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Multi-modal nanoprobe based on upconversion nanoparticles for monitoring implanted stem cells in bone defect of big animal

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ABSTRACT. Monitoring implanted stem cells in bone regeneration and other cell therapies is of great importance to reveal the mechanism of tissue repair and to optimize clinical treatments. However, big challenge still remained in lacking an imaging nanoprobe. Herein, we designed surface modified upconversion nanoparticles (UCNs) with multi-modal imaging capabilities of fluorescence, magnetic resonance imaging (MRI) and dual-energy computed tomography (CT). It was found that the UCNs can label stem cells in an efficient (over 200 pg/cell) and long-term (at least 14 days) manner, with almost no influence on the viability, cell cycle, apoptosis, and multi-lineage differentiation. Thus, clinical dual-energy CT and MRI were successfully applied to observe the migration of labeled cells on a bone-defect model of rabbit for at least 14 days. The results visualized the gathering of stem cells at the defect site of cortical bone, and the in vivo images were well correlated with the in vitro fluorescence observation without extra staining. Therefore, a potentially translatable nanoprobe was developed for non-invasive and real-time tracking of cells, which may be meaningful for understanding the bone regeneration in clinic and shed light on the visualization of cells in other cell therapies.
1. Introduction

Cell therapy, in which stem cells and other mammalian cells are infused or transplanted into tissue, has been pre-clinically applied in diabetes, myocardial deceases, liver impairment, muscle and skeleton deceases, neurodegenerative diseases, and tumor, showing promising prospects in clinical applications. Among these areas, using stem cells to directly treat bone defects or as seed cells to assist tissue engineering scaffold for bone tissue repairing has attracted intensive attentions in recent years. However, so far there have been no definite results to clearly depict the exact mechanism of the role of stem cells in bone regeneration. For example, low concentration of viable stem cells at the defect area was considered as the reason for the failure of autologous bone marrow transplantation. Some reports, however, showed that successful bone regeneration cases relied only on a very small amount of viable implanted stem cells. In addition, researchers reported inconsistent results concerning whether the implanted stem cells were actively transformed to bone tissue, or only acted as a medium to release related factors to promote endogenous cellular repair. The unclear mechanism on the role of stem cells severely hampers the development of clinical treatment, and it should be largely ascribed to the lack of a protocol for monitoring the behavior of implant cells in vivo.

Optical imaging techniques are of great importance for investigating the behavior of implanted cells. For example, the imaging technique based on the insetion of an optical-reporter-gene is a classical strategy for stem cells monitoring. It generally can offer veracious information on the distribution and proliferation of stem cells via optical imaging in histological analysis. However, the optical observation in vivo was limited to small animals owing to the high absorption and scattering of visible light in bio-tissues. Besides, this approach is difficult to be translated due to the requirements for DNA modification, transfection of genetic material, and
the epigenetic gene silencing owing to DNA methylation as well, all of which may lead to the raised concern of adverse cellular effects\textsuperscript{15}.

Positron emission tomography (PET) and magnetic resonance imaging (MRI) are the imaging techniques potentially applicable for tracking cells in big animal or human being. For example, PET possesses extremely high sensitivity to direct observe several tens of cells\textsuperscript{16-18}. However, owing to the limited half-lives of radionuclides, PET techniques are not ideal for long-term imaging studies such as bone regeneration\textsuperscript{19}. Besides, radio-labelling involving chemical conjugation of emitters may be unstable in the body environment. Thus, the PET technique could not correlate with \textit{ex vivo} studies at a relative long-term endpoint and was limited to the short time \textit{in vivo} tracking\textsuperscript{20}. An alternative approach is employing superparamagnetic iron oxide nanoparticles to direct label cells\textsuperscript{21-22}. By doing so, tracking of the labeled cells by MRI was widely studied in nervous system disease\textsuperscript{23-24}, myocardial infarction\textsuperscript{25-26} and angiogenesis in cancer\textsuperscript{27}, etc. In spite of these promising outcome in soft tissues, skeleton system as a biological hard tissue with relatively high density and low hydrogen proton concentration is not suitable for individual MRI imaging techniques\textsuperscript{28-29}. Thus, the main issue for studying the role of stem cells in bone regeneration is lack of an appropriate nanoprobe that can non-invasively track the cells behavior \textit{in vivo} for a relatively long time in big animals.

