Biochemical and Biophysical Research Communications 381 (2009) 54–58

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Cyclic RGD peptide-labeled upconversion nanophosphors for tumor cell-targeted imaging

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ARTICLE INFO

Article history:
Received 30 January 2009
Available online 8 February 2009

Keywords:
Upconversion
Bioimaging
Nanoparticles
Tumor imaging
Cancer
PEG modification
Integrin $\alpha_v\beta_3$
RGD peptide

ABSTRACT

One of the great challenges of oncology is to improve methods for early tumor detection. Thus tumor cell-targeted optical imaging has been intensively studied. Bioimaging with upconversion (UC) phosphors (UCPs) is of considerable interest due to a variety of possible applications taking advantage of infrared-to-visible luminescence. Here we report for the first time tumor cell-targeted UC imaging using UCPs modified with cyclic RGD peptide (RGD-Y_2O_3). Cyclic RGD peptide binds specifically to integrin $\alpha_v\beta_3$ which is highly expressed in a tumor cell surface of certain cancer types but not in normal tissues. Since UC emission from RGD-Y_2O_3 was observed for U87MG cancer cell (high integrin $\alpha_v\beta_3$ expression), but not for MCF-7 cancer cell (low integrin $\alpha_v\beta_3$ expression), this UC imaging is considered to be integrin $\alpha_v\beta_3$ specific. The non-invasive imaging of integrin $\alpha_v\beta_3$ expression using UCP-based probes will have great potential in cancer imaging in general in living subjects.

Bioimaging technique has received particular attention as an essential tool in the field of biomedical research through the observation of biological phenomena both *in vivo* and *in vitro*. However, current imaging methodologies utilizing organic dyes or fluorescent proteins remain problematic. They allow us only short observation periods since they are easily photo-bleached [1]. Their use in biological tissues is also restricted due to limited light penetration depth associated with strong scattering of the excitation light of short wavelength [2]. Furthermore, short wavelength excitation with high quantum energy results in tissue photo-toxicity. Although the use of quantum dots may solve the first problem [3], it cannot resolve the latter two concerns.

Fluorescence imaging utilizing near-infrared (NIR) excitation is expected to have a strong impact on biomedical imaging since the NIR (800–1500 nm) is located within the so-called biological window, where water and biological tissues have minimal absorbance and autofluorescence [4,5]. Another advantage is that NIR light can penetrate deeper into tissues due its lower scattering.

Recently, upconversion (UC) phosphors (UCPs) have been used for bioimaging [6–17]. UCPs are ceramic materials containing rare earth ions. The materials can absorb IR radiation and upconvert it to emit visible light by the stepwise excitation among discrete energy levels of the rare earth ions [18]. For example, yttrium oxide (Y_2O_3) matrix containing several atomic% of erbium (Er-Y_2O_3) exhibits upconversion emission at 550 nm (green) and 660 nm (red) following excitation at 980 nm. We have previously reported that covalent attachment of poly(ethylene glycol) (PEG) to the surface of Er-Y_2O_3 nanoparticles drastically improved dispersion stability in aqueous milieu [10]. The PEG-modified Er-Y_2O_3 nanoparticles showed upconversion emission without cell-toxicity [10].

In this study, we report for the first time that targeting and UC imaging of integrin $\alpha_v\beta_3$-positive tumor cells using Er-Y_2O_3 nanoparticles labeled with cyclic arginine–glycine–asparatic acid (RGD) peptide via bi-functional PEG. Integrin $\alpha_v\beta_3$, which binds to RGD-containing components of interstitial matrix, plays a key role in tumor angiogenesis and metastasis. Its expression is significantly upregulated in invasive tumor cells of certain cancer types (glioblastoma, melanoma, breast, ovarian, and prostate cancers, and in almost all tumor vasculature) but not in quiescent endothelium and normal tissues [19,20]. Because integrin $\alpha_v\beta_3$ is a potential therapeutic target in cancer research, RGD peptide-modified compounds have been successfully used for applications such as molecular imaging, gene therapy, radiotherapy and targeted drug

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doi:10.1016/j.bbrc.2009.02.004

delivery [21–23]. Cyclic RGD peptide has also been used for tumor cell targeting due to its high selectivity and affinity [5]. More recently, Cai et al. reported *in vivo* targeting and imaging of tumor vasculature using cyclic RGD peptide-modified quantum dots [4]. Because of the advantages of UCPs over quantum dots [10,24–26], non-invasive imaging of integrin $\alpha_v\beta_3$ expression using UCP-based probes will have great potential in cancer imaging in general in living subjects.

