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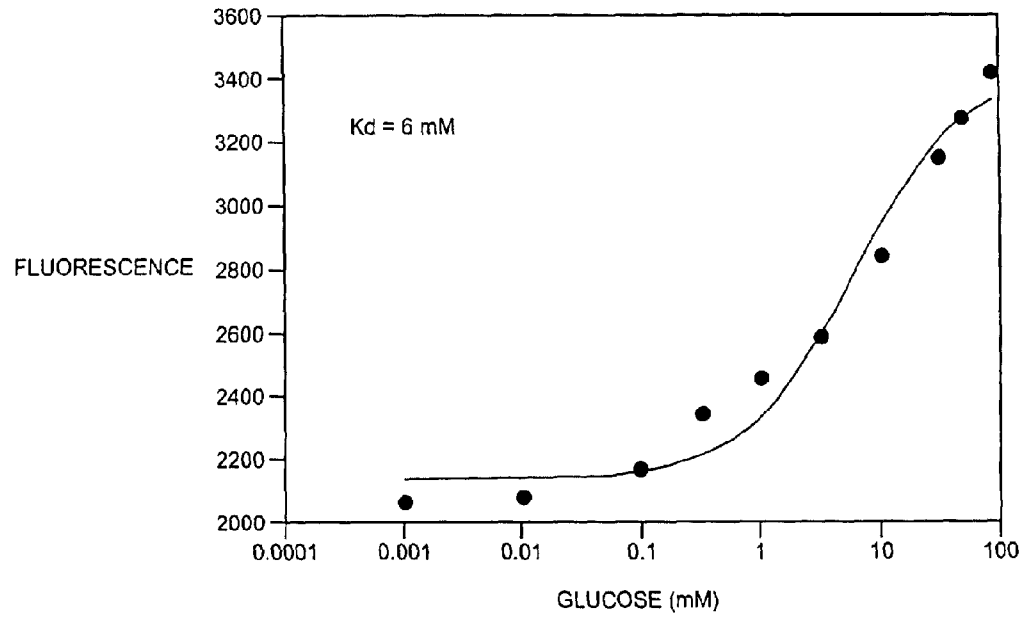
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[Continued on next page]

(54) Title: ENTRAPPED BINDING PROTEINS AS BIOSENSORS



(57) Abstract: The invention is directed to entrapped binding proteins, mutated binding proteins containing reporter groups, compositions of mutated binding proteins containing reporter groups in analyte permeable matrices, and their use as analyte biosensors both in-vitro and in vivo. The binding proteins are covalently bound to a fluorescein dye so that the concentration of an analyte (e.g., glucose) in a given sample is proportional to the fluorescence signal on a scale.

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ENTRAPPED BINDING PROTEINS AS BIOSENSORS

BACKGROUND OF THE INVENTION

1. Field Of the Invention

[0001] The invention is in the field of biotechnology. Specifically, the invention is directed to entrapped mutated binding proteins, mutated binding proteins containing reporter groups, compositions of mutated binding proteins containing reporter groups in analyte permeable matrices, and their use as analyte biosensors both *in vitro* and *in vivo*.

2. Description of Relevant Art

[0002] Monitoring glucose concentrations to facilitate adequate metabolic control in diabetics is a desirable goal and would enhance the lives of many individuals. Currently, most diabetics use the "finger stick" method to monitor their blood glucose levels and patient compliance is problematic due to pain caused by frequent (several times per day) sticks. As a consequence, there have been efforts to develop non-invasive or minimally invasive *in vivo* and more efficient *in vitro* methods for frequent and/or continuous monitoring of blood glucose or other glucose-containing biological fluids. Some of the most promising of these methods involve the use of a biosensor. Biosensors are devices capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element that is combined with a transducing (detecting) element.

[0003] The biological recognition element of a biosensor determines the selectivity, so that only the compound desired to be measured leads to a signal. The selection may be based on biochemical recognition of the ligand where the chemical structure of the ligand (e.g. glucose) is unchanged, or on biocatalysis in which the biological recognition element catalyzes a biochemical reaction of the analyte.

[0004] The transducer translates the recognition of the biological recognition element into a semi-quantitative or quantitative signal. Possible transducer technologies are optical, electrochemical, acoustical/mechanical or colorimetrical. The optical properties that have been exploited include absorbance, fluorescence/phosphorescence, bio/chemiluminescence, reflectance, light scattering and refractive index. Conventional

reporter groups such as fluorescent compounds may be used, or alternatively, there is the opportunity for direct optical detection, without the need for a label.

[0005] Biosensors specifically designed for glucose detection that use biological elements for signal transduction typically use electrochemical or colorimetric detection of glucose oxidase activity. The