USE OF IR-TO-VISIBLE UPCONVERSION FLUORESCENT NANOPARTICLES FOR TRACKING OF SIRNA DELIVERY

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ABSTRACT
IR-to-visible upconversion fluorescent nanoparticles (UCNs) were developed to track in vitro delivery of small interference RNA (siRNA) and meanwhile efficiently transfect siRNA to the cells. NaYF₄ upconversion nanoparticles co-doped with Yb/Tm (NaYF₄:Yb,Tm) were conjugated with GL3 siRNA through electrostatic attraction at the weight ratio of 8:3. Then, siRNA-conjugated UCNs were incubated with the cultured human breast cancer MCF-7 cells. After 24h-incubation the successful cellular uptake of siRNA was visualized through the emitted blue fluorescence of siRNA-conjugated UCNs under confocal laser scanning microscopy. After 48h-incubation the efficiency of delivered GL3 siRNA in the inhibition of luciferase gene was ~60% that was determined by luciferase assay. This article provided initial insights into the effectiveness and suitability of NaYF₄:Yb,Tm upconversion nanoparticles as an easily detectable and self-tracking delivery vector of siRNA.

KEY WORDS
upconversion nanoparticles, siRNA delivery, imaging, biomaterials

1. Introduction
The rapid advance in diagnostics and monitoring of infectious and genetic diseases are undermining the traditional biological fluorescent labels, and the novel development of more efficient and ultrasensitive fluorescent materials is becoming a forceful trend. The IR-to-visible upconversion fluorescent nanoparticles (UCNs) with week autofluorescence, high light penetration depth, low toxicity, little photodamage and long-time photostability were developed as potential biological imaging probes. Upconversion nanoparticles (UCNs) are phosphors that absorb two or more infrared-light photons and emit visible-light photons. The fundamental mechanisms of UCNs are to utilize a combination of an absorber ion and an emitter ion that belong to rare earth lanthanides in a crystal. The absorber ion is excited by an infrared light source and then transfers this energy nonradiatively to the emitter ion that radiates a detection photon [1]. Compared with conventional down-conversion phosphors including organic dyes and quantum dots that were widely used for biological studies, the upconversion phosphors show chemical stability, low toxicity, biocompatibility and resistance to photobleach. In particular, the background autofluorescence from cells or tissues upon illumination with near-infrared (NIR) light is extremely low, which makes UCNs more suitable and sensitive for qualitative and especially quantitative detection [2]. Furthermore, the NIR excitation light of UCNs has highest light penetration depth in tissues, which increases their in vivo feasibility. It is because the tissue transmissivity is highest in the NIR spectral range due to low inherent scattering and absorption properties within the region [3]. UCNs are relatively new materials, while they have the potential to become a new class of fluorescent probes for some biological applications based on the advantages stated above [4-8]. Here the fluorescent UCNs were utilized as imaging probes of small interference RNA (siRNA). siRNA are a class of 21-23 nucleotide-long double-stranded RNA molecules that play a variety of roles in biology. Most notably, siRNA is involved in the RNA interference (RNAi) pathway where the siRNA induce efficient sequence-specific silencing of gene expression [9,10]. The discovery of RNAi has generated tremendous interest by scientists to evaluate its application as a useful approach in gene therapy. However, the complex spatial and temporal interplay of siRNA and other biomolecules or organs from the cellular to the integrative level is a challenging prehension. Some typical strategies have been applied to track and monitor the in vitro delivery of siRNA, involving fluorescently end-modified siRNAs [11], co-transfecting reporter plasmids [12] and co-transfecting unmodified siRNA with semiconductor quantum dots (QDs) [13]. But rapid photobleaching of organic fluorophores, the limited selection of available reporters and the cytotoxicity of QDs currently prevent RNAi tracking from being feasible in either long-term or multiplexed studies. Therefore, in this article upconversion fluorescent nanoparticles which are photostable, biocompatible, highly luminescent and ultrasensitive were developed to efficiently transfect siRNA as delivery system and meanwhile track siRNA delivery as imaging probes.
Nanometer-sized silica-coated NaYF₄ upconversion nanoparticles (UCNs) co-doped with lanthanide ions such as Yb/Er or Yb/Tm were synthesized like the previous article [14]. Then amino groups were coated on the silica shell, which made UCNs with positive charge at neutral PH suitable for the attachment of siRNA. The internalization of UCNs conjugated siRNA into cells was imaged using confocal laser scanning microscopy and inhibition activity of transfected siRNA was analyzed.

