IN VIVO FLUORESCENT IMAGING USING CYCLIC-ARGININE -GLYCINE-ASPARTIC ACID PEPTIDE AND PEG MODIFIED EUROPIUM NANOPARTICLE

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Abstract- we report a novel strategy to prepare Eu3+ nanoprobes for in vivo HepG-2 tumor-bearing mice imaging by conjugation of cyclic-arginine-glycine-aspartic acid peptide (cyclic-RGD) for tumor specific recognition, and PEG to improve biocompatibility and increase the circulation time. These Eu3+ nanoprobes exhibit excellent luminescent properties, high dispersion stability in water solutions and no cytotoxicity in living tumor cells after 24 h incubation. The Eu3+ nanoprobes can be completely cleared after 13 days from liver to feces and not be sequestered in blood and tissues which ensure the security of the nanoprobes for in vivo applications. The results report here provides a new perspective for the Eu3+ nanoprobes in biomedical applications.

Index Terms- Nanoparticles; In vivo imaging; tumor specificity; Excretion pathway; Nontoxicity.

I. INTRODUCTION

The development of biocompatible, high specificity and low toxicity lanthanide nanoparticle probes for in vivo tumor diagnosis and targeted treatment is currently an area with more and more consideration. Lanthanide nanoparticles, especially europium luminescent nanoparticles, have the unique optical properties such as large Stokes shift, long luminescent lifetime, characteristic sharp-line emission as well as excellent photostability [1], [2]. These features open new possibilities for tumor cellular specific imaging and clinical tumor diagnosis.

Nanoscale Eu3+ luminescent complexes have been linked with biological recognition molecules such as antibodies to endow them with the expected function of bio-recognition and specific targeting for biomedical applications [3], [4]. However, the relatively large size of antibodies suffers from the steric hindrance obstruction on the surface of nanoparticles. In view of this problem, arginine-glycine- aspartic acid (RGD) peptide is selected as an alternative choice. RGD peptide has been widely used as a promising approach for delivering anticancer drugs or contrast agents for cancer therapy and diagnosis clinically [5]-[7] which specifically interacted with integrin $\alpha_v\beta_3$ on the surface of tumor cells. So far, there still lack the research concerning in vivo RGD peptide modified Eu3+ nanoprobes for tumor specifically imaging.

Despite the immense potential in medical applications of Eu3+ luminescent nanoparticles, it is still unclear about their toxicity. In fact, Eu3+ is known to be relatively nontoxic compared to other heavy metal nanoparticles such as quantum dots. Published work regarding Eu3+ nanoparitcles has so far only effectively assumed that they are safe in vitro [8]. There are a large number of studies reporting that the nanoparticles can deposit in organs and tissues for several months [9]-[11]. Therefore, potential toxicity of Eu3+ luminescence nanoparticles in vivo is also expected to be determined such as organs distribution and excretion pathway because deposition and retention of the nanoparticles in organs and blood circulation will block further clinical diagnosis. However, because of the behavior of Eu3+ nanoparitcles under in vivo conditions is unknown and the chemical composites of nanoparitcles are variance between different reports, the distribution and toxicity data from previous reports are difficult to assess. Here, we report a novel approach to prepare desired Eu3+ luminescent nanoprobes which Eu3+ nanoparticles are linked with RGD and PEG for in vivo tumor specific targeting and imaging. These Eu3+ nanoprobes exhibit excellent luminescent properties, high dispersion stability in water solutions. Then they are successfully applied to living tumor cells image in vitro and tumor specific imaging in vivo. The Eu3+ nanoparticles display no cytotoxicity in living tumor cells after 24 h incubation and can be completely cleared after 13 days from liver to feces without being sequestered in blood and tissues which ensured the security of the Eu3+ nanoprobes for in vivo applications. These results have opened up new possibilities of Eu3+ nanoprobes for ultrasensitive imaging and diagnosis of tumors in the future.

II. MATERIALS AND METHODS

Chemicals. Amphiphilic copolymer styrene-maleic an- hydride (SMA, Sigma-Aldrich), 1-ethyl-3-(3-dimethylamino- propyl) carbodiimide hydrochloride (EDC, Sigma- Aldrich), N-hydroxysulfosuccinimide sodium salt (Sulfo-NHS, Sigma- Aldrich), RGD

(cyclo(Arg-Gly-Asp-D-Tyr-Lys),GL Biochem Ltd, Shanghai), NH_2-PEG_{5000}-NH_2 (GL Biochem Ltd, Shanghai).

