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Near-infrared fluorescence glucose sensing based on glucose/galactose-binding protein coupled to 651-Blue Oxazine

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ABSTRACT

Near-infrared (NIR) fluorescent dyes that are environmentally sensitive or solvatochromic are useful tools for protein labelling in *in vivo* biosensor applications such as glucose monitoring in diabetes since their spectral properties are mostly independent of tissue autofluorescence and light scattering, and they offer potential for non-invasive analyte sensing. We showed that the fluorophore 651-Blue Oxazine is polarity-sensitive, with a marked reduction in NIR fluorescence on increasing solvent polarity. Mutants of glucose/galactose-binding protein (GBP) used as the glucose receptor were site-specifically and covalently labelled with Blue Oxazine using click chemistry. Mutants H152C/A213R and H152C/A213R/L238S showed fluorescence increases of 15% and 21% on addition of saturating glucose concentrations and binding constants of 6 and 25 mM respectively. Fluorescence responses to glucose were preserved when GBP-Blue Oxazine was immobilised to agarose beads, and the beads were excited by NIR light through a mouse skin preparation studied *in vitro*. We conclude GBP-Blue Oxazine shows proof-of-concept as a non-invasive continuous glucose sensing system.

1. Introduction

Fluorescence-based glucose sensing is being researched by a number of groups as a potential technology for continuous glucose monitoring (CGM) in patients with diabetes mellitus [1,2], particularly as it may overcome many of the problems associated with commercially available sensors, which employ needle- or wire-type subcutaneously implanted amperometric enzyme electrodes or microdialysis probes [3,4]. Though offering significant clinical benefits in diabetes management [5], the performance of current electrochemical CGM technology is limited by signal drift, impaired responses and suboptimal accuracy when used *in vivo*.

Fluorescence *in vivo* glucose sensors have potential advantages compared to electrochemical sensors, stemming from high sensitivity, freedom from interference from electrochemically active substances in the tissues and the option to measure fluorescence lifetimes as well as intensity [1]. One of the most important benefits of fluorescence for sensing is that excitation and emission of fluorescence in the near-infrared (NIR) spectral range potentially allows non-invasive glucose monitoring from the skin surface of implanted sensors, since the tissues are transparent through several centimetres of NIR light illumination [1].

Various receptors for glucose have been investigated for fluorescence glucose sensing in recent years, including the lectin concanavalin A (Con A) [6–9], the enzymes glucose oxidase [10] and hexokinase [11], and a number of boronic acid derivatives [12,13]. We and others have focused on glucose recognition using glucose/galactose-binding protein (GBP), one of the superfamily of bacterial periplasmic binding proteins [14–25]. GBP is notable for sensing purposes because it is not oxygen-dependent (unlike the commonly used glucose oxidase of present CGM systems) and the large conformational change in the molecule that occurs on glucose binding can be transduced into a fluorescence signal proportional to glucose concentration via a site-specifically attached fluorophore [25]. In previous work, we have engineered mutants of native GBP with increased binding constants (K_d) that allow sensing in the pathophysiological range appropriate to diabetes [15], and we have demonstrated glucose sensing systems using GBP where the solvatochromic (environmentally or polarity-sensitive) fluorophore, badan, is covalently linked to a cysteine residue introduced near the binding site using site-directed mutagenesis [14–17,25]. Addition of glucose and closing of the lobes of GBP around the binding site, reduces the polarity in the microenvironment of the site, thus increasing the fluorescence intensity and lifetime of the fluorophore [25].

Abbreviations: CGM, continuous glucose monitoring; DMF, dimethylformamide; FRET, fluorescence resonance energy transfer; GBP, glucose/galactose-binding protein; NIR, near-infrared; Ni–NTA, nickel nitrolotriacetic acid; PBS, phosphate-buffered saline.