Materials and methods

Reagents. $Y(NO_3)_3{\cdot}6H_2O$ (99.99% purity) and Urea (99.0% purity) were purchased from Kanto Chemicals (Tokyo, Japan). $Er(NO_3)_3{\cdot}5H_2O$ (>99% purity) was from Kojundo Chemical Laboratory (Saitama, Japan). Urease was from Wako (Osaka, Japan). Cyclo(RGDyK) peptide was from ANASPEC (CA, USA). Eagle's minimal essential cell culture medium with Earle's salts (E-MEM) was purchased from Wako (Saitama, Japan). All other cell culture reagents, including fetal bovine serum (FBS), non-essential amino acids (NEAA), sodium pyruvate and penicillin-streptomycin were purchased from GIBCO-Invitrogen (CA, USA).

Preparation of UCP nanoparticles. Yttrium oxide (Y_2O_3) nanoparticles including 10% Er with size range from 30 to 60 nm were synthesized using an enzymatic decomposition method using Urea and urease as previously described [10]. The Er-Y_2O_3 nanoparticles were generated by calcinating the hydroxycarbonate precursors at 1000 °C for 1 h.

The surface of the Er-Y_2O_3 nanoparticles was modified using 3-aminopropyltrimethoxysilane (APTES) and PEG as illustrated in Fig. 1. The Er-Y_2O_3 nanoparticles (50 mg) were suspended in 45 mL of 2-propanol and subjected to ultrasonication. After 300 µl of APTES was added, the mixture was stirred for 24 h at 70 °C. Particles were then isolated, washed five times with ethanol by centrifugation, and finally dried in air at room temperature.

The APTES-modified Er-Y_2O_3 (APTES-Er-Y_2O_3) nanoparticles (20 mg) were suspended in 10 mL of dry-dimethyl sulfoxide (DMSO, Wako, Tokyo, Japan), to which was added 500 µM heterofunctional PEG containing *N*-hydroxysuccinimide (NHS) and maleimide (MA) at the both ends (NHS–PEG–MA) (MW = 5000, Sunbright MA-050HS, NOF Corp., Tokyo, Japan) and stirred for

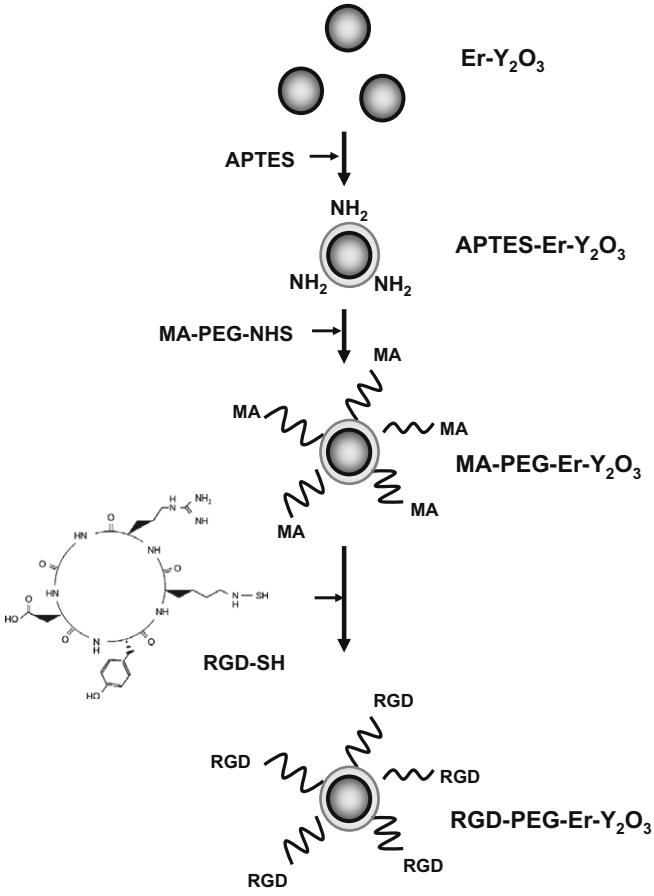

Fig. 1. Schematic illustration outlining the preparation of RGD-modified Er-doped Y_2O_3 nanoparticles.

