

Insulin-based regulation of glucose-functionalized nanoparticle uptake in muscle cells†

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Effective regulation of nanoparticle (NP) uptake facilitates the NP-based therapeutics and diagnostics. Here, we report the use of insulin and 2-deoxyglucose (2-DG) to modulate the cellular uptake of glucose-functionalized quantum dots (Glc-QDs) in C2C12 muscle cells. The cellular uptake of Glc-QDs can be modulated up to almost two-fold under insulin stimulation while be down-regulated in the presence of 2-DG. These results demonstrate the use of secondary regulators to control the cellular uptake of NPs through membrane protein recognition in a specific and fine-tunable fashion.

Introduction

Regulating cellular uptake of nanoparticles (NPs) in biological systems is critical for the development of NP-based therapeutics. NP delivery vehicles can increase delivered doses of drugs and imaging agents, while diminishing undesired off-target effects. NPs with tailorable structures provide a potential means of controlling therapeutic efficacy¹ as well as concomitant toxicity.² For example, the surface charge and hydrophobicity of NPs determine their cellular uptake, facilitating the NP-assisted intracellular delivery³ and bioimaging.⁴ In particular, targeting motifs on the NP surface can effectively guide them to cells through membrane protein recognition, providing receptor/transporter-mediated cellular uptake⁵ that can be modulated by external chemicals or biomolecules.⁶

Glycomaterials are important nanoplatfoms in biomedical applications,⁷ where the carbohydrate motifs allow these materials to be solubilized under physiological conditions⁸ and recognized by cell membrane proteins.⁹ For example, galactose-functionalized NPs can be selectively accumulated in hepatocellular carcinoma cell line HepG2 that expresses asialoglycoprotein receptor (ASGP-R).¹⁰ Also, 2-deoxyglucose-functionalized NPs can target mammary tumor cells¹¹ and migrate across the blood brain barrier¹² where glucose transporters (GLUTs) are highly expressed. The use of carbohydrate-functionalized NPs for active targeting depends on the level of receptors and transporters that are in a state of flux, providing a potential

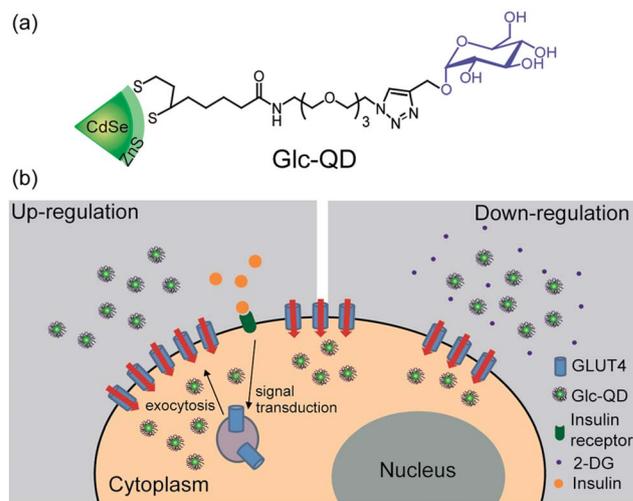
strategy to control the cellular uptake of carbohydrate-functionalized NPs through modulation of ASGP¹³ and GLUT¹⁴ levels.

Here, we report the use of insulin to modulate the cellular uptake of glucose-functionalized quantum dots (Glc-QDs) through control of the GLUT4 level on the membrane of C2C12 muscle cells (Scheme 1). The cellular uptake of Glc-QDs was investigated in both non-differentiated and differentiated muscle cells, where the Glc-QD uptake was enhanced in differentiated cells by insulin stimulation. We also demonstrated that the uptake efficiency of Glc-QDs can be inhibited in the presence of the competitive molecule 2-deoxyglucose (2-DG). Therefore, both insulin and 2-DG act as regulators to control the cellular uptake of Glc-QDs in a specific fashion, demonstrating the potential of secondary regulators for tuning the cellular uptake of NPs. Glucose-functionalized NPs can

