Nanoparticles speckled by ready-to-conjugate lanthanide complexes for multimodal imaging
Nanoscale speckled by ready-to-conjugate lanthanide complexes for multimodal imaging

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Multimodal and multifunctional contrast agents receive enormous attention in the biomedical imaging field. Such contrast agents are routinely prepared by the incorporation of organic molecules and inorganic nanoparticles (NPs) into host materials such as gold NPs, silica NPs, polymer NPs, and liposomes. Despite their non-cytotoxic nature, the large size of these NPs limits the in vivo distribution and clearance and inflames complex pharmacokinetics, which hinder the regulatory approval for clinical applications. Herein, we report a unique method that combines magnetic resonance imaging (MRI) and fluorescence imaging modalities together in nanoscale entities by the simple, direct and stable conjugation of novel biotinylated coordination complexes of gadolinium(III) to CdSe/ZnS quantum dots (QD) and terbium(III) to super paramagnetic iron oxide NPs (SPION) but without any host material. Subsequently, we evaluate the potentials of such lanthanide-speckled fluorescent-magnetic NPs for bioimaging at single-molecule, cell and in vivo levels. The simple preparation and small size make such fluorescent-magnetic NPs promising contrast agents for biomedical imaging.

1. Introduction

In vivo multimodal imaging has the potential to precisely pinpoint the anatomy and physiology associated with the onset and progression of diseases such as cancer. With the infiltration of molecular and nanomaterials contrast agents into the clinical settings, the quality and precision of biomedical imaging technology have significantly reformed in the recent past. Integration of two or even more contrast agents of individual imaging modalities, each complementing the limitation of other agents, into a nanoscale entity is fundamental to multimodal imaging and the extraction of the finest information about pathophysiological conditions. Multimodality in the clinical settings can be readily accomplished by the combination of techniques such as magnetic resonance imaging (MRI), ultrasonography, positron and single photon emission tomography (PET/SPECT), and X-rays-based computed tomography (CT). Nonetheless, some of these techniques pose a radiation hazard. Thus, nanomaterials incorporated with contrast agents for fluorescence and magnetic resonance attract considerable attention for non-invasive in vivo multimodal imaging but without any concern about hazardous radiation.

Fluorescent and paramagnetic materials for the construction of multimodal NPs are routinely recruited from among visible/NIR dyes, semiconductor quantum dots (QDs), upconversion NPs (UCNPs), carbon NPs, noble metal QCs, lanthanide ions, SPION, oxides, or vanadates of gadolinium, and coordination complexes of lanthanides. Host materials, such as NPs of silica, polymers, lipids and carbon, have been extensively exploited for accommodating two or more such contrast agents into nanoscale entities through host-guest interactions, involving hydrogen bonding, hydrophobicity, π-stacking, nanopore-filling, covalent or coordinate bonding, and stimuli-responsive molecular gates. In addition, the large surface area, unique pores, and versatility of the host materials allow one to recruit drugs, antibiotics and peptides for targeted labeling, and stimuli for the release/delivery of contrast agents, drugs, or genes. Nonetheless, the large size of the final product, which is largely contributed by the host material, poses a major challenge in both their distribution to the parenchymal target organ/lesion and clearance from the body. Therefore, simple
and small-size multimodal contrast agents are constantly sought-after towards practical applications in the clinical settings by the direct doping, co-precipitation, adsorption, or non-covalent or covalent conjugation of a fluorophore to a paramagnetic agent or vice versa.

