Targeting polymeric fluorescent nanodiamond-gold/silver multi-functional nanoparticles as a light-transforming hyperthermia reagent for cancer cells†

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This work demonstrates a simple route for synthesizing multi-functional fluorescent nanodiamond-gold/silver nanoparticles. The fluorescent nanodiamond is formed by the surface passivation of poly(ethylene glycol) bis(3-aminopropyl) terminated. Urchin-like gold/silver nanoparticles can be obtained via one-pot synthesis, and combined with each other via further thiolation of nanodiamond. The morphology of the nanodiamond-gold/silver nanoparticles thus formed was identified herein by high-resolution transmission electron microscopy, and clarified using diffraction patterns. Fourier transform infrared spectroscopy clearly revealed the surface functionalization of the nanoparticles. The fluorescence of the materials with high photo stability was examined by high power laser irradiation and long-term storage at room temperature. To develop the bio-recognition of fluorescent nanodiamond-gold/silver nanoparticles, pre-modified transferrin was conjugated with the gold/silver nanoparticles, and the specificity and activity were confirmed in vitro using human hepatoma cell line (J5). The cellular uptake analysis that was conducted using flow cytometry and inductively coupled plasma mass spectrometry exhibited that twice as many transferrin-modified nanoparticles as bare nanoparticles were engulfed, revealing the targeting and ease of internalization of the human hepatoma cell. Additionally, the in situ monitoring of photothermal therapeutic behavior reveals that the nanodiamond-gold/silver nanoparticles conjugated with transferrin was more therapeutic than the bare nanodiamond-gold/silver materials, even when exposed to a less energetic laser source. Ultimately, this multi-functional material has great potential for application in simple synthesis. It is non-cytotoxic, supports long-term tracing and can be used in highly efficient photothermal therapy against cancer cells.

1 Introduction

The design of nanoparticles (NPs) for biological applications is one of the most important fields of research in both chemistry and biology. Interest in the development of NPs for the labeling, targeting, and therapy for tumors is great. In recent decades, quantum dots have been developed with unique fluorescence and tunable optical properties that are controlled by varying their size. They have been extensively utilized for various purposes, including water splitting, hydrogen storage, biosensing, or as bio-labeling markers. However, toxicity associated with possible leakage remains a risk when they are applied in vitro or in vivo because they contain heavy metals and chalcogenides. Fluorescent carbon-based nanoparticles have attracted increasing attention recently for use in biological systems due to high biocompatibility. Photoluminescent carbon dots have been generated by laser ablation of the graphite powder and cement mixture, followed by surface passivation with diamine-terminal poly(ethylene glycol). Luminescent amorphous carbon dots have been synthesized by the pyrolysis of anhydrous citric acid with N-(β-aminoethyl)-γ-aminopropyl methyl-dimethoxy silane at 240 °C. Microwave assisted photoluminescent carbon dots were obtained by adding glycerol under microwave illumination for various periods. However, investigations of carbon dots have focused on their fabrication rather than their application.
With respect to other carbon allotropes, nanodiamond (ND) exhibits high biocompatibility and anti-corrosive property of carbon-based materials and more potential biological applications.\textsuperscript{13,14} Ultra-nanocrystalline diamond (UNCD) has been used to culture neural stem cells on the surface-modified UNCD films. UNCD coatings improve the efficiency of expansion and differentiation of neural stem cells more effectively than does the commercial polystyrene platform, even when the cells are preserved in a differentiation reagent-free medium.\textsuperscript{15} In a subsequent investigation, ND-coated substrates for neuronal growth performed remarkably well, with an effectiveness similar to that of the standard protein-coated platform.\textsuperscript{16} Moreover, NDs have been used as outstanding transporters for delivering drugs or genes in organisms.\textsuperscript{13,17,18} ND-conjugated chemotherapy has overcome drug efflux and improved the inhibition of tumor growth, and so represents a significant improvement in chemotherapeutic efficacy and safety.\textsuperscript{19} Hence, the NDs have been proved to be bio-friendly, anti-corrosional, and tumor-inhibiting substrates, and suitable for use in biological systems.

