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Modulating the Glucose Transport by Engineering Gold Nanoparticles

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Abstract

Gold Nanoparticles (GNP) have been widely used as a delivery vehicle for the targeted release of the drugs, vaccine and therapeutics. Literature evidence indicates that besides acting as a carrier, gold nanoparticles can alter the uptake mechanism of a targeting agent to the cancer cell. Therefore, designing a carrier plays a critical role to modulate various internalization pathways of a cargo. Given the role of malfunctioning reporter in various Mendelian diseases, it will be important to come up with a synthetic bio-mimetic transporter that can optimize the transport deficit. Glucose transport is impaired in hyperglycemia and glucose possibly faces identity crisis to enter into the cell. Using GNP for transport management of various metabolites is emerging. We have reported a design, synthesis and *in vitro* demonstration of an artificial Nano Glucose Transporter (NGT) to capture extracellular glucose and carry them to the cell when added exogenously, independent of endogenous cellular transporter pathways. This strategy could be extended for future application to the transport problems where extracellular biologic and metabolite burdens could be potential threat to the physiology.

Keywords: Artificial glucose transporter; Gold nano particles; Nano delivery; Design; Mammalian cell; Hyperglycemia

Introduction

Designing nanoconstruct with therapeutic agents for biomedical purposes has gained tremendous application for drug delivery applications. Using monoclonal antibody (mAB, such as Herceptin, Cetuximab), peptide (Bombesin, Arginylglycylaspartic acid (RGD) and other small molecule drugs for attaching to the nanoparticles are among the few of many application by GNPs assisted targeted drug delivery [1-3]. Gold nanoparticles is exclusively used by researchers as a carrier of several drugs, vaccines, and other payloads to release at site specific locations [4]. Recent report has demonstrated that based on the cargo design gold nanomaterial can alter the cellular uptake mechanism when used for targeted delivery of therapeutics [5,6]. Transporter defect can lead to various diseases caused by genetic alteration or mutation of the transporter protein [7]. Various transporters can be constructed with appropriate bioactive ligands to target biological systems where transporter defect is responsible for pathophysiological phenotypes [8]. Diabetics can be caused by several reasons, primarily for insufficient insulin production or insulin resistance. Insulin resistance can occurs even there is sufficient insulin production by islet cell, due to various defects in insulin signalling pathways [9]. In diabetics, the ultimate end point hallmarks the excess amount of blood glucose or extracellular glucose. This causes metabolic burden and glucotoxicity to various organs for non-clearance of excess glucose level due to disrupted transport [10]. Glucotoxicity generates reactive oxygen species (ROS), unfolded protein response, islet amyloid deposition, inflammation, hypoxia and many other pathological phenotypes while starving the cell from the fuel obtained from metabolism [10]. Additionally, hyperglycaemia can cause damage to the kidney (diabetic nephropathy), eyes (diabetic retinopathy) and other organs. High fat diet can also induce hyperglycaemia and obese people often develop diabetics as well [11]. This co-relation suggests that lipid and sugar transport to cell work either in a synergistic or in an antagonist fashion to each other. Defects in glucose transporters family are found to be primarily responsible for diminished pancreatic beta cell glycosylation [12,13]. Glut-4 (glucose transporter protein 4, solute carrier protein, SLC2A4) knockout mice spontaneously develop diabetics, indicating the role of impaired glucose transport, and a key cause of hyperglycaemia [14]. Hyperglycaemia is a metabolic disorder affecting health of millions of people across the world. Impaired glucose transport plays determining role in the progression of the diabetes and among them, Glut-1 to Glut-4 protein translocation are important for the maintenance of the glucose homeostasis [12]. Efforts to build a nanoscale transporter are emerging, even though the initiative requires additional efforts to come up with advancement. Using GNP to transport important biologics and metabolite to the cell is not well explored. Herein we report a design (Figure 1A), synthesis and *in vitro* evaluation of an artificial nano gold glucose transporter capable of carrying glucose to cell by an endogenous glucose transporter independent pathway. Our goal is to synthesize a bio-mimetic transporter at nano scale that can bypass the requirement of endogenous glucose transporter protein. As a proof of concept, we have imposed a different identity to the glucose, so that it can easily enter the cell without requiring its bonafide carrier protein, rather using different route for internalization to meet the possible metabolic demand of a starving cell.