Preparation of Eu3+ nanoparticles. An acetone solution (15 mL) containing hydrophobic Eu3+ luminescent complexes (1 mg) and amphiphilic copolymer styrene- maleic anhydride (10 mg) dissolve into 30 mL deionized water are mixed in the bottle under a reduced pressure condition. After rotary evaporation at 35 ^oC for 1 h, the acetone in the bottle is removed and the product Eu3+ nanoparticles are stored at 4 ^oC refrigerator for further use.

Preparation of Eu3+ nanoprobes. Eu3+ nanoparticles in water (8 mL) is mixed with a PBS solution (0.1 mL, pH 7.4) containing EDC (10 mg) and Sulfo-NHS (10 mg), and then the mixture is incubated for 30 min at 25 °C in a Thermomix shaker (Eppendorf, Germany) at 300 rpm. After being separated by centrifugation and washed with PBS solution (pH 7.4), the resulting activated Eu3+ nanoparticles are dispersed in PBS solution (10 mL, pH 7.4) by ultrasonic oscillation. Furthermore, RGD (Fig. 5b) solution (0.38 mg of RGD in PBS buffer, 0.2 mL) and NH_2-PEG_{5000}-NH_2 (MW: 5000; 1 mg) is added to this colloidal solution. The obtained mixture is incubated in the Thermomix shaker for 5 h at 25 °C, 300 rpm. After being separated by centrifugation and washed with PBS solution (pH 7.4) at least 3 times, the resulting Eu3+ nanoprobes are dispersed in PBS (5 mL) by ultrasonic oscillation and stored at 4 °C for further use.

AFM imaging. AFM imaging is conducted on a MultiMode atomic force microscope equipped with a Type IV controller (Veeco). About 10 □L of Eu3+ nanoparticles and Eu3+ nanoprobes solution in DI water is pipetted onto a fresh cleaved mica surface and incubated for about 10 min. The specimen is gently rinsed with water and dried under a flow of nitrogen. AFM images are recorded in “tapping mode” exposed to air and analyzed by using Nanoscope III v. 5.12 r2 (Veeco).

In vitro cytotoxicity measurement. HeLa and HepG-2 cells are trypsinized and resuspended in Dulbecco’s modified Eagle’s medium (DMEM) containing fetal bovine serum (FBS, 10%) and penicillin/streptomycin (1%). The cells are seeded at a density of 0.2-1.0 million cells per well in a 96-well plate. After 24 h of incubation at 37 °C with 5% CO_2 (the following steps are carried out under the same conditions), the cells are washed with phosphate buffered saline (PBS, 0.01mol L^{-1}, pH7.4). The Eu3+ nanoparticles solutions with different concentrations (10 □L, 0.0125-0.2 mg mL^{-1}) and DMEM (90 □L) are added to the wells. After 24 h of incubation, the supernatant is removed, and the cells are washed with PBS for three times. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (10 □L, 0.5 mg mL^{-1}) and DMEM (90 □L) are then added to each well. After 4 h of incubation, the medium is discarded, and the intracellular water-insoluble formazan blue is collected by DMSO (100 □L). The optical absorbance is measured at 490 nm on a Synergy H4 Hybrid Multi-Mode Microplate Reader. Each data point is collected by averaging that of ten wells, and the untreated cells are used as controls. The data analyses are conducted by Student’s t-test. Differences with P values <0.05 are considered to be statistically significant.