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Although we have fabricated a prototype fibre-optic glucose sensor using badan-GBP that is suitable for subcutaneous implantation over several days [17], badan cannot be used for non-invasive glucose sensing since the excitation/emission wavelengths are not in the NIR range (ex 400/em 550 nm). A number of NIR fluorophores are commercially available, but only the minority are known to be environmentally sensitive and they do not have a functional group that allows covalent linking to a protein such as GBP. The most studied environmentally sensitive NIR dye is Nile Red and, to be usable for sensing, Thomas et al., synthesised special thiol-reactive Nile Red derivatives [21].

In the present study, we investigate for use in GBP-based glucose sensing systems the NIR fluorescent dye, 651-Blue Oxazine, which has not previously been reported as a solvatochromic fluorophore. We also demonstrate for the first time the use of 'click chemistry' as a method for simple, high-yield covalent linking of Blue Oxazine to GBP mutants of different K_d, and show *in vitro* proof-of-concept for non-invasive transcutaneous glucose sensing using GBP-Blue Oxazine.

2. Materials and methods

2.1. Reagents

651-Blue Oxazine azide was purchased from Active Motif Chromeon GmbH (Tegernheim, Germany). Click Chemistry reagent kits and iodoacetamide alkyne were from Invitrogen (Paisley, UK). Zeba desalting columns were from Thermo Fisher Scientific Inc (Rockford, IL, USA) and nickel-nitrilotriacetic acid (Ni–NTA) agarose was from Qiagen (Crawley, UK). All other chemicals were molecular biology grade and were purchased from Sigma–Aldrich (Poole, UK). Mutant GBP proteins from *Escherichia coli* were over expressed and purified as described previously [14,15].

2.2. Preparation of Blue Oxazine-labelled GBP

GBP mutants were covalently labelled with 651-Blue Oxazine in a two-step process (Fig. 1). Firstly, GBP was labelled with iodoacetamide alkyne by mixing GBP (final concentration 100 μM) with tris(2-carboxyethyl(phosphene) (final concentration 2.5 mM) in phosphate-buffered saline (PBS) at pH 7.4 and a 10-fold molar excess of iodoacetamide alkyne and allowed to react for 2 h at room temperature. Excess reagent was removed using Zeba desalting columns. In the second step, Blue Oxazine azide was conjugated to GBP-alkyne using the Click-iT® Protein Reaction Buffer Kit in the following manner: 100 μl GBP-alkyne (final concentration 100 μM), 20 μl Blue Oxazine azide (500 μM) in 200 μl reaction buffer, 60 μl distilled water, 20 μl copper sulphate reagent and 20 μl Kit Additive 1 were mixed, vortexed and incubated at room temperature for 1 h. Excess dye was removed using Zeba desalting columns.

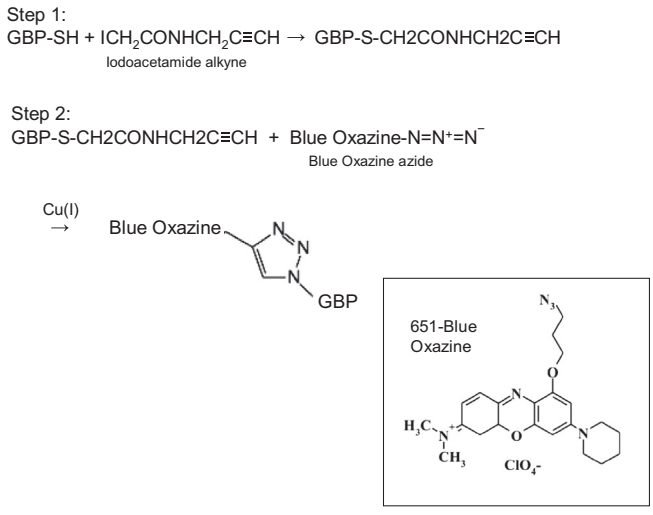

Fig. 1. Two-step labelling of GBP with 651-Blue Oxazine dye using click chemistry. Inset: molecular structure of Blue Oxazine.

