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Quantum Dots Conjugated with Transferrin for Brain Tumor Cell Imaging

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Abstract

Malignant brain cancers derived from the cerebral parenchyma are difficult to remove completely due to the impossibility of eliminating the entire cancerous area, thus the eradicating brain cancer at the cellular level is strongly desired in order to obtain a good prognosis. In this study, we assessed the efficiency of Quantum Dots (QDs) conjugated with Transferrin (Tf) (QDs-Tf) for specific imaging of cancer cells. The expression of Transferrin Receptor (TfR) was confirmed on the surface of U87 cells (human glioblastoma cells, a major type of malignant brain cancer cells) at a high level; however the expression in NHA (a normal human astrocyte cell line) was extremally low. The labeling efficiency of U87 cells by QDs-Tf was 99.8%, while that of QDs alone was 8.4%. In addition, the red fluorescence derived from U87 cells labeled with QDs-Tf was clearly detected and the intensity was maintained for at least two days. These data suggest that imaging glioblastoma cells using QDs-Tf is useful for the detecting cancer at the cellular level with clinical applications.

Keywords: Quantum dots (QDs), Transferrin, Brain tumor cells, Glioblastoma cells, U87 cells

Introduction

Quantum dots (QDs), also known as semiconductor nanocrystals possess the unique optical properties in comparison to common organic fluorophores [1,2]. In fact, QDs consisting of a CdSe/ZnS-core/shell have several optical advantages, such as a high photoluminescence quantum yield, narrow emission band, and wide range of excitation wavelengths, size-dependent emission spectra and resistance photobleaching [3-7]. QDs also enable multiplex imaging due to the simultaneous excitation of mixed populations of QDs with broad absorption cross-sections. In addition, QDs are less affected by chemical modification on their surface [1].

According to these luminescence properties, QDs are used as fluorescent agents in various diagnostic tests, such as ELISA (Enzyme-Linked ImmunoSorbent Assay) particularly to detect minor components and perform fluorescent immunostaining [8,9]. Recently, QDs have been applied as fluorescent probes of biomolecules and live cells [10]. We previously addressed the *in vivo* imaging of transplanted Adipose Tissue-Derived Stem Cells (ASCs) labeled with QDs modified by octa-arginine (R8) or cationic liposomes, Lipofectamine®, and succeeded in observing the behavior and quantifying the rate of accumulation of transplanted cells in major organs [11-13]. On the other hand, highly selective labeling using QDs of cancer cells with high-grade a malignancy and metastatic nature is expected to enable clinicians to detect cancer cells at early stages and increase the rate of successful surgery [14-18].

Malignant brain cancers derived from the cerebral parenchyma are difficult to remove completely due to the impossibility of eliminating the entire cancerous area, therefore, malignant brain cancers exhibit recurrence at local sites confined to the immediate vicinity of the area of operation, and the five-year survival rate is very low [19,20]. Moreover, current clinical diagnostic imaging methods such as Positron Emission Computed-Tomography (PET), Magnetic Resonance Imaging (MRI), ultrasonic sound waves and X-ray Computed Tomography (X-ray CT) were developed to diagnose tissues and organs, thus it is almost impossible to detect brain cancers at the cellular level. Fluorescence Imaging (FI) technology, which can detect cancers at the cellular level, is expected to efficiently diagnose malignant brain cells, although this technology has not yet been applied in the clinical setting.

Transferrin (Tf) is a type of plasma glycoprotein, present in the blood and that carries ferric ions. The Tf Receptor (TfR) is known to be overexpressed on the surface of tumor cells, whereas the expression in normal tissue cells is extremally low [1,3,15,17,18]. The identification and diagnosis of tumors can thus be achieved using targeted Tf. In fact, Wang et al. [18] showed the labeling of HepG2 cells (a human hepatocyte carcinoma cell line) with water-soluble QDs conjugated with transferrin (QDs-Tf) [18], and Schieber et al. [1] demonstrated the labeling of HeLa cells (a human cervical carcinoma cell line) with azide-modified QDs-Tf [1]. However, the effects on labeling malignant brain cancer cells, such as glioblastomas, using QDs-Tf are largely unknown.