Fluorescent nanodiamond (FND) that is formed by modifying ND can fluoresce when irradiated under light, and it has all of the favorable properties of ND. The FND can be synthesized by three processes: (1) FND with a nitrogen vacancy (N-V)\textsuperscript{−} center is produced by irradiating synthetic ND with an ionic beam.\textsuperscript{20} The N-V\textsuperscript{−} FND exhibits stable and bright fluorescence with little photobleaching and photoblinking. However, the high production cost of FND limits its use, and even simplifying the fabrication procedure does not eliminate the need for high charges and a complex synthesis process.\textsuperscript{21} (2) The second method of producing FND is to conjugate organic fluorophores with ND.\textsuperscript{22} The surfaces of ND have plenty of groups and so are easily functionalized via a chemical reaction.\textsuperscript{23} Nevertheless, their photobleaching of organic fluorophores restricts their bio-application for the preservation or long-term tracing. (3) An ND surface can be passivated by conjugation with a polymer, and the fluorescence is probably attributed to the combination of electrons and holes that are confined on the surface of the material, in a process similar to that in semiconductors.\textsuperscript{24,25} FND is fabricated through a simple wet chemical route, but its hydrophobic property and the required toxic solvents, toluene and chloroform, restrict its application to biological systems. Before FND can be used in more organisms, the problems of its large size, photobleaching, photoblinking and required solvent must be solved. Furthermore, conjugating ND with metallic materials that are used in energetic or biological fields is of great interest for development in the future,\textsuperscript{25,26} but the related investigations are still limited.

In this work, the urchin-like Au/Ag NPs conjugating with FND are first combined for cell-labeling and photothermal therapy, and the combining NPs also equip an ability of specific cell targeting. The wet chemistry synthesis of FND provides an easy and sample route for production, and the production rate is in a high yield. The small (5 nm) hydro-dispersible FND is fabricated by surface passivation. It retains the unique properties of ND and emits fluorescence without photobleaching or photoblinking under long-term irradiation by a high power laser. FND is combined with pre-modified urchin-like Au/Ag NPs via thiol-metal bonding, for use as a long-term tracing probe in \textit{in vivo} or \textit{in vitro} experiments. The simple, convenient and specific thiolating reaction includes the ring opening of 2-iminothiolane by FND terminal primary amino-group nucleophilic attacking, forming the terminal thiol group, which is subsequently involved in conjugation. The FND-Au/Ag NPs are proven to be a photothermal therapeutic material under near infrared (NIR) irradiation. The time-dependence of the cell uptake ratio of the combined NPs is simply monitored \textit{via in situ} flow cytometry, which is performed simultaneously with the quantification of gold by inductively coupled plasma mass spectrometry (ICP-MS) detection. The material is further bonded with the bio-recognized human transferrin (Tf) \textit{via} a simple thiolation process, and it promotes the cell uptake ratio to the specifically targeted J5 cell, simultaneously enhances fluorescent intensity and reduces the therapeutic power of NIR laser. The photothermal therapeutic behavior can easily be detected \textit{via} fluorescence microscopy. A decline of the emitted luminescence from calcein AM indicates an increase in temperature, which destroys the J5 cell. The injured cells reveal the same damaged area after trypan blue staining analysis. Therefore, the multi-functional FND-Au/Ag-Tf NPs exhibit specifically targeted, long-term tracing and photothermal therapeutic properties against the human heptoma cell, while, outstandingly, the required materials can be synthesized simply.

2 Experimental

2.1 Chemicals and materials

Chlorauric acid (HAuCl\textsubscript{4}), silver nitrate (AgNO\textsubscript{3}) and ascorbic acid (AA) were purchased from Acros. Poly(ethylene glycol) bis(3-aminopropyl)-terminated (PEG\textsubscript{1500})\textsubscript{N}, low-molecular-weight chitosan, monochloroacetic acid and 2-iminothiolane hydrochloride were obtained from Sigma-Aldrich. Calcein AM, trypan blue, Hoched 33342 and human transferrin (Tf) were purchased from Jackson ImmunoResearch Laboratories. Nanodiamond was obtained from RiteDia Corporation. All chemicals were obtained from a local chemical retailer and used as received. All of the water that was used in this investigation was reagent-grade, and produced using a Milli-Q SP ultrapure-water purification system from Nihon Millipore Ltd., Tokyo.

2.2 Preparation of FND

FND was synthesized using a method similar to a previously used fluorescence carbon dot synthesis procedure.\textsuperscript{27} FND was fabricated by the surface passivation. The synthesis of the material involved mixing 200 mg bare ND with 2000 mg PEG\textsubscript{1500} in a flask and reacted at 120 °C for three days. After the reaction, the mixture was washed with 40 mL H\textsubscript{2}O to remove unreacted precursors, and centrifuged at 7000 ppm. The washing process was repeated twice. The FND was dried in an oven or re-dispersed into H\textsubscript{2}O for further use.