2.3. Fluorescence measurements

Steady-state fluorescence was recorded using a Gemini EM Fluorescence Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The excitation wavelength was set at 645 nm for Blue Oxazine, and emission spectra recorded between 650–750 nm. All data were obtained at room temperature. The labelled protein was incubated with increasing amounts of D-glucose for 5–10 min before fluorescence was recorded.

2.4. In vitro transcutaneous sensing

To immobilise GBP-Blue Oxazine (using mutant H152C/A213R/L238S) on Ni–NTA agarose beads, 20 μl Ni–NTA agarose beads in suspension as supplied by the manufacturer were washed in PBS and incubated with 40 μl of GBP–Blue Oxazine (30 μM) for 2 h at room temperature. The beads were then washed extensively with PBS to remove any unbound protein and re-suspended in PBS. Change in fluorescence intensity on addition of saturating glucose concentration (100 mM) was measured in a microplate reader. To establish detection of fluorescent light through skin, shaved dorsal skin from a sacrificed mouse was placed at the bottom of the well (skin surface against well surface), followed by a layer of the GBP-Blue Oxazine labelled beads (subcutaneous side) in PBS or glucose and fluorescence was measured by exciting the beads from the bottom of the well through the skin. For controls, Ni–NTA agarose beads with no immobilised GBP-Blue Oxazine were used, with skin as before.

2.5. Estimation of binding constants

The apparent binding constant, K_d, for each labelled GBP mutant was calculated by fitting the data of fluorescence intensity at different glucose concentrations to sigmoidal curves using Prism 5 software (GraphPad, San Diego, CA, USA).

3. Results

3.1. Solvatochromic nature of 651-Blue Oxazine

We first studied the effect of solvents of different polarity on the fluorescence intensity of the dye 651-Blue Oxazine, the molecular structure of which is shown in Fig. 1. The maximal fluorescence intensity of the dye showed a graded reduction to about 40% when the solvent environment was changed by steps from less polar, 100% dimethylformamide (DMF) to the more polar 90% water/10% DMF (Fig. 2a). The emission spectra in more polar solvents also showed a red shift, with the emission maximum changing from 666 nm in DMF to 675 nm in 90% water (Fig. 2a).

3.2. Labelling of GBP mutants with 651-Blue Oxazine

We site-specifically and covalently attached 651-Blue Oxazine to GBP at the thiol group of a cysteine residue introduced at position 152 (near the glucose-binding site) in a number of mutants of GBP of different K_d, previously synthesised by protein engineering

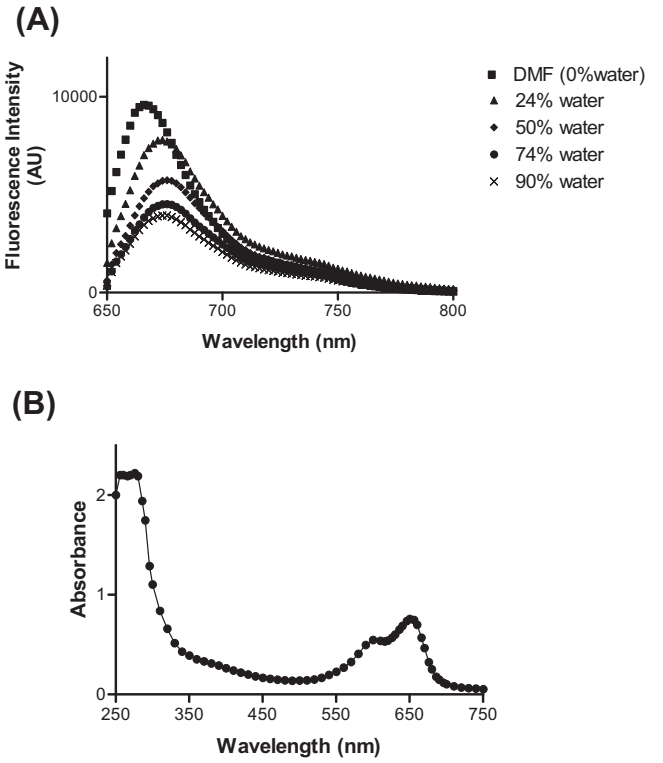

Fig. 2. (A) Fluorescence emission spectra of 651-Blue Oxazine in varying concentrations of DMF and water. (B) Absorption spectrum of H152C/A213R/L238S-Blue Oxazine.

