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N-Acetylglucosamine biofunctionalized CdSeTe quantum dots as fluorescence probe for specific protein recognition†

Fang-Fang Cheng,[a] Guo-Xi Liang,[ab] Yuan-Yuan Shen,[a] Rohit Kumar Rana[c] and Jun-Jie Zhu*[a]

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Hsp70 proteins are implicated in resistance to chemotherapy in cancers, the detection of which is important for cancer treatment and prognosis. In this work, we report the study on the detection of specific intracellular target protein in fixed cells using GlcNAc-conjugated CdSeTe QDs. The QDs were coupled with Con A *via* a carbodiimide reaction and then were further assembled with GlcNAc by lectin–carbohydrate interaction between Con A and GlcNAc. The obtained QDs-Con A-GlcNAc conjugates have an emission wavelength at 650 nm that is close to the near-infrared (NIR) regions and a specific recognition for Hsp70. These results show that the QDs-Con A-GlcNAc probe can be a promising tool for direct localization of the Hsp70 protein.

Introduction

Fluorescence immunostaining is widely used in cell biology for probing structure and localizing proteins of interest on the molecular scale.[1] Conventional immunofluorescence techniques rely on labeling with the primary antibody and visualization by secondary staining with dye-labeled IgG (such as fluorescein isothiocyanate (FITC), Texas Red or Alexa *etc.*) which are prone to interfering factors, unstable sensitivity, high discrepancy between laboratories and subjective interpretation.[2,3] Moreover, under a UV light source, these organic fluorescent dyes usually suffer photobleaching and have a wide emission range so that the spectra of different dyes may overlap and may be difficult for simultaneous observation.[4] Compared with the traditional organic fluorophores (*e.g.*, organic dyes and fluorescent proteins), quantum dots (QDs) have unique optical properties, such as brighter fluorescence, greater photostability, and multiple colors with a single excitation.[5–8] In addition, the large surface area of QDs is easy to be further modified by biorecognition molecules, such as peptides, antibodies, nucleic acids, carbohydrates or small-molecule ligands as fluorescent probes.[5,9–15] In the basis of these above properties, QDs are of great interest to biological applications such as fluorescent biosensors and biolabels.[16–20]

70 kDa Heat Shock Protein (Hsp70), a stress-inducible protein, is involved with tumor cell proliferation (breast, uterine cervix and lung), lymph node metastasis (breast and colon), increased tumor size (uterine cervix), and presence of mutated p53 (breast and endometrium).[21] Hsp70 overexpression in cells can enhance tumorigenicity and limit the effectiveness of cancer therapy. In cancer cells, the expression of Hsp70 is abnormally high, thus Hsp70 is considered to be a potential biomarker of a variety of cancers, making it significant to evaluate Hsp70 for cancer diagnosis and therapy.[22] Recent results have shown that Hsp70 possesses lectin-like activity with a strong affinity for *N*-acetylglucosamine (GlcNAc), and the GlcNAc-directed lectin activity of Hsp70 can be modulated by the presence of ATP.[23] Some efforts have been exploited to detect Hsp70 proteins *via* QDs fluorescence. Streptavdin-QDs 605 conjugates were used for immunofluorescence labeling of mortalin, a member of the heat shock 70 proteins, to show different staining patterns in normal and transformed cells.[24] However, this work mainly focused on the remarkable photostability of Streptavdin-QDs 605 conjugates which show a somewhat lack of specificity and probably bound with other proteins. Later, GlcNAc modified CdTe QDs were synthesized through a mercapto exchange process using thiol-GlcNAc and used for tracking Hsp70 proteins. This method was based on the premise that a complicated organic reaction is needed to acquire biocompatible and water-soluble thiol-GlcNAc.[25] Thus, it is highly desirable to establish simple and effective protocols to modify the QDs with the biorecognition molecules as fluorescent reporters for the specific identification of Hsp70 proteins.