The covalent conjugation offers stability to the multimodal contrast agent when compared with those prepared by doping, co-precipitation, or adsorption. For example, stable contrast agents for MRI and fluorescence imaging are prepared by the conjugation of Gd(III) complexes to organic dyes such as porphyrin,6,7 fluorescein8 and Cy5.5,9,10 QDs such as CuInS2/ ZnS,17,18 silicon,19 InP,20 CdSeTe/CdS,21 CdSe/ZnS22 and CdTe/ ZnS23 and persistent luminescence NPs,24 UCNP,27–29 and gold QCs.32–35 Similarly, NPs prepared by the conjugation of dye molecules such as Cy5.5,11 rhodamine,12,13 Congo red13 and Eu(III) complex,14 as well as QDs such as CdSe/ZnS24 and CdTe,25 and Au QCs,26–28 to SPION, have been found attractive for the combined MRI and fluorescence imaging. Gd(III) complexes employed in multimodal NPs are prepared using ligands such as 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) or diethyltriamine pentaacetic acid (DTPA), which are functionalized with N-hydroxysuccinimide (NHS) or biotin for the successive conjugation to amine or streptavidin functionalized fluorophores. Although the synthesis of such ligands can be carried out in a typical organic chemistry laboratory, simple and cost-effective preparation of ready-to-conjugate complexes of lanthanides, such as Gd(III), Eu(III) and Tb(III), is highly desirable for biomedical applications. Herein, we report simple and small-size fluorescent-magnetic bimodal NPs prepared by the direct conjugation of novel biotinylated DOTA-Gd(III) complexes to streptavidin-functionalized CdSe/ZnS QDs and biotinylated DOTA-Tb(III) complexes to streptavidin-functionalized SPION but without any sophisticated chemical reaction or host material. A simple biotinylated-DOTA derivative prepared by the direct condensation between one of the carboxylic acid groups in DOTA and ω-bromopropyl ester of biotin is utilized in the preparation of the complexes and the fluorescent-magnetic NPs. Subsequently, these NPs were bioconjugated using epidermal growth factor (EGF), and the potentials of the EGF-conjugated NPs for single-molecule, cell and in vivo imaging were evaluated in human lung epithelial adenocarcinoma (H1650) cells or B6 mice.

2. Results and discussion

Two types of novel fluorescent-magnetic NPs prepared by the direct tethering of coordination complexes of biotinylated Gd(III)-DOTA complexes to streptavidin-functionalized CdSe/ ZnS QDs and biotinylated Tb(III)-DOTA complexes to streptavidin-functionalized SPION enabled us to obtain the combined MRI and fluorescence images in vitro and in vivo. The biotinylated complexes are prepared by the simple chemical reactions shown in Fig. 1. Initially, an ω-bromopropyl ester of biotin (2), which was prepared by the 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)-catalyzed reaction between 1,3-dibromopropane and biotin, was conjugated to one of the carboxylic groups of 1,4,7,10-tetraazacyclododecane 1,4,7,10-tetraacetic acid (DOTA) to obtain the biotinylated ligand 3. Subsequently, biotinylated Gd(III)-DOTA complex (4) was prepared by the reaction of 3 with GdCl3. Similarly, biotinylated Tb(III)-DOTA complex (5) was prepared using TbCl3·6H2O. The complexes were prepared by a procedure similar to that reported by Brittain and Desreux.55 We followed the formation of 5 by recording the absorption and fluorescence spectra (Fig. 2A) of the reaction mixture at different time intervals. Moreover, MALDI-TOF mass spectroscopy was employed for the characterization of the complex. The characteristic fluorescence band of Tb(III) complex with the main band ca. 545 nm, which is due to the 5D4 → 7F5 transition in Tb(III),16,57 was helpful for the detection of 5. Furthermore, we confirmed the formation of 5 from an increase and saturation in the fluorescence intensity (inset of Fig. 2A) of 5 with time under reaction, which is due to the replacement of water ligands of Tb(III) by oxygen (COOH groups) and nitrogen (cyclen ring) atoms of 3 and the associated absorption-energy transfer-emission (AETE) mechanism.57 On the other hand, the fluorescence intensity of an aqueous solution of Tb(III) ions, but without 3, remained intact upon heating. These results confirm the formation of 4 and 5. Details of preparation and characterization of 2–5 are provided in the Experi-
mental section. The fluorescent-magnetic NPs, 6 and 7, were prepared by the direct tethering of 4 and 5 to the biotin-binding sites of streptavidin moieties on QD and SPION, respectively (Fig. 1). Fig. 2B and C shows the TEM images of 6 (B) and 7 (C). The wavelength of excitation light was 230 nm in A.