Upconversion nanoparticles (UCNs), especially doped NaLnF\textsubscript{4} (Ln=lanthanides), can convert near infrared (NIR) light to visible and have found wide applications in various biomedical fields owing to their advantages such as low toxicity, resistance to photobleaching and deep penetration of NIR in biotissues\textsuperscript{30-31}. Furthermore, with choosing appropriate element of lanthanides, NaLnF\textsubscript{4} can simultaneously possess the imaging contrast of computed tomography (CT) and MRI\textsuperscript{32-34}. Thus, as shown in Figure 1, we herein designed a nanoprobe based on the surface modification
of core-shell structured UCNs with fluorescence/MRI/CT multimodal imaging capabilities, resulting in the efficient and stable label of bone marrow mesenchymal stem cells (BMSCs). With such a nanoprobe, BMSCs were tracked by multiple imaging techniques in a bone-defect model of big animal (rabbit), especially by the dual-energy CT that can discriminate the signal from bone and nanoparticles\textsuperscript{35-38}. Meanwhile, the labeled UCNs directly gave corresponding fluorescent images in the \textit{in vitro} observation without further histological staining. The current work may suggest a non-invasive and real-time route with potential promise in illustrating the mechanism underlying in bone regeneration, and in clinically tracking the implanted cells in cell therapy as well.

2. Results and Discussion

2.1 UCNs and multi-modal imaging capabilities

The core-shell upconversion nanoparticles (UCNs in this work) were synthesized with Yb and Tm co-doped NaYF\textsubscript{4} as a core and undoped NaGdF\textsubscript{4} as a shell, following our previously reported successive hot-injection approach\textsuperscript{39}. Fig. 2A and 2B show the transmission electron microscopy (TEM) images of synthesized NaYF\textsubscript{4}:Yb, Tm core and NaYF\textsubscript{4}:Yb, Tm@NaGdF\textsubscript{4} core/shell nanocrystals, respectively. Core nanoparticles were spherical with a diameter of \(\sim\)40nm. After coating with a shell layer, UCNs presented hexagonal-plate shape with a diameter of about 50 nm. High-resolution TEM image in the inset of Figure 2A indicated the highly crystalline nature of the core nanoparticles, whilst Figure 2B inset showed obvious difference in the crystal lattice orientation between the inner part and the outer part, suggesting the core-shell structure. The scanned elemental spectra across a single NaYF\textsubscript{4}:Yb,Tm@NaGdF\textsubscript{4} core-shell nanocrystal with the direction along the line marked in Figure 2C inset demonstrated that Gd mainly distributed at the edges while Y mainly located in the core. Two-color element distribution of the core/shell
nanocrystals further revealed the obvious Y signal in the cores and Gd signal on the shell (Figure 2D). Then the UCNs were coated by a thin layer of silane with amino group (denoted as UCNs-NH$_2$) and the resultant UCNs-NH$_2$ were mixed with a commercially available transfection agent called DNA Transfectin 3000 (TS) for the final nanoparticles (denoted as UCNs-TS). The surface charge of UCNs-NH$_2$ and UCNs-TS was 10.3 and 18.6 mV, respectively, which suggests that TS modification further improved hydrophilicity of the UCNs-NH$_2$.