Cell imaging. HeLa cells and HepG-2 cells are propagated in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (1%). Then the cultured cells are trypsinized and resuspended in this DMEM at a concentration of about 1×10^5 mL^{-1}. The cell suspension (100 □L) is transferred to a confocal dish (35 mm). After incubation for 24 h at 37 °C in 5% CO_2, the cells are carefully rinsed with PBS solution (pH 7.4). Then a colloidal solution of Eu3+ nanoprobes (100 □L, 0.2 g L^{-1}) and the same concentration of Eu3+ nanoparticles are added, separately. After incubation for 3 h at 37 °C in 5% CO_2, the dish is rinsed three times with PBS solution (pH 7.4) and then fresh serum-free medium (1 mL) is added. The plates are incubated for another 10 min at 37 °C. As for block experiment, 10 □M RGD first adds into the confocal dish, and then incubates Eu3+ nanoprobes and Eu3+ nanoparticles as described above. Differential interference contrast (DIC) and fluorescent imaging are performed on an IX 71 inverted microscope with a 60×oil immersion objective (NA 1.45; Olympus), equipped with a digital camera (DVC 1412-B/W, DVC Co., USA). The digital images are collected and analyzed using IP Lab software (BD Biosciences Bioimaging).

In vivo and ex vivo imaging with spectra unmixing technique. Athymic nude mice (8 weeks, 20±2 g) obtained from Department of Laboratory Animal Science (Peking University Health Science Center) are injected via the tail vein with the Eu3+ nanoprobes (1 mg mL^{-1}, 0.2 mL) and Eu3+ nanoparticles (1 mg mL^{-1}, 0.2 mL), respectively. In vivo fluorescence images of the mice are obtained with Maestro 2 in vivo with spectra unmixing system (Cambridge Research & Instrumentation, Woburn, MA). The excitation filter is set as 435-480 nm; the emission filter is 620 nm long-pass filter. The liquid crystal tunable emission filter (LCTF, with a bandwidth of 20 nm and a scanning wavelength range of 500-950 nm) is automatically stepped in 10 nm increments from 500-720 nm, while the CCD captured images at each wavelength with constant exposure. At the special time points of 3 h and 13 days after injection, the mice are sacrificed, and the organs are removed for ex vivo fluorescence imaging on the same Maestro 2 in vivo system. All animal experiments are approved by the Animal Ethics Committee of the Medical School, Peking University.

ICP-AES assays. Tissues distribution of Eu3+ nanoparticles and Eu3+ nanoprobes are assessed by measuring europium levels by ICP-AES (PROFILE SPEC, Leeman). Tissues are removed, washed, and weighed. For each sample, the whole tissue is digested in nitric acid and heated at 140 °C for several hours until the tissues are completely digested. Continued stirring to react until the solution is colorless and clear. Afterwards, the solution volumes of every organ are adjusted to 10 mL using deionized water. And then the europium content is analyzed using ICP-AES.

Clearance studies. The Eu3+ nanoparticles and Eu3+ nanoprobes (1 mg mL^{-1}, 0.2 mL) are injected intravenously via the tail vein, and blood samples (0.1 mL) are retrieved from the inner canthus at 1h, 3h, 6 h, 24 h, respectively. The samples are then analyzed by time-resolved spectrofluorometer (Synergy H4, Biotek).

Excretion studies. To address whether the Eu3+ nanoprobes are eliminated into feces and urines, athymic nude mice are given tail-vein injection of Eu3+ nanoprobes (1 mg mL^{-1}, 0.2 mL) under sterile conditions, and are kept in Nalgene metabolic cages. Feces and urines are obtained at 0, 1 h, 2 h, 3 h, 6 h, respectively. For preparation of the fecal samples, a 3×excess weight of double-distilled water (DD-H2O) is added to facilitate softening upon soaking for three days. Samples are analysis by time-resolved spectrofluorometer (Synergy H4, Biotek).