To evaluate whether the conjugation of 4 to QDs affects the properties of QDs, we recorded and analyzed the steady-state absorption (traces a–d in Fig. 3A) and photoluminescence (PL, traces a′–d′ in Fig. 3A) spectra and the nanosecond PL decay profiles (inset of Fig. 3A) of QD655 and QD705 before and after the conjugation of 4. As seen in Fig. 3A, the absorbencies of QDs (QD655 or QD705) are not affected by 4. The slight difference in the optical density (<300 nm) of 6 when compared with that of pristine QD is attributed to the absorption of UV light by 4. Furthermore, the changes in the PL quantum efficiencies ($\Phi_F = 79\%$ for QD655 and $\Phi_F = 51\%$ for QD705) and PL lifetimes ($\tau_{PL} = 12$ ns for QD655 and $\tau_{PL} = 41$ ns for QD705) of QDs after conjugation of 4 were negligible. Herein, the $\Phi_F$ values of QDs were provided by the manufacturer, whereas the PL lifetime values were experimentally determined by fitting the PL decay profiles to the third order kinetics. We assume that the spatial separation between the complex and the QD, which is provided by the polymer and streptavidin coating on QD, preserves the physical properties of the QD core in 6. These observations suggest that stable multimodal NPs can be routinely constructed by the direct tethering of ready-to-conjugate lanthanide complexes to NPs such as QDs and SPIONS, but without considerably affecting the size or properties of the NPs.

Lanthanide-speckled fluorescent-magnetic NPs for cell labeling and in vivo applications were prepared by the recruit-ment of 1 equivalent of biotinylated EGF ligand to the surface of QD or SPION, which was prior to the tethering of multiple coordination complexes (4 or 5). Herein, EGF is selected by considering the over-expression of EGF receptors (EGFR) in the cell-line used, which is human lung epithelial adenocarcinoma cells (H1650). First, human recombinant EGF was biotinylated using biotin sulfo-NHS ester and purified by gel-filtration on a Sephadex G25 column. The biotinylated EGF molecules were tethered (at 1:1 EGF–biotin:QD/SPION) to streptavidin on QD/SPION over 30 min at room temperature, which was followed by the conjugation of 4 to QD and 5 to SPION at 10:1 equivalences (4:QD and 5:SPION).
The binding of biotinylated EGF molecules to streptavidin on the NPs was tested by Förster resonance energy transfer (FRET) measurements. Herein, a FRET system was constructed using CdSe/ZnS QD as the energy donor and AlexaFluor633 as the acceptor. First, AlexaFluor633-labeled EGF was prepared by the reaction between biotinylated EGF (50 μL, 6 μM) and NHS ester of AlexaFluor633 (50 mL, 30 μM) over 1 h at room temperature. The labeled EGF molecules were purified by repeated (3 times) dialysis against a membrane for 2 kDa, during which low molecular mass compounds such as free AlexaFluor633 NHS ester were eliminated. The binding of biotinylated AlexaFluor633 conjugate to streptavidin–QD conjugate \( (\text{Em}_{\text{max}} = 605 \text{~nm}) \) was analyzed by obtaining the fluorescence spectra of mixtures of the two at different concentrations. As the concentration of EGF-AlexaFluor633 was increased in the mixture, the PL intensity of QDs (energy donor) was decreased, which was associated with the appearance and enhancement of the characteristic fluorescence band of AlexaFluor633 (Fig. 3B). These results suggest FRET from QD to AlexaFluor633, which is due to the binding of biotinylated EGF to streptavidin on QDs.

