Aptamer Conjugated Quantum Dots for Imaging Cellular Uptake in Cancer Cells

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The fluorescence labeling of aptamers is a useful technology for aptamer tracking and imaging on the cells. The aptamer SL2-B, against the heparin binding domain (HBD) of VEGF165 protein, was linked to QDs, producing the QD-SL2-B aptamer conjugate. The QDs and the QD-aptamer conjugate were characterized and photobleaching effect was studied prior to the cellular incubation. Fluorescence imaging showed that the QD-SL2-B aptamer conjugate could specifically recognize Hep G2 liver cancer cells and is taken up by the cells without addition of any external transfecting or cell permeabilizing agent. In addition, the results also indicate that incubation time is important for the aptamer cellular uptake and to exhibit its antiproliferative activity on Hep G2 liver cancer cells. This QDs labeling technique provides a new strategy for labeling aptamer molecules for aptamer detection, imaging and understanding their cellular uptake.

Keywords: Aptamer, Quantum Dots, VEGF, Fluorescence, Cellular Uptake.

1. INTRODUCTION

In 1990, an in vitro selection process called systematic evolution of ligands by exponential enrichment (SELEX) was developed to screen single-stranded nucleic acid molecules from random pool of library against the target ligand.1,2 This class of single-stranded molecules is referred to as “aptamers.” They possess high binding affinity and specificity that are comparable to monoclonal antibodies. However, unlike prevalent monoclonal antibodies, aptamers are small in size, non-immunogenic and can be easily modified which makes them promising candidates on therapeutic front.3,4 Furthermore, different SELEX methods have been developed to meet the needs of the researchers such as cell-SELEX, microfluidic SELEX and capillary electrophoresis SELEX (CE-SELEX).5,6,7

Apart from susceptibility to degradation by nucleases attack and rapid renal filtration, another limitation for in vitro and in vivo application of aptamers is their inefficient cellular uptake.8,9 However, this limitation is applicable to aptamers where cellular uptake is important for its therapeutic functions. Presently, most of the selected aptamers have very poor cellular uptake and cannot be taken up by the cells without external aid of transfecting and cell permeabilizing agents. Several inherent factors such as size and nucleic acid charge acts as potent barrier to cellular uptake.10 Due to the presence of negatively-charged phosphate backbone, aptamer experience electrostatic repulsion from negatively-charged cell membrane surface and thereby resulting in inadequate cellular association and hence, affecting their cellular uptake. Moreover, sequences more than 25 bases are difficult to import into the living cells because of their size and self-hybridization tendency. To date, very few aptamers, such as anti-PSMA (prostate-specific membrane antigen) aptamer, ACT-GRO-777 (or AS1411) aptamer that binds to nucleolin protein, and sgc8 aptamer that binds to acute lymphoblastic leukemia (ALL) T-cells, can directly be internalized without the use of external agents.11–15 Moreover, the cell internalization property of anti-PSMA aptamer has enabled them to deliver siRNAs and anti-cancer drugs into the cells.14,15 For other aptamers, currently the cellular uptake problem is countered by use of various transfection methods such as complexation with liposomes and vector delivery systems.16–19

In the past few years, nanometer sized semiconductor crystals referred to as quantum dots (QDs) are attracting more and more attention for their potential applications in biosensors, cell labeling, and biological imaging.20–22 QDs exhibit several advantages over the conventional organic fluorophores such as high quantum...
yields, long-term photostability, single-wavelength excitation, and size-dependent tunable emission. More recently, several groups have engineered QDs and conjugated them with antibodies, peptides, and oligonucleotides resulting in the development of highly sensitive imaging probes for in vitro or in vivo applications. However, the presence of toxic elements such as cadmium and selenium in the core part of QDs makes these nanoparticles inherently toxic and, and concerns have been raised about the toxicity effects of QDs to both cell cultures and live animals. This problem has been mitigated by use of mechanism such as polymer encapsulation and core-coatings, which reduces the toxicity to certain extent.