Material and Methods

Cell culture and chemicals

Primary Pancreatic Cancer (PANC-1) cell was purchased from ATCC (American type culture collection) and cultured under standard mammalian tissue culture condition as per the instructed protocols. In brief, PANC-1 was grown in DMEM (Dulbecco's Modified Eagle's Medium) media with 10% FBS, 1% Penicillin and streptomycin antibiotics in complete media. Before, NGT mediated glucose transport experiment, high glucose DMEM and color free DMEM for fluorescent measurement were used in order to mimic a hyperglycemic situation. Glucose oxidase assay kit (Sigma), GSH (glutathione, sigma), Cytochalasin B (Sigma), $CT(PEG)_2$ (Carboxy-PEG12-Thiol) from (Thermo scientific), cell based glucose assay kit (Cayman chemicals), NBD labeled glucose (2-(N-(7-Nitrobenz-2-oxa-1,3-diazole-4-yl)Amino)-2-Deoxyglucose) from (Cayman chemicals), NaBH4 (Sodium borohydride), Benzoxaborole (5 amino-2-hydroxymethyl phenyl boronic acid, Sigma), Bioluminescent Enzylight TM ADP/ADP ration assay kit (ELDT-100), High Glucose DMEM, Glucose free DMEM were purchased.

Synthesis, DCC-NHS coupling reaction and characterization of the Nanoconjugate (NGT)

Naked gold particle was prepared and characterized as reported in our previous publications [5,6]. In brief, gold precursor salt (Sodium tetra chloroaurate (III) dehydrate, $NaAuCl_4$) solution, 2 mL (10 mM) was diluted in 1:100 mili Q water and $NaBH_4$ (13.6 mg) in 100 mL was added to the gold solution. The reaction color changed from purple to red wine with an indication of the formation of spherical shape 5 nm size GNP. The reaction mixture was stirred for 6 h at RT and then $CT(PEG)_2$ (30 mg) was added to the GNP solution. The resulting dark red solution was further stirred for overnight at room temperature. The solution was centrifuged at 80,000 g for 2 h at 10°C to purify the conjugate and collect the concentrated loose pellet. Resulting GNP-$CT(PEG)_2$ conjugates were diluted (1:5) with miliQ water and then 250 µM N,N'-Dicyclohexylcarbodiimide (DCC) and 250 µM N-Hydroxysuccinimide (NHS) were added. The resulting mixture was stirred for 1-2 h at RT in order to the formation of the complex and the Benzoxaborole (500 µM) was added to the reaction mixture and stirred for overnight. The solution was centrifuged and washed one more time with miliQ water in order to remove the unreacted reagents and purify the final GNP-PEG-Benzoxaborole (GNP-PEG-BRXL).

Stability of various NGT conjugates

To test the stability of the various NGT prepared, the constructs were added to the high glucose media and incubated for 3 h and overnight. The pictures were taken for the solution containing the nanoconjugate treated solution. Most of the conjugates were aggregated while GNP-GSH conjugates were stable up to 1.5 h. Only the gold PEG conjugates showed the integrity of the typical nanoparticle physical characteristics in terms of colors due to Surface Plasmon Resonance.

Glucose oxidase assay

The assay was carried out according to the manufacturer protocol. For test tube assay, color free high glucose media was used. Nanoconjugates (100 µM) were added to the media with untreated control and glucose standard. After 3 h incubation, the media was spanned down at ultra-centrifuge with 50,000 g for 30 minute at 15°C in order to separate the nanoparticle bound glucose from the free glucose in the media. After, separating nanogold pellet at the bottom, 10 µL of the supernatant was aliquoted and added to the test tube having 990 µL DI water. Reconstituted glucose oxidase assay mixture 2 mL was added to the 1.0 mL of the sample solution. Untreated control and known glucose standard (1 mg/mL) was used as positive control for quantification. After 30 minute incubation at 37°C, reaction was quenched with 12 N H_2SO_4 (sulfuric acid) which led to formation of orange solution. The final solution content was measured by UV-Vis at 540 nm and quantified as manufacturer protocol.

For cell based experiments, (1×10^5 cells/mL) was grown in 24 well cultured plates. Next day cell were serum starved for 4 h and then serum free, color free high glucose media was added to the cell. For inhibitor experiments, cells were pre-incubated with inhibitor cytochalasin B (120 µg/mL) about an hour. Then the nanoconjugate (50 µM) were added to the cultured well (1 mL) and incubated for 2 h. After 3h incubation, 10 µL of the media was withdrawn from the cultured media and added to the test tube having 990 µL DI water. Then the glucose oxidase assay was exactly carried out as described above. The glucose oxidase assay mixture was added, then reaction was quenched after 30 min and measured for absorbance at 540 nm.