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† Electronic supplementary information (ESI) available: Synthesis of glucose-terminated ligands, characterization of Glc-QDs (*i.e.* mass spectrum, emission spectrum and DLS data) and negative controls (*i.e.* PEG-QDs and TMA-QDs) for the insulin effect on regulating QD uptake in differentiated C2C12 cells. See DOI: 10.1039/c4tb00608a

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Scheme 1 (a) Molecular structure of glucose-functionalized quantum dot (Glc-QD). (b) Schematic illustration of the cellular uptake of Glc-QDs regulated by insulin and 2-deoxyglucose (2-DG) in C2C12 muscle cells.

potentially be applied for GLUT4 targeting as well as controllable NP uptake in GLUT4-expressing cells such as adipocytes, skeletal and cardiac muscle cells.

Results and discussion

Glucose-conjugated ligands presenting dithiol anchoring groups were synthesized for the surface functionalization of QDs featuring (1) dihydrolipoic acid (DHLLA) as a stable bidentate anchor,¹⁵ (2) a tetra(ethylene glycol) (TEG) spacer to minimize non-specific interactions with proteins and cells,¹⁶ and (3) a glucose headgroup conjugated to the ligand through azide-alkyne cycloaddition “click chemistry” (Scheme 1a). CdSe/ZnS core-shell QDs were used to prepare glucose-functionalized QDs (Glc-QDs) through a ligand exchange process. (See ESI† for the Glc-QD synthesis and characterization.) The emission peak of Glc-QDs was observed at 555 nm and the dynamic light scattering (DLS) data showed that the hydrodynamic size of Glc-QDs was *ca.* 8 nm (Fig. S2, ESI†).

The regulation of Glc-QD uptake was investigated in C2C12 cells, a widely used skeletal muscle cell line. It is well-established that insulin stimulates the GLUT4 translocations from intracellular storage compartments to the cell membrane to enhance the glucose uptake in C2C12 cells.¹⁷ Both non-differentiated and differentiated cells were cultured for Glc-QD uptake studies through the choice of culture media. The morphology of C2C12 cells was changed from spindle-shaped myoblasts (Fig. 1a) to fiber-shaped myotubes (Fig. 1b) after differentiation.¹⁸ The cellular uptake and intracellular distribution of Glc-QDs can be visualized using confocal microscopy. Glc-QDs were internalized and punctate fluorescence was observed in the confocal images, consistent with the expected entrapment of Glc-QDs in endosomal/lysosomal compartments after cellular uptake.¹⁹ In parallel, the cytotoxicity of Glc-QDs was determined through an alamarBlue[®] assay, where the results showed no significant toxicity was observed up to 1 μ M of Glc-QDs in both non-differentiated and differentiated cells after incubation for 4 h (Fig. 2).

The cellular uptake efficiency of Glc-QDs was next investigated under insulin stimulation. Flow cytometry was used to quantify the uptake efficiency of Glc-QDs based on the fluorescence properties of QDs. Both non-differentiated and

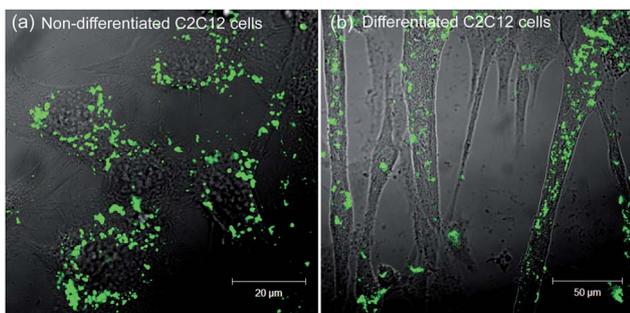


Fig. 1 Confocal images of Glc-QDs (250 nM) in (a) non-differentiated and (b) differentiated C2C12 cells after incubation for 4 h.