The fluorescence spectra of NaYF$_4$:Yb, Tm and NaYF$_4$:Yb, Tm@NaGdF$_4$ nanoparticles under 980 nm continuous-wave excitation were shown in Figure 3A. The emission bands were clearly resolved at ~360 nm, ~450 nm, and ~803 nm, respectively. The intensity of core-shell structured nanoparticles was obviously higher than that of the core, which should be ascribed to the suppressing of surface quenching effect. Under NIR irradiation, the as-prepared UCNs displayed visible purple-blue color on the photograph (Figure 3A inset).

Owing to the existence of gadolinium, the core-shell nanoparticles showed obvious MRI and CT imaging contrast. T1-weighted MR images were brightened while T2-weighted images were darkened as the Gd concentration increased over the tested range (Figure 3C and 3D). According to the linear fitting of the experimental data, longitudinal relativity (R1) and transverse relativity (R2) were around 0.86 and 37 mM$^{-1}$ s$^{-1}$, respectively. For CT images of the core-shell nanoparticles, the values of the Hounsfield unit (HU) were measured as a function of the lanthanide concentration in the range of 5 to 80 mM, and Iopamiro was used as a control. Both of the samples displayed a well-correlated linear relationship (Figure 3B inset). Importantly, the UCNs-TS possessed a higher HU value than Iopamiro at same molar concentration, indicating the potential of UCNs-TS as a CT contrast agent. We further evaluated the capability of signal separation in the mixture of UCNs-TS and bone tissue together in ethanol by clinical dual-energy
CT. Owing to that ethanol is a poor-solvent for UCNs-TS, the nanoparticles tend to quickly deposit at the bottom of the container. As shown in Figure 3, dual-energy CT scan acquired the image data simultaneously at two X-ray with the tube voltage of 80 kV and 140 kV, respectively. In each separated dataset, both UCNs-TS and the bone tissue showed enhanced signals. By post-processing the dual-energy CT data with decomposition method, UCNs-TS and bone tissue could be set apart from each other and presented as individual maps of distribution, as shown in Figure 3. This result provides us the possibility to distinguish the implanted nanoparticles-labeled stem cells and normal bone tissue by dual-energy CT technique.

2.2 The influence of nanoparticles on BMSCs

Figure 4a presented that the viability of BMSCs would not decrease after incubating with different concentration of nanoparticles unless it increased to ~500 µg/ml. Even if the concentration was increased to 1000 µg/ml, the cell viability still kept above 80%. The half maximal inhibitory concentration (IC50) of the nanoparticles is about 1500 µg/ml. It should be noted that in our previous work for gold nanoparticles38, the IC50 is only about 500 µg/ml under the same surface modification, indicating that the UCNs show much less cytotoxicity than gold nanoparticles. Therefore, the incubating concentration of 1000 µg/ml for UCNs-TS that may ensure an efficient labeling of cells was used in the following experiments.

For further identifying the biocompatibility of UCNs nanoparticles on BMSCs, we detected the apoptosis of BMSCs by Annexin V-FITC/PI apoptosis detection kit after incubation with UCNs-TS for 24 hours. The percentage of alive cells of the control group and the UCNs-TS group were 96.9% and 96.7% respectively (Figure 4B), which demonstrated that the nanoparticles did not induce obvious apoptosis. In order to determine the effect of UCNs-TS on the proliferation of BMSCs, CCK-8 assay was conducted at the 1st day, 3rd day, 5th day and 7th day after labeling
with the UCNs, the observation at 450 nm had no difference between the control group and UCNs-TS group (Figure 4C). After transfected with UCNs nanoparticles for 24 hours, the cell cycle of BMSCs was detected. It was found that there was almost no difference between each group at G0/G1 phase, G2/M phase and S phase, demonstrating that the nanoparticles did not cause cell cycle arrest (Figure 4D).