Fluorescently labeled glucose uptake using confocal microscopy

PANC-1 cell (1×10^5 cell/mL) was grown in 4 well chamber slides under normal tissue culture conditions overnight. Next day cell were serum starved for 4 h with glucose free and color free DMEM media. Then, fluorescently labeled NBD glucose (150 µg/mL) were added in the chamber in presence of GNP-PEG-BRXL (50 µM) and inhibitor individually or co-incubation with appropriate control of each component. The cells were allowed to co-incubated with the treatments for 40 min in the incubator and the media was removed immediately after 1 h. Chamber was washed with cell based assay buffer 3X times and then fixed with 4% paraformaldehyde solution for 20 min at room temperature. The chamber was further washed with 1X PBS (phosphate buffered saline) for 3 times and then mounted in a mounting media containing DAPI (4',6-diamidino-2-phenylindole, nuclear binding stain) and attached with a cover slides.

Measurement of ADP/ATP ratio

PANC-1 cell (2×10^4) were seeded in 96 well tissue cultured treated clear bottom plate. Next day, cells were serum starved for 4 h in glucose free media and then supplemented with serum free high glucose media. Cells were then treated with NGT (50 µM) for 2 h compare to untreated control. Cellular media was changed after 2 h and glucose free starving media was added for additional one hour. To measure the ATP (adenosine tri phosphate) and ADP (adenosine di phosphate), we used Enzylight^{TM} ADP/ATP bioluminescent assay kit and followed the manufacture protocol. In brief, cells were treated with ATP reagent for 1 min containing (Assay buffer, substrate and ATP enzyme). The content of the cellular well was transferred into a tube to measure the luminescence (RLUA) in a luminometer tube. Ten minutes after the reading (RLUA), luminescence of the same samples were measured (RLUB) and this provide the background before measuring ADP. Then, ADP reagent (containing ADP enzyme) was added to each cellular content of the 96 well that was treated previously with ATP reagent. After 1 min, luminescence was recorded (RLUC) on

a luminometer and calculated ADP/ATP ratio according to the kit protocol as: ADP/ATP ratio=RLU C-RLU B/RLU A

Results and Discussion

To prepare NGT (Figure 1A), we have synthesized a series of NGT nanoconjugate with various ligands (Glutathione, Mercaptoundodecanoic acid, Triopronein, mercaptophenyl boronic acid, $CT(PEG)_2$ (carboxy-PEG_{12}-thiol) and then used DCC-NHS coupling (N,N'-Dicyclohexyl-carbodiimide and N-Hydroxy-succinimide) to install the glucose sensing chemical nose to the linker [15]. For the demonstration of the concept, we have used benzoxaborole compound (5 amino-2-hydroxymethyl phenyl boronic acid acid) as a glucose binding motif (Figure 1B) [16].