[a]*State Key Lab of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing, 210093, P.R. China. E-mail: jjzhu@nju.edu.cn; Fax: +86-25-83597204; Tel: +86-25-83597204*

[b]*School of the Environment, Jiangsu University, Zhenjiang 212013, P.R. China*

[c]*Nanomaterials Laboratory, Inorganic and Physical Chemistry Division, CSIR-Indian Institute of Chemical Technology, Tarnaka, Hyderabad, 500607, India*

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Herein, we designed a protein-targeted fluorescence probe by incorporating GlcNAc onto Con A-conjugated CdSeTe QDs *via* specific binding affinity between lectins and carbohydrates. The obtained QD-Con A-GlcNAc conjugates have a maximal emission wavelength at 650 nm close to the near-infrared (NIR) regions and exhibit specific a binding capability to Hsp70 proteins for their detection.

Experiments

Materials

Selenium powder (Se, 99.5%, 200 mesh) and tellurium powder (Te, 99.8%, 200 mesh) were purchased from Acros Organics (New Jersey, USA). Cadmium chloride was purchased from Tingxin Chemical Reagent (Shanghai, China). Sodium borohydride was purchased from Tianjin Chemical Research Institute (Tianjin, China). *N*-Acetylglucosamine (GlcNAc), Concanavalin A (Con A), L-cysteine (98%), Trixton X-100, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), and *N*-hydroxysuccinimide (NHS), were purchased from Sigma-Aldrich. 4′,6-Diamidino-2-phenylindole (DAPI), mouse primary polyclonal anti-Hsp70, FITC-labeled goat anti-mouse secondary antibody and fluorsafe mounting medium were purchased from Beyotime Institute of Biotechnology (Nantong, China). Phosphate buffered saline (PBS, 0.01 M, pH 7.4) contained 136.7 mM NaCl, 2.7 mM KCl, 8.7 mM Na_2HPO_4 and 1.4 mM KH_2PO_4. All other reagents were of analytical grade and used without further purification. Ultrapure water with 18.2 MΩ cm^{-1} (Millipore Simplicity, USA) was used throughout the experiments.

Synthesis of the L-cysteine-capped CdSeTe QDs

L-Cysteine-capped CdSeTe QDs were synthesized as reported previously.[26] Briefly, 0.25 mmol of $CdCl_2$ and 0.6 mmol of L-cysteine were dissolved in 200 mL water and the pH adjusted to 11.5 with 1 M NaOH. The precursor solution was then loaded into a three-necked flask clamped in a heating mantle. The solution was heated to 95 °C under argon flow to obtain a colorless solution. At this temperature, 2.0 mL of fresh NaHTe aqueous solution prepared from $NaBH_4$ and Te powder (0.125 mmol) was injected into the reaction system under vigorous stirring; the solution was then refluxed at 95 °C. After heating for 0.5 h, the freshly prepared sodium hydroselenide (NaHSe) solution after premixing with an appropriate amount of L-cysteine was added into the CdTe precursor solution. The solution pH value was then adjusted to 11.5 with an appropriate amount of 1 M NaOH solution. Further nucleation and growth of the QDs proceeded upon refluxing the solution at 95 °C under open air conditions with a reflux condenser for 6 h. The as-prepared CdSeTe QDs were precipitated and washed with 2-propanol. The QDs were dried overnight at room temperature in a vacuum. The final product in powder form could be resuspended in water.

Preparation of QDs-Con A-GlcNAc conjugates

Con A was conjugated with carboxyl-terminated QDs *via* an EDC/NHS-mediated reaction. First, 100 μL of 5 μM QDs solution was added into 800 μL PBS solution containing 2 mM EDC and 5 mM NHS, and was incubated for 30 min at room temperature. Then 100 μL of Con A (1 mg mL^{-1}) was added to this mixture and reacted for 12 h at 4 °C. The QDs-Con A conjugates were ultrafiltrated using Millipore filtration tube (CMW = 100 kDa) at 5000 *g* for 10 min at 4 °C to remove the non-conjugated QDs and by-product. The concentrated QDs-Con A conjugates were dispersed in 100 μL PBS. 100 μL of the resultant QDs-Con A was dissolved in 800 μL Tris–HCl (0.1 mM, pH 7.4) containing 0.1 mM Ca^{2+} and 0.1 mM Mn^{2+}. Then 100 μL of 100 mM GlcNAc was added. After incubation for 2 h at 37 °C, free GlcNAc, Ca^{2+} and Mn^{2+} were removed by ultrafiltration with Millipore filtration tube (CMW = 100 kDa) at 5000 *g* for 30 min. For control experiments, QDs-Con A-mannose was prepared by the same procedure.