The potential of EGF-conjugated fluorescent-magnetic NPs for bioimaging in vitro is tested in H1650 cells. The cells cultured up to 70% confluence were washed with phosphate buffered saline (PBS) and treated with 1 nM solutions of EGF–QD, EGF–6 or EGF–7 conjugate for 1 h under ice-cold conditions. During this step, the cells were labeled by the conjugate, which is due to the specific binding of EGF ligands to EGFR molecules over-expressed in the plasma membrane of H1650 cells. The labeled cells were copiously washed with PBS, harvested using trypsin, and made into pellets for MRI and fluorescence imaging. We selected cell pellets instead of single cells because of the technical challenge associated with MRI of single cells. On the other hand, MRI of cell pellets labeled with biomarker-specific contrast agents has been exploited in the characterization of biomolecules, tumor cells, and apoptosis. Fig. 4A–E shows the fluorescence image and MRI of QDs, SPION, 4–7, and H1650 cell pellets with or without labeling using EGF–6 or EGF–7. The NIR (705 nm) PL of QDs (Fig. 3A and 4B) and the enhancement of \( T_1 \)- and \( T_2 \)-weighted MRI contrasts of cells labeled with EGF–6 show the potentials of NIR QD speckled by Gd[III] complex for multimodal bioimaging. The bright MRI contrast of labeled cells (Fig. 4Cb), when compared with cells that are not labeled (Fig. 4Cc) and solutions of 4 (Fig. 4Cd) and 6 (Fig. 4Ca), is attributed to changes in the magnetic relaxation of the protic organelles such as EGFR and endosomes of labeled cells. Nonetheless, the \( T_1 \)-weighted contrasts of 4 and 6 in the solution phase are comparable to that of a QD solution (Fig. 4Ce) or the buffer (Fig. 4Dh). Enhancement in the \( T_2 \)-weighted MRI contrast was also observed for cells labeled with EGF–7. As SPION-based particles are ideal \( T_2 \)-contrast agents, we have recorded and analyzed the \( T_2 \)-weighted MRI of sample solutions (5, SPION, and 7) and H1650 cells labeled with EGF–7. Interestingly, an enormous increase (darkening) in the MRI \( T_2 \) contrast of labeled cells [Fig. 4Dg \( (T_1 \) to 4E \( (T_2 \)] was observed, which is comparable to the darkening of \( T_2 \)-weighted MRI contrast of a solution of SPION [Fig. 4Di \( (T_1 \) to 4E \( (T_2 \)]. Nevertheless, prominent absorption in the UV region (Fig. 2A) and emission in the blue-green region (insert of Fig. 2A) are major limitations of 5 and 7 for fluorescence-based bioimaging. Thus, we focus our further studies on EGF–6, where the properties of QDs, such as exceptionally bright and stable PL, broad absorption of light in the Vis-NIR region, large Stokes shift and NIR PL (655 or 705 nm), are combined with the MRI contrast of Gd(III) complex for bioimaging.

Fluorescence microscopy was employed for the detection of endocytosis of EGF–6 conjugate in living cells. Herein, H1650 cells cultured up to 70% confluence in 60 mm tissue culture plates were labeled using a 1 nM solution of EGF–6 or EGF–QD conjugate. The labeling was carried out under ice-cold conditions, which minimizes the endocytosis of the conjugate during the labeling step. Furthermore, the nuclei of the cells were stained with Syto 21 or Syto 13 dye by following the methods reported in the literature. The labeled cells were copiously washed with PBS, the medium was changed to Dulbecco’s modified Eagle’s medium (DMEM), and the cells were excited with 400 nm (for both QDs and Syto dyes) or 532 nm (for QDs alone) laser beam. The labeled cells were observed in an inverted optical microscope. Fluorescence image of the cells labeled using EGF–6 conjugate (Fig. 5A) indicates efficient intracellular delivery of the conjugate, which is comparable to the endocytosis of EGF–QD conjugate (Fig. 5B and C). On the other hand, control cells incubated with 6 or QD, but without any EGF, do not show any intracellular fluorescence. The intracellular delivery of the conjugate takes place by binding to EGFR, which is overexpressed in the plasma membrane of H1650 cells, and subsequently the EGFR–EGF–6 assembly is engulfed by receptor-mediated endocytosis.