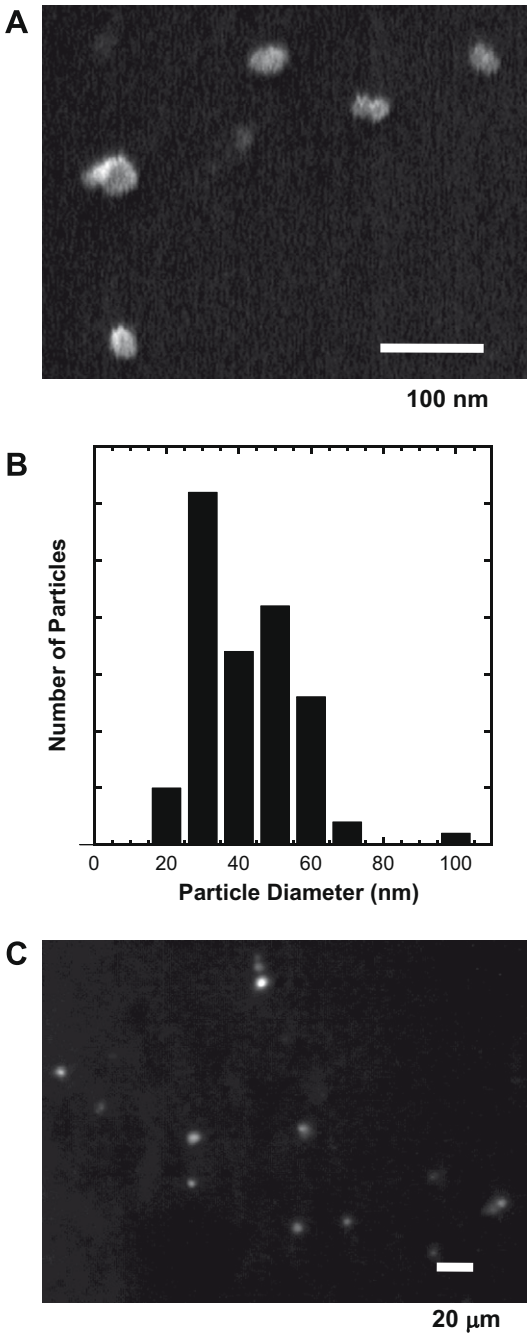

Fig. 2. (A) SEM image of RGD-PEG-Er-Y_2O_3 nanoparticles. The scale bar represents 100 nm. (B) Histogram of the particle sizes obtained from ~100 RGD-PEG-Er-Y_2O_3 nanoparticles in SEM images. (C) UC emission image of RGD-PEG-Er-Y_2O_3 nanoparticles. The nanoparticles were excited using an IR laser (λ_{ex} = 980 nm) and the UC emission between 660 and 740 nm was observed. The scale bar represents 20 µm.

24 h at room temperature. The MA-PEG modified APTES-Er-Y_2O_3 (MA-PEG-Er-Y_2O_3) nanoparticles were isolated, washed three times with dry DMSO by centrifugation, and suspended in 10 mL of dry DMSO.

In order to introduce a thiol group into cyclo(RGDyK) peptide (potent integrin $\alpha_V\beta_3$ antagonist), 1 mg of cyclo(RGDyK) was dissolved in 500 μL of dry DMSO, to which was added 1 mg of *S*-acetylthioglycolic acid *N*-hydroxysuccinimide ester (SATA), and stirred over night at room temperature Then 1 mL of 10% hydroxylamine was added and stirred for 3 h to deprotect a thiol group and to yield the thiolated RGD peptide cyclo(RGDy(ε-acetylthiol)K), denoted as RGD-SH. The MA-PEG-Er-Y_2O_3 was allowed to react with RGD-SH for 12 h at room temperature in dry DMSO. The final conjugate (RGD-PEG-Er-Y_2O_3) was isolated, washed three times with distilled water by centrifugation. The size of RGD-PEG-Er-Y_2O_3 was confirmed by SEM observation as described before [10]. UC emission was also confirmed as described before [10].