III. RESULTS AND DISCUSSION

Preparation of Eu3+ nanoprobes. In this work, we use Eu3+ nanoparticles as the luminescent nanoparticles, which particle sizes are almost uniform (Fig. 1a). The Eu3+ nanoparticles are modified with RGD and PEG by a standard procedure that had been widely used in functionalization of nanoparticles. The excessive EDC and sulfo-NHS to the carboxylic groups on Eu3+ nanoparticles form active ester leaving groups, which can react subsequently with free amino groups present in RGD peptide and PEG to form the resulting Eu3+ nanoprobes [12]. The prepared Eu3+ nanoprobes exhibit excellent spectral quality as compared to Eu3+ nanoparticles. Fig.1c shows the photo of Eu3+ nanoparticles and Eu3+ nanoprobes (λ_{em}= 620 nm) which have similar luminescent intensity in water under UV irradiation (λ_{ex}= 365 nm). PL spectral and absorbance spectral have no broaden and shift observed for Eu3+ nanoprobes as compared to the Eu3+ nanoparticles dispersed in water (Fig.1e, 1f). Because of the low contrast, it is difficult to observe the RGD and PEG encapsulation of Eu3+ nanoparticle in TEM analysis. Therefore, they are confirmed by AFM images. The AFM results of Eu3+ nanoparticles and Eu3+ nanoprobes reveal after connecting with RGD and PEG, the prepared Eu3+ nanoprobes are homogeneously globular in shape and could also be well-dispersed in aqueous solutions without aggregation in contrast with Eu3+ nanoparticles (Fig.1a, 1b). Cross-section height analyses show vertical heights of these particles are 12.15 nm of Eu3+ nanoparticles (Fig. 1a) and 54.45 nm of Eu3+ nanoprobes (Fig. 1b) (n=100). The size increase of Eu3+ nanoprobes is also supported by the observation of gel electrophoresis image in which Eu3+ nanoprobes run significantly slower than Eu3+ nanoparticles (Fig.1d). These results together with diameter analysis imply that RGD and PEG are grafted on the Eu3+ nanoparticles surface.

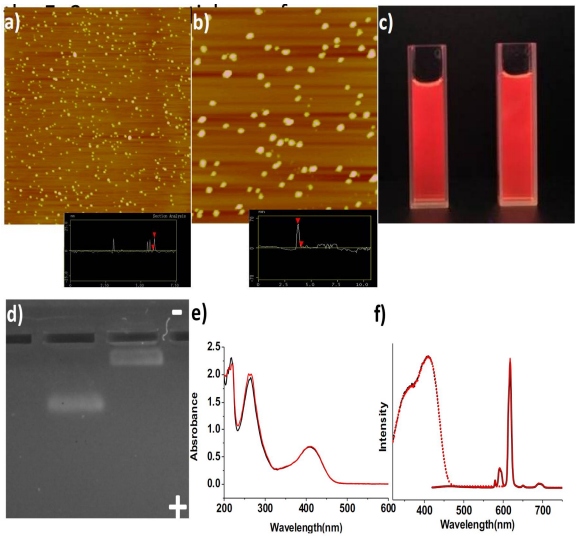


Fig. 1. a) AFM scanning of Eu3+ nanoparticles (7.5 × 7.5 ·m) and section analysis insert in the picture; b) AFM scanning of Eu3+ nanoprobes (5 × 5 ·m) and section analysis insert in the picture; c) Eu3+ nanoparticles (left) and Eu3+ nanoprobes (right) solution in water under UV irradiation at 365 nm; d) Electrophoresis image of Eu3+ nanoparticles (left line) and Eu3+ nanoprobes (right line); e) Absorption spectra of Eu3+ nanoparticles (black line) and Eu3+ nanoprobes (red line) in water solution; f) Luminescence excitation spectrum (dashed lines, λ_{em}=620 nm) and photoluminescence spectrum (solid lines, λ_{ex}=415 nm) of Eu3+ nanoparticles (black line) and Eu3+ nanoprobes (red line) in water solution.

Specificity targeting of Eu3+ nanoprobes to tumor cells. To demonstrate the binding specificity of Eu3+ nanoprobes to tumor cells, Eu3+ nanoparticles and Eu3+ nanoprobes are incubated with HeLa cells and HepG-2 cells. We prepare Eu3+ nanoprobes by covalent reaction and confocal fluorescent microscope images show their strong red luminescent signals. After 3 h of incubation at 37 °C with Eu3+ nanoparticles and Eu3+ nanoprobes, it can be seen clearly that Eu3+ nanoparticles do not exhibit any significant binding to the HeLa cells and HepG-2 cells (Fig. 2c), whereas Eu3+ nanoprobes show strong

fluorescent signals to both cells lines (Fig. 2a). Furthmore, in order to confirm the specificity targeting of Eu3+ nanoprobes to tumor cells through the $\alpha_v\beta_3$ integrin on the surface of tumor cells, we also conduct RGD blocking control experiments, which we treat the cells with 10 □M RGD first to block the $\alpha_v\beta_3$ integrin and then incubate Eu3+ nanoprobes with tumor cells as described above. As shown in Fig. 2b, this pretreatment drastically reduce the targeting of Eu3+ nanoprobes. From the tumor cell labeling experiment, it can be concluded that Eu3+ nanoprobes exhibit efficiently affinity and specificity to tumor cells through the $\alpha_v\beta_3$ integrin in vitro.