Fluorophore-labeled HeLa cells and confocal fluorescence imaging

The HeLa cells were cultured on a circular cover slip at a seeding density of 10 000 cells per well in a DMEM high glucose medium, which contained 10% fetal calf serum, L-glutamine (2 mM), penicillin (100 units mL^{-1}), and streptomycin (100 mg mL^{-1}). After 24 h incubation in 5% CO_2 at 37 °C, cells were then fixed with 4% (w/v) paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 for 5 min at room temperature. They were washed with 0.01 M PBS (pH 7.4) three times and blocked with 1% BSA in PBS for 1 h. 250 μL solution of QDs-Con A-GlcNAc (1 μ mol) containing ATP at a concentration of 300 μg mL^{-1} was then added to each cover slip (prefixed and blocked as mentioned above), and allowed to incubate at 37 °C for 2 h. Slips were counterstained with 4-6-diamidino-2-phenylindole (DAPI). Afterward, the cells were washed three times with 1 mL of 1% Tween 20-PBS buffer and fixed to glass slides with fluorsafe mounting medium. The excitation of QDs-Con A-GlcNAc was performed under an excitation wavelength of 488 nm from an argon laser while the excitation of DAPI stained chromosomal DNA was performed under an excitation wavelength of 405 nm from a diode laser with a confocal laser scanning microscope. The control experiments were carried out under the same conditions.

Apparatus

The fluorescence spectra were obtained with an Edinburgh FLS920P fluorescence spectrometer (Edinburgh Instruments Ltd, UK). The optical absorption spectra were measured using a Shimadzu 3600 UV-vis spectrometer (Shimadzu, Japan). Fourier transform infrared (FT-IR) spectra were recorded on a Nicolet 6700 FT-IR spectrometer using KBr pressed disks. The TEM images were acquired using a JEOL JEM-2100 (JEOL, Japan) transmission electron microscope operating at an acceleration voltage of 200 kV. Confocal laser scanning microscopy (CLSM) studies were performed using a Leica TCS SP5 microscope (Germany).

Results and discussion

Characterization of QDs-Con A-GlcNAc conjugates

CdSeTe QDs stabilized by L-cysteine were synthesized according to the reported method and characterised by their UV-vis spectrum, fluorescence spectrum and transmission electron microscopy. Fig. 1A presents the absorption and fluorescence spectrum of the prepared CdSeTe QDs. The CdSeTe QDs in aqueous solution show three absorption bands and have a maximum emission wavelength at 626 nm. The high-resolution transmission electron microscopy (HRTEM) image revealed an average diameter of 5 nm as shown in Fig. 1B. The close-up in the inset of Fig. 1B shows lattice planes. In the case of DLS measurement, as shown in Fig. S1, ESI,† the mean size of the QDs has a narrow distribution of 4 ± 2 nm. The obtained CdSeTe QDs have excellent water solubility, stability and a high quantum yield of 0.5.

Moreover, to achieve an active protein-targeting probe, QDs were modified by Con A to enable the conjugation of target molecules GlcNAc. The process was as shown in Fig. 2A, Con A was covalently attached to L-cysteine-coated QDs using NHS and EDC as cross-linkers. Carboxyl groups in the L-cysteine were activated to react with amine groups in Con A *via* amide reaction. GlcNAc was linked to QDs-Con A according to the strong affinity between GlcNAc and Con A, a saccharide-binding lectin from jack beans, which shows reversible strong affinity for non-reducing α-D-mannose, α-D-glucose, *N*-acetylglucosamine and polysaccharide with unmodified hydroxyl groups at C-3, C-4 and C-6.[27,28] To verify the successful conjugation, UV-vis and FTIR measurements were carried out. The presence of the typical peak of Con A at 280 nm in the absorption spectrum indicated the successful covalent attachment of Con A to the QDs as shown in Fig. 2B. It was also confirmed by the presence of two amide bonds (I and II) at 1650 cm^{-1} and 1541.1 cm^{-1}, which are characteristics of amides mainly in protein, as shown in Fig. 2C.[29,30] After the QDs were modified by GlcNAc, the peak of GlcNAc at 1400 cm^{-1}, corresponding to the bond of CO–NH, can be observed.