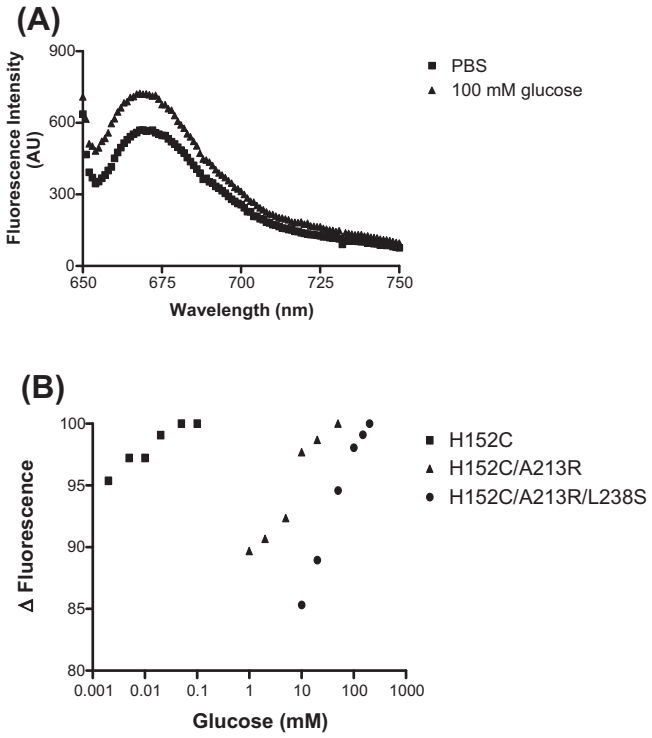

Fig. 3. (A) Fluorescence emission spectrum of H152C/A213R/L238S-Blue Oxazine in the absence and presence of 100 mM glucose. (B) Glucose-induced fluorescence changes for GBP mutants labelled with Blue Oxazine.

techniques. In a two-step, click-chemistry procedure, described in detail in the Methods and illustrated in Fig. 1, we first linked iodoacetamide alkyne to the single GBP thiol at position 152; this was followed by Cu(I)-catalysed cycloaddition of 651-Blue Oxazine azide to form a stable triazole conjugate.

Fig. 2b shows the absorbance spectrum of the GBP triple mutant (H152C/A213R/L238S)-Blue Oxazine conjugate, purified from excess dye using a desalting column. An absorption peak at 280 nm was observed which is consistent with protein absorption (tryptophan residues in GBP), and a peak in the 600–750 nm region is consistent with the Blue Oxazine fluorophore, linked to protein.

We used click chemistry to label three mutants with Blue Oxazine: H152C, H152C/A213R and H152C/A213R/L238S. The fluorescence intensity showed an increase for all mutants with addition of glucose: Fig. 3a shows, as an example, the emission spectra of H152C/A213R/L238S-Blue Oxazine in buffer of 0 and 100 mM glucose concentration. Fig. 3b compares the change in fluorescence intensity with addition of glucose for these labelled single, double and triple mutants of GBP. There was an almost a three order of magnitude change in K_d between the mutants, with a K_d of 7.4 μM, 6.5 mM and 25.5 mM.

The percent changes in fluorescent intensity from zero glucose to the saturating glucose level and the binding constants for all mutants, are shown in Table 1. The single mutant showed a fluorescence change of about 8%, whilst fluorescence increased by about 15% for H152C/A213R and by 21% for the triple mutant H152C/A213R/L238S.