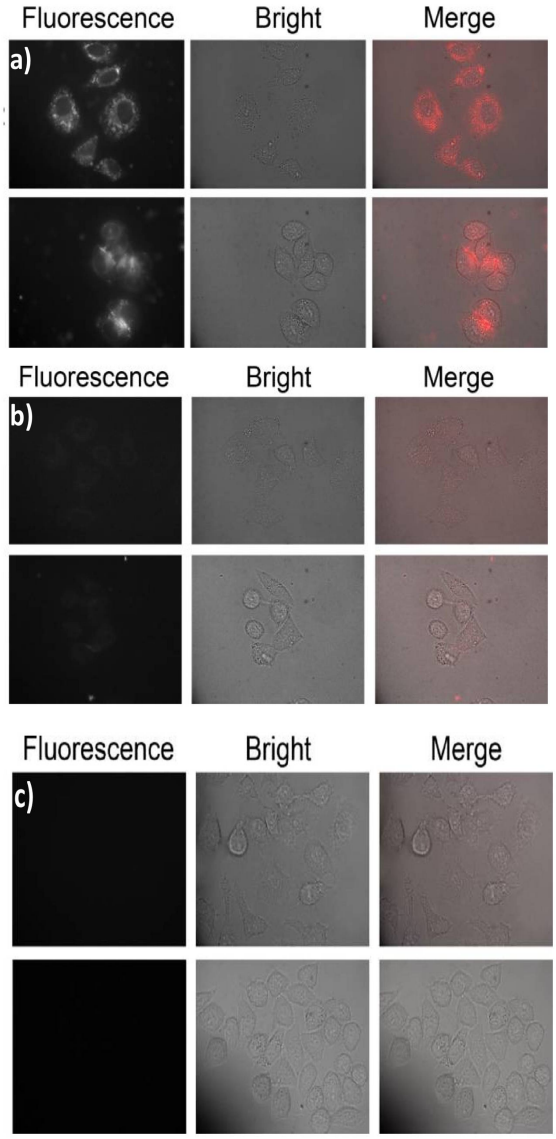


Fig. 2. In vitro cells staining experiments of HepG-2 cells (the upper row) and HeLa cells (the following row). a) Staining HepG-2 cells and HeLa cells with Eu3+ nanoprobes; b) HepG-2 and HeLa cells are blocked by 10 □M RGD first and then staining with Eu3+ nanoprobes; c) Staining HepG-2 cells and HeLa cells with Eu3+ nanoparticles.

In vivo tumor imaging. The application of fluorescence imaging with Eu3+ nanoprobes in vivo for sensitive cancer diagnostic application has been highly desirable. However, animal tissue absorb scatter photons and generate strong autofluorescence is one of the obstacles which will obscure signal collection. Besides, the nanoparticles have to be biologically stable enough in vivo conditions and preferentially accumulate to the tumor sites. In order to overcome these problems, we design RGD and PEG conjugated Eu3+ luminescent nanoparticles, in which RGD can actively target $\alpha_v\beta_3$ integrin on tumor cells and PEG can improve biocompatibility and increase the circulation time in vivo. Furthermore, we use Maestro 2 in vivo spectrum imaging system with spectral unmixing technique, which can reduce the effect of native tissue autofluorescence in fluorescence imaging [13]. In our experiments, athymic nude mice bearing subcutaneous HepG-2 tumor (Fig. 3a) are administered with Eu3+ nanoprobes and Eu3+ nanoparticles through intravenous injection, respectively. Images are taken at 0.5 h, 1 h, 3 h, 6 h, 1 day, 2 days, 4 days and 13 days by the Maestro 2 in vivo imaging system. Using spectral unmixing technique, we separate the autofluorescence background signals from the Eu3+ nanoprobes signals (Fig. 3d). Fig. 3b depicts spectral unmixing results obtained from the superimposed images of pure mice autofluorescence and Eu3+ nanoprobes signals. The composite image clearly show the whole animal and the tumor sites which indicate that the Eu3+ nanoprobes can be visualized, with little or no interference from the mouse autofluorescence. Because of the high signal to noise ratio, good luminescent intensity and excellent photostability, Eu3+ nanoprobes designate the tumor obviously. On the other hand, control group inject with Eu3+ nanoparticles have relatively low accumulation in tumor sites, and the luminescent signals are too weak to be detected.