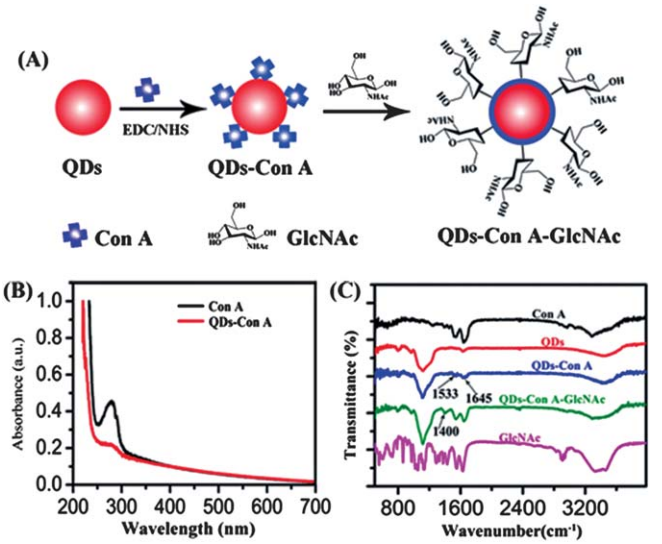


Fig. 2 (A) Schematic illustration of the preparation of QDs-Con A-GlcNAc. (B) UV-vis absorption spectra of QDs-Con A. (C) IR spectrum of QDs, Con A, QDs-Con A, and QDs-Con A-GlcNAc, respectively.

Furthermore, compared with the original QDs solution, the emission peak of the QDs-Con A and QDs-Con A-GlcNAc conjugates each undergoes a distinct change due to the modification of Con A and GlcNAc as illustrated in Fig. 3. The reason might be due to the increase of QDs-Con A-GlcNAc sizes after the modification (Fig. S1, ESI†).[31] The emission peak positions were red-shifted from 626 to 650 nm, close to the near-infrared (NIR) region (650–900 nm), allowing deep photon penetration as a result of the relatively high transparency, reduced scattering, and low autofluorescence of biological tissues.[32–35] In addition, QDs-Con A-GlcNAc still retain a high quantum yield of 0.32. Therefore, the as-prepared QDs-Con A-GlcNAc can be used as a promising candidate for versatile fluorescent probes in biological research.

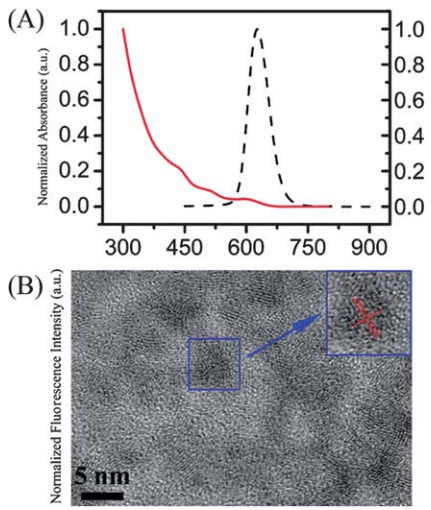


Fig. 1 (A) Absorption spectra (solid lines) and fluorescence spectra (dashed lines) of CdSeTe QDs. (B) HRTEM images of CdSeTe QDs.

