PpIX-GNR-MBA compositions, and then generate remarkable enhancement for photo treatment effect under the local overheating, ROS generation and cell apoptosis. Importantly, this FA receptor-mediated PpIX-GNR-MBA-FA nanoprobe will provide a novel strategy for the PTT/PDT synergistic treatment.

Materials And Methods

Chemicals and Materials

1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, 98%), Chloroauric chloride trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, 99%) were provided by Sigma–Aldrich; Silver nitrate (AgNO_3 , 99.99%), Sodium borohydride (NaBH_4 , 99.99%), hexadecyl trimethyl ammonium bromide (CTAB, $\text{C}_{16}\text{H}_{33}(\text{CH}_3)_3\text{NBr}$, 98%), and N-hydroxysuccinimide (NHS, 98%) were achieved from Macklin; protoporphyrin IX (PpIX, 95%), cysteamine hydrochloride (Ch, 98%) were purchased from BELLAN LIFE SCIENCES DEP; PBS 1X, DMEM 1X and 0.25% Trypsin were provided by Corning; 4-mercaptobenzoic acid (MBA, Xiya Reagent, 98.0%); folic acid (FA, Aladdin, 97%); DMSO (Tianjin Zhiyuan Chemical Reagent Co., Ltd.); fetal bovine serum (FBS, Gibco); CCK-8 kit (Cell Counting Kit-8, DOJINDO); 4% paraformaldehyde (meilunbio, MA0192-OCT-23D). Each reagent was used as received without any dispose. Ultrapure water used in the whole experiments with the resistivity higher than 18.2 MΩ.cm.

CO_2 incubator (2406-2, SHEL LAB); freeze dryer (LABCONCO, Guangzhou Keqiao Experimental Technology Equipment Co., Ltd.); Nicolet 6700 Fourier transform infrared (FTIR) spectrometer (Thermoelectric Nico, USA); fluorescence spectrophotometer (FL-2500); UV-Visible spectrophotometer (UV-2700); confocal Raman spectroscopy STREAM MAPPING (inVia, RENISHAW); JEM-2100HR transmission scanning microscope (Japan Electronics, Japan); plasma emission spectrometer (SPECTRO ARCOS MV, Spike, Germany); JOANLAB HS-12 magnetic stirrer; centrifuge (TDZ5-WS, manufacturer: Changsha Xiangzhi Centrifuge Instrument Co., Ltd.); HC-2518 High Speed Centrifuge

(Anhui USTC Zonkia Scientific Instruments Co, Ltd); electronic balance (JA2003N Internal School, JINGHRI, Shanghai Jinghai Instrument Co., Ltd.); ultrasonic cleaning machine (JP 020, manufacturer: Shenzhen Jiemeng Cleaning Equipment Co., Ltd.); digital thermostatic water bath (HH-4, manufacturer: Jintan Ronghua Instrument Manufacturing Co., Ltd., Jiangsu Province); iMark Microplate (Bio-Rad) enzyme Standard instrument; dialysis bag (D34MM, molecular weight cutoff 500); Cuvette (model 751/722, size 10 m/m, in accordance with JB760-68 cuvette regulations).

Synthesis of PpIX-SH and MBA-FA

According to the previous reports [51], the thiolated photosensitizer was prepared as followings: PpIX (6mg) was added into 2 mL DMSO. After completely dissolved, 4 mg NHS was added to the solution and stirred in N₂ for 2 h at room temperature. The mixture of 7 mg EDC, 3.6 mg cysteamine hydrochloride, and 44.6 μ L TEA were stirred under 400rpm in darkness for 24 h. The result product was removed into a dialysis membrane with a molecule weight of 500 and purified by dialysis against deionized water for 3 days, and then freeze-dried to reddish brown powder.

In order to achieve the conjugation of the amino acid (-NH₂) of FA and the activated carboxylic acid of MBA, the synthesis of the thiolated FA was also referred to the above method [51]. Briefly, 1mL of MBA (40 μ M) activated by EDC/NHS was reacted with 1mL of FA (50 μ M) at room temperature for 24 h, following the unconjugated MBA and FA were removed through dialysis.

