Application of liposome-encapsulated ceramic phosphors for cancer cell imaging under near infrared excitation

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Abstract. Bioimaging with fluorescent probes is used as an invaluable tool in a biomedical field both in vivo and in vitro. However, organic dyes have some problems such as photo-breach and cytotoxicity due to short wavelength with high quantum energy. Recently, a new approach using rare-earth-doped ceramic nanophosphors (RED-CNP) shows that fluorescence from RED-CNP in both visible (upconversion) and near infrared (NIR) wavelength region under NIR excitation is available for bioimaging. In order to efficiently introduce the RED-CNP into cancer cells, in this study we have developed a lipid nano-particles of liposome-encapsulated erbium (Er) ion-doped Y2O3 (lipoxyY2O3). Cationic lipo-Y2O3 could clearly visualize the intracellular region of human hepatocellular carcinoma Huh-7 cells by a fluorescence microscope measurements equipped with near-infrared excitation source scanning. The results imply that the lipo-Y2O3 would potentially be useful material for imaging of cancer cells. The embedded Y2O3 in the liposome having cancer-specific ligands and/or antibodies on its surface should have a great potential for cancer cell imaging in general in living subjects.

1. Introduction
Fluorescence bioimaging is an important technique to visualize phenomena and migration of substances in biological systems by using fluorescent agents. Many of the problems in fluorescence bioimaging, such as damages on biological substances, color fading, autofluorescence and light scattering are caused by the irradiation of ultraviolet (UV) or visible (VIS) light for excitation. The use of rare-earth-doped ceramic nanophosphors as a fluorescent agent can solve the above problems, because they are known to exhibit upconversion (visible) and efficient NIR fluorescence under NIR excitation [1]. Fluorescence bioimaging in the NIR range of 0.8 to 2.0 [μm attracts a great deal of attention for the visualization of deeper lying images in living body without damage, because the use of the NIR light can reduce light scattering which is a major cause of the loss of both excitation and fluorescence lights in a living body in vivo. The emission intensity of the fluorescence of these nanophosphors does not decay. Therefore, the long term observation is possible. To apply them as biophotonic devices, the dispersion stability under physiological conditions, the control of specific interaction with a target substance and biocompatibility must be introduced to the probe. Liposome is bilayer vesicle structured by phospholipids that have similar composition of biological membrane, and show low toxicity and antigenicity. Also, both hydrophilic and hydrophobic agents can be
encapsulated in it and a variety of targeting ligands can be introduced on its surface. In this study, cationic liposome encapsulating erbium (Er) ion-doped \( \text{Y}_2\text{O}_3 \)-ceramic nanoparticle (lipo-\( \text{Y}_2\text{O}_3 \)) were prepared from dipalmitoyl phosphatidylcholine (DPPC), stearylamine (SA) and cholesterol by using a complex emulsion method. NIR-to-visible emission was observed from the lipo-Y\(_2\)O\(_3\) under 980-nm NIR excitation in the field of biological microscope. We evaluated the potential ability of lipo-Y\(_2\)O\(_3\) on the cancer cell imaging by the detection of intracellular Y\(_2\)O\(_3\).

2. Experimental Section

2.1. Preparation of PEGylated erbium ion-doped \( \text{Y}_2\text{O}_3 \) ceramic nanoparticles (PEG-Y\(_2\)O\(_3\)-CNP)

The erbium ion-doped \( \text{Y}_2\text{O}_3 \)-CNP were prepared by calcinating precursors at 900°C for 3 h. The precursors, \( \text{Y}_2\text{Er} \text{(CO}_3\text{)}\text{OH} \), was precipitated using homogeneous precipitation. A mixture of urea and nitrates of yttrium and erbium was dissolved water as a starting solution. By heating solution up to 80°C, the urea decomposed into \( \text{CO}_3^{2-} \) and \( \text{NH}_4^+ \) as precipitants for the precursor [2]. The PEG-Y\(_2\)O\(_3\)-CNP was prepared as previous described [3].

2.2. Preparation of lipo-Y\(_2\)O\(_3\)

Liposomes of cationic were prepared by using complex emulsion method [4]. The DDPC (40 mol%), cholesterol (40 mol%) and SA (20 mol%) were diluted in chloroform solution. PEG-Y\(_2\)O\(_3\)-CNP were suspended to 5 mg/ml in HEPES buffer (20 mM HEPES pH7.4, 150 mM NaCl). Added PEG-Y\(_2\)O\(_3\)-CNP suspension in lipid solution and emulsified a mixture with sonicator to form a water in oil (w/o) type emulsion. The above emulsion was then added to HEPES buffer agitated with homogenizer to form a complex water in oil in water (w/o)/w-type emulsion. Residual chloroform was removed by stirring for 12 h. The emulsion were centrifuged at 20,000g for 20 min at 10°C and then washed twice and resuspended to 1 mg/ml in HEPES buffer.