2.3 Preparation of Au/Ag bimetallic NPs

Urchin-like Au/Ag NPs were synthesized as described elsewhere.\textsuperscript{28} Briefly, Au/Ag NPs were obtained by mixing 2.5 mL of...
10 mM HAuCl₄ and 0.2 mL of 10 mM AgNO₃ in 100 mL H₂O, and then adding 0.4 mL of 100 mM AA rapidly with stirring at 400 rpm for 10 s. After one minute at rest, 100 mL of 1 wt% as-made aqueous chitosan solution was added to the solution to stabilize the urchin-like Au/Ag NPs. The NPs thus formed could be stored at 4 °C in a refrigerator for at least half a year. Before they can be reacted with the other materials, the NPs should be centrifuged at 3000 rpm and re-dispersed into H₂O twice to eliminate unwanted surfactant.

2.4 Preparation of FND-Au/Ag NPs

To synthesize FND-Au/Ag NPs, as-prepared FND was functionalized to be terminated by a thiol group. A 0.1 mg mass of FND was dispersed in 1 mL H₂O and sonicated for 30 minutes. A 1 mg mass of 2-iminothiolane hydrochloride was added to the well dispersed FND solution and reacted for three hours at room temperature. After centrifugation at 3000 rpm and two re-dispersions, 1 mL of Au/Ag urchin-like NPs was added to the thiol-terminated FND solution and allowed to react for three hours. For further use, the product must be centrifuged at 3000 rpm and re-dispersed into an aqueous solution twice to remove undesired precursors.

2.5 Preparation of FND-Au/Ag-Tf NPs

Human transferrin was functionalized by 2-iminothiolane hydrochloride before reacting with FND-Au/Ag NPs. A 0.4 mL volume of 0.0625 ppm Tf was mixed with 1 mg of 2-iminothiolane hydrochloride at room temperature for three hours. A 1 mL volume of aqueous FND-Au/Ag NPs reacted with decorating Tf solution at room temperature for a further three hours. To remove unnecessary Tf, the product was washed by H₂O and re-dispersed into the aqueous solution twice.

2.6 Cell viability of the materials

The cell viability experiment involved a single cell line. Human hepatoma cell line J5 was maintained in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) that contained fetal bovine serum (10%), L-glutamine (2.9 mg mL⁻¹), streptomycin (1 mg mL⁻¹), and penicillin (1000 units mL⁻¹). Briefly, exponentially growing cells were placed in 96-well plates at an initial density of 2000 cells per well. A 1 mL volume of aqueous FND-Au/Ag NPs reacted with FND for 12 hours in 6-well plates with cover glass at 37 °C and 5% CO₂ humidity atmosphere. For imaging, 5 × 10⁴ cells were seeded in a well of 6-well plate with a 24 mm collagen-coating round cover slide. For cell uptake, 5 × 10⁴ cells were seeded in a well of 12-well plate. The preparation of samples on the glass slides from which confocal images were obtained involved several steps. Briefly, the culture medium was vacuumed to expel free FND-Au/Ag-Tf NPs. Exponentially grown cells were fixed on the cover glass using 1 mL of 2% and of 4% paraformaldehyde per well for five minutes. The nuclei in the cells were stained with Hoechst 33342 for a further five minutes to label the cells, and the lysosome was stained with Lyso-Tracker® Green DND-26 for 1 hour. The stained cells were then observed by confocal microscopy. After the staining procedure, the pieces of cover glass over the cells and nanoparticles were fixed by nail polish to the top of the slides with a moderate amount of mounting oil between them. The cells were excited using an ultra-violet laser at 408 nm and emitted wavelength region 450–500 nm were detected to image nuclei. The Lyso-Tracker® Green DND-26 was excited using a green-blue laser at 488 nm and emitted wavelength region 520–550 nm was detected to image lysosome. The FND was excited using a 561 nm laser and the emission was detected in the 600–650 nm range.

2.7 Photo stability of FND NPs

The photo stability experiment was incubated under the same conditions as above. Human hepatoma cell line J5 was incubated with FND for 12 hours in 6-well plates with cover glass at 37 °C and 5% CO₂ humidity condition. After vacuum the medium were refilled, calcein AM was added to stain the cells. A cover glass was fixed on the slides with amount of moderate mounting oil. The cells were excited using a 488 nm (output power: 30 mW), and then 561 nm (output power: 50 mW), laser for one minute or three hours, and emission in the wavelength regions of 520–570 nm and 600–650 nm was detected.