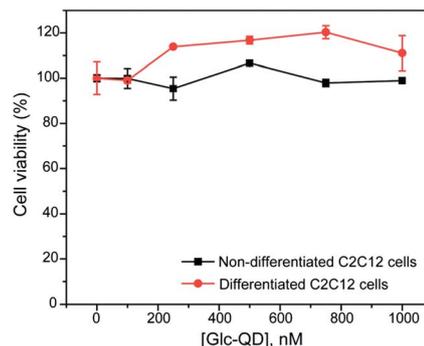


Fig. 2 Cytotoxicity studies of Glc-QDs in both non-differentiated and differentiated C2C12 cells after incubation for 4 h.

differentiated cells were treated with insulin for Glc-QD uptake studies. In these studies, a substantial increase of Glc-QD uptake was observed in differentiated cells in an insulin dose-dependent manner, and the cellular uptake of Glc-QDs can be over an almost two-fold range (Fig. 3a and b). However, there was no noticeable change of Glc-QD uptake in non-differentiated cells under the insulin-stimulated conditions (Fig. 3c and d).

It has been reported that GLUT4 is expressed in differentiated C2C12 cells but not in non-differentiated C2C12 cells.²⁰ The presence of insulin can increase the level of GLUT4 on the cell membrane of differentiated C2C12 cells to facilitate the cellular uptake of glucose.²¹ Therefore, the enhanced Glc-QD uptake under insulin stimulation is presumably due to the sufficient GLUT4 level in differentiated C2C12 cells. These

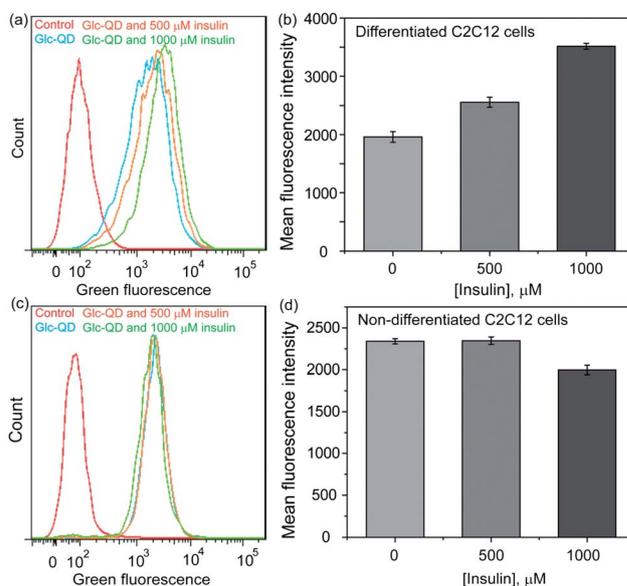


Fig. 3 Flow cytometric analysis of Glc-QD uptake in differentiated and non-differentiated C2C12 cells. Fluorescence intensity histogram and correlative mean fluorescence intensity of Glc-QDs in the presence of different amounts of insulin in (a and b) differentiated and (c and d) non-differentiated C2C12 cells. Glc-QDs (250 nM) were incubated in C2C12 cells for 4 h with the treatment of insulin.

results also indicated that the glucose motifs on the QD surface can be recognized by GLUT4, and the Glc-QD uptake can be fine-tuned through the control of the insulin-responsive GLUT4 level on the cell membrane.

This insulin effect on regulating Glc-QD uptake was further confirmed with negative controls including QDs with different functionalities (*i.e.* galactose-functionalized QDs (Gal-QDs), polyethylene glycol-functionalized QDs (PEG-QDs) and trimethylammonium-functionalized QDs (TMA-QDs)) and different cell lines (*i.e.* NIH/3T3 cells, mouse embryonic fibroblast cells). From these control studies, no noticeable change of the fluorescence distribution was observed with insulin treatment (Fig. 4 and S3, ESI†), indicating the specificity of the insulin stimulation in increasing the cellular uptake of Glc-QDs in differentiated C2C12 muscle cells.