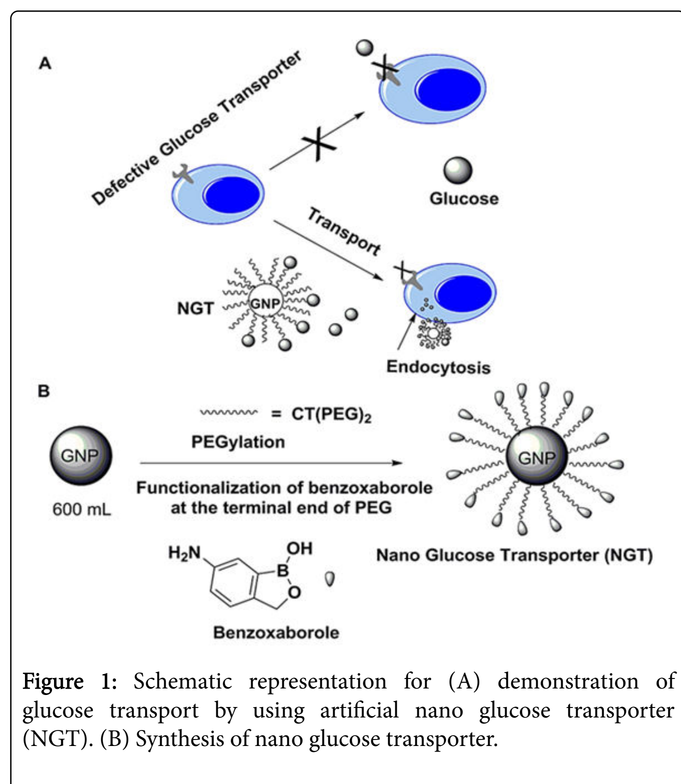

Figure 1: Schematic representation for (A) demonstration of glucose transport by using artificial nano glucose transporter (NGT). (B) Synthesis of nano glucose transporter.

The synthetic process is followed as described in our previously published literature (materials and method) [17]. After successful synthesis, the construct was characterized by using UV-Vis, Zeta potential and DLS measurement (Figure 2A and Table 1).

Compounds	λ_{max} (nm)	DLS (nm)	ζ (mV)
GNP-PEG	510	23.92	-17.6
GNP-PEG-BRXL	520	88.29	-44.2

Note: The DLS and zeta potential values are not average.

Table 1: Maximum absorbance (λ_{max}), dynamic light scattering (DLS) and zeta potential (ζ) of GNP-PEG and GNP-PEG-BRXL.

The λ_{max} shift of the surface Plasmon band (SPR) is observed in the UV-Vis spectra as surface of the naked gold particles are functionalized with PEG benzoxaborole ligands (Figure 2A).

We first evaluated the stability of the nanoconjugate in the high glucose media. We have found that the most of the NGT are unstable in the DMEM media except the gold-PEG conjugate. The majority of the nanoconjugate were aggregated in high glucose media within 30 min (glutathione conjugate survived about 1.5 h) except the gold PEG conjugate showed stability for prolonged time (Figure 2B). Thus, the stability of NGT conjugates for the other ligand was poor in the high glucose media except for the $CT(PEG)_2$ gold conjugate. PEG is well known for its non-immunogenicity and higher retention time during circulation when injected *in vivo* [18]. We continued with GNP-PEG-BRXL for further study as PEG has wider biomedical application and the PEG-NGT showed reasonable stability. We evaluated the glucose binding ability of the construct by using glucose oxidase colorimetric enzyme assay in test tubes. The functional assay shows the glucose carrying capacity of the nanoconstruct in test tube as evident from the decrease of the total glucose level (Figure 2C).

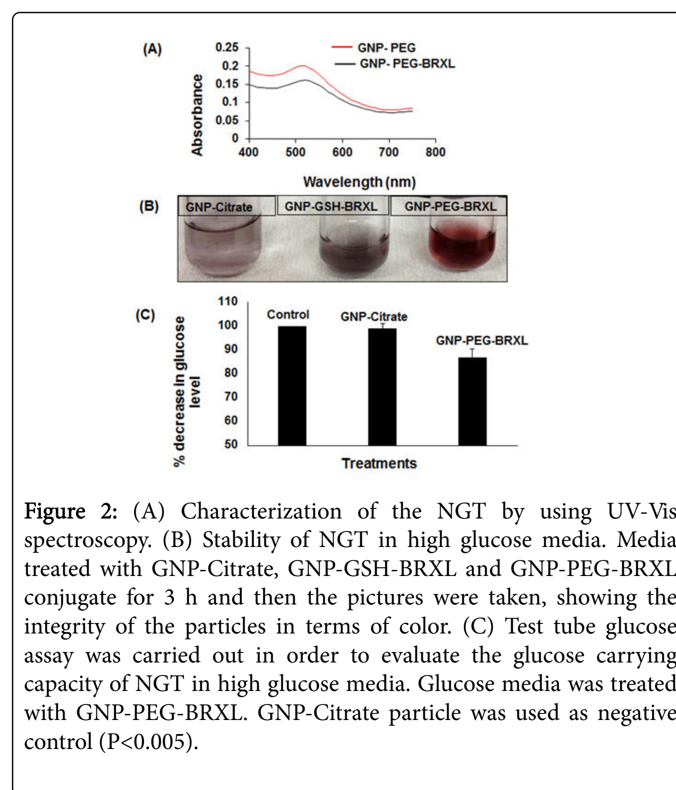

Figure 2: (A) Characterization of the NGT by using UV-Vis spectroscopy. (B) Stability of NGT in high glucose media. Media treated with GNP-Citrate, GNP-GSH-BRXL and GNP-PEG-BRXL conjugate for 3 h and then the pictures were taken, showing the integrity of the particles in terms of color. (C) Test tube glucose assay was carried out in order to evaluate the glucose carrying capacity of NGT in high glucose media. Glucose media was treated with GNP-PEG-BRXL. GNP-Citrate particle was used as negative control ($P<0.005$).