In this study, we evaluated the expression of TfR on the surface of U87 cells (human glioblastoma cells). We then prepared new QDs655-Tf (using QDs with an emission peak at 655 nm) and assessed whether U87 cells can be efficiently labeled using QDs655-Tf.

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Materials and methods

Cells and Materials

U87 cells (human glioblastoma cells) and Normal Human Astrocytes (NHA) was purchased from Lonza (Basel, Switzerland). Qdot® ITK™ 655 carboxyl Quantum Dots (QDs655), antibiotics (penicillin and streptomycin) and transferrin obtained from human serum fluorescein conjugate (fluorescein-Tf) were purchased from Life Technologies™ (Grand Island, NY). N-hydrocylsulfosuccinimide (Sulfo-NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Thermo scientific (Yokohama, Japan). Minimum Essential Medium (MEM) eagle and Phosphate-Buffered Saline (PBS) were obtained from Sigma-Aldrich (St. Louis, MO). Human transferrin was purchased from R&D Systems, Inc. (Minneapolis, MN). Sephadex G-100 was purchased from Wako (Osaka, Japan). L-glutamine and 100×non-essential amino acids for MEM eagle were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). A Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). All chemicals were reagent grade and used as received without further purification.

Analysis of the expression of transferrin receptor

To confirm the expression of transferrin receptor on the surface of the NHA and U87 cells, both cells were treated with trypsin and collected. The cells were incubated with fluorescein-Tf (50 μg/mL) in sodium azide water solution (0.4 mg/mL) for 20 minutes at 37°C in the culture medium (MEM eagle with 10% FBS, 1% L-glutamine (200 mM), 1% penicillin/streptomycin and 1% 100×non-essential amino acids for MEM eagle), then incubated for 60 minutes on ice. Both cells were washed with PBS and observed using phase-contrast fluorescence microscopy. The expression efficiency was analyzed using FACS caliber flow cytometry (BD Biosciences).

Synthesis and column filtration of QDs conjugated with transferrin

QDs655 conjugated with transferrin (QDs655-Tf) was synthesized according to previous reports [15,18]. A total of 10 μL of QDs655 (8 μM), 10 μL of EDC PBS solution (1 mg/mL) and 10 μL of Sulfo-NHS PBS solution (0.02 mg/mL) were mixed for 15 minutes. A total of 7.5 μL of transferrin PBS solution (1 mg/mL) was added to the mixture, and which was shaken for two hours, and then 60 μL of PBS was added. There was no precipitate, and the sample was prepared for column filtration. A Sephadex G-100 column (1.4 cm diameter×30 cm) with PBS was prepared, and then the sample, including QDs655, transferrin and QDs655-Tf, was loaded onto the column bed at 0.22 mL/min. The fluorescence of 655 nm was used to monitor the specimens eluting from the column, and the eluted latter solution was collected. Thereafter, following evaporation and refrigeration, the red sample was sealed in a refrigerator (Figure 2A).

Characterization of QDs-Tf

The absorbance spectra, photoluminescence spectra, size distribution, and zeta potential of QDs655 and QDs655-Tf in water were measured. The absorption spectra were measured using an Agilent Technology 8453A UV-visible spectrophotometer (Agilent Technology, Santa Clara, CA, U.S.A.). The photoluminescence spectra were measured using a photonic multichannel analyzer (Hamamatsu, PMA-12) (Hamamatsu Photonics, Shizuoka, Japan). The size distribution and zeta potential were measured using a dynamic light-scattering spectrophotometer (ZETASIZER Nano-ZS, Malvern Instruments Limited).