The effects of UCNs-TS on the differentiation capability of BMSCs to osteoblasts, chondrocytes and adipocytes was respectively investigated. As shown in Figure 5a inset, in osteogenic differentiation assay, alizarin red was used to label the formed osteoblast. The results showed that UCNs-transfected experimental group and positive control group were almost same in color, while the negative control group did not show the color of alizarin red. At the same time, as an important signal that presents the osteogenic differentiation, ALP did not differ significantly in the expression between the UCNs-transfected experimental group and the positive control group, while the expression level in the negative control group was significantly lowered. These results demonstrated that the UCNs-TS nanoparticles hardly affect the osteogenic differentiation of stem cells. Similarly, in the study of chondrogenic differentiation, alcian blue staining on cartilage cells and the expression of cartilage cell gene marker, SOX9, showed that the UCNs-TS nanoparticles had no effect on the chondrogenic differentiation of stem cells. In adipogenic differentiation assay, oil red O staining on adipose cells and the expression of adipose cells marker gene, PPARγ, also showed that the UCNs-TS nanoparticles had no effect on the adipogenic differentiation of stem cells, as shown in Figure 5c.

We further investigated the effect of UCNs-TS on the differentiation of BMSCs into osteoblasts at different time points. The expression of key osteoblast differentiation genes, OSX, OCN and Collagen I, were detected. As shown in Figure 5d-f, after being co-cultivated with UCNs-TS
nanoparticles for 1 day, 7 days, 14 days and 21 days, the expression of OSX, OCN and mRNA of Collagen I were almost identical with that in the control group. The above results showed that UCN-TS nanoparticles have excellent biocompatibility and no effect on the differentiation of stem cells in different stages of osteoblast differentiation.

2.3 Efficient labeling of BMSCs

The results of inductively coupled plasma mass spectrometry (ICP-MS) showed that the number of nanoparticles uptaken by BMSCs were 244 pg/cell for UCNs-TS after incubated for one day. In contrast, for UCNs-NH2 as a control, the uptaken dose at the 1st day was 134 pg/cell. After 24 h of incubation, the labelled cells were transferred to fresh culture media without nanoparticles to measure the uptaken stability of UCNs-TS. It was found that the uptaken dose of UCNs-TS nanoparticles per cell had been around 200 pg without perceptible change within 14 days observation. On the contrary, without modification of the transfection agent TS, UCNs-NH2 nanoparticles were obviously excreted by the BMSCs in 14 days to be a remained dose of ~20 pg/cell. These results implied that the modification of TS rendered the retention of UCNs in BMSCs.

For further identifying the long-term labeling of nanoparticles, CT images were obtained for the labeled cells in culture solution at different time, as shown in Figure 6b. It should be noted that before the imaging of the solution at each time-point, extracellular nanoparticles were completely washed out, thus the signal of UCNs-TS directly represents those labeled stem cells. The corresponding volumes of deposited labeled cells were 22.8 mm³, 24.7 mm³, 25.2 mm³, 24.6 mm³, and 24.1 mm³, and the HU values were 1620, 1310, 1344, 1295, 1277 for 1, 4, 7, 10, 14 days, respectively. Owing to that the HU value is directly related to the local concentration of contrast agent, the slightly increase of the volume of the labelled cells, resulting in the decrease
of HU value, indicate that the fluctuation of the number of nanoparticles were imperceptible. Meanwhile the signal from MRI T1 weight also kept the same with time extending, as shown in Figure 6c, indicating that the local concentration of labelled cells would not change. Therefore, the results further evidenced that with the help of transfection agent, the UCNs-TS nanoparticles can be retained in BMSCs for at least 14 days.