Enhancing the accumulation of Eu3+ nanoprobes in the solid tumors is crucial for realizing their unique feature in tumor diagnosis and treatment. As shown in Fig. 3c, the fluorescent intensities of the unmixed Eu3+ nanoprobes with the increase of time are examined. After 30 min postinjection, the fluorescence of Eu3+ nanoprobes is observed in the subcutaneous tumor. There are steady increase with the time in the tumor fluorescent intensity after injection in Eu3+ nanoprobes group and the fluorescent signal of tumor reach the highest intensity at 6 h postinjection and then the fluorescent intensity begin to gradually reduce until completely disappear after 13 days. Some reports regard nanoparticles with small size usually less than 50 nm [14], [15] will increase the accumulation in tumors because small nanoparticles have the ability to penetrate deep into the tumor tissue [16]. However, other reports also show small nanoparticles could re-enter the blood

stream and deplete the nanoparticles accumulated within tumors [17]. Although the accumulation behavior of nanoparticles is still controversial, our results show the Eu3+ nanoprobes can maintain in the tumor for several days, partly because the stable coating of PEG protect them from being decomposed and prolonging their accumulation times.

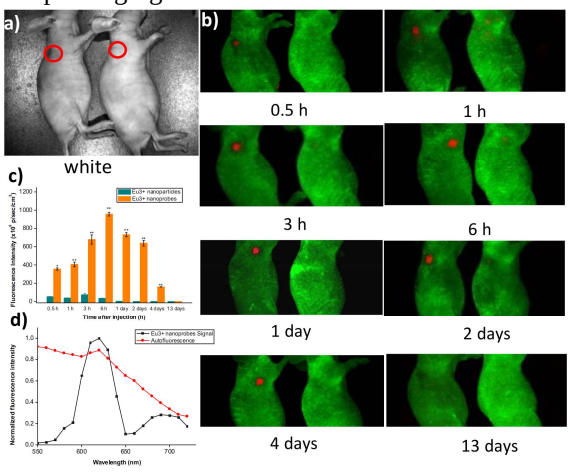


Fig. 3. In vivo spectra unmixing imaging of HepG-2 tumor-bearing mice (right shoulder inside red circles) inject with Eu3+ nanoprobes (left mouse) and Eu3+ nanoparticles (right mouse) via the tail vein. a) White image of HepG-2 tumor-bearing mice; b) Unmixed fluorescent images of mice. The mice autofluorescence color green while the unmixed Eu3+ nanoparticles signals color red; c) Comparing of fluorescence intensities of mice injected with Eu3+ nanoprobes and Eu3+ nanoparticles. The data are represented as mean ± standard deviation. Using one-tailed paired Student's t-test (n=5), "*" denotes where $P<0.05$, "**" denotes where $P<0.01$ as compared Eu3+ nanoprobes to the mice injected with Eu3+ nanoparticles. d) The "pure" autofluorescence (red line) and Eu3+ nanoprobes (black line) spectra used for spectral unmixing imaging. The autofluorescence spectrum is obtained from normal mouse without injection Eu3+ nanoprobes. The Eu3+ nanoprobes spectrum is obtained by deducting of the autofluorescence signal from the mixture signal of Eu3+ nanoprobes -administrated mouse.