2.8 Confocal microscopic images in vitro

Human hepatoma cell line J5 was incubated with FND-Au/Ag-Tf NPs for 12 hours in 6-well plates with cover glass inside at 37 °C and 5% CO₂ humidity atmosphere. For imaging, 5 × 10⁴ cells were seeded in a well of 6-well plate with a 24 mm collagen-coating round cover slide. For cell uptake, 5 × 10⁴ cells were seeded in a well of 12-well plate. The preparation of samples on the glass slides from which confocal images were obtained involved several steps. Briefly, the culture medium was vacuumed to expel free FND-Au/Ag-Tf NPs. Exponentially grown cells were fixed on the cover glass using 1 mL of 2% and of 4% paraformaldehyde per well for five minutes. The nuclei in the cells were stained with Hoechst 33342 for a further five minutes to label the cells, and the lysosome was stained with Lyso-Tracker® Green DND-26 for 1 hour. The stained cells were then observed by confocal microscopy. After the staining procedure, the pieces of cover glass over the cells and nanoparticles were fixed by nail polish to the top of the slides with a moderate amount of mounting oil between them. The cells were excited using an ultra-violet laser at 408 nm and emitted wavelength region 450–500 nm were detected to image nuclei. The Lyso-Tracker® Green DND-26 was excited using a green-blue laser at 488 nm and emitted wavelength region 520–550 nm was detected to image lysosome. The FND was excited using a 561 nm laser and the emission was detected in the 600–650 nm range.

2.9 Photothermal ablation test

The photothermal ablation experiment proved that the NPs converted light to heat. Human hepatoma cell line J5 was incubated in 6-well plates with cover glass. For thermal therapy, cells were cultured until more than 95% confluency. The cells were divided into three categories. They were the cells that were incubated without any nanomaterials (which served as a control); the cells that were incubated with FND-Au/Ag and the cells that were incubated with FND-Au/Ag-Tf NPs in the atmosphere described above. Following incubation for 12 hours to allow uptake by the cell, the culture medium was exchanged to remove the free nanomaterials. To visualize the photothermal behavior during the experiment, the cultures were stained with calcein AM. A Leica TCS SP5 confocal microscope was used to observe the system. The target cells were irradiated at various powers (output power densities of 46, 28 and 24 W cm⁻²) by a near infrared femtosecond pulse laser at a wavelength of 800 nm with a pulse width of 88 fs and a period between consecutive pulses of 20.5 ns. The irradiated area was 500 μm × 500 μm. After irradiation, trypan blue, a dye that is commonly used to color selectively dead tissues or cells, was added to the cell dishes for 30 minutes.

2.10 Characterization

The morphology of the samples was studied using transmission electron microscopy (TEM) with an electron gun that was...
operated at 100 keV. The TEM images and high-resolution transmission electron microscopic (HRTEM) images were collected using a JEOL JEM-2100F electron microscope. The zeta potential and particle size were obtained at room temperature using a MALVERN ZETASIZER-3000. The photoluminescence excitation and emission spectra were obtained at room temperature using a FluoroMax-3 and FluoroMax-P spectrophotometer with a 1 cm wide quartz cell at room temperature. The transmittance Fourier transform infrared (FTIR) spectra of FND, decorated FND and FND-Au/Ag NPs that were dispersed in KBr pellets, were obtained using a Varian FTIR-640 spectrometer. The Au concentration in the as-prepared colloid solution was determined using a Thermo X series II ICP-MS. A FACS AriaU flow cytometry was used to detect the uptake behavior of the cells. A Leica TCS SP5 confocal microscope was used to imagine the confocal in vitro images and perform in photothermal ablation experiments.