In our previous work, a SL$_2$-B aptamer sequence was identified that binds to VEGF$_{165}$ protein with $K_d = 0.5$ nM. The aptamer was further chemically modified to enhance its stability against nucleases enzymes in the biological fluids and antiproliferative potential of modified aptamer was demonstrated in Hep G2 liver cancer cells under hypoxia conditions. This chemically modified aptamer was referred to as PS-modified SL$_2$-B aptamer. In the current study, the cellular fate of PS-modified SL$_2$-B aptamer was investigated after binding to VEGF$_{165}$ protein in Hep G2 cancer cells using confocal imaging. QDs were used as fluorescent probes for imaging purpose due to their superior fluorescent properties above-mentioned. Different techniques including transmission electron microscope (TEM), dynamic light scattering (DLS), agarose gel electrophoresis, and fluorescence spectroscopy were first used to characterize QDs and QD-aptamer conjugates. The cellular uptake of the synthesized QD-aptamer conjugates was imaged using laser scanning confocal microscopy. The results demonstrate that PS-modified SL$_2$-B aptamer after binding to VEGF$_{165}$ protein can be taken up by Hep G2 cancer cells without the presence of any external transfecting or cell permeabilizing agent in the medium. Furthermore, the data suggest this cellular uptake may be involved for SL$_2$-B aptamer to exhibit its antiproliferative activity on Hep G2 cancer cells.

2. EXPERIMENTAL DETAILS

2.1. Materials

Cadmium oxide (CdO, 99.99%), selenium powder (Se, 100 mesh, 99.99%), trioctylphosphine (TOP, >90%), trioctylphosphine oxide (TOPO, 90%), hexadecylamine (HDA, >99%), diethylzinc solution, tetramethyldisilathiane, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccimide (NHS), fluorescein were purchased from Sigma-Aldrich. Glutathione (GSH, reduced) and tetramethylammonium hydroxide (TMAH) were purchased from Merck. The HPLC grade unlabeled and Texas red-labeled PS-modified SL$_2$-B aptamer sequence (5’-[NH$_2$-C$_6$-T]TTCCGTTGTAACCCGTTACGGG+3’, where * is phosphorothioate linkage) were purchased from Sigma-Aldrich. The sequence of scrambled aptamer used in the study is 5’-[NH$_2$-C$_6$-T]TTCCGTTGTAACCGTTACGGG+3’. Nanosep spin filters were purchased from Pall Filtrations. Agarose gel and tris-borate-EDTA buffer (0.089 M tris, 0.089 M borate and 0.002 M EDTA) were purchased from 1st Base. Human hepatocellular carcinoma (Hep G2) cell line was a gift from Dr. Tong Yen Wah’s lab. The hypoxia chamber was purchased from Billups-Rothenberg. Dulbecco’s modified eagle’s media (DMEM) and fetal bovine serum (FBS) were purchased from Caisson Laboratories. Trypsin-EDTA and 1% penicillin/streptomycin mixture were purchased from PAN biotech. The chamber slides were purchased from Thermo Fisher Scientific. Hoechst 33342 trihydrochloride trihydrate dye and wheat germ agglutinin, Alexa Fluor 488 conjugate dye were purchased from Invitrogen. Para-formaldehyde was purchased from Sigma-Aldrich. Phosphate buffer saline (PBS, 137 mM NaCl, 2.7 mM KCl, and 10 mM phosphate buffer) was purchased from 1st Base. Deionized water was used for all the experiments.