Cell line. Human MCF-7 (low integrin $\alpha_V\beta_3$ expression) breast carcinoma cells and U87MG (high integrin $\alpha_V\beta_3$ expression) glioblastoma cells were purchased from European Collection of Cell Cultures. U87MG cells were grown in E-MEM medium with 10% FBS, 1% NEAA, 1% sodium pyruvate and 1% penicillin–streptomycin

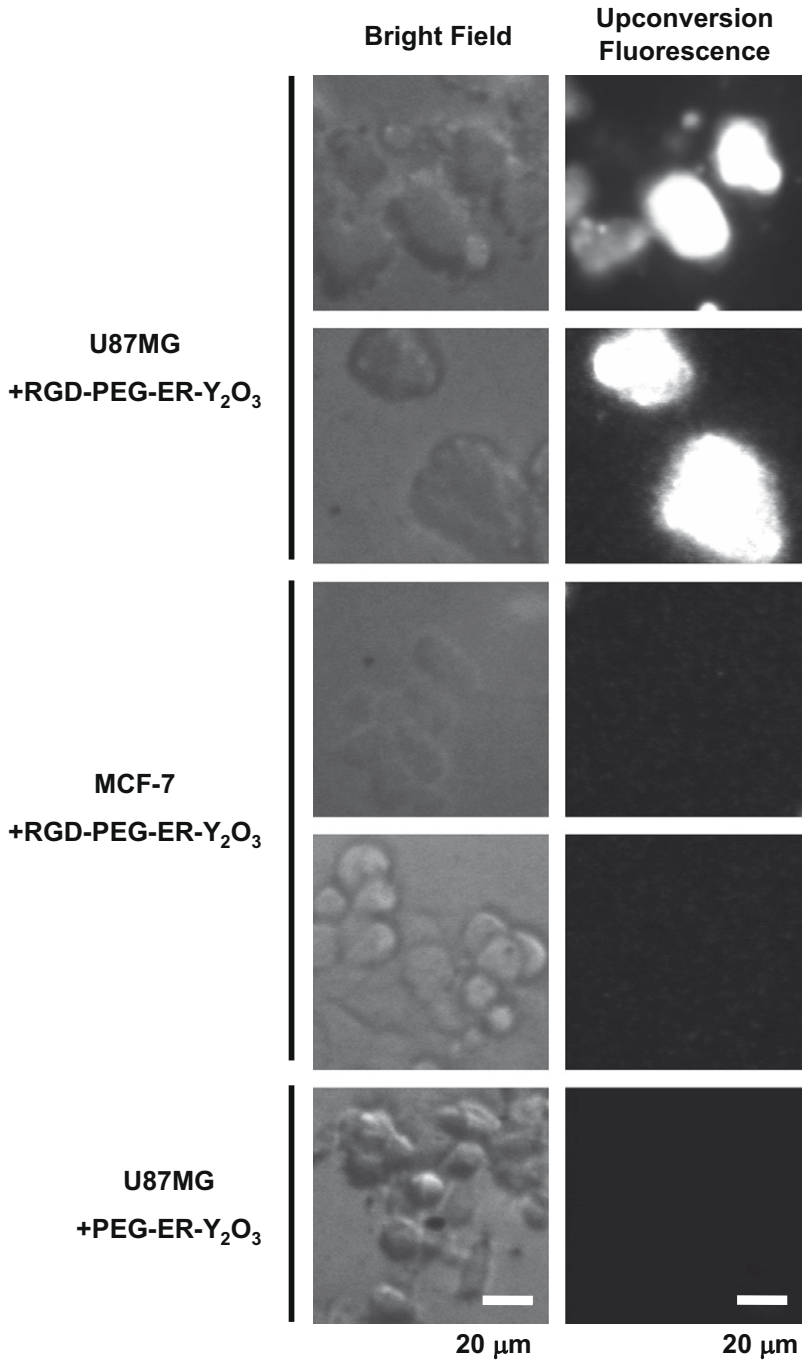

Fig. 3. *In vitro* staining of human glioblastoma U87MG (high integrin $\alpha_V\beta_3$ expression) and human breast cancer MCF-7 (integrin $\alpha_V\beta_3$ negative) using RGD-modified UCP nanoparticles (RGD-PEG-Er-Y_2O_3) (right columns). Staining of U87MG with unmodified PEG-Er-Y_2O_3 nanoparticles is also shown as a control. All fluorescence images were acquired under the same condition and displayed under the same scale. The UCP nanoparticles were excited using an IR laser (λ_{ex} = 980 nm) and the UC emission between 660 and 740 nm was observed. The scale bar represents 20 μm.

in 5% CO_2 at 37 ℃. MCF-7 cells were grown in E-MEM medium with 10% FBS, 1% NEAA and 1% penicillin–streptomycin in 5% CO_2 at 37 °C. Cells were detached from cell culture dish with trypsin-EDTA for passage.