Distribution and clearance of Eu3+ nanoprobes in vivo. Despite of the efficient labeling of Eu3+ nanoprobes to tumor, we know little about the organ distribution and clearance of Eu3+ nanoprobes in vivo. With regard to nanoprobes for tumor diagnosis in vivo, the adverse effects of the particles such as toxicity have been extensively concerned. The possibilities of the toxicity are caused by the sequestration of Eu3+ nanoprobes in organs. In our experiments, the method of spectral unmixing imaging have excellent sensitivity, thus it is possible to detect the presense of Eu3+ nanoprobes in organs and tumors. High fluorescent signals is obtained for the tumor in Eu3+ nanoprobes group but rarely in Eu3+ nanoparticles group after 3 h postinjection (Fig. 4b). Other organs with readily detectable fluorescent signals distribution include livers, spleens, kidneys and hearts which are also observed to have lower intensity comparing to tumor of Eu3+ nanoprobes group at the same time point in both groups (Fig. 4a). After 13 days, the fluorescence of both groups disappear (Fig. 4d). Further experiments of the fluorescence in the organs are carried out after 13 days postinjection in comparison to the 3 h. The results show significant differences in the fluorescent siganls nearly none in all organs (Fig. 4c). As reported by Daou group, the substantial loss of fluorescence can be due to either the clearance of the nanoparticles from the body or the slow degradation of the nanoparticles [18]. Therefore, we use Inductively Coupled Plasma-Atomic Emission Spectrometer (ICP-AES) to confirm the level of europium element in the variety of organs that injected with the same amount of Eu3+ nanoprobes and Eu3+ nanoparticles as spectral unmixing imaging experiments. As shown in Fig. 4e, the results of ICP-AES after 3 h demonstrated tumor in Eu3+ nanoprobes group have much higher element europium levels compared to Eu3+ nanoparticles group. Other organs such as spleen, liver, kidney and heart in both groups have much lower element europium levels comparing to tumor in Eu3+ nanoprobes group. After 13 days, europium element cannot be detected in all organs and tumors of the two groups by ICP-AES. Therefore, the biodistribution of Eu3+ nanoprobes by ICP-AES in animals after intravenous injection prove to be consistent with the research of organs and tumors samples by the methods of spectral unmixing imaging.

The clearance of Eu3+ nanoprobes out of the body was potential important when they are injected intravenously in vivo as clinical applications. However, the excretion pathway of Eu3+ nanoprobes is still unclear so far. In order to confirm the excretion pathway of Eu3+ nanoprobes, animals are sacrificed and dissected after 3 h postinjection, and then their major organs such as liver, intestine and bladder are exposed in order to observe the fluorescent signals of the Eu3+ nanoprobes. After surgically exposing and imaging by spectral unmixing imaging, Eu3+ nanoprobes are found mainly in liver and intestine but none in bladder (Fig. 3f, 3g). As we know, the main organs responsible for excretion are the kidneys and the liver. Localization of Eu3+ nanoprobes in the liver and intestine indicates that the nanoprobes are excreted by liver into feces. It is worth noting that there are no Eu3+ nanoprobes excreted in bladder which signify that Eu3+ nanoprobes are incapable of crossing the renal filtration. In our estimated, it is probably because PEG in the complex increases the hydrodynamic diameter which surpasses the renal filtration threshold [19] and leads to the block of Eu3+

nanoprobes from kidney pathway. Blood of mice are also taken after administration of Eu3+ nanoprobes and Eu3+ nanoparticles immediately (Fig. 4h) by the time-resolved spectro- fluorometer. Eu3+ nanoparticles signals are detected gradually decease till 24 h in both groups, which show that the Eu3+ nanoparticles have been cleared from the blood circulation after administration and no traces of these signals are detected after 24 h. Furthermore, feces and urines are collected every one hour after injection of Eu3+ nanoprobes and analyzed by the time-resolved spectro- fluorometer. The samples of luminescence of urines are measured and there are not significantly different from the control mice. In the consequence of feces samples, the luminescent intensities of Eu3+ nanoprobes are gradually increased for up to 5 h postinjection (Fig. 4i), this explicitly explain the clearance of Eu3+ nanoprobes is through liver to feces. Therefore, our studies have demonstrated efficient excretion of Eu3+ nanoprobes by mice from liver to feces and will not be sequestered in blood.