Down-regulation of Glc-QD uptake was investigated by the treatment of 2-deoxyglucose (2-DG), a glucose analog that has been used to decrease the glucose uptake inside the cell.²² In the presence of 2-DG, greatly reduced uptake of Glc-QDs was observed in differentiated cells (Fig. 5). Again, these results demonstrate that the glucose motifs on the QD surface can be recognized by GLUT4 and 2-DG becomes the competing molecule to inhibit the cellular uptake of Glc-QDs.

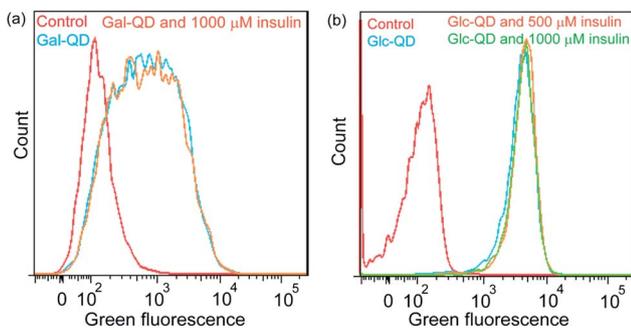


Fig. 4 (a) Galactose-functionalized QDs (Gal-QDs) were incubated in differentiated C2C12 cells for 4 h with the treatment of insulin. (b) Glc-QDs were incubated in NIH/3T3 fibroblast cells for 4 h with the treatment of insulin. The concentration of QDs used in these experiments was 250 nM.

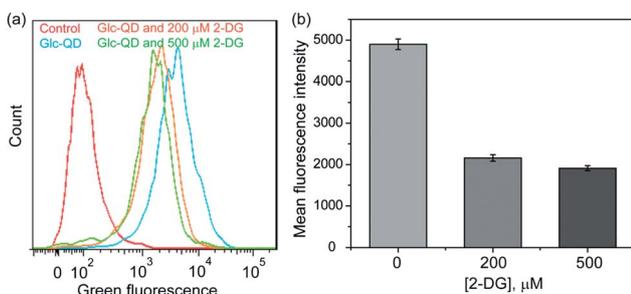


Fig. 5 (a) Fluorescence intensity histogram and (b) correlative mean fluorescence intensity of Glc-QDs in the presence of different amounts of 2-DG in differentiated C2C12 cells. Glc-QDs (250 nM) were incubated in C2C12 cells for 4 h with the treatment of 2-DG.

Conclusions

We have demonstrated that the glucose motifs on the QD surface coupled with secondary regulators provide a means of modulating nanoparticle uptake by muscle cells. The cellular uptake of Glc-QDs can be up-regulated through insulin stimulation and down-regulated in the presence of 2-DG. These findings provide insight for the design of surface-functionalized NPs that can use cellular signalling to provide controllable uptake systems for use in the intracellular delivery and imaging applications.

Experimental section

General

All chemicals used for the fabrication of QDs were purchased from Sigma-Aldrich, Fisher Scientific or Stream, unless otherwise specified. The chemicals were used as received. Dichloromethane (DCM) used as solvent for chemical synthesis was dried according to the standard procedure. ¹H NMR spectra were recorded at 400 MHz on a Bruker AVANCE 400. Fluorescence from the alamarBlue[®] assay was detected using a SpectraMax M5 microplate reader (Molecular Device Inc., USA). Dynamic light scattering (DLS) measurement was performed using a Malvern Zetasizer (Nano Series, Malvern Instruments Inc., USA) and the data analysis was done using Malvern PCS software. Flow cytometry analysis was performed in a BD LSR-II flow cytometer equipped with FACSDiva (BD Sciences, USA) by counting 10 000 events, and the cell debris were excluded from analysis by proper dot plot gating.