2. Experimental

100μl of siRNA (300μg/ml) and 800μl of UCNs (100μg/ml) are mixed up 1ml of solution with 100μl of DI water, and incubated for 30min at room temperature under stirring. The weight ratio of UCNs and siRNA is 8:3. Subsequently, the siRNA-conjugated UCNs (UCN-siRNA) were spun down at 10,000rpm for 10min and washed twice with DI water. The pure siRNA-conjugated UCNs was formed at the concentration of 92.7μg/ml. The absorbance of supernatant of spun-down mixture and the total siRNA used in this process was analyzed with UV-vis spectrophotometer at 260 nm wavelengths. Conjugation efficiency is expressed as 100% minus the percentage of the absorbance of free siRNA remaining in the supernatant to the absorbance of the total amount of siRNA. 1% agarose gel was used to visualize the siRNA in the supernatant of the samples at different weight ratio of 1:3, 4:3 and 8:3.

8.4μg/ml of UCNs was added into 5 x 10⁵ MCF-7 cells cultured in a 25cm² flask with 5mL of medium and incubated for 24 hours. The medium was removed after incubation and the cells were washed with 5ml of PBS for 5 min. The cells were then trypsinized, spun down and resuspended in 5 ml of PBS. The treated cells were washed with PBS for three times to remove all the free UCNs. 1 x 10⁴ of cells were seeded in each well of chambered glass plate and next day were fixd using 4% buffered paraformaldehyde. The cells were imaged with confocal laser scanning microscopy under excitation of NIR laser at 980nm wavelength. For Luciferase assay, MCF7 cells cultivated in a 25cm² flask with 90% confluency were transfected with pGL3-control luciferase reporter vectors according to the procedures of Invitrogen lipofectamine 2000. The transfected cells were trypsinized and 10 000 cells placed into 3 sets of 5 wells each in the 96-well plate with 100μL of medium. 8.4μg/ml of UCN-siRNA was added into 2 sets and incubated for 24h and 48h respectively, and the third set is the control cells without UCN-siRNA. 100μL of Promega Bright Glo solution was added to each well and the luminescence was obtained using the Optima Fluostar with Luminescence Optic.

3. Results and Discussions

3.1 Characterization of siRNA-conjugated UCNs

NaYF₄:Yb,Tm upconversion nanoparticles (UCNs) emit blue fluorescence under the excitation of near-inferred light at 980nm. The size of UCN was 50nm at diameter being analyzed by Transmission Electron Microscope (TEM) and 190nm measured by Zetasizer Nano ZS. The zeta potential of UCNs is +27.6mV because of coated amino groups. GL3 siRNA was conjugated to the UCNs through the electrostatic attraction. The conjugation efficiency of siRNA was 42.25% being analyzed with UV-vis spectrophotometer. Meanwhile, gel electrophoresis results of unbound siRNA in the supernatant after the spinning procedure confirmed the conjugation of siRNA (Figure 1). Lane 1 represented control siRNA at 30μg/ml concentration, Lane 2 represented the supernatant of spun down unconjugated UCNs and Lane 3-5 represented the supernatant of spun down UCN-siRNA with the weight ratio of 1:3, 4:3 and 8:3. The decreasing luminescence from lane 3-5 indicated that higher concentration of UCNs was used, higher conjugation efficiency was achieved. After conjugation of siRNA, the size of UCN-siRNA increased to 261nm and the zeta-potential decreased to -21.3mV, and the fluorescence spectra did not change as expected.