For the techniques based on nanoparticulate contrast agent, the capability of labeling cells with long-term, high-dose internalization and non-cytotoxicity is of great importance. Higher intracellular concentration of nanoparticles generally results in a better imaging contrast. In last two decades, researchers reported various approaches to tailor the internalization of nanoparticles. For example, positively charged nanoparticles are more favored for being uptaken than their negatively charged counterparts in different cancer cell types, such as MCF-7 cells, endothelial cells, and HeLa cells\(^{40-41}\). Several kinds of cell penetrating peptides (CPPs), including trans-activating transcriptional activator (TAT)\(^ {42-44}\), herpes simplex virus type 1 (HSV-1)\(^ {45-46}\), model amphipathic peptide (MAP)\(^ {47}\), were modified to guide the nanoparticles traversing through the cellular membranes in a so-called "protein transduction" process. Alternatively, modification with targeting ligands, such as folic acid\(^ {48}\), albumin\(^ {49-50}\) and cholesterol\(^ {51}\), also results in the uptaken of nanoparticles by caveolin-mediated endocytosis. Despite of these significant achievements, the intracellular dose of contrast agent for cellular imaging still has plenty room for the improvement. For example, the cellular uptake of gold nanoparticles recently was increased from \(~30\) pg/cell\(^ {52}\) to \(~88.98\) pg/cell\(^ {53-54}\), and thus can give an observable contrast on a microCT. However, it is still insufficient for the imaging on clinical CT so that the challenge still remains for the cellular monitoring in big animals. In our work, a commercially available transfection agent TS and thin layer of SiO\(_2\) significantly improved the
biocompatibility of the nanoparticles. Therefore, the labeling dose was tremendously increased to above 200 pg/cell. This result shows promising potentials in sensitive and precise imaging of labeled cells, especially for investigation of big animals and the applications of clinical imaging techniques. Besides, high-dose of cellular uptake also benefits for the area of drug delivery, as the cargo such as drugs, proteins, peptides or nucleic acids could be released to directly exert therapeutic effects on cytoplasmic and nuclear targets\textsuperscript{55}.

On the other hand, exocytosis of the contrast agent is expected to be avoided as it may give artifacts, or gradually lower the contrast for long-term observation. However, high-dose of cellular uptake did not ensure the retention of nanoparticles in cells for a relatively long term. For example, about 84%, 66% and 49% of mesoporous silica nanoparticles modified with phosphonate, folate and polyethylenimine, respectively, were found to be excreted from cells after 6h\textsuperscript{56}. In contrast, our experiments showed that UCNs-TS almost would not be excreted in 14 days. The excretion or retention of nanoparticles in cells may relate to their surface modifications and cell lines, and the mechanism should be further studied in the future. Nevertheless, our results suggest that long-term and high-efficient labeling of BMSCs without side-effect was achieved by UCNs based on the surface modification, leading to the potential of UCNs as the multi-modal imaging nanoprobe.

2.4 \textit{In vivo} clinical imaging and the histological analysis

Based on the above results, with the injection of UCNs-TS labeled BMSCs in a bone defect model of rabbit, we further explored the capability of tracking these cells by multiple imaging methods including dual-energy CT and MRI. As shown in Figure 7a-d and g-j, the signals of UCNs-TS labeled BMSCs were extracted by dual-energy CT scanning from the bone of rabbit as
a big animal model. At the first day after the implantation, some of the labeled cells were kept at
the injected place, while contrast signal was also observed far from the bone defect. Over 14
days following up the implantation, the contrast signals of UCNs-TS labeled BMSCs near the
bone defect maintained and the distribution of these cells has the obvious tendency to migrate to
defect site, as observed from the difference between Figure 7c and i. In the meantime, the signal
far from the bone defect gradually vanished. This result might be attributed to two possible
reasons: first, the nanoparticles-label cells at the other end of bone may enter the body cycle and
therefore were invisible on CT owing to the low concentration, and second, they may be homed
to the defect site and the signal was combined to that of the cells originally at there.