2.3. Cell lines and culture

Human cancer cell lines, HeLa (cervical cancer cell line) and Huh-7 (liver cancer cell line) were obtained from the RIKEN Cell Bank. Human normal diploid cell line, Hs68 (foreskin cell line) were from the Human Science Research Resource Bank (HSRRB). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C under 5% CO\(_2\) in a humidified atmosphere. To visualize the incorporated lipo-Y\(_2\)O\(_3\), Huh-7 cells were seeded 1 \( \times \) 10\(^5\) cells/well in a Lab-Tek II Camber slide and cultivated for 24 h. Then the cells were treated with 0.05 mg/ml lipo-Y\(_2\)O\(_3\) or PEG-Y\(_2\)O\(_3\)-CNP in serum free DMEM for 6 h at 37°C. Following the reagent treatments, the medium was exchanged to 10% FCS DMEM. After 18 h, the cells were washed in PBS, fixed in 4% paraformaldehyde. The cells were observed under a fluorescent microscope equipped with near-infrared excitation source (TCLDM9, Thorlabs, Newton, NJ).

2.4. Cytotoxicity assay

Normal and cancer cell lines were seeded 2 \( \times \) 10\(^4\) cells/well in 24-well plate and cultured for 24 h. Various doses of lipo-Y\(_2\)O\(_3\) were added to cells and incubated for 48 h and cytotoxicity of lipo-Y\(_2\)O\(_3\) was examined by trypan blue dye exclusion assay.

3. Results and Discussion

3.1. Observation of lipo-Y\(_2\)O\(_3\)
Figure 1 shows scheme (a) and SEM images (b, c) of lipo-Y$_2$O$_3$ obtained by complex emulsion method. The resulting lipid vesicles were mostly spherical and unilamellar in appearance. The mean diameter of lipid vesicles determined by analysis of electron micrographs was about 500 ± 200 nm. PEG-Y$_2$O$_3$-CNPs of the size of about 200 nm from 5 to 10 were embedded in the liposome. Thus, we successfully prepared lipo-Y$_2$O$_3$.

3.2. Cytotoxicity of lipo-Y$_2$O$_3$

To investigate the cytotoxicity of lipo-Y$_2$O$_3$, human hepatoma Huh-7 cells, human cervical carcinoma HeLa cells and normal human foreskin cell line Hs68 cells were treated with various concentrations of lipo-Y$_2$O$_3$ for 24 h and their cell viabilities were assayed by trypan blue dye exclusion procedure. In both cancer and normal cells, severe damage caused by lipo-Y$_2$O$_3$ was not observed (Figure 2), showing that this dose-range of lipo-Y$_2$O$_3$ using for bioimaging does not affect various cell viabilities.

3.3. Detection of lipo-Y$_2$O$_3$ in intracellular region of cancer cell

To investigate the incorporation of PEG-Y$_2$O$_3$-CNP into cancer cells, Huh-7 cells were incubated with 0.05 mg/ml lipo-Y$_2$O$_3$ or PEG-Y$_2$O$_3$-CNP at 37°C for 6 h and observed under a fluorescence microscope equipped with NIR excitation source. As shown in Figure 3, NIR fluorescence was clearly
detected as a dot in the intracellular region of lipo-Y$_2$O$_3$-treated cells, suggesting that lipo-Y$_2$O$_3$ was incorporated into the cells by endocytosis. On the other hand, based on the fluorescence pattern of PEG-Y$_2$O$_3$-CNPs, the CNPs were thought to be nonspecifically adsorbed on the cell surface. These results indicated that lipo-Y$_2$O$_3$, but not PEG-Y$_2$O$_3$-CNP, was efficiently incorporated into Huh-7 cells. In addition, efficient labeling of the cells was observed by the treatment with 0.01 - 0.1 mg/ml lipo-Y$_2$O$_3$. In this dose-range any cytotoxic effects were not detected (Figure 2).

![Figure 3. Detection of lipo-Y$_2$O$_3$ in intracellular region of cancer cells.](image)

**Figure 3.** Detection of lipo-Y$_2$O$_3$ in intracellular region of cancer cells. Huh-7 cells were incubated with 0.05 mg/ml Lipo-Y$_2$O$_3$ or PEG-Y$_2$O$_3$-CNP at 37°C for 6 h. The cells were examined under a fluorescence microscope equipped with a 980 nm laser diode for the NIR excitation source. Scale bars present 30 µm.

4. **Conclusion**

The present data indicate that the lipo-Y$_2$O$_3$ might potentially be useful material for imaging of cancer cells. The NIR fluorescence imaging in living nude mice which are subcutaneously inoculated with Y$_2$O$_3$-CNP-labeled cancer cells leads to elucidation of the inversion and metastasis processes of malignant tumor cells. Moreover, functional modification of Lipo-Y$_2$O$_3$ by cancer-specific ligands and/or antibodies will confer a great potential for cancer cell imaging in general in living subjects. Furthermore, efficacious cancer therapy without any adverse effects will be enabled by enclosing an anticancer agent in liposome.

**References**

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