We found that GNP-PEG-BRXL can bind to glucose in the media and survived for long period of time in the solution as evident from the characteristic SPR color of the NP. Given a static system, this data shows the formation of GNP-PEG-BRXL glucose complex in addition to the functional potency of the NGT. We used gold citrate particles (5 nm sizes, spherical shape) as control in order to rule out if the biding of the glucose was gold mediated non-specific interaction between glucose and GNP. We found that glucose binding to the NGT is due to exclusive attachment of benzoxaborole grafted on PEG terminal. Our next goal was to determine whether it can carry the extracellular glucose inside the cell without requiring endogenous glucose transporter proteins. To test that, we have used cytochalasin B, a known inhibitor that block the endogenous glucose transporter mediated cellular glucose uptake [19,20]. To achieve that, we incubated the PANC-1 cell with the NGT construct for 3 h both in complete media and serum starved media (phenol red free high glucose media). The experiment was monitored by taking aliquot from the supernatant

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to measure the remaining glucose by using glucose oxidase assay. We observed a decrease (20%) of glucose level upon NGT treatment to the cell (Figure 3A). The window of glucose concentration change didn't vary much upon serum starvation. This shows the robustness of the NGT in complex cellular environment, thus potential for drug delivery application to modulate the therapeutic window. There was not much difference between the amount of glucose level in presence and absence of cytochalasin B. This result indicates that NGT is carrying glucose to the cell without using cellular transporter pathway. We observed that there is similar change in glucose content with half an amount of the NGT added in cellular assay compare to test tube assay. Next, we wanted to understand whether this alteration in glucose concentration upon NGT addition is just due to the chemical complex formation between glucose with NGT in the culture medium. Or this decrease of glucose concentration is due to the transport of the glucose by NGT from media to cell is independent of endogenous glucose transporter. To investigate that possibility, we seeded the cell in a 4 well chamber slide and grown overnight. Then cells were serum starved in glucose free and phenol red free media for 3 hour and glucose transport inhibitor (cytochalasin B) was pre-incubated in appropriate chamber for 1 h (60 µg/500 µL). Then, NBD-labeled glucose was added to the entire chamber having control, inhibitor and no inhibitor as well. After that NGT was added to the appropriate chamber without and with inhibitor. After 40 min incubation, cell were fixed and used for confocal imaging. There was a decrease in NBD-glucose uptake upon only inhibitor treatment while glucose was internalized to the cell upon NGT treatment even in presence of inhibitor (Figure 3B).