To analyse the binding of EGF to EGFR and the subsequent intracellular pathway of EGF–6/EGF–QD conjugates, H1650
cells were labeled with EGF–QD conjugate and the time- and intensity-gated single-molecule fluorescence of EGF–EGFR complexes were recorded. Here, EGFR in the plasma membrane of the H1650 cell was activated by applying a solution (1 nM) of EGF–QD conjugate. As seen in Fig. 5C, immediately (~15 min) after the activation, EGF–EGFR complexes were present preferentially in the cell membrane, mostly attached to the filopodia or lamellipodia. The blinking and PL intensities of individual fluorescence spots in the cell membrane were comparable to that of pristine QDs tethered on a glass substrate (Fig. 5D and E), validating the detection of EGFR single-molecules. Fig. 5F shows PL intensity histograms of fluorescence spots detected at different time intervals after the activation of EGFR with EGF–QD conjugate. Interestingly, uniform distribution of EGFR molecules, with fluorescence intensities equivalent to 1 or 2 QDs, was detected immediately (~15 min) after the activation (Fig. 5Fb). However, brighter fluorescence spots were emerged with time under incubation. Temporal changes in the intensities of fluorescence spots, which are assigned to the dimerization and clustering of receptors, were examined by the correlation of the time- and intensity-gated fluorescence images of >500 single-molecule receptors in the cell membrane with that of QDs tethered on a glass substrate. Within 15 min of post activation of EGFR, the majority (>80%) of the receptors were bound with one [(QD-EGF)-(EGFR)], or two [(QD-EGF-EGFR)] EGF–QD conjugates (Fig. 5Fb). During the next 15 to 30 min, the occurrences equivalent to dimers [(QD-EGF-EGFR)] and larger clusters increased. As a result of this increase, the histogram shifted to the higher intensity side (Fig. 5Fc and d). The fluorescence spots with intensity equivalent to that of a single QD are attributed heterodimers of EGFR, i.e. EGFR pre-dimers (Fig. 5Fe) ligated by only one EGF–QD conjugate (QD-EGF-(EGFR))2 (Fig. 5Ff). An increase in the number of fluorescence spots with intensity equivalent to that of two QDs suggests the formation of signaling dimers [(QD-EGF-EGFR)], which is the result of either the activation of each pre-dimer by two EGF–QD conjugates (Fig. 5Fg) or association and disproportionation of two heterodimers (Fig. 5Ff) into a signaling dimer and a pre-dimer, which is indicated in Fig. 5Ff–h. The high intensity fluorescence spots, which dominated with time under incubation, suggest clustering of dimers. The larger clusters are eventually transported into the cytoplasm and subsequently accumulated in the perinuclear lysosome organizing region (Fig. 5A and B). These results suggest that EGF–QD and EGF–6 conjugates allow for the detection of dimerization and clustering of EGFR in living cells.

Despite the unique optical and magnetic properties of nanomaterials, toxicity is an unsolved central issue that hinders their biological applications. Toxicity of nanomaterials can be size-, surface- or materials-related. Therefore, a general solution to the toxicity of nanomaterials is far from reality, and case-by-case analysis is often necessary. We examined the cytotoxicity of EGF–6 conjugate by the assay of mitochondrial reductase enzyme activity (MTT assay) in H1650 cells labeled with the conjugate and compared the results with that of unlabeled cells and cells labeled with EGF–QD conjugates. The viability of cells is retained above 90% (Fig. 6A) when treated with EGF–6 solutions having concentrations up...
to 10 nM, which is an order of magnitude higher than that employed in cell labeling and imaging (Fig. 5A) experiments. As seen in Fig. 6A and B, the viability of cells labeled with EGF–6 conjugate is comparable or marginally higher than that of those labeled with EGF–QD conjugates. These observations not only confirm the biocompatibility of 6 but also suggest that multiple Gd(III) complexes on QDs do not induce any cytotoxic effect.