Synthesis of GNRs

GNRs were synthesized according to the seed-mediated method [18]. Briefly, the seed solution was prepared as following: First, 7.5 mL of 0.1 mM CTAB solution was mixed with 250 μ L of 0.01 mM HAuCl₄ aqueous solution. Second, 0.60 mL of 0.01 mM ice-cold NaBH₄ was rapidly added into the above mixture with stirring. Third, the solution gradually turned light brown with stirring, and maintained in a water bath at 30°C for 2 h. Meanwhile, 1.7 mL of 10 mM HAuCl₄ aqueous solution, 250 mL of 10 mM AgNO₃ aqueous solution, 0.80 mL of 1M HCl and 270 μ L of 100 mM ascorbic acid aqueous solution were successively added to 40 mL of 100 mM CTAB aqueous solution with stirring. Finally, when the solution turned colorless, 420 μ L seed solution was added to trigger the reaction for 12 h, and then centrifuged at 7500 rpm for 10 min.

Preparation of PpIX-GNR-MBA

Primarily, 200 μ L of 40 μ M MBA was added to 1 mL GNR solution with stirring at 40 rpm for 2 h, and then 200 μ L PpIX-SH solution was added and stirred at 600 rpm in dark at room temperature for 12 h. Consequently, the solution was centrifuged to remove the superfluous MBA, PpIX and PpIX-SH at 5000 rpm for 10 min, and the PpIX-GNR-MBA suspension was achieved and stored for use.

Preparation of PpIX-GNR-MBA-FA nanoprobe

First, 200 μ l prepared MBA-FA were added to 1 mL GNR solution and stirred for 2 h. 200 μ l prepared PpIX-SH was added to the mixture and stirred for 12 h. Then, the product was centrifuged at 5000 rpm for 10 min, and the preparation of PpIX-GNR-MBA-FA was completed.

Characterization of PpIX-GNR-MBA-FA nanodrugs

First, the infrared spectrum of the prepared PpIX-GNR-MBA-FA/SERS nanoprobe is measured by a FTIR spectrometer, and the corresponding ultraviolet-visible absorption spectra is achieved by a ultraviolet-visible spectrophotometer; the fluorescence emission spectrum is performed with a photoluminescence spectrophotometer; the TEM image was implemented with the transmission electron microscope; the cell viability is tested by CCK-8 assay. Specially, to assess quantitative characterization, we measure the intracellular transport and uptake amount of the prepared PpIX-GNR-MBA-FA/SERS nanoprobe by using confocal micro-Raman spectral imaging and inductively coupled plasma-mass spectrometry (ICP-MS) [53], respectively.

Cell culture

HeLa cells, an immortalized cell line with excessive FR expression[18] were cultured in the culture medium containing 90% DMEM, 10% FBS, as well as 1% double resistance (penicillin streptomycin) in 37 °C containing 5% CO₂.

Characterization of nanoprobe internalization and localization

HeLa cells were seeded into 6-well plate of 1.5 mL DMEM (1×10^5 cell/well). After cells attachment, the culture medium was changed to 1.5 mL medium containing GNR-MBA, GNR-MBA-FA, PpIX-GNR-MBA-FA, and then incubated for 2 h and 4 h to confirm the time of co-culturing. Following, HeLa cells were washed with PBS and then fixed with 4% glutaraldehyde for 20 min. Subsequently, Raman spectral imaging at the peaks of 1077 and 1583 cm^{-1} was implemented, in which a laser with wavelength of 785 nm and power of 2 mW, the objective of 50 $\times$, the spectral range of 400-2000 cm^{-1} , the acquisition time of 0.1 s and the scanning range in x and y directions of 0.5 μ m were chosen.

ICP emission spectrometer was also employed for quantitative characterization of the internalized probes, in which HeLa cells (1×10^5 cells/well) were cultured in the 6-well plate of 1.5 mL DMEM for 24 h. After cells attachment, the culture medium was changed as 1.5 mL medium containing GNR-MBA, GNR-MBA-FA, PpIX-GNR-MBA-FA, and co-cultured for 4 h. Subsequently, HeLa cells were digested by trypsin (0.25%) and re-dispersed in PBS after centrifugation; and then the cell suspension was transferred into a 15 mL shrinkable flask, 2 mL HNO₃ and 0.5 mL HCl were added drop by drop to dispel the cells. Following, the boiling water bath was used to remove the acid until the solution of cell suspension was clarified. Finally, the solution was constant to 10mL and analyzed by ICP emission spectrometer to obtain the content of gold after dissolution, which can be used to calculate the number of GNR.