Cancer cell imaging. Cells were plated in glass-bottom dish at a density of 20,000 cells/mL. Cells were then incubated in 2.0 mL medium in the presence of 100 μg/mL RGD-PEG-Er-Y_2O_3 nanoparticles for 3 h. Cells were washed three times with distilled water, and then 2 mL of medium was added.

Imaging of the RGD-PEG-Er-Y_2O_3 nanoparticles attached to cancer cells by detecting the UC emission under IR excitation was performed using an inverted microscopy system (IX71, Olympus, Tokyo, Japan). The UCP nanoparticles were illuminated with a continuous-wave laser diode (1200 mA, 980 nm). The UC emission between 660 and 740 nm was collected with ×40 microscope object lens (UPlanFLN, Olympus) through a bandpass emission filter (HQ700/75, Chroma Technology, VT, USA). Images were taken using a CCD camera (MC681SPD-R0B0, Texas Instruments, TX, USA) coupled to an image intensifier (C8600-05, Hamamatsu Photonics, Shizuoka, Japan).

Results and discussion

In order to develop an UCP probe for tumor cell-targeted imaging, the thiolated cyclic RGD peptide was covalently attached on the surface of MA-PEG-modified Er-Y_2O_3 nanoparticles following the covalent attachment of heterofunctional NHS-PEG-MA on the APTES modified Er-Y_2O_3 nanoparticles Fig. 1. Since the cyclic RGD peptide binds to integrin $\alpha_V\beta_3$ that highly expressed in invasive tumor cells but not in normal tissues, it is expected that the RGD modified Er-Y_2O_3 nanoparticles also can recognize integrin $\alpha_V\beta_3$ expression level.

Fig. 2A shows a SEM image of the RGD-PEG-Er-Y_2O_3 nanoparticles. Non-agglomerated nanoparticles were successfully obtained. From a detailed particle size analysis of approximately 100 particles from several SEM micrographs, the average particle size was 39.8 nm with a standard deviation of 21 nm (Fig. 2B). The UC emission image of RGD-PEG-Er-Y_2O_3 nanoparticles excited by the IR laser was also successfully observed (Fig. 2C).

Tumor cells were stained with 100 μg/mL RGD-PEG-Er-Y_2O_3 nanoparticles for 3 h and washed three times to remove unbound nanoparticles. UC emission with IR excitation was observed under the microscope. The representative brightfield and UC fluorescence images are shown in Fig. 3. To determine whether it is integrin $\alpha_V\beta_3$ expression level dependent, two different cell lines, MCF-7 (human breast cancer, integrin $\alpha_V\beta_3$ negative) and U87MG (human glioblastoma, high integrin $\alpha_V\beta_3$ expression) cells were used. When the integrin negative cells (MCF-7) were used, no upconversion fluorescence signal from RGD-PEG-Er-Y_2O_3 nanoparticles was observed. Whereas, the integrin positive cells (U87MG) were clearly visualized (Fig. 3). In order to further confirm whether it is RGD-integrin interaction specific, unmodified PEG-Er-Y_2O_3 nanoparticles were incubated with the integrin positive U87MG cells as a negative control experiment. As shown in the figure, no fluorescence was observed for unmodified PEG-Er-Y_2O_3 nanoparticles, which support the specific interaction of RGD-PEG-Er-Y_2O_3 nanoparticles with the integrin-positive tumor cells.

In conclusion, we report for the first time tumor cell-targeted UC imaging using UCPs modified with cyclic RGD peptide via bifunctional PEG with heterogeneous ends. One of the great challenges of oncology is to improve methods for early tumor detection. Thus tumor cell-targeted optical imaging has been extremely studied. On the other hand, bioimaging with UCP is of considerable interest due to a variety of possible applications taking advantage of infrared-to-visible luminescence. Thus non-invasive imaging of integrin $\alpha_V\beta_3$ expression using UCP-based probes will have great potential in cancer imaging in general in living subjects.

Acknowledgments

This work is financially supported by RIKEN and the Ministry of Education, Science, Sports, Culture, and Technology (MEXT) of Japan. K.S. was financially supported by "Development of upconversion nano-particles for bio-nano-photonics" from New Energy and Industrial Technology Development Organization (NEDO) of Japan.

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