Immunofluorescence labeling of Hsp70-expressed cells

The cell targeting and luminescence imaging of the GlcNAc bioconjugated QDs-Con A were explored with HeLa cells that

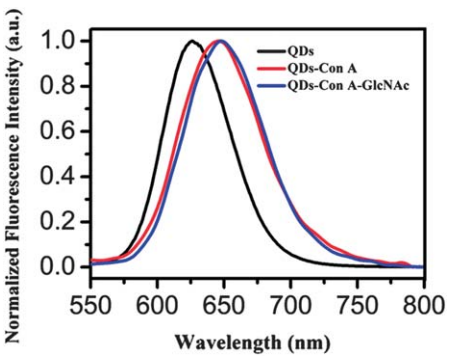


Fig. 3 Fluorescence spectra of QDs, QDs-Con A and QDs-Con A-GlcNAc.

were a representative Hsp70-overexpressed cell line. The mechanism was as illustrated in Scheme 1. HeLa cells were firstly pretreated with 4% (w/v) paraformaldehyde to fix the proteins and Triton X-100 to increase the cell permeabilization so that the QDs-Con A-GlcNAc can enter into cells across the hole of the cell membrane and specifically interact with Hsp70 protein mainly distributed in the cytoplasm and nucleus surface of cells *via* the recognition action of GlcNAc for Hsp70 proteins. The N-terminal domain of Hsp70 protein possesses ATPase activity and the activity of Hsp70 proteins is ATP-dependent. Therefore, ATP was added to activate the activity of Hsp70 coupling with the QD-Con A-GlcNAc.

In order to confirm the targetability of the QDs-Con A-GlcNAc, confocal microscopy observation was used to evaluate the cell association of the QDs-Con A-GlcNAc by Hsp70-positive HeLa cells. And non-active targeted QDs such as bare QDs were chosen as the negative controls. As shown in Fig. 4, cells treated with L-cysteine QDs fluorescence cannot be observed after BSA and Tween 20-PBS were added to avoid non-specific interactions of L-cysteine QDs with the cells. In contrast, QDs-Con A-GlcNAc showed a high fluorescence, indicating that the QDs labeled with the GlcNAc can specifically and tightly target the cells through the interaction between Hsp70 and GlcNAc and cannot be displaced by BSA and washed away by Tween 20-PBS. It is quite obvious that cells can be effectively stained by the QDs-Con A-GlcNAc and exhibit bright red fluorescence that distributed in the cytoplasm and relatively weak purple fluorescence that is the co-localization of QDs with the DAPI signal surrounding the nucleus, indicating that Hsp70 proteins were located in both the cytoplasm and outside the cell nuclei surface, consistent with the previous researches.[21] Meanwhile, we use mouse primary polyclonal anti-Hsp70 and FITC-labeled goat anti-mouse secondary antibody, coupled with QDs-Con A-GlcNAc, to detect the Hsp70 protein. As shown in Fig. 5, the co-localization of red fluorescence (QDs-Con A-GlcNAc) and green fluorescence (FITC-IgG) was observed, indicating that QDs-Con A-GlcNAc can target the Hsp70 protein. An additional control experiment was carried out by incubating

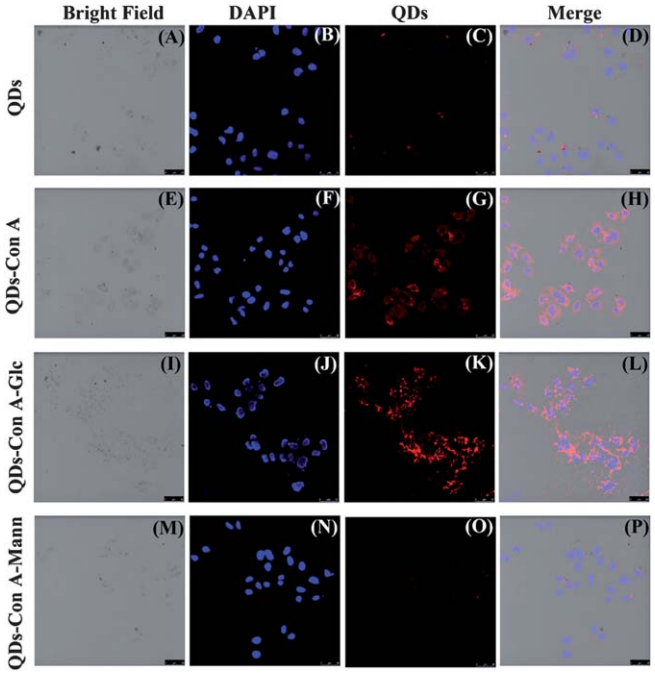
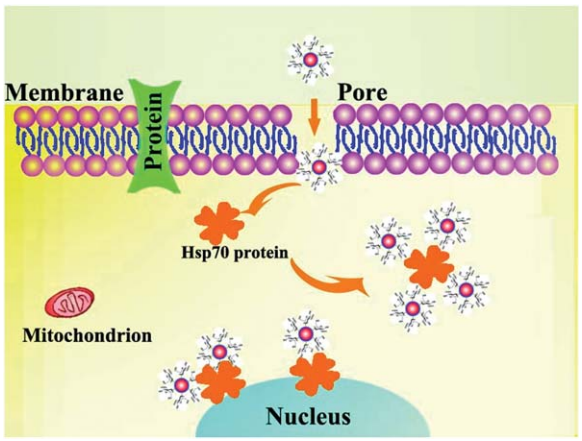