![Figure 1](image)

The cell viability of UCN-siRNA on MCF-7 cell line was analyzed using MTT assay (Figure 2). The initial data based on the absorbance value obtained with Microplate Reader was compared as a percentage to the positive control, cells untreated with UCN-siRNA. Sample 1-5 represented cells treated with UCN-siRNA at different concentration of 2.1μg/ml, 4.2μg/ml, 8.4μg/ml, 12.6μg/ml and 16.8μg/ml respectively. From the graph, we found that the cell viability hardly decreased as the concentration of UCN-siRNA added increased. The 24h incubation period of UCN-siRNA did not result in any significant cell death. UCN-siRNA at 8.4μg/ml concentration with cell viability of ~100% is selected to use for cellular imaging and siRNA delivery.
3.2 Cellular Imaging of UCN-siRNA

Cellular uptake of siRNA-conjugated UCNs was studied in MCF-7 human breast cancer cell lines. Blue-fluorescent NaYF₄:Yb,Tm UCNs were used for microscopic imaging by confocal laser scanning microscopy and monitoring intracellular trafficking of conjugated siRNA. After the incubation of UCN-siRNA with cells, the bright field and NIR confocal microscopic images of treated cells were obtained and overlaid to localize the UCN-siRNA (Figure 3). The blue fluorescence emitted from UCNs was visible inside the MCF-7 cells under excitation by NIR laser at 980nm, suggesting UCN-siRNA were internalized by the cells. In comparison to the visualization of unconjugated UCNs (data not shown), the confocal microscopic images for UCN-siRNA are dimmer and not as well distributed in the cytoplasm, and the internalization time of UCN-siRNA is much longer. While it shows that UCN-siRNA have been taken into the cell, it may not have been taken up as easily compared to the positively charged UCNs, as evident in past research claiming that strongly negative particles are not easily taken up into cells. This may be a consideration for future design of specific targeted delivery carriers.

3.3 siRNA-mediated inhibition of luciferase gene

Gene-silencing activity of preparations was determined with cell culture assays of reporter gene-targeted siRNA using exogenous luciferase gene expression assays. After transfected with pGL3-control luciferase reporter vectors, MCF-7 cells were incubated with 8.4μg/ml of UCNs functionalized with commercial GL3 siRNA (UCN-GL3) that specifically inhibit luciferase report gene. The percentage luminescence based on the control that is MCF-7 cells transfected with luciferase reporter vectors but not incubated with UCN-GL3 were analyzed and presented graphically in the Figure 4. The delivered GL3-siRNA would decrease the luminescence by inhibiting the luciferase expression in the cytoplasm. The cells incubated for 1 day with UCN-GL3 was found to emit ~100% luminescence, while the cells incubated for 2 days emitted ~40% luminescence, less than all other samples. Analysis of the results between these 2 samples suggests that it may take longer for the RNAi effect from the delivered siRNA to take effect. This may be due to the longer time, not only 24h, required for UCN-siRNA to be
internalized into the cells, congruent with the findings of confocal images. In addition, it is highly likely that the release of siRNA from UCNs occurs mainly in the 24 h after internalization of UCNs, resulting in the steep decrease in luminescence of UCN-GL3.

4. Conclusion

Non-toxic and self-tracking NaYF₄:Yb,Tm upconversion nanoparticles (UCNs) have been developed to efficiently monitor and deliver siRNA to cells. siRNA was easily conjugated to the surface of UCNs and visualized to be taken up by MCF-7 cells under confocal microscopy through the conjugated blue-fluorescence UCNs. Meanwhile, the GL3 siRNA was delivered into cells and silence specific luciferase gene expression as much as ~60%.

Reference