Imaging of U87 cells using QDs-Tf

To confirm the efficiency of labeling U87 cells using QDs655-Tf, U87 cells were treated with QDs655 alone (1.0 nM) or QDs655-Tf (1.0 nM) for four hours at 37°C in the culture medium. The cells were washed with PBS three times to remove the free QDs655 or QDs655-Tf, and then were observed using a phase-contrast microscope. The ImageJ software program was used to measure the fluorescent intensity. The cells were subsequently collected using trypsin, and the labeling efficiency was analyzed using flow cytometry.

Confocal laser scanning microscopy

A confocal laser scanning microscopy (FV1000, OLYMPUS) analysis was conducted to confirm the location of QDs655-Tf (0.5 nM) in the labeled NHA and U87 cells. Images obtained from the bottom of the coverslip to the top of the cells were recorded, and each image was superimposed on a PC to quantify the total brightness and pixel area of each region of interest. Before imaging, the cells were washed three times with PBS and replaced with fresh transduction medium.

Cytotoxicity of QDs-Tf to U87 cells

U87 cells (1×10^4 cells) were seeded in 96-well plates (BD Biosciences) with 100 μL of the culture medium for two days, and then incubated with 100 μL of the culture medium, including QDs655-Tf (0, 0.01, 0.1, 0.5 and 1.0 nM), at 37°C. After four hours incubation, the medium was replaced with the labeling medium (MEM eagle with 2 % FBS, 1% L-glutamine (200 mM), 1% penicillin/streptomycin and 1% 100×non-essential amino acids for MEM eagle). Viable cells were counted using the Cell Counting Kit-8 (CCK-8). The CCK-8 reagent (10 μL) was added to each well and the reaction was allowed to proceed for up to four hours. The absorbance of the sample at 450 nm was measured against a background control using a microplate reader (BMG LABTECH, Offenburg, Germany).

Statistical analysis

Numerical values are presented as the mean ± SD. Each experiment was repeated three times. Statistical significance was evaluated using unpaired Student's t-test for comparisons between the two groups; p-values of <0.05 were considered to be statistically significant.

Results

Confirmation of the expression of transferrin receptor on the surface of the NHA and U87 cells

NHA and U87 cells were investigated with respect to whether the transferrin receptor is expressed on their surface using fluorescein-Tf. Green fluorescence derived from the fluorescein was detected in the U87 cells, but not in the NHA (Figure 1A). The level of the expression of transferrin receptor was estimated using flow cytometry. The expression efficiency in the U87 cells was proved to be higher than 98% (Figures 1B and 1C). These data suggest that U87 cells express transferrin receptors at a high level, although the expression of receptors was extremely low in the NHA.

Synthesis and characterization of QDs655-Tf

QDs655-Tf was synthesized and purified as shown in Figure 2A. The size distribution of QDs655-Tf is also presented (Figure 2B). The peak in size of QDs655-Tf was much bigger than that of QDs655 (15-20 nm, Life Technologies™ data) due to the conjugation with Tf. The zeta potentials of QDs655 and QDs655-Tf were -7.97 mV and -10.2