2.2. Synthesis of Core–Shell CdSe/ZnS QDs

Core–shell CdSe/ZnS QDs were synthesized by reacting organometallic precursors at high temperatures in a coordinating solvent mixture. 0.068 g of CdO, 5.91 g of TOPO and 6.02 g of HDA were kept under vacuum for 1 hour and the heated at 110 °C for 2 hours. After degassing, the cadmium precursor solution was then heated at 330 °C under nitrogen atmosphere until it forms colorless solution and then cooled to 300 °C for stabilization for 2 hours. The degassed Se powder dissolved in 3.5 ml of TOPO was rapidly injected into cadmium solution. Subsequently, overcoating CdSe core with thin layer of ZnS formed a CdSe/ZnS core–shell QDs. The ZnS precursor solution was added drop wise to passivate CdSe core surface. When the temperature reaches 80 °C, toluene was added to the product mixture. The synthesized organic QDs were centrifuged at 10,000 rpm for 15 minutes to remove excess of TOPO and redissolved back in toluene.

2.3. Synthesis of Glutathione (GSH)-Capped CdSe/ZnS QDs

The organic QDs were made hydrophilic using GSH as capping agent by ligand exchange. The GSH consisting of both the sulphhydryl and carboxylic group forms a disulphide (S–S) bond with ZnS coating on CdSe/ZnS QDs thereby exposing the carboxylic group. GSH dissolved in TMAH, a phase transfer reagent, was added dropwise to 1 ml of organic QDs dissolved in 10 ml of chloroform. Obtained upper aqueous layer was centrifuged twice at 7500 rpm for 5 minutes and finally the pellet was dissolved in 8 ml of DI water. The quantum yield of QDs was calculated relative to fluorescein (whose quantum yield is 95%) by measuring integrated fluorescence intensity of QDs and the fluorescein and then plotting graph between


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absorbance versus integrated fluorescence intensity. The slope obtained from the graph was used to calculate quantum yield using the equation,

$$\Phi = \Phi_{\text{fluorescein}} \left( \frac{S}{S_{\text{fluorescein}}} \right) \left( \frac{\eta^2}{\eta_{\text{fluorescein}}^2} \right)$$

where $\Phi$ = quantum yield of the QDs, $\Phi_{\text{fluorescein}} = \text{quantum yield of fluorescein}$, $S = \text{slope of the graph}$, and $\eta = \text{refractive index of the solution}$.

2.4. Conjugation of PS-Modified SL2-B Aptamer Sequence to QDs

The GSH-capped CdSe/ZnS core–shell QDs were conjugated with SL2-B aptamer sequence using EDC/NHS activation chemistry. The aptamer was added to the activated QDs in molar ratio of 1:10, followed by incubation for 3 hours at room temperature. The unconjugated aptamer sequence was removed through Nanosep filters by centrifuging at 10,000 g for 5 minutes. The final QD-aptamer bioconjugates were resuspended in PBS buffer (pH 7.4).

2.5. Characterization of QDs and QD-Aptamer Bioconjugates

The fluorescence spectrum of QDs was obtained with a Perkin Elmer LS 55 fluorescence spectrometer using 1 cm quartz cuvette, 480 nm excitation wavelength with excitation slit width set at 10 nm. The size of QDs was determined by using a JEOL 2010F high resolution transmission electron microscope. The samples (15 µl) were dropped on carbon-coated copper grid and dried overnight before taking the measurement. The zeta potential of QDs and QD-aptamer conjugates was measured using a Malvern Instruments Nano-ZS zeta sizer machine. 15 µl of QDs and QD-aptamer conjugates were loaded to separate wells and ran on 0.7% agarose gel at 100 V in TBE buffer for 1.5 hour using BIORAD gel electrophoresis apparatus and gel was illuminated using the GeneSnap software from a Syngene bioimaging gel documentation system. The photobleaching experiment of QDs and QD-aptamer conjugates was performed using a Nikon A1Rsi laser scanning spectral confocal microscope system.