3.3. Potential for non-invasive glucose sensing

Since each mutant had an oligohistidine tag at the C terminus, for proof-of-concept of NIR-based transcutaneous glucose sensing using GBP-Blue Oxazine, we immobilised H152C/A213R/L238S-Blue Oxazine to Ni–NTA agarose beads via Ni-histidine binding. Addition of a saturating concentration of glucose to bead-immobilised GBP-Blue Oxazine resulted in an increase in fluorescence intensity that was comparable in magnitude (26.5%) to that seen with the labelled protein in free solution (21%) (Fig. 4a). GBP-Blue Oxazine-attached beads were then irradiated with NIR light through mouse skin placed *in vitro* in a fluorescence microplate reader. Skin from a sacrificed mouse was placed at the bottom of the well, followed by a layer of the GBP-Blue Oxazine labelled beads in PBS or glucose (beads on subcutaneous side of skin) and fluorescence was measured by exciting the beads from the bottom of the well through the skin and detecting the emitted fluorescence from the top. Fluorescence from a control well of unlabelled beads and skin was subtracted from the test fluorescence. Fig. 4b shows that NIR fluorescence was detected through the skin, with a spectrum similar to that of GBP-Blue Oxazine in free solution.

Table 1
Change in fluorescence intensity on addition of a saturating concentration of glucose to GBP-Blue Oxazine mutants of different K_d.

GBP mutant	Δ Fluorescence (%)	K_d (mM)
H152C	8	0.0074
H152C/A213R	15	6.5
H152C/A213R/L238S	21	25.5

4. Discussion

We describe here for the first time the solvatochromic, polarity-sensitive nature of the NIR fluorophore 651-Blue Oxazine. We have previously shown that labelling of GBP with a solvatochromic, non-NIR fluorescent dye, badan, attached near the binding site of the protein, allows monitoring of glucose concentrations due to the changed conformation of the protein [14–17]; we now extend that

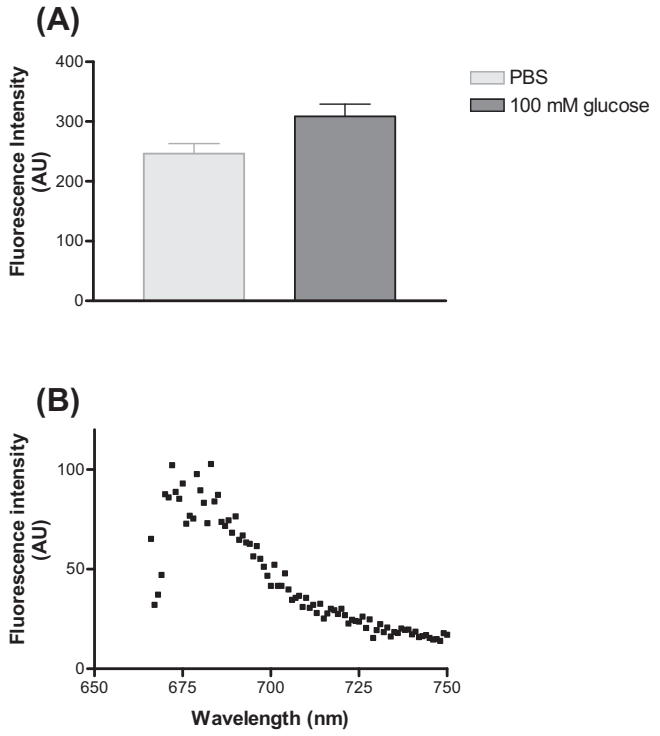

Fig. 4. (A) Glucose-induced fluorescence response of H152C/A213R/L238S-Blue Oxazine attached to Ni-agarose beads. (B) Fluorescence emission spectrum of the GBP-Blue Oxazine immobilised to beads and irradiated through mouse skin.

work by substituting Blue Oxazine for badan and thus show proof-of-concept for NIR-based glucose sensing using GBP-Blue Oxazine. Such a system might be used for non-invasive glucose sensing and might be envisaged as, for example, a 'smart tattoo' where GBP-Blue Oxazine is encapsulated in micro- or nanovesicles impregnated in the dermis or subcutaneous tissue, or as a completely implanted capsule, with excitation and interrogation of glucose-dependent NIR light from the skin surface [26].