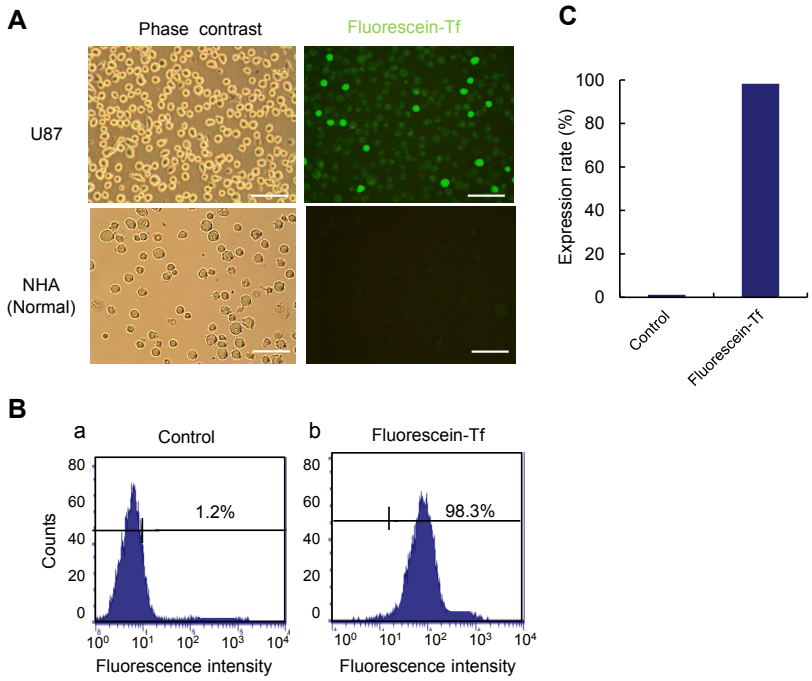

Figure 1: Analysis of the expression of transferrin receptor on the NHA and U87 cells. (A) Phase and fluorescent images of NHA and U87 cells after treatment with fluorescein-Tf. The detected green fluorescence shows the expression of transferrin receptor on the U87 cells. The white bar indicates 100 μm. (B) The analysis of the expression of transferrin receptor on the surface of U87 cells using flow cytometry. (C) The expression of transferrin receptor on the U87 cells.

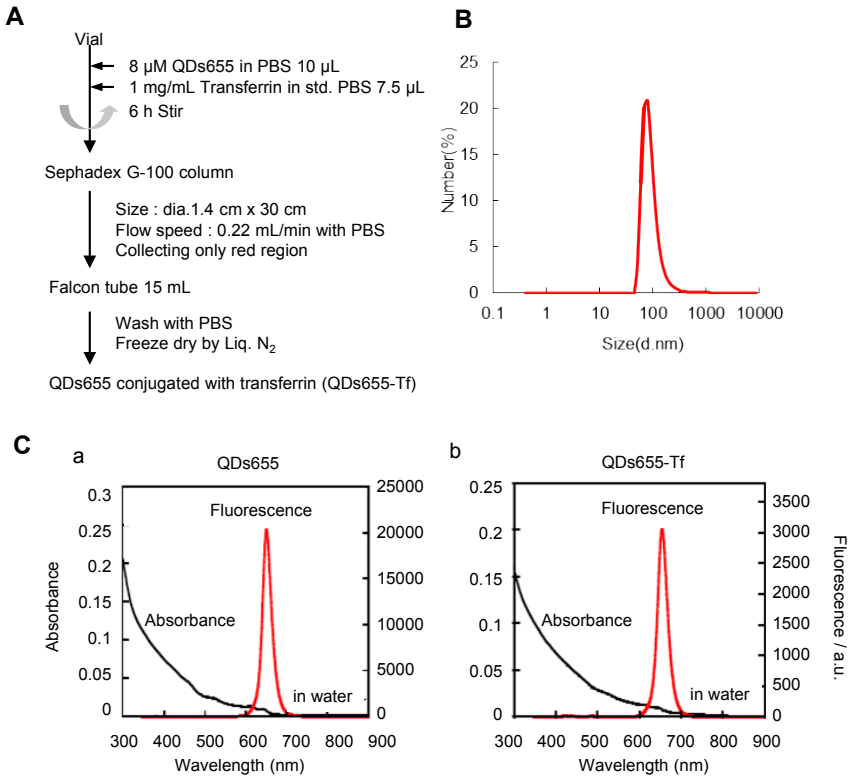

Figure 2: Synthesis and characterization of QDs655-Tf. (A) A scheme of the synthesis and column filtration of QDs655-Tf. (B) The size distribution QDs655-Tf in water. The peak size of QDs655-Tf was approximately 90 nm. (C) The absorbance and fluorescence spectra of QDs655 and QDs655-Tf in water.

		Quantum yield (365 nm excitation)	Zeta potential (mV)
QDs655	651	0.53	-7.97
QDs655-Tf	654	0.11	-10.2

Table 1: Characterization of QDs655 and QDs655-Tf.