Despite the in vitro non-cytotoxic nature, when applied in vivo, most nanomaterials interact with the reticuloendothelial systems and inflame complex pharmacokinetics, hindering regulatory approval for clinical applications. In other words, uniform biodistribution and efficient renal or hepatobiliary excretion are fundamental prerequisites for in vivo applications of nanomaterials – from biomedical imaging to drug delivery and cancer therapy. We evaluated the potentials of lanthanide-speckled fluorescent-magnetic NPs for in vivo imaging in B6 mice and analyzed the in vivo fate of the NPs. Here, a 20 nM solution of EGF–6 conjugate was administered in nude mice by intravenous injection. The MRI and fluorescence images of the mice were acquired before and at different time intervals after the injection. Within 30 min post injection (Fig. 6E and F), the NPs accumulated mostly in the liver. Moreover, an increase in the MRI contrast of the liver was detected in the T1-weighted image (Fig. 6G). The fluorescence from the liver diminished over 2 h (Fig. 6H) and touched the background level within 24 h post injection. These results show not only the efficient detection of the NPs from deep tissues, such as the liver, but also the clearance of the NPs. The decrease and disappearance of in vivo fluorescence can be assigned to excretion through the renal pathway, which was heretofore observed for QDs and QD-based fluorescent-magnetic NPs. However, we do not rule out a possibility that the NPs were degraded by the liver enzymes. While we focus the current studies on the preparation of novel lanthanide-speckled NPs for multimodal imaging, long-term pharmacokinetics and toxicity of such NPs need further attention.

3. Conclusions

We demonstrate the preparation of simple fluorescent-magnetic NP contrast agents by the direct conjugation of simple ready-to-bioconjugate complexes of Gd(III) or Tb(III) to the surface of QDs or SPION. Furthermore, by the recruitment of EGF hormone to the surface, these NPs were efficiently delivered in human lung epithelial adenocarcinoma cells wherein EGFR is overexpressed. The potentials of the lanthanide-speckled NPs composed of QDs/SPION and ready-to-bioconjugate coordination complexes of Gd(III)/Tb(III) for bioimaging are realized by obtaining MRI and fluorescence images of sample solutions, EGFR single-molecules, live cells and mice. The straightforward preparation, T1- and T2-weighted MRI contrast, bright and stable NIR fluorescence, and biocompatibility make NPs decorated with multiple coordination complexes promising for biomedical imaging and image-guided therapy.

4. Experimental section

4.1. Materials

All the chemicals and solvents used in the preparation, purification and characterization of the complexes and fluorescent-magnetic NPs were of analytical grade. Streptavidin-functionalized CdSe/ZnS QD samples (Emλmax = 605/655/705 nm) and AlexaFluor633-NHS ester were obtained from Life Technologies, and SPION was obtained from NANOCS. Biotin and DOTA were obtained from Wako Chemicals; lanthanide salts and DBU were obtained from Sigma-Aldrich; and 1,3-dibromopropane was obtained from Tokyo Chemical Industries.

Steps involved in the preparation of biotinylated Gd(III) and Tb(III) complexes and the lanthanide-speckled fluorescent-
magnetic NPs are summarized in Fig. 1. First, o-bromopropyl ester of biotin (2) was prepared by the reaction between biotin (1 g, 4.09 mmol) and 1,3-dibromopropane (2.42 g, 12.0 mmol), which was catalyzed by DBU (980 μL, 6.55 mmol). Here, biotin was dissolved in acetonitrile (25 mL) by the addition of DBU, which was followed by the addition of 1,3-dibromopropane. This reaction mixture was heated at 82 °C for 12 h with vigorous stirring. Subsequently, the reaction mixture was cooled at room temperature, during which a white precipitate was formed. The amount of the precipitate was increased with the drop-wise addition of n-hexane (less than 4 mL) to the crude reaction mixture. The white precipitate was collected by filtration through a Whatman grade 1 filter paper, re-dissolved in chloroform and purified by column chromatography on silica gel (200--300 mesh). The product was crystallized as an o-white heavy liquid in the ice-cold reaction mixture. After the supernatant was decanted, the crude product was dissolved in 5 mL methanol. Subsequently, upon addition of 15 mL acetone, Biotinylated DOTA (3) was prepared by the DBU-catalyzed condensation between one of the carboxylic groups of DOTA and the o-bromo group of 2. In this condensation reaction, a mixture of DOTA (200 mg, 0.495 mmol), 2 (200 mg, 0.619 mmol) and DBU (300 μL, 2.01 mmol) was dissolved in acetonitrile (50 mL) and stirred at 82 °C for 18 h (Fig. 1). Here, the amount of DBU was set at 300 μL for the complete solubilization of DOTA. After 18 h, the reaction mixture was cooled by placing it at room temperature and then in an ice bath. Upon addition of a dilute solution of trifluoroacetic acid (300 μL in 10 mL dichloromethane), the crude product was separated as an off-white heavy liquid in the ice-cold reaction mixture. After the supernatant was decanted, the crude product was dissolved in 5 mL methanol. Subsequently, upon addition of 15 mL acetone, 3 was collected as a white precipitate, which was further purified by repeated precipitation from a methanol : acetone mixture (1:4) to provide 3 in 65% yield.