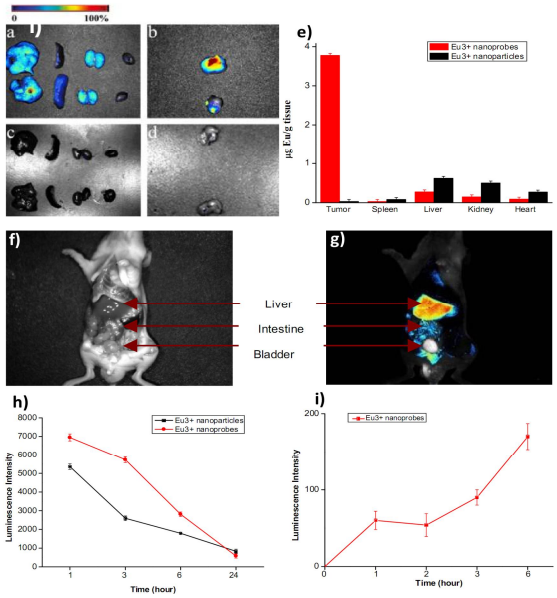


Fig. 4. a) Organs (from left to right: liver, spleen, kidneys and heart) and b) Tumor fluorescent images of Eu3+ nanoprobes (the upper row) and Eu3+ nanoparticles (the following row) get out from the living mice after administration via the tail vein for 3 h; c) Organs (from left to right: liver, spleen, kidneys and heart) and d) Tumor luminescence images of Eu3+ nanoprobes (the upper row) and Eu3+ nanoparticles (the following row) get out from the living mice after administration via the tail vein for 13 days ; e) ICP-AES analysis shows element europium level in various tissues at 3 h after injection of Eu3+ nanoprobes and Eu3+ nanoparticles, respectively; Surgically exposing and imaging for f) White image and g) Fluorescent image of the main organs and the filling bladder of the HepG-2 tumor-bearing mice using spectral unmixing technique after administration of Eu3+ nanoprobes via the tail vein; h) Time-resolved luminescence detection in blood of mice after administration with Eu3+ nanoprobes and Eu3+ nanoparticles via the tail vein; i) Time-resolved luminescence detection of mice feces after administration with Eu3+ nanoprobes via the tail vein.

Cytotoxicity. In order to test the toxicity of Eu3+ nanoparticles in the living system, HepG-2 cells and HeLa cells are grown for 24 h in the presence of Eu3+ nanoparticles. The HeLa cells and HepG-2 cells are exposed to DMEM with different concentrations of Eu3+ nanoparticles and the percentages of the cells viability are quantified. Results show no toxicity is found based on cell proliferation count when compared to control cells that are not incubated with Eu3+ nanoparticles (Fig. 5a). These results are similar to the previous reports that Eu(tta)$_3$dpbt nanoparticles showed noncytotoxicity at 7.4 □M for HeLa cells and Lewis cells [8]. Thus, Eu3+ nanoparticles are demonstrated nontoxic for biological applications.

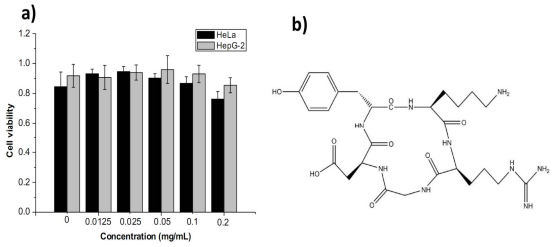


Fig. 5. a) Cytotoxicity of Eu3+ nanoprobes at different concentrations after 24 h incubation with HeLa cells and HepG-2 cells by MTT assay. The data are represented as mean ± standard deviation. Using one-tailed paired Student's t-test (n=10), "*" denotes where P<0.05, "" denotes where P<0.01. b) Molecular structure of Cyclo (Arg-Gly-Asp-D-Tyr-Lys) (molecular formula: $C_{27}H_{41}N_9O_8$).**

CONCLUSION

In summary, we have developed a conspicuously new strategy to modify Eu3+ nanoprobes which exhibit essential properties requisite for in vivo tumor imaging, such as biocompatibility, comparatively small size and nontoxicity. The Eu3+ nanoprobes can target to tumor efficiently and specifically both in vitro and in vivo. The Eu3+ nanoprobes can be eliminated clearly after 13 days from liver to feces which ensure the security of the probes for in vivo applications. The results report here exploit new vision for Eu3+ nanoprobes in vivo imaging and will have great potential in tumor diagnosis in future.

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