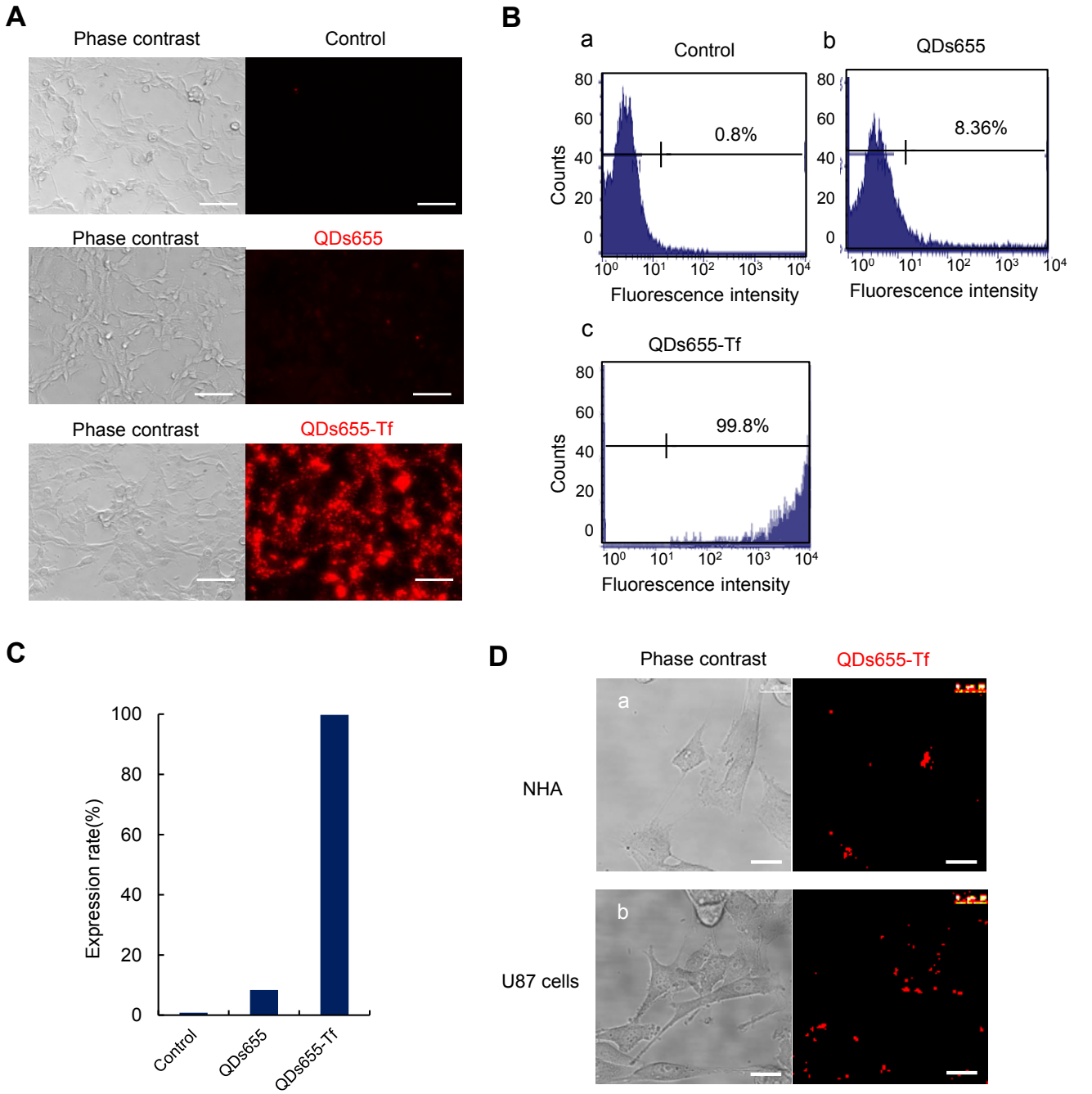

Figure 3: Labeling of U87 cells using QDs655-Tf. (A) Phase contrast and fluorescent images of NHA and U87 cells after treatment with QDs655-Tf. The red fluorescence could be detected only in the condition of labeling using QDs655-Tf. The white bar indicates 100 μm. (B) The labeling efficiency of QDs655 and QDs655-Tf for U87 cells using a flow cytometry analysis. (C) A graph of the labeling efficiency of QDs655 and QDs655-Tf in the U87 cells. (D) Observation of the internalization of QDs655-Tf in the NHA (a) and U87 cells (b) using confocal laser scanning microscopy. The white bar indicates 25 μm.

mV, respectively. The absorbance and fluorescence spectra of QDs655 and QDs655-Tf are shown (Figure 2C). The maximum fluorescence wavelength was approximately 655 nm in both particles. As a result, little change in the fluorescence spectra of QDs655-Tf following conjugation with Tf was observed. The quantum yields of Qds655 and QDs655-Tf were 0.53 and 0.11, respectively (Table 1).

Imaging of U87 cells using QDs655-Tf

To investigate whether U87 cells can be efficiently labeled with QDs655-Tf, the morphology and fluorescence derived from the U87 cells after treatment with QDs655-Tf were observed using phase-contrast fluorescence microscopy. The red fluorescence derived from the U87 cells labeled with QDs655-Tf was detected efficiently. On the other hand, no signals were detected in the U87 cells labeled with QDs655 (Figure 3A). The labeling efficiency was estimated and compared using flow cytometry. The labeling efficiency of QDs655 and QDs655-Tf after four hours of treatment was 8.36% and 99.8%, respectively (Figures 3B and 3C). Moreover, the differences of labeling efficiency of QDs655-Tf in NHA and U87 cells were detected using confocal laser scanning microscopy (Figure 3D). The labeling efficiency of QDs655-Tf in U87 cells was confirmed to be higher than that in NHA.

Concentration dependency on labeling of QDs655-Tf in U87 cells