Cytotoxicity and therapeutic efficacy assays

Cytotoxicity assay of the prepared PpIX-GNR-MBA-FA nanoprobe was performed through cell counting assay kit-8 (CCK-8). First, HeLa cells were incubated in the 96-well plate (1×10^5 cells/well) for 24 h; After cells attachment, 10 μ l PpIX-GNR-MBA-FA (the concentrations of GNR were respectively set as 0, 2, 5, 10, 20 μ g/mL) were added to each well and incubated for 24 h. Subsequently, the cells were treated with 10 μ l CCK-8 solution, and incubated at 37°C for 2 h. And then the corresponding absorbance is measured by an iMark Microplate (BioRad) enzyme Standard instrument using 490 nm light excitation.

The phototherapeutic efficacy is tested by CCK-8 assay under 490 nm light excitation. HeLa cells were respectively treated with PpIX-GNR-MBA-FA (0, 2, 5, 10 μ g/mL), and incubated for 24 h. And then the cells were irradiated with a 785 nm laser (177 mW/cm², 20 min) for PTT stimulus, a 633 nm laser (6.54 mW/cm², 15 min) for PDT stimulus, and the combination therapy (PTT/PDT) of a 785 nm laser then a 633 nm laser. Finally, the viability was evaluated with CCK-8 assay as described above.

HeLa cells (1×10^5 cells/well) were seeded on 6-well plate with 1.5 mL DMEM and incubated for 24 h. After cells attachment, the culture medium was changed to 1 mL fresh culture medium containing PpIX-GNR-MBA-FA (10 μ g/mL) and cultured for 24 h; the in-internalized probes were removed by washing with PBS, and then 1 mL fresh culture solution was added. Finally, HeLa cells were respectively irradiated by a laser of 633 nm (0.37 mW, 10 s) for PDT or 785nm (10 mW, 10 s) for PTT or combined PTT/PDT with the spot diameter of 1.5 μ m. The cell morphological changes during laser irradiation were recorded with an optical microscope.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent of publication

All authors agreed to submit this manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this published article; the conflict of interest was shown in supplementary.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

MSX performed the majority of the experiments. LYZ, MSX, PT, and WDC involved in writing the manuscript. LYZ, XXL, PT, and SDL co-ordinated experiments and provided important advices for the experiments along with financial support. MSX, PT, and WDC were involved with the design, interpretation and data analysis. All authors read and approved the final manuscript.

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Figures

Figure 1

A) (B) Sketch of the synthesis reaction of PpIX-SH and FA-MBA, respectively; (C) the structure comparison of PTT, PTT/PDT and FA-PTT/PDT.

Figure 2

(A)(B) Fourier transform infrared (FTIR) spectra of different compositions; (C) UV-Vis absorption spectra of different compositions; (D) Raman spectra of different compositions; TEM images of (E) GNRs; (F) PpIX-GNR-MBA-FA, respectively.

Figure 3

The average internalized number of the GNR-MBA, PpIX-GNR-MBA and PpIX-GNR-MBA-FA by HeLa cell measured by ICP method.

Figure 4

Raman spectra of HeLa cells (A) control group; cocultured with the nanodrugs PpIX-GNR-MBA-FA for (B) 2h; (C) 4h, respectively.

Figure 5

Raman spectral imaging results of HeLa cell (a1-a5): without treatment; incubated with (b1-b5) GNR-MBA; (c1-c5) PpIX-GNR-MBA; (d1-d5) PpIX-GNR-MBA-FA, respectively.

Figure 6

Relative viability of HeLa cells (A) control group without laser irradiation; (B) treated with PTT or PDT or PTT/PDT by different concentrations of PpIX-GNR-MBA-FA nanoprobe for 24h; (C) Bright field images of HeLa cells (a1,a2,a3) without treatment; treated with (b1, b2, b3) PTT; (c1, c2, c3) PDT; (d1, d2, d3) PTT/PDT, in which the first, second, third rows represent the results of HeLa cells irradiated with laser (before treatment, treatment for 0 and 3min respectively).

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