Fig. 4 Specificity of QDs probes to detect HSP70 proteins in fixed HeLa cells. (A)–(D) QDs alone, (E)–(H) QDs-Con A conjugate, (I)–(L) QD-Con A-GlcNAc, (M)–(P) QD-Con A-mannose. Blue fluorescence shows DAPI stained nuclei and red fluorescence shows the localization of QDs. The scale bar: 50 μm.

QDs-Con A-mannose with HeLa cells. Cells cannot be effectively stained by QDs-Con A-mannose and bright red fluorescence cannot be exhibited, further indicating the specific affinity of Hsp70 for GlcNAc. Unfortunately, cells treated with QDs-Con A also showed high red fluorescence because of the affinity between Con A and the cell surface carbohydrate. To confirm the target binding of QDs-Con A-GlcNAc from the presence of GlcNAc rather than Con A, a competitive inhibition assay was performed. The HeLa cells were incubated with the nanovehicles and free Con A in the medium (Fig. S2, ESI†). As expected, the binding of QDs-Con A is effectively suppressed in the presence of Con A at a concentration of 50 μg mL^{-1} while the QDs-Con A-GlcNAc conjugate is not suppressed. One possible explanation is that the cell surface carbohydrate of the Con A is hindered to lead to a decrease of the binding for QDs-Con A. Moreover, cells incubated with QDs-Con A-GlcNAc showed no fluorescence as well in the presence of GlcNAc because the binding sites were blocked. Therefore,



Scheme 1 Schematic illustration of the mechanism of Hsp70-positive HeLa cells labeled with QDs-Con A-GlcNAc.

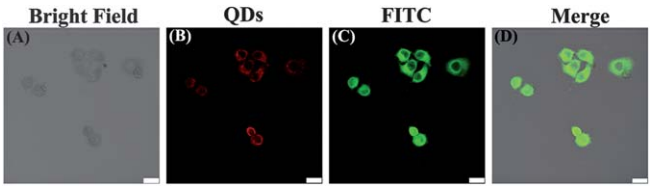


Fig. 5 CLSM of HeLa cells after incubation with QD-Con A-GlcNAc and anti-Hsp70 antibody/IgG-FITC. The scale bar: 25 μm.

QDs-Con A-GlcNAc was a excellent candidate for detecting Hsp70 proteins.

Conclusions

In summary, a simple and convenient strategy was developed to synthesize GlcNAc-decorated QDs that can generate a conjugate capable of targeting disease-related proteins, Hsp70 proteins. Con A was covalently attached to QDs through an EDC/NHS-activated amide reaction, while GlcNAc was subsequently coupled through the specific interaction between Con A and the carbohydrates of GlcNAc. In comparison with the previous research, the modified QDs maintain excellent optical properties with a longer emission wavelength at 650 nm close to the NIR range. More importantly, the biofunctionalized QDs specifically labeled Hsp70 proteins, making them a promising candidate as an attractive bioprobe for application in biological research.

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