U87 cells were treated with QDs655-Tf at various concentrations (0, 0.01, 0.1, 0.5 and 1.0 nM) in the labeling medium. The morphology and red fluorescent images of U87 cells labeled with QDs655-Tf were observed (Figure 4A). The relative fluorescent intensity was dependent on the concentration of QDs655-Tf (Figure 4B). In addition, no significant cytotoxicity was observed under any of the conditions (Figure 4C). These data suggest that U87 cells can be labeled with QDs655-Tf without cytotoxicity under at least 1 nM of QDs655-Tf.

Time dependency on labeling of QDs655-Tf in U87 cells

To investigate the labeling time of QDs655-Tf in the U87 cells, U87 cells were treated with QDs655-Tf at 1.0 nM in the labeling medium, and the morphology and red fluorescence were observed. The morphology and red fluorescent images are presented in Figure 5. The red fluorescence of QDs655-Tf was maintained until day 2; however, the intensity of red fluorescence decreased slightly at days 5 and 7. These data suggest that the labeling time of QDs655-Tf in U87 cells is adequate to detect human glioblastoma cells during surgery for at least two days.

Discussion

Cancer cell labeling using fluorescent probes, such as QDs, is very useful for the diagnosing and treating malignant tumors as a result of effective detection at the cellular level [1]. Transferrin Receptor (TfR) [1,3,15,18], Epidermal Growth Factor (EGFR) [14], HER2 [21] and folate receptor [22,23] are considered to be target molecules for detecting cancer cells. In this study, we selected TfR as the target receptor for cancer cells due to the cost and convenience of transferrin. Transferrin is reported to be over-expressed in many types of human cancer cells, such as gastric, colon, breast and lung cancer cells, while being only minimally distributed in normal tissues [24]. We confirmed that TfR is expressed on the surface of U87 cells (a glioblastoma cell line) at a high rate, but not in NHA (normal human astrocytes). We thus addressed the synthesis and purification of QDs655-Tf.