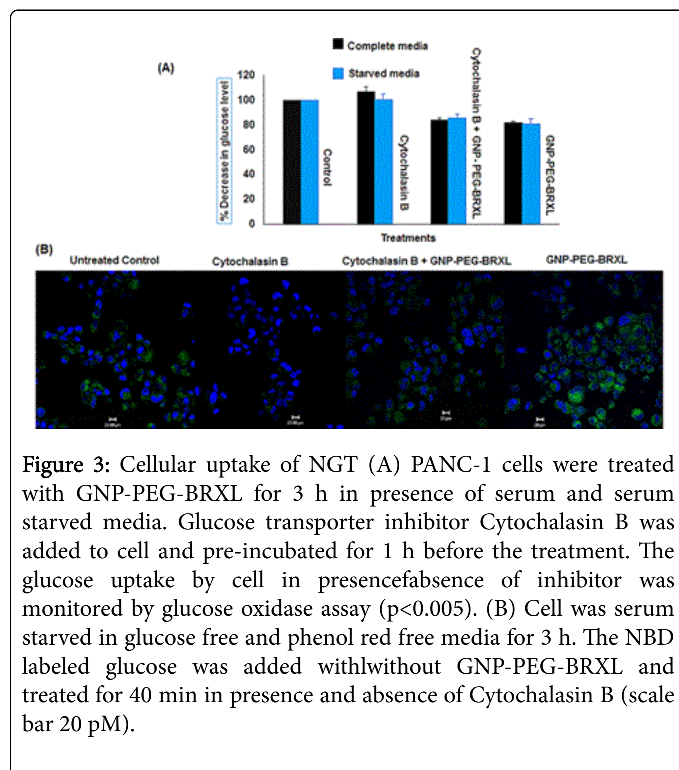

Figure 3: Cellular uptake of NGT (A) PANC-1 cells were treated with GNP-PEG-BRXL for 3 h in presence of serum and serum starved media. Glucose transporter inhibitor Cytochalasin B was added to cell and pre-incubated for 1 h before the treatment. The glucose uptake by cell in presencefabsence of inhibitor was monitored by glucose oxidase assay (p<0.005). (B) Cell was serum starved in glucose free and phenol red free media for 3 h. The NBD labeled glucose was added withlwithout GNP-PEG-BRXL and treated for 40 min in presence and absence of Cytochalasin B (scale bar 20 pM).

The uptake for the NGT treatment with inhibitor was less compare to NGT treatment without inhibitor, possibly due to the decrease of free basal level of glucose uptake by cellular endogenous glucose transport pathway. Thus exogenous NGT can carry the glucose from the extracellular matrix to the cell as a bio-mimetic glucose transporter.

We wanted to understand the possible consequence of the NGT mediated cellular uptake of glucose. We measured ATP level and ADP/ATP ratio index to understand whether NGT mediated internalized glucose can be metabolically incorporated to meet the cellular energetics. We found that NGT treated cell has a higher the ADP/ATP ratio compare to untreated control (Figure 4).

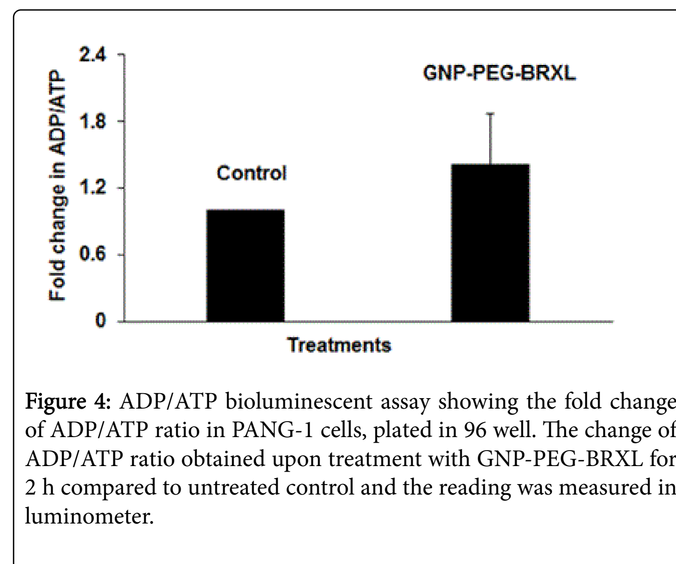

Figure 4: ADP/ATP bioluminescent assay showing the fold change of ADP/ATP ratio in PANG-1 cells, plated in 96 well. The change of ADP/ATP ratio obtained upon treatment with GNP-PEG-BRXL for 2 h compared to untreated control and the reading was measured in luminometer.

Indicating possible utilization of extracellular glucose to meet the energy demand for the highly proliferating cancer cells. This result is also indicating that NGT internalized glucose to cytosol may rapidly escape endosomal sorting.

Conclusion

Our study demonstrates the synthesis of an artificial nanoscale glucose transporter that carry a different identify of glucose. As a proof of concept we have shown that the same glucose when reassembled in a nano formulation can overcome the inhibition of endogenous glucose transport. As the literature evidence implicate that tuning size, charge, and shape of a nanoscale cargo, nano formulation can modulate the cellular uptake mechanism of a delivering agent [21,22]. Therefore, the same glucose that requires an endogenous cellular transporter protein can internalized in different pathway with aid of an exogenous nano glucose transporter as evident from the inhibitor treatment. Thus NGT can act as a glucose sponge or a biomimetic glucose pump. Similar strategy can be applied for the cell which has endocytosis defect or inherent malfunctioning transporter to overcome the possible pathophysiological phenotype associated with this [23,24]. Further study is required to precisely understand the molecular mechanism how NGT works at the cellular interface and come up with next generations of nano transporter with better performance.

Notes

The authors declare no competing financial interests.

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