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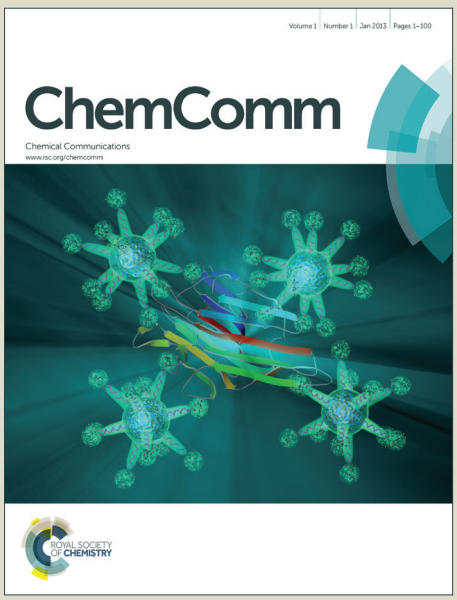

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Impact of molecular charge on GLUT-specific cellular uptake of glucose bioprobes and *in vivo* application of the glucose bioprobe, GB2-Cy3

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The molecular charge of fluorescence bioprobes has recently received much attention due to its influence on cellular uptakes. Herein, we demonstrated the effect of molecular charge of glucose bioprobes to their GLUT-specific cellular uptake. We also applied GB2-Cy3 to *in vivo* imaging in the zebrafish model.

Glucose is the main energy source in our body and is transported into cells through glucose transporters (GLUTs). Glucose metabolism is tightly regulated by either insulin-dependent and independent pathways.[1] The imbalance of glucose homeostasis is the key feature for various diseases, such as cancer, diabetes and obesity.[2] Therefore, the development of glucose bioprobes that can monitor cellular glucose uptake has drawn research attention as diagnostic tools and for the discovery of novel therapeutic agents to treat metabolic disease.[3] The widely used glucose tracer for *in vivo* analysis is [^{18}F]-2-fluoro-2-deoxy-D-glucose, using positron emission tomography (PET).[4] However, the utilization of radioactive bioprobes is quite limited in cellular imaging and in drug discovery research. In this context, various fluorescence glucose bioprobes have been developed since 2-NBDG was first reported.[5–13]

The fluorescence glucose bioprobes consist of three parts; glucose, linker, and fluorescence dye. The design strategy of reported glucose bioprobes is based on the modification of each part. The glycosylation site on D-glucose is one of the key elements that determine the cellular uptake pattern of glucose bioprobes.[9] The linker between glucose and fluorescence dye also influences the GLUT-specific cellular uptake of glucose bioprobes and linker effects in glucose bioprobes have been a key interest in bioprobe design.[11] The final element of glucose bioprobe design is the fluorescent dye. Although various glucose bioprobes labeled with different fluorophores were reported,[5–13] there was no systematic report on the effect of fluorophore structure on these glucose bioprobes. Thus, it is worth exploring the influence of fluorescence dyes on the cellular uptake of glucose bioprobes.

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Recently, the molecular charge of fluorescence dyes has been studied due to their potential influence on cellular uptake. Frangioni group and Weissleder group reported that the zwitterionic fluorophore shows better performance in optical *in vitro* and *in vivo* imaging compared with cationic or anionic dyes.[14–16] Without structural differences of the dyes, molecular charge might affect the non-specific cellular uptake of fluorophores. Inspired by their work, we envisioned that the molecular charge of fluorophores in glucose bioprobes could affect their GLUT-specific cellular uptake. Considering the design strategy of glucose bioprobes and previous reported probes, we first examined the relationship between molecular charge of glucose bioprobes and their GLUT-specific uptake *in vitro* and *in vivo*.

To test this hypothesis, we synthesized two GB2-Cy3 derivatives with zwitterionic Cy3 (GB2-Cy3-S1) and −1 charged Cy3 (GB2-Cy3-S2) to modify the molecular charge of original GB2-Cy3[11] with +1 charged Cy3 (Fig. 1). Three glucose bioprobes have different charges on Cy3 (+1, 0, −1) with minimal structural differences. Expected net charge of these glucose bioprobes in water at pH 7.4 is consistent with calculated molecular charges using the Marvin Sketch program (Fig. 1). Prior to the cellular application of these three glucose bioprobes,