The diameter of QDs655-Tf was increased in comparison to that of QDs655 due to conjugation with transferrin. The zeta potential of QDs655-Tf was maintained at the same level as that of QDs655. The absorbance and fluorescent spectra of QDs655 were not influenced by conjugation with transferrin, and the fluorescent wavelength of QDs655-Tf peaked at approximately 655 nm. The quantum yield was decreased following conjugation with transferrin, however, the decrease was the same as previously reported decreases, and the strong red fluorescence was maintained [1,3,15,18]. Therefore, QDs655-Tf is

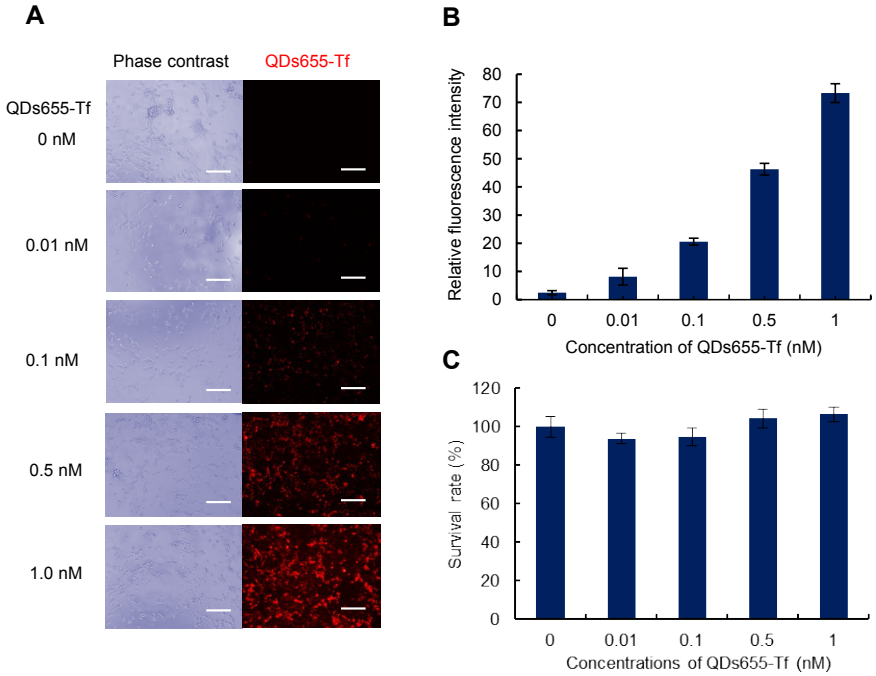

Figure 4: Concentration dependency and cytotoxicity of QDs655-Tf in labeling U87 cells. (A) Phase and fluorescent images of U87 cells labeled with QDs655-Tf at various concentrations (0, 0.01, 0.1, 0.5 and 1.0 nM). The white bar indicates 100 μm. (B) The relative fluorescent intensity of U87 cells labeled with QDs655-Tf. (C) The survival rates of U87 cells labeled with QDs655-Tf at various concentrations (0, 0.01, 0.1, 0.5 and 1.0 nM). No significant differences were observed between the concentrations.