Apart from tissue transparency to NIR light, other advantages of NIR glucose sensing include reduction in interference from tissue autofluorescence and from Raman and Rayleigh light scattering, which chiefly occur below wavelengths of about 600 nm. Photodecomposition is also less at higher wavelengths. Moreover, excitation in the NIR region can be accomplished using cheap, stable and compact diode lasers, so sensitivity can be increased considerably. Although we did not study glucose-induced fluorescence lifetime changes of GBP-Blue Oxazine in this first report, time-resolved NIR fluorescence may offer additional advantages for *in vivo* sensing since lifetimes are relatively independent of light scattering, photobleaching and fluorophore concentration, so that any coating or encapsulation of sensors in the tissues should not lead to signal drift [1,25].

So-called 'click chemistry' was used for linking the dye to GBP. The advantages of this methodology for fluorophore labelling of sensing proteins include rapid, simple and high-yield procedures which are selective for the azide–alkyne components and which occur under mild aqueous conditions unlikely to adversely affect the biofunctionality of the protein [27,28]. In this respect, we confirmed that GBP-Blue Oxazine retained glucose-dependent fluorescence increases in solution and when immobilised to agarose beads. The simplicity of click chemistry using commercially available Blue Oxazine azide contrasts with the relative complexity of synthesising thiol-reactive derivatives of Nile Red or squaraine in previous research on NIR-based GBP glucose sensing. [21,22].

In addition to the Nile Red- or squaraine-GBP sensors mentioned above [21,22], a limited number of NIR-based glucose sensors have been described previously, including a boronic acid functionalized squarylium cyanine dye-based sensor [29], a sensing system using single-walled carbon nanotubes [30] and several fluorescence energy transfer (FRET)-based systems using Alexa Fluor 633 or Alexa Fluor 647-labelled Con A in a competitive assay with dye-loaded Sepharose [31], or allophycocyanin-labelled Con A as donor and malachite green-labelled dextran as FRET acceptor [8]. A FRET-based system with dual labelling of GBP with nitrobenzoxadiazole and Texas Red fluorophores has also been described [32]. A more complex system based on competitive binding and FRET with apo-glucose oxidase as the glucose receptor has been recently reported, where Alexa Flour-647-aminodextran is bound to QSY-21-apo-glucose oxidase (GOX), whereby fluorescence is quenched. When glucose is introduced into the system, it displaces the Alexa Fluor-647-tagged aminodextran from QSY-21 dye-conjugated apo-GOx, resulting in an increase in the Alexa Fluor-647 fluorescence [33]. Considering all these NIR sensors, we see the advantages of using GBP as a glucose receptor include the fact that it avoids the toxicity and aggregation tendency of Con A, and use of GBP labelled with an environmentally sensitive fluorophore avoids the need to encapsulate competitive FRET assays within a membrane.

For CGM in patients with diabetes, a sensor must operate in the region of about 0.5–20 mM or greater. Native GBP and the H152C GBP mutant labelled with badan have a K_d in the micromolar range [14] and are thus not suitable for clinical use. We found here that H152C-Blue Oxazine had a similar K_d of 7.4 μM. The mutant GBP (H152C/A213R)-Blue Oxazine showed a K_d of 6.5 mM and the triple mutant GBP(H152C/A213R/L238S)-Blue Oxazine had a K_d of 25.5 mM, This last compares with a K_d of 11 mM for GBP(H152C/A213R/L238S)-badan [15]; presumably the different molecular structure and size of Blue Oxazine slightly impairs glucose binding compared to GBP-badan and thus increases the K_d.

We therefore show that Blue Oxazine is a solvatochromic fluorophore and GBP linked to Blue Oxazine shows proof-of-concept as a non-invasive continuous glucose sensing system for use in diabetes.

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