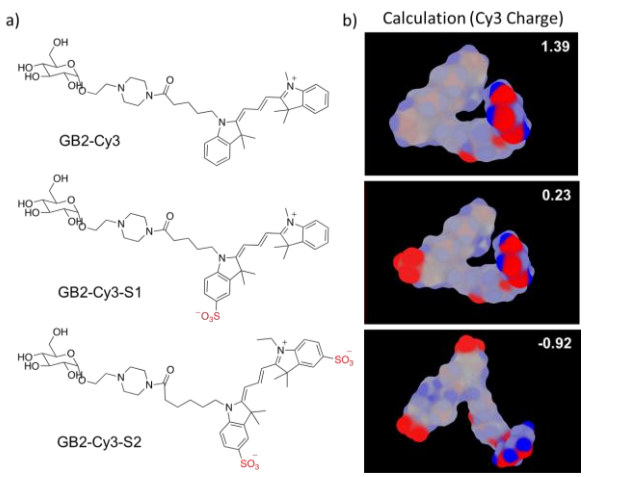

Fig. 1 (a) Chemical structure of GB2-Cy3, GB2-Cy3-S1, and GB2-Cy3-S2. (b) Molecular charge calculation and surface charge area modeling of three glucose bioprobes using Marvin sketch 6.1.3. Molecular charge of Cy3 in pH 7.4 is displayed in white numbers in the picture.

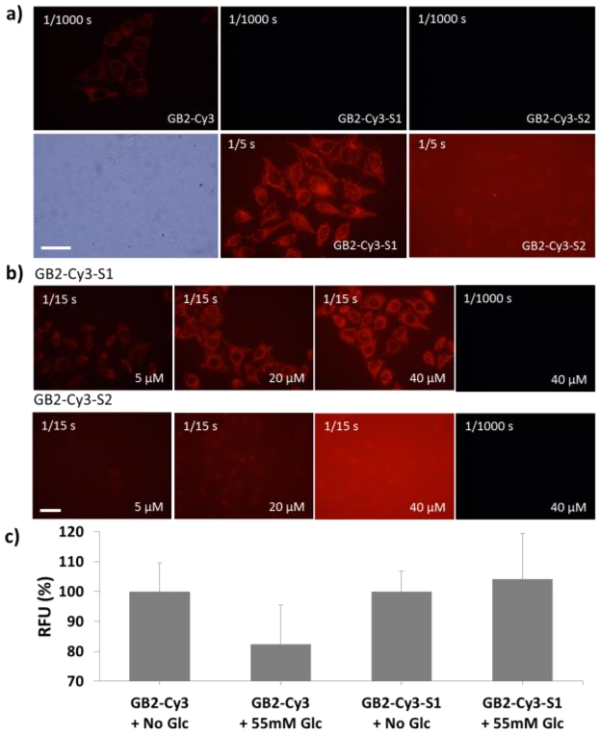

Fig. 2 (a) Fluorescence microscopic images after cellular uptake of GB2-Cy3, GB2-Cy3-S1, and GB2-Cy3-S2 (10 μM each) in HeLa cells. The images were captured after 1/1000 and 1/5 sec exposure time. The scale bar represents 20 μm. (b) Dose-dependent cellular uptake of GB2-Cy3-S1 and GB2-Cy3-S2. The images were captured after 1/1000 and 1/5 sec exposure time. The scale bar represents 20 μm. (c) Glucose competition assay with GB2-Cy3, and GB2-Cy3-S1 with 55 mM D-glucose.

we measured their photophysical properties. Fluorescence intensity and quantum yield of glucose bioprobes in methanol and water was increased as the molecular charge of Cy3 become negative, which is consistent with previous reports (Fig. S1).[17] The increased number of sulfonate groups on Cy3 dyes improved their photophysical properties. Initially, we expected that the ameliorated photophysical properties of GB2-Cy3-S1 and GB2-Cy3-S2 might lead to an improved version of glucose bioprobe compared to the original GB2-Cy3.

To find an optimized condition for cellular imaging, we treated 10 μM concentration of each probe to the human cervical carcinoma HeLa cell line for 30 min and acquired fluorescence images with a 1/1000 sec exposure time (Fig. 2a). Interestingly, cellular uptake of GB2-Cy3-S1 and GB2-Cy3-S2 were not observed, in contrast to that of GB2-Cy3. When the exposure time is increased up to 1/5 sec from 1/1000 sec, cellular uptake of GB2-Cy3-S1 was barely observed, but not in the case of GB2-Cy3-S2. Even though, unlike GB2-Cy3-S2, GB2-Cy3-S1 shows a dose-dependent cellular uptake pattern (Fig. 2b), cellular uptake of GB2-Cy3-S1 was still not observed at the 1/1000 sec exposure time, even at the highest concentration. Based on these experiments, we concluded that the cellular uptake of GB2-Cy3 is superior to that of GB2-Cy3-S2 and GB2-Cy3-S1, which is exactly opposite to our expectation concerning the improved photophysical properties of these GB2-Cy3 derivatives. Although cellular uptakes of two glucose bioprobes were reduced compared to that of GB2-Cy3, new bioprobes might have better selectivity for GLUT-specific cellular uptake. To test this hypothesis, we performed the competition assay and showed that cellular uptake of GB2-Cy3 was reduced as previously reported,[11] but that of GB2-Cy3-S1 was not affected, even in the presence of a high concentration of free D-glucose (Fig. 2c). In contrast to the observation of Frangioni group and Weissleder group,[14–16] zwitterionic property in fluorophores was not beneficial for the GLUT-specific cellular uptake of GB2-Cy3. The positively charged version of glucose bioprobe, original GB2-Cy3, showed the best performance in *in vitro* cellular imaging. These interesting results could be a clue for the cellular behavior of previously reported glucose bioprobes. Zwitterionic[7] and negatively charged[8] glucose bioprobes, reported in 2004 and 2006, were not GLUT-specific and showed no competition with free D-glucose (Fig. S4). Other reported glucose bioprobes with positive or no net charge generally showed good performance as a glucose tracer with GLUT specificity (Table S1).[5,6,9–13] On the basis of our findings, along with previously reported glucose bioprobes, we are confident that the variation of molecular charge on the GB2-Cy3 is important and can serve as one of the considerations for the rational design of new glucose bioprobe.