2.6. Cell Binding Studies and Fluorescence Quenching Assay

Human hepatocellular carcinoma Hep G2 cells (0.1 × 10^5 cells/ml) were grown on chamber slides in DMEM media supplemented with 10% FBS and 1% penicillin-streptomycin. The cells were incubated with 100 µl of 0.01 nM of QD-aptamer conjugates and Texas red-labeled aptamer conjugates at 37 °C in hypoxia condition (5% CO₂, 1% O₂, and 94% N₂) inside the hypoxia chamber. No transfecting or cell permeabilizing agent was added to enhance the cellular uptake. After incubation, cells were washed with PBS buffer (pH 7.4) three times. The cell nuclei were stained with Hoechst dye and plasma membrane with Alexa fluor dye using standard staining procedures. The cells were then fixed using 4% paraformaldehyde solution. Localization of QDs and QD-aptamer conjugates were performed using laser scanning spectral confocal microscopy (Nikon A1Rsi, 60× objective). The cellular uptake was determined by measuring the fluorescence intensity of the conjugates in 50 cells using Image J software.

3. RESULTS AND DISCUSSION

3.1. Characterization of QDs

Before using QDs for biological studies, their optical properties should first be characterized. Highly photoluminescent aqueous core–shell CdSe/ZnS QDs were synthesized with bright red color emission at 610 nm wavelength (Fig. 1(A)). The QDs were photostable for more than 2 months, with narrow emission spectra and full width half maximum (FWHM) as narrow as 28 nm. Figure 1(B) illustrates the high resolution TEM image of synthesized QDs. The TEM images confirm the size of QDs in 3–5 nm range. The TEM images shows well resolved
lattice structure of monodisperse QDs. However, no information can be deduced about the CdSe/ZnS interface from the images.

Quantum yield calculations of QDs determine the quality of the synthesized QDs. The quantum yield is highly influenced by the coating agent used, its charge, and type of solvent in organic QDs. According to the reported literature, the quantum yield of aqueous QDs decreases drastically in comparison to the original organic QDs upon phase transfer. In this work, QDs showed very high quantum efficiency with quantum yield dropping by only 13.4% on phase transfer from organic to aqueous phase ($\Phi_{\text{organic}} = 70.11\%$, $\Phi_{\text{aqueous}} = 57.71\%$). These values are better or comparable to the reported quantum yield results for aqueous QDs.\textsuperscript{31, 32}

3.2. Characterization of QD-Aptamer Conjugates
The formation of QD-aptamer conjugates was confirmed using agarose gel electrophoresis and zeta potential measurements. Lane 1 and Lane 2 in Figure 2 show the gel images of QDs before and after the conjugation with the aptamer respectively. It is apparent that the electrophoretic mobility of the QD-aptamer conjugates is faster than the QDs alone, confirming the formation of QD-aptamer conjugates. The conjugates run faster due to higher charge to mass ratio after the attachment of the negatively-charged DNA aptamer molecules to the QDs surface. In addition, conjugation of 5’end NH$_2$-modified aptamer with –COOH group of aqueous QDs increased zeta potential by 57.3 mV (Fig. 3). The increase is due to the conjugation of highly negatively-charged DNA aptamer molecule to the QDs surface that increases the overall surface charge on QDs.

Figure 2. Agarose gel electrophoresis analysis of QDs conjugation with aptamer. Shown here is QDs (Lane 1) and QD-aptamer conjugates (Lane 2).

Figure 3. Zeta potential distribution of QDs ($-25.1$ mV) and QD-aptamer conjugates ($-82.4$ mV).

3.3. Photobleaching Effect of QD-Aptamer Conjugates and Texas Red-Labeled Conjugates
Photobleaching is one of the major limitations of the conventional organic dyes and fluorescent proteins being used for cellular and medical imaging. The photobleaching effect was compared on the synthesized QD-aptamer conjugates and commercially available Texas red-labeled aptamer conjugates. Both conjugates were exposed to laser light continuously using laser scanning confocal microscopy. The fluorescence intensity of the Texas red aptamer conjugates dropped to zero within 5 minutes but the fluorescence intensity of the QD-aptamer conjugates was stable even after more than 60 minutes of continuous exposure to laser light (Fig. 4). The result clearly indicates the high photostability of QD-aptamer conjugates compared to the Texas red-labeled aptamer conjugates and their potential as fluorescent probe for long period cellular imaging.