Fabrication of QDs

QDs were decorated with glucose-functionalized ligands by following the reported two-step procedure.¹⁵ In the first step, trioctylphosphine oxide/trioctylphosphine (TOPO/TOP) coated QDs (10 mg) were mixed with HS-C5-TEG ligands (30 mg) in methanol (MeOH, 5 mL). The reaction mixture was stirred at 35 °C for 24 h under an inert atmosphere. In this step, amphiphilic ligands replaced the native hydrophobic ligands from the surface of QDs, and the resulting amphiphilic QDs became soluble in MeOH. The next step involved the purification of QDs with hexane and addition of glucose-functionalized ligands (30 mg) to the amphiphilic QDs in MeOH. As a result, dithiol ligands slowly substituted monothiol ligands from the QDs surface due to their better chelating capability. After 24 h of stirring, MeOH was evaporated and QDs were dispersed in water. Finally the aqueous QD sample was purified by dialysis. Gal-QDs were prepared by following the same procedure as Glc-QDs. The syntheses of PEG-QDs and TMA-QDs were followed as in the reported literature.^{23,15}

Cell culture

C2C12 muscle cells and NIH/3T3 fibroblast cells (American Type Culture Collection (ATCC)) were cultured at 37 °C, 5% CO₂ in a humidified atmosphere. C2C12 cells were grown in the high glucose Dulbecco's Modified Eagle's Medium

(DMEM) supplemented with 10% fetal bovine serum and 1% antibiotics (100 U mL⁻¹ penicillin and 100 mg mL⁻¹ streptomycin). NIH/3T3 cells were grown in the high glucose DMEM supplemented with 10% bovine calf serum and 1% antibiotics. Differentiation of C2C12 cells was followed as in the reported procedure.²⁴ Briefly, cells were transferred to a 24-well plate (6 × 10⁴ cells per well) and incubated for 48 h in growth media to about 100% confluence and then switched to differentiation media. Differentiation medium was prepared in low glucose DMEM supplemented with 2% horse serum and 1% antibiotics. The differentiation medium was changed when a significant amount of cell death/floating or cell debris was present. Large multinucleated myotubes would be visible after C2C12 cells were cultured in differentiation medium for 4 days.

Confocal microscopy studies of Glc-QDs in C2C12 cells

C2C12 cells were seeded in glass-bottomed dishes (1.8 × 10⁵ cells per dish, MatTek Corporation, 14 mm microwell) 24 h prior to the confocal experiment. On the next day, the old medium was removed and cells were washed with cold phosphate buffered saline (PBS). Glc-QD solution (250 nM in low glucose DMEM) was incubated with cells for 4 h. Thereafter, cells were washed with PBS three times before taking the images. Confocal microscopy images were obtained on a Zeiss LSM 510 Meta microscope by using a 63 × objective. Settings of the confocal microscope: green channel of QD: λ_{ex} = 488 nm and λ_{em} = BP 505–550 nm (BP: band pass).

Cytotoxicity study

Cytotoxicity was determined by an alamarBlue[®] assay (Invitrogen Biosource, USA), which is based on the conversion of resazurin to resorufin *via* a reduction reaction dependent on cellular metabolic activity.²⁵ Non-differentiated and differentiated cells were cultured in a 24-well plate (6 × 10⁴ cells per well). The cells were incubated with Glc-QD solutions (various concentrations in low glucose DMEM) for 4 h. After incubation, cells were washed with PBS and treated with 10% alamarBlue[®] dye in low glucose DMEM with 10% fetal bovine serum (500 μL per well) for 2 h. The fluorescence intensity was recorded using a SpectroMax M5 microplate reader to determine cell viability. Settings of the microplate reader: λ_{ex} = 560 nm, λ_{em} = 590 nm.

Insulin and 2-DG treatment

Non-differentiated and differentiated cells were cultured in a 24-well plate (6 × 10⁴ cells per well). The cells were incubated with low glucose DMEM for 1 h as in the serum starvation process. Different amounts of insulin or 2-DG were prepared in glucose-free DMEM to be incubated with cells for 30 min. Glc-QDs (250 nM) were prepared along with different amounts of insulin or 2-DG in low glucose DMEM and incubated with cells for 4 h before the flow cytometry measurement.

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