After studying the charge effect in glucose bioprobes, we moved our attention to the *in vivo* application of GB2-Cy3. Although cell-based monitoring of glucose uptake is still in huge demand for metabolic disease research, the *in vivo* validation of drug candidates is an essential step, due to the complexity of glucose homeostasis triggered by insulin-dependent and independent signaling pathways. In this context, the zebrafish can be considered as a 'powerhouse' animal model for drug discovery research, because it is amenable for vertebrate-based compound screening and validation. We recently developed an *in vivo* glucose uptake screening system using 2-NBDG in zebrafish.[18] However, 2-NBDG cannot be considered as an ideal bioprobe for *in vivo* glucose uptake monitoring due to its high treatment concentration, low sensitivity, and fast photobleaching.

To establish a fluorescence-based monitoring system with GB2-Cy3 in zebrafish larvae, we first tested whether GB2-Cy3 uptake can be adequately measured time-dependently. 72 hour post fertilization (hpf) larvae were used because they allow drug testing in a 96 well plate format. In addition, GLUT transporter expression and glucose metabolism has been established at this stage of development.[18] We measured probe uptake into the zebrafish larval eye, because this is known to be a rich source of expression of GLUTs[19] and the eye shape in 72 hpf larvae is amenable for image analysis of fluorescent intensity. We observed that 3 h incubation with GB2-Cy3 is appropriate for microscopic observation of uptake into the zebrafish larval eye (Fig. S6). Next, we compared the dose-dependent *in vivo* uptake of GB2-Cy3 with that of 2-NBDG, using a fluorescent microplate reader, which would be advantageous for drug screening and validation. As shown in Fig. 3, GB2-Cy3 uptake can be observed at a dose of 5 μM and the fluorescence signal continued to increase dose-dependently

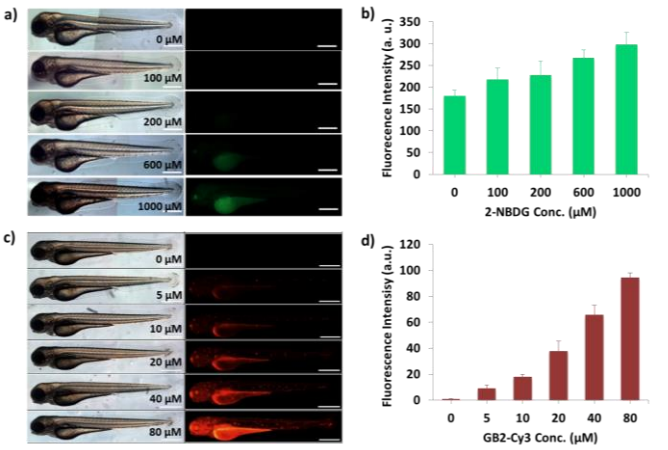

Fig. 3 Dose-dependent uptake of 2-NBDG in zebrafish (a) and its quantification data (b). Dose-dependent uptake of Cy3-GB2 in zebrafish (c) and its quantification data (d). The scale bar represents 500 μm.