3 Results and discussion

3.1 Fabrication and characterization of FND-Au/Ag-Tf NPs

Bio-functional FND-Au/Ag-Tf NPs were synthesized by a simple tandem synthesis process and subsequently characterized by photoluminescence spectroscopy, transmission electron microscopy (TEM) and thermally stable Fourier transform infrared (FTIR) spectroscopy. The synthesis is composed of five steps. (1) Synthesis of FND and functionalization of the primary amino terminal group to thiol. (2) Fabrication of Au/Ag urchin-like NPs. (3) Combination of FND and Au/Ag NPs through Au–S bonding. (4) Thiolation of Tf terminal from primary amino to thiol. (5) Conjugation of FND-Au/Ag NPs with thiolated bio-recognition Tf group. At the beginning of the tandem synthesis, the polymeric FND was prepared in a manner similar to that of the preparation of carbon dots (CDs), reported elsewhere. ND particles with a diameter of 5 nm were covalently bonded to poly(ethylene glycol) bis[3-aminopropyl] terminated (PEG1500N). In this bonding reaction at 120 °C, the carboxylic acid terminals of bare ND formed amide FND. The absorption of FND shows broad absorption in the visible region, as present in Fig. 1. In Fig. S1,† the photoluminescence spectra of FND are obviously broad and related to the excitation wavelength. The excitation wavelength is progressively shifted from 375 nm to longer wavelength 475 nm in 25 nm increments, and the emission of FND simultaneously shifted from 530 nm to 550 nm. The synthesis of polymeric FND by surface passivation modification is simpler than the FND with NV– center. The functionalization of carbon materials with various reagents through amide bonds has been presented in other studies. Thiolation reagent 2-iminothiolane is selected to react specifically with primary amine, which was widely distributed over the surface of FND, to enable it to conjugate with Au/Ag NPs. In the thiolating reaction, the nucleophilic primary amino groups attack the electrophilic cyclic thioimidate groups including the ring opening of 2-iminothiolane, forming the terminal thiol group, which is subsequently involved in conjugation. Herein, urchin-like Au/Ag bimetallic NPs were fabricated by a time-saving one-pot synthesis process. O-Carboxymethylchitosan is chosen to stabilize the irregular thermodynamically disfavored NPs owing to the coordination capacity of the carboxylic acid group and the effect of chelation. Furthermore, O-carboxymethylchitosan eliminates the problem of the lack of solubility of bare chitosan, but it retains its non-cytotoxic and bio-friendly properties, which are exploited in various applications. Thiolated FND provides activated sites for the replacement of some chitosan by the formation of stronger covalent bonds between Au and thiol groups, without destroying the stability of the NPs. The FND-Au/Ag NPs have been proved to be highly stable for at least one month in aqueous solution at room temperature. 2-iminothiolane modification before the combination of FND-Au/Ag NPs with bio-molecular Tf, causing the thiol terminal groups to covalently conjugate with the FND-Au/Ag NPs. As presented in Scheme 1, the FND-Au/Ag-Tf is a multi-functional nanomaterial; FND fluoresces, a property which can be exploited in long-term tumor tracing, urchin-like Au/Ag NPs possess efficiency NIR light to heat for cancer therapy, and Tf recognizes the transferrin receptor of a specific cell, increasing the number of NPs that are engulfed by cells through endocytosis. As the number of NPs inside the targeted cells increases, more intense

| Fig. 1  Absorption spectrum of nanomaterials. Insets display TEM images of FND and FND-Au/Ag NPs. Both inset TEM images include scale bars that represent 50 nm. |

Scheme 1 Synthesis and application of FND-Au/Ag-Tf.
fluorescence passes through the cell membrane, and the photothermal therapeutic damage to the malignant tumor cell is thus increased.

FND and FND-Au/Ag-Tf NPs are characterized by UV-Vis absorption spectroscopy and TEM (Fig. 1). FND has a similar absorption spectrum to that of bare ND at visible wavelengths, but exhibits greater absorption/scattering of lower wavelengths. However, metal materials exhibit plasmon resonance at their surfaces when irradiated with light whose wavelength greatly exceeds the size of the materials. The oscillating electrons, when illuminated, generate electromagnetic waves and thus increase absorption/scattering. The surface plasmon oscillation varies with the size and geometry of the metal nanoparticles. In our previous investigation, the aspect ratio of the thorns on the urchin-like Au/Ag NPs was tuned by changing the metal precursors that were used in the synthesis. NIR light has been proven to penetrate 7 cm of muscle and at least 10 cm of breast tissue. In this work, NPs that absorb intense NIR were chosen to convert light to heat. They exhibit a transverse and a longitudinal plasma wavelength of 652 nm and 982 nm, respectively. The longitudinal SPR band of the urchin-like NPs is red-shifted by 31 nm to 1013 nm when the FND is combined with the Au/Ag NPs, indicating variation in the local dielectric constants of the various chemicals that attached to the surface of NPs. Variations in the Au/Ag surroundings induce variations in the electron density, and various shifts in the surface plasmon absorption maximum. The same phenomenon is also detected in the conjugation of FND-Au/Ag and Tf, which cause a red-shift of 4 nm to 1017 nm. The intense absorption of blue light region was associated with the combination of the Au/Ag NPs absorption and the FND absorption/scattering. The TEM images show the morphologies of FND and FND-Au/Ag-Tf (insets in Fig. 1). ND and FND have the same form, with a diameter of approximately 5 nm (see Fig. S2†). The shape of FND-Au/Ag is almost identical to that of FND-Au/Ag-Tf (see Fig. S2†), because the amorphous Tf biomolecules cannot easily be visualized using TEM.