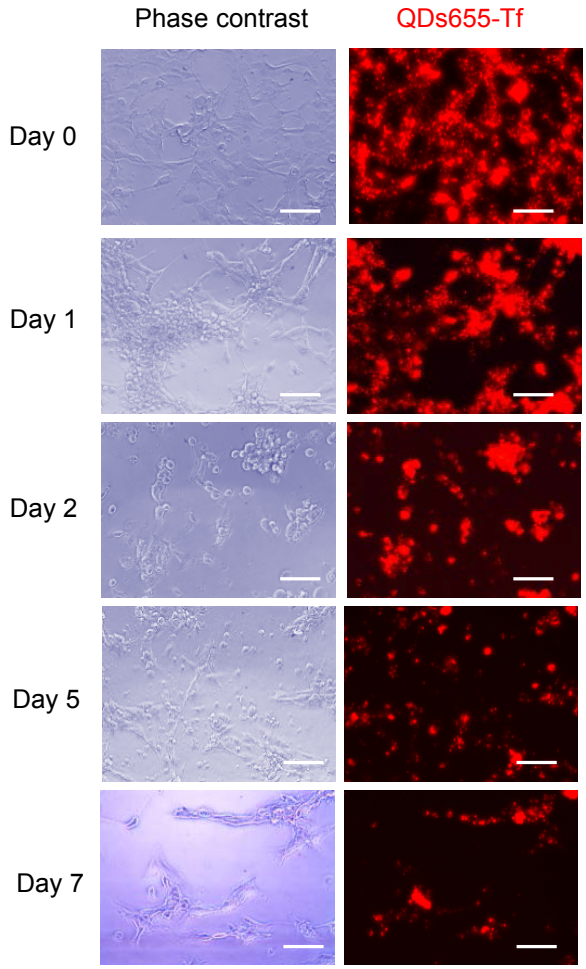

Figure 5: Time dependency of QDs655-Tf labeling in the U87 cells. Phase and fluorescent images of U87 cells labeled with QDs655-Tf at a concentration of 1.0 nM after various time periods of incubation (day 0, 1, 2, 5 and 7). The red fluorescence derived from QDs655-Tf was maintained until at least day 2. The white bar indicates 100 μm.

expected to be a fluorescent probe of U87 cells expressing Transferrin Receptor (TfR).

The labeling efficiency in U87 cells was compared between QD655 and QDs655-Tf. The efficiency of QDs655 labeling in the U87 cells was lower than 10% in this study, thus, QDs655 alone did not label the U87 cells efficiently. Comparable results were obtained in our previous examinations of stem cell labeling [12]. QDs655 alone appears to fail to interact with the surface of U87 cells sufficiently. On the other hand, the labeling efficiency of QDs655-Tf in the U87 cells was 99.8%. We thus confirmed that conjugating Tf is useful for targeting U87 cells.

The fluorescent intensity of labeling of QDs655-Tf in the U87 cells was dependent on the concentration of QDs655-Tf. In addition, no cytotoxicity of labeling with QDs655-Tf was observed in the U87 cells within 1.0 nM. We previously reported that murine ASCs can be labeled with QDs655 using octa-arginine (R8) without cytotoxicity up to 8.0 nM of QDs655 [12]. A recent study was carried out to investigate the *in vivo* imaging of transplanted murine ASCs labeled with QDs655 without laparotomy, therefore, a fairly strong fluorescent intensity was demanded. This study aimed to evaluate imaging of glioblastoma cells in the brain during surgery. In addition, the labeling intensity of U87 cells by QDs655-Tf was maintained for at least two days. Therefore, a labeling concentration of QDs655-Tf of 1.0 nM is thought to be adequate to detect glioblastomas. However, the efficiency of labeling of glioblastoma cells using QDs655-Tf *in vivo* remains unclear. In our laboratory, work continues on the in vivo imaging of glioblastomas with QDs655-Tf for clinical application.

Conclusion

In this study, U87 cells (human glioblastoma cells, a major type of malignant brain cancer cell line) expressed transferrin receptors at a high level; however, the expression in NHA (normal human astrocytes) was very low. The synthesis and purification of QDs655-Tf was successful, and the red fluorescence derived from QDs655-Tf was confirmed. The labeling efficiency of QDs655-Tf in the U87 cells was 99.8%, whereas that of QDs655 alone was 8.4%. The red fluorescence derived from U87 cells labeled with QDs655-Tf was clearly detected, and the intensity was maintained for at least two days. These data suggest that imaging glioblastoma cells using QDs-Tf is useful for detecting at the cellular level with clinical applications.

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