By using clinical MRI, axial and coronal scanning of T1 weight contrast can also image the
UCNs-TS labeled BMSCs in a bone defect model of rabbit at the 1st day (Figure 7e and f) and
the 14th day (Figure 7k and l), respectively. The contrasts of UCNs-TS labeled BMSCs on MRI
images were marked in red dashed lines, while the defect of the bone was marked by red arrow.
It can be observed that at the first day after implantation, the contrast area was larger than that on
the CT image. After 14 days, the movement of UCNs-TS labeled BMSCs towards bone defect
areas was also observed as same as the result of CT imaging. Moreover, it was found that the
contrast areas of UCNs-TS labeled BMSCs are somewhat larger than those of CT imaging for
both the first day and after 14 days. This may be owing to the higher sensitivity of MRI than CT
so that the circumjacent area of UCNs-TS labeled BMSCs with lower concentration was detected
by MRI. Furthermore, the bright area increased (as shown in Figure 7k and l, pointed by the
arrow), which reveals the initiation of regenerated callus.

On the 14th day after the implantation, the rabbit was sacrificed and the slices at the site of
bone defect were obtained. As shown in Figure 8, for the control group without UCNs-TS
labeled BMSCs, tissue with the morphology of directional alignment to some extent can be observed around the defect area, which should be attributed to the new bone in terms of trabecula. For the experiment group, the blue-purple fluorescence of the UCNs-TS labeled BMSCs can be observed without any further staining. Interestingly, the UCNs-TS labeled BMSCs only appears along the interface between normal tissues and bone defect area and tended to an orientation array that would be related to the initiation of regenerated trabecula. This result strongly suggested that a part of the nanoparticle-labeled cells might integrate into or transform to new tissue, and therefore the nanoparticles were integrated into bone tissue. The fluorescence observation provided further evidence for the results of clinical imaging that the contrast signal indicated the migration of BMSCs, and the signal around defect area could last for at least 14 days after implantation in contrast to the disappearance on the other end. Thus, the histological analysis identified the reliability of the clinical imaging, and demonstrated UCNs-TS as a cellular nanoprobe for long term multi-modal imaging.

In fact, taking advantages of the NIR excited fluorescence, upconversion nanoparticles have found wide applications in the tracking of cells, including BMSCs\textsuperscript{57}, human amniotic fluid stem cells\textsuperscript{58}, and dendritic cells\textsuperscript{59}. However, the observation of cells by fluorescence is limited to \textit{in vitro} studies or small animals so far. Besides fluorescence imaging, Liu et. al.\textsuperscript{60} designed NaYb\textsubscript{4}:Er nanoparticles to combine upconversion fluorescence and CT contrast capabilities together and act as the contrast agent for angiography and lymph node mapping. On the other hand, the clinical imaging capability such as MRI was also integrated into the lanthanides-based nanoparticles with MRI contrast agents or by lanthanide itself. Shi et. al.\textsuperscript{61} conjugated gold and upconversion nanoparticles together to form MRI and CT imageable nano-balls, which increased signal-to-noise ratio of tumors after the local-injection \textit{in vivo}. However, CT/MRI imaging of
UCNs was limited in tissue-scale, such as tumors and blood pool. Thus, this paper for the first time developed a cellular nanoprobe based on the appropriate design of upconversion nanoparticles for the non-invasive monitoring implanted cells by using the clinical CT/MRI imaging techniques. More importantly, the fluorescence signal \textit{in vitro} can be well correlated with the \textit{in vivo} CT/MRI imaging results especially for the model of big animals.

3. Conclusion

In summary, we developed a cellular nanoprobe that not only visualized the integration of the implanted UCNs-TS labeled BMSCs in rabbit \textit{in vivo} with clinical instruments of dual-energy CT and MRI, but also directly observed the histological graphs without further staining. The protocol is based on a facile modification of UCNs with commercially available transfection agents to tailor the internalization. The resultant UCNs-TS nanoparticles as the cellular nanoprobe can label BMSCs with a dose of over 200 pg/cell without affecting the cell cycle, apoptosis, cell proliferation and multi-lineage differentiation, and were almost not excreted for at least 14 days, which ensures the possibility and veracity of cellular monitoring in big animal model. By the correlation between both clinical imaging results of dual-energy CT & MRI and the histological observation, we found that the implanted BMSCs tended to migrate to the bone defect border and were related to the initiation of regenerated callus tissue. Thus, the current study provides a translatable proof-of-concept for tracking implanted cells based on the clinical CT and MRI instruments, and allows the longitude research on big animal without sacrificing at certain endpoints.