Fig. 2 displays the HRTEM images and diffraction pattern of FND-Au/Ag NPs to reveal the relationship and the morphologies of FND and Au/Ag NPs. The urchin-like Au/Ag NPs were fabricated using a silver-tailed mechanism, and the amount of silver precursor can be easily changed to alter the morphology. The mean length of the NPs is approximately 100 nm, and the mean transversal and longitudinal lengths of the tips are 12 and 40 nm, respectively (Fig. 2a). The aspect ratio and aperture angle of the tip strongly affects the resonance frequency, but the number of tips marginally affects the SPR band. However, a previous experimental and calculation-based study revealed that the energy intensity increases with the number of tips on the NPs. Furthermore, urchin-like NPs with many low aperture angle tips provide more intense electric fields than NPs with other morphologies, and so can be exploited in future applications in surface-enhanced Raman spectroscopy (SERS) and photothermal therapy. The FND particles that are smaller than 5 nm are observed to attach to the Au/Ag NPs. The selected area of high-resolution image of FND-Au/Ag NPs in Fig. 2b shows clear lattice fringes of the Au/Ag NPs and FND. Two sets of lattice planes with d-splittings of 0.230 and 0.205 nm correspond to the (111) lattice planes of the face-centered cubic (FCC) crystal of the Au/Ag NPs and the cubic crystal of FND. Selected area electron diffraction (SAED, inset of Fig. 2b) clearly shows the (111), (200), (220) and (311) planes, revealing that the Au/Ag NPs are crystalline and randomly oriented. The (111) plane of the FND overlaps the (200) plane of Au/Ag NPs. Together, these results provide clear evidence that FND are conjugated with the Au/Ag NPs and the crystalline structures of the FND-Au/Ag NPs.

To confirm the thiolation of FND and the combination of Au/Ag NPs, FTIR is utilized to visualize the functional groups following the chemical synthesis. FND exhibits C–N stretching and N–H bending at 1271 cm⁻¹, C=O stretching at 1731 cm⁻¹, C–H stretching at 2923 and 2863 cm⁻¹ and terminal amine N–H stretching at 3427 cm⁻¹ (associated with the red area in Fig. 3a). However, the thiolation of FND results in the conversion of terminal amines to acetamidine groups and the formation of thiol terminals by the ring opening of 2-iminothiolane. The thiol terminal group is difficult to detect herein, but the acetamidine group is clearly observed at 3145 cm⁻¹ (red area in Fig. 3b). The FND-Au/Ag NPs yield peaks at 3414 and 3127 cm⁻¹, which correspond to the O–H stretching of the O-carboxymethylchitosan and the acetamidine group of FND (blue and red areas, respectively, in Fig. 3c). The functional groups detected herein would ensure the success of the modifications of FND and the combination of FND with Au/Ag NPs. Moreover, the zeta potentials are obviously changed in the tandem synthetic procedure as presented in Table S1. The zeta potential is decreased from 13.5 to 8.4 after thiolation of FND, and the conjugation of FND with urchin-like Au/Ag NPs and Tf is greatly shifted to 48.1 and 43.0 due to the chitosan surrounding. The high zeta potential also prevents aggregating of NPs in aqueous atmosphere and can be stored at room temperature for at least 3 months. In addition, the DLS size of the samples is slightly increased from 210.0 nm to 319.1 nm or 375.5 nm in the synthetic procedure. The size of FND is surprisingly different in DLS and TEM measurements due to the easy-aggregation nature of ND.

3.2 Cell viability of the materials

Cytotoxicity tests are performed to determine whether the nanomaterials herein can be utilized in biological systems. The
selected materials that were used to synthesize the bio-functional FND-Au/Ag-Tf NPs were all highly bio-compatible. The ND and Au/Ag NPs have been proven elsewhere to be bio-friendly nanomaterials and they have been utilized in numerous investigations. The surface passivation reagent, PEG1500N, and the connector of the materials, 2-iminothiolane, have also been proven to be bio-compatible. Human transferrin (Tf) can be produce by human organs, such as the liver and brain. Although the synthetic chemicals are all found to be non-toxic, the combination of materials for use in biological systems must be carefully considered. In vitro cell viability was evaluated to ensure the biosafety of the materials (Fig. 4). The human hepatoma cell line (J5) was separately incubated with various concentrations of FND, FND-Au/Ag and FND-Au/Ag-Tf NPs. After 72 hours of reaction, the cell viability was similar with control, even at high concentrations. The original concentrations of the materials were 10 \( \mu \text{g mL}^{-1} \) (FND), 200 \( \mu \text{g mL}^{-1} \) (Au/Ag NPs) and 0.625 \( \mu \text{g mL}^{-1} \) (Tf). The tandem cell viability experiments were diluted 10 fold for testing. The highest concentrations of FND-Au/Ag shows higher cell viability than lower concentrations are in reasonable error. The test confirms that the materials are all non-toxic toward J5 cells and can be used in the applications described below.