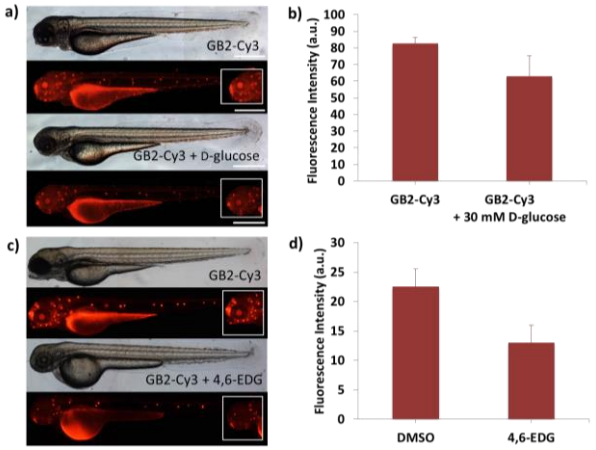

Fig. 4 (a) Glucose competition assay of GB2-Cy3 in zebrafish. (b) Fluorescence intensities of zebrafish larval eyes. (c) GB2-Cy3 uptake was deteriorated by GLUT inhibitor, 4,6-EDG (4,6-*O*-ethylidene-α-D-glucose, 2.5 mM) in zebrafish. (d) Fluorescence intensities of GB2-Cy3 uptake into the zebrafish larval eye. The scale bar represents 500 μm.

up to 80 μM, with superb signal to noise ratio. In contrast, at least 600 μM concentration of 2-NBDG was required for optimal glucose uptake observation. We also confirmed that GB2-Cy3 uptake in the zebrafish larval eye occurred in a dose-dependent manner (Fig. S7, see ESI†). With these results in hand, we tested whether *in vivo* uptake of GB2-Cy3 is GLUT-specific or not. In the presence of 30 mM D-glucose, *in vivo* uptake of GB2-Cy3 was effectively competed out by excess D-glucose (Fig. 4a,b). Furthermore, the GLUT inhibitor, 4,6-*O*-ethylidene-α-D-glucose (4,6-EDG), reduced GB2-Cy3 signal (Fig. 4c,d), which provided the additional confirmation that GLUT activity is an important component of GB2-Cy3 uptake. Collectively, these results confirmed that *in vivo* uptake GB2-Cy3 in zebrafish larvae is controlled by GLUT-specific processes.

As a final test to demonstrate that a GB2-Cy3-based assay system is a suitable for *in vivo* monitoring of glucose uptake regulators as anti-diabetic agents, we tested GB2-Cy3 uptake in zebrafish using glucose uptake enhancers with different modes of action; insulin-independent AMP kinase activator (ampkinone)[20] and insulin-dependent PPARγ agonist (rosiglitazone). Upon treatment with two anti-diabetic agents, GB2-Cy3 uptake in zebrafish larvae significantly increased, which was quantified by measuring fluorescence intensities of the zebrafish larval eye and fluorescent microplate reader analysis

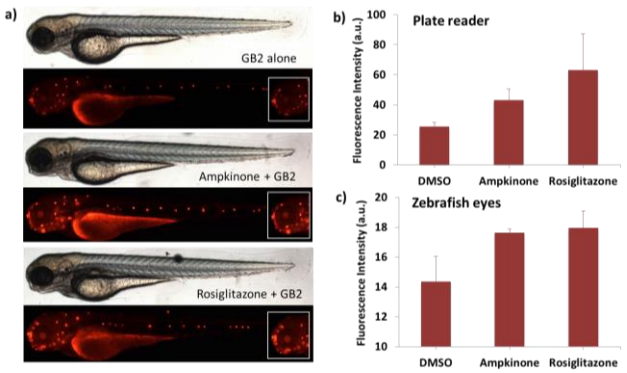

Fig. 5 (a) GB2-Cy3 uptake increases in zebrafish after treatment with glucose uptake enhancers, Ampkinone (10 μM) and Rosiglitazone (10 μg/ml). (b) GB2-Cy3 uptake quantification of lysed larvae using a plate reader. (c) GB2-Cy3 uptake quantification by measuring fluorescence intensities of the zebrafish larval eye.

of lysed larvae (Fig. 5). To further confirm that GB2-Cy3 can detect anti-diabetic drugs, an additional series of glucose uptake enhancers (insulin, GAPDS[21] and emodin[18]) were shown to increase the *in vivo* uptake of GB-Cy3 (Fig. S8). These results confirmed that GB2-Cy3 is an excellent bioprobe for *in vivo* monitoring of perturbed glucose uptake upon treatment with regulators of glucose homeostasis.

In this study, we demonstrated the importance of the molecular charge of glucose bioprobes on their uptake behavior. Cellular uptake of the positively charged GB2-Cy3 is GLUT-specific, but that of negative charged and zwitterionic GB2-Cy3 are not. This observation would be helpful for the design of new glucose bioprobes and the mechanistic understanding of GLUT-specific uptake of GB2-Cy3. We also confirmed GB2-Cy3 as a successful *in vivo* glucose tracer for GLUT-specific *in vivo* monitoring of glucose uptake in zebrafish model with significantly improved properties compared to the widely used glucose bioprobe, 2-NBDG. Considering the complexity of glucose homeostasis, we anticipate the *in vivo* application of GB2-Cy3 would provide a new insight for metabolic disease study and a valuable, vertebrate-based assay for anti-diabetes drug discovery.

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