Figure 4. Comparison of fluorescence emission intensity of QD-aptamer conjugates (smooth line) and Texas red-labeled aptamer conjugate (dashed line) on continuous exposure of laser light ($\lambda = 488$ nm).

3.4. Confocal Imaging of QD-Aptamer Conjugates in Hep G2 Cancer Cells
As demonstrated in the previous study, the PS-modified SL$_2$-B aptamer displays sequence dependent inhibition on cellular proliferation of Hep G2 cells by binding to VEGF$_{165}$ protein.\textsuperscript{33} Keeping this in consideration, the cellular uptake of this aptamer in Hep G2 cells was investigated using synthesized QD-aptamer conjugates. To quantitate the conjugates uptake by Hep G2 cells, a time interval experiment was conducted and evaluated...
by measuring the fluorescence intensity signal obtained using laser scanning confocal microscopy. Results shown in Figure 5 demonstrated significant differences in the cellular uptake of conjugates based on the incubation time. After 24 hours of incubation, the confocal image indicated the presence of conjugates on the cell membrane surface without any substantial cellular uptake of conjugates (Fig. 5(A)). However, the amount of intracellular conjugate population increased with the increase in incubation time. The percentage of conjugates inside the Hep G2 cells increased by 6-fold after 36 hours of incubation (Fig. 5(B)). After 48 hours, the cellular uptake was further increased by 2-fold (Fig. 5(C)). The enhanced uptake of conjugates with increase in the incubation time correlates with the stronger antiproliferative activity observed at 72 hours of incubation in the previous study. The data shows that PS-modified SL$_2$-B aptamer after binding to VEGF$_{65}$ protein get internalized without any external aid and appears to exhibit its antiproliferative activity in Hep G2 cells with increase in the cellular uptake. This finding is in agreement with the correlation observed between cellular uptake and antiproliferative activity in ACT-GRO-777 (AS1411) aptamer targeted against nucleolin protein.$^{34-36}$ Moreover, no cellular uptake was observed with scrambled aptamer conjugates (Fig. 5(D)). This confirms the cellular internalization of conjugates is aptamer-specific and not dependent on QDs. However, further experimental studies are warranted to elucidate the correlation between cellular uptake of SL$_2$-B aptamer and its antiproliferative activity in Hep G2 cells. Moreover, from the confocal images, the conjugates appear to be localized in the cell cytoplasm and around the nuclei.

Compared to QD-aptamer conjugates, a weak fluorescence signal was observed for Texas red-labeled aptamer on incubation for 48 hours with Hep G2 cells (Fig. 5(E)). Furthermore, autofluorescence effect studied using only Hep G2 cells as control displayed a negligible fluorescence in comparison to the QD-aptamer conjugates (Fig. 5(F)). These results illustrate the use of QD-aptamer as fluorescent probes for cellular imaging and demonstrate the effect on the cellular uptake of aptamer at the extended time periods in cancer cells. However, further studies are needed to elucidate the molecular mechanism of the PS-modified SL$_2$-B aptamer for its antiproliferative activity in cancer cells.

4. CONCLUSION

In conclusion, this study shows that Hep G2 cancer cells can uptake PS-modified SL$_2$-B aptamer without addition of any external transfecting or cell permeabilizing agents in the medium. Moreover, this cellular uptake study provides valuable information that cellular uptake of aptamer with increase in incubation time seems to be important for it to exhibit its antiproliferative activity in cancer cells. Thus, conjugation of this aptamer with other anti-cancer drugs can be useful for specific intracellular delivery and to further enhance the inhibitory action on cancer cells. Furthermore, long-term photostability of the QDs probes makes them potentially useful for in situ studies when long periods of monitoring are required. Thus, the coupling of high fluorescence emission and photostability of the QDs with high degree of specificity of the aptamers can be a powerful tool for biolabeling and targeting studies.

References and Notes

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