### 3.3 Photo-stability of FND

Organic dye for many decades has been widely used for labeling in solar cells, probes and the tracing of tumors, among other applications. However, the photobleaching and photoblinking of organic dye limit the usage in long-term tumor tracing. The degree of photobleaching in this work was determined by illumination under a confocal microscopic laser at 488 and 561 nm with output powers of 30 mW and 50 mW. The J5 cells were incubated with FND for 12 hours, and stained by adding calcein AM for labeling. Calcein AM emits green fluorescence when illuminated at 488 nm irradiation, and FND emits red when irradiated at 561 nm (Fig. 5a). Clearly, calcein AM is fully bleached after one minute of irradiation (white dashed circles), but the FND remains unchanged. The ratio of the intensity to the original intensity \( \left( \frac{I}{I_0} \right) \) is plot as a function of time (Fig. 5b). The \( \frac{I}{I_0} \) of the organic dye, calcein AM, falls to less than 20% in 10 s of irradiation, and then to 10% after 30 s. Fluorescence of calcein AM was bleached to invisible in 30 s irradiation (Fig. 5b insets). In contrast, the fluorescence of FND remains constant without any bleaching even when the period of irradiation is increased to three hours (Fig. S3†). The fluorescence of FND remains detectable following storage at room temperature for one month (Fig. S4†), revealing that FND can be applied as a long-term tracing material.
3.4 Analysis of cellular uptake and cell–TF-NPs interaction

The location of NPs in a cell directly influences the degree of photothermal therapy provided to a group of cells. The internalization of fluorescence materials is used to study the uptake of NPs through cell endocytosis. If NPs attach only to the surface of cells, then they would be easily removable using a vacuum in vitro, or by blood flow in vivo, reducing the effectiveness of any remedy. So, the internalization of NPs in cells would lead to their possible aggregation, therein increasing the intensity of emission, improving labeling and therapeutic efficacy against cancer. Confocal microscopy is used herein to locate the fluorescent species Hoechst and FND (Fig. 6). Bright field confocal microscopy reveals the skeleton of the cell. The cell nuclei, stained by Hoechst, can absorb 405 nm light and exhibits blue fluorescence, indicating the tangent plane of the cell. In this work, FND emitted red fluorescence when irradiated with 561 nm green light. The merge plot revealed the location of NPs and the J5 cells, indicating that many FNDs were engulfed by cells. In Fig. S5,† the lysosome were further strained by LysoTracker® Green DND-26 as green fluorescence. The positions of red fluorescence which emit from the samples are obviously colocalized with LysoTracker® green fluorescence, and present at least 55.86% colocalization rate, suggesting that the samples in J5 cell were accumulated in lysosomes. These results show that the FND and FND-Au/Ag can be taken up naturally by cells, also indicating the biocompatibility of the bare NPs. However, the direct uptake of unrecognized NPs by a cell pose the risk that the therapeutic procedure may seriously harm an untargeted cell or organ. Therefore, bio-recognized Tf must be conjugated with NPs before therapy. Then, the therapy will focus on the targeted cells and reduce the damage to healthy tissue. Moreover, as a cell engulfs more NPs, the emissive and therapeutic properties will be strengthened. The rate of uptake can be easily quantified by either evaluating fluorescence by

![Confocal microscopic images of J5 cell line incubated with nanomaterials.](image6)

![Cell uptake behavior as revealed by (a) flow cytometry and (b) ICP-MS.](image7)

Fig. 6 Confocal microscopic images of J5 cell line incubated with nanomaterials.

Fig. 7 Cell uptake behavior as revealed by (a) flow cytometry and (b) ICP-MS.
flow cytometry or by measuring the concentration of Au by inductively coupled plasma mass spectrometry (ICP-MS). Flow cytometry yields the fluorescent intensity of FND against incubation time, without interference by the auto-fluorescence of the J5 cell (Fig. 7a). FND-Au/Ag-Tf NPs were initially taken up rapidly almost twice as fast as FND-Au/Ag, and this difference increases with incubation time. The difference between rates contributed to the overexpression of transferrin receptor (TfRc) of the J5 cell, improving the uptake ratio of FND-Au/Ag-Tf NPs by receptor-mediated endocytosis. More intense fluorescence corresponds to accumulation of a higher Au concentration inside the cell, which can be identified by ICP-MS (Fig. 7b). Similarly, the concentration of Au increases with incubation time, and after one day, the cell has accumulated twice the amount of NPs when FND-Au/Ag-Tf is used, compared with FND-Au/Ag. The higher concentration in the cell reveals greater damage during therapy. The two plots verify that the FND and Tf combine with Au/Ag NPs with equal effectiveness, and the amount of NPs can be easily monitored by ICP-MS or flow cytometry.