ASSOCIATED CONTENT
Supporting Information. The Supporting Information is available free of charge on the ACS Publications website, including the experimental section.

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Notes

The authors declare no competing financial interest.

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Figure 1. Schematic illustration of the design for the non-invasive imaging of implanted stem cells with MRI/dual energy CT and the “in situ” observation of the fluorescence of UCNs by a confocal laser scanning microscopy (CLSM).
Figure 2. TEM images of a) NaYF₄:Yb, Tm core nanoparticles, b) NaYF₄: Yb, Tm @NaGdF₄ core/shell nanoparticles. Insets show the magnified high-resolution images of the corresponding nanoparticles. c) The chemical mapping of core/shell structure with marking Gd in green and Y in red, respectively, and d) the distribution of Gd and Y along the line marked in c).
**Figure 3.** a) The fluorescence spectra of the NaYF$_4$:Yb, Tm core nanoparticles and NaYF$_4$: Yb, Tm@NaGdF$_4$ core/shell nanoparticles (UCNs), respectively. Inset shows the photograph of UCNs under the irradiation of a 980nm excitation laser. b) CT contrast of UCNs and commercial Iopamiro in terms of Hounsfield Unit and (inset) contrast images. The T1 weight (c) and T2 weight (d) of the UCNs in the form of relaxation time and contrast images. e) Dual energy CT imaging and signal separation of UCNs and bone scaffold in ethanol.
Figure 4. a) Concentration-dependence of cell viability after co-cultivate with UCNs-TS, and the influence of 1000 µg/mL UCNs-TS on the b) apoptosis, c) cell proliferation, and d) cell cycle of BMSCs.
Figure 5. The influence of 1000 µg/mL UCNs-TS on the multi-differentiation capability: Expression of key mRNA in (a) osteogenic differentiation, (b) chondrogenic differentiation, (c) adipogenic differentiation. Insets show the immunostaining of BMSCs of corresponding sample. (d-f) the expression of mRNA in osteogenic differentiation when incubated for different time.
Figure 6. The maintenance of UCNs-TS nanoparticles in cells within 14 days after incubation with 1000 µg/mL UCNs for 24 h: a) The dose of nanoparticles retained in cells at various time points, with a control group of UCNs-NH₂; b) CT and c) MRI T1 weight images at 1st, 4th, 7th, 10th and 14th after 24-hour cultivation, respectively.
**Figure 7.** CT (a, b, g, h), decomposed dual energy CT images (c, d, i, j), and MRI T1 weight images (axial: e, k, and coronal: f, l) of UCNs-TS nanoparticles-labelled stem cells in rabbit bone-defect model.
Figure 8. Observation of the slices at bone defect at 14th day after implanting cells with (lower panel) and without (upper panel) UCNs under a two-photon fluorescent confocal microscopy.
REFERENCES


34. Shen, J. W.; Yang, C. X.; Dong, L. X.; Sun, H. R.; Gao, K.; Yan, X. P., Incorporation of computed tomography and magnetic resonance imaging function into NaYF$_4$:Yb/Tm upconversion nanoparticles for in vivo trimodal bioimaging. *Analytical Chemistry* **2013**, *85*(24), 12166-12172. DOI: 10.1021/ac403486r.


55. Betzer, O.; Shwartz, A.; Motiei, M.; Kazimirsky, G.; Gispan, I.; Damti, E.; Brodie, C.; Yadid, G.; Popovtzer, R., Nanoparticle-Based CT Imaging Technique for Longitudinal and
Quantitative Stem Cell Tracking within the Brain: Application in Neuropsychiatric Disorders. 

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