**4.2. Methods**

1H and 13C NMR spectra were acquired in a JEOL 400 MHz spectrometer, and MALDI/LDI-TOF mass spectra were recorded in a BRUKER Microflex spectrometer. Fluorescence images of labeled H1650 cells were acquired in an inverted optical microscope (Olympus IX70) equipped with a 40x objective lens, long-pass or band-pass filters for Syto dyes and QDs, and an iXon3 EMCCD (Andor Technology) or a digital CCD camera (Olympus). The excitation light used was either 400 nm femtosecond pulse (details are provided below) for Syto dyes or 532 nm cw laser (Millennia II, Spectra Physics) for QDs. Intensities of fluorescence spots were obtained by analyzing the PL intensity trajectories of single-molecule videos recorded using the EMCCD camera. Details of single-molecule measurements are reported elsewhere.70–73 Fluorescence images and MRI of sample solutions, cell pellets and mice were acquired using a small animal imaging system (Maestro, Perkin-Elmer) and a MRI machine (MR Technology, Inc., Japan). Fluorescence images of samples, cell pellets and mice were analyzed using the Maestro or Image-Pro Plus software (Roper Industries, Inc., USA).

Steady-state fluorescence/PL spectra were recorded using a fluorescence spectrophotometer (Hitachi FL4500). PL decay profiles were recorded using an assembly of a polychromator (Chromex-250IS) and a streak-scope (Hamamatsu-C4334). Details of PL lifetime measurements are reported elsewhere.72–75 The samples were excited using 400 nm pulses generated from the SHG crystal of an optical parametric amplier (Coherent OPA 9400), and the fluorescence signals collected through suitable band-pass or long-pass filters were focused at the entrance slit of the polychromator and recorded using a streak-camera. **Cytotoxicity assay.** Cytotoxicity of NPs was evaluated by MTT assay (MTT cell proliferation kit, Roche Diagnostics). Here, ca. 1 million H1650 cells per plate were inoculated into 96-well tissue culture plates (FALCON) containing DMEM supplemented with 10% FBS and cultured at 37 °C for 48 h. After the cells were copiously washed with PBS, the medium was exchanged with DMEM (without FBS) supplemented with different concentrations (0.1 to 10 nM) of EGF–QD or EGF–6 conjugate. After 1 h incubation, the cells were copiously washed with PBS and supplemented with MTT solution (10 μL per well, 5 mg mL−1) and DMEM-FBS medium. After 4 h incubation at 37 °C, the cells were lysed using the lysis buffer (100 μL per well), which is 10% sodium dodecyl sulfate (SDS) in 0.01 M HCl. The intracellular formazan crystals formed from MTT, which was the result of the metabolic activity of mitochondrial reductase enzyme, were dissolved overnight at 37 °C. The viabilities of unlabeled cells and those labeled with EGF–QD or EGF–6 were determined by the measurement of the absorbance of formazan at 550 nm.
Acknowledgements

This study was carried out under the Precursory Research for Embryonic Science and Technology (PRESTO) program (to VB) of Japan Science and Technology Agency (JST). Moreover, this study was supported by the Japan Society for the Promotion of Science under the Kaken-hi Grant 24750029. MH acknowledges a graduate research fellowship from JSPS. We thank Ayami Nishioka of Kagawa University for TEM experiments.

Notes and references