3.5 Photothermal therapy

The FND-Au/Ag-Tf is herein proved to be a long-term photo-stable fluorescent material, and the fact that it is specifically recognized by J5 cells increases its rate of uptake by cells and the quantity taken up. Moreover, FND-Au/Ag-Tf NPs can act as a photo-translating hyperthermia reagent that destroys targeted malignant tumor cells. The J5 cells were incubated herein at the same concentration as FND-Au/Ag and FND-Au/Ag-Tf NPs for 12 hours. The sample cells were washed with PBS several times to eliminate undesired materials on cells. Before the

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**Fig. 8** The photothermal experiments. (a) Microscopic images of J5 cell line incubated with nanomaterials, irradiated using 800 nm near-infrared laser at various powers (2.3 W, 1.4 W and 1.2 W) and stained by trypan blue. Inset plots display the fluorescence of calcein AM after irradiation. Both scale bars are 500 μm. (b) Fluorescence bleaching ratio of the calcein AM fluorescence after the NIR irradiation obtained using confocal microscopy.
photothermal therapeutic experiments were performed, the cells were stained by calcein AM for the in situ monitoring of the degree of photothermal behavior by observation of the extent of quenching. After the experiments, the cells were stained with trypan blue, which selectively turns dead cells blue (Fig. 8a). In the photothermal experiments, the samples were irradiated for one minute with an 800 nm NIR pulse laser with various output power densities (46, 28 and 24 W cm$^{-2}$). The bare J5 cells or cells with FND all remained alive under the irradiation, even at highest power, revealing that the non-damaging NIR laser power can be used as a very safe source of power in photothermal therapy. The cells exhibited almost no blue color, and the $F_i/F_0$ ratio therefore remained at almost unity (Fig. 8b), revealing that the power source was friendly to cells. However, the J5 cells that were incubated with FND-Au/Ag were damaged at the highest laser power of 46 W cm$^{-2}$ illumination, yielding a 500 μm × 500 μm black square that had quenched fluorescence (see insets in Fig. 8a). After the experiments, the blue square that was stained with trypan blue was identical to the quenched area, indicating the damage to the J5 cells. The area of damage decreased as the power radiation was reduced (28 or 24 W cm$^{-2}$), and the fluorescent intensity was also correlated with the damage at 20% and 10% quenching at 28 or 24 W cm$^{-2}$, respectively. The J5 cells that took up with FND-Au/Ag-Tf were damaged at 20% and 10% quenching at 28 or 24 W cm$^{-2}$, consistent with the reciprocal of the ratio of the concentrations of NPs. The bio-recognized Tf influences all of the properties of the FND-Au/Ag-Tf material, which fluoresces intensely. This property can be exploited in labeling and enhancement of internalization, for reducing the therapeutic power. The multi-functional FND-Au/Ag-Tf NPs that effectively convert light to heat have potential for future biological applications.

4 Conclusions

Multi-functional FND-Au/Ag-Tf NPs are fabricated by a simple synthetic route and proven to be biocompatible materials. Samples thereof exhibit remarkable photo-stability without any photobleaching or photoblinking, and so are excellent long-term cancer tracing materials. The bio-recognized Tf enhances the rate and quantity of uptake of the NPs. It is therefore useful in cancer cell labeling and photothermal therapy. The detections of the samples by ICP-MS and flow cytometry proved that cells took up twice as many of the Tf-modified NPs as bare NPs, whether measured by FND or Au concentration. The limiting power density for photothermal therapy when human hepatoma cells are incubated with FND-Au/Ag-Tf is almost half of that when they are incubated than FND-Au/Ag, suggesting that the bio-recognized NPs have high therapeutic efficiency. The degree of photothermal ablation can be easily investigated in situ by observing $F_i/F_0$, the reduction in intensity over time. The results thus obtained are identical to those obtained using the trypan blue dying process. Herein, the use of versatile FND-Au/Ag-Tf NPs in the labeling, and targeting of tumor cells, as well as high-efficiency photothermal therapy against them. The labeling and therapeutic properties can be conveniently monitored in situ by confocal microscopy. In the future, these NPs will be easily modified by conjugation with various specific target materials, such as genes or proteins, for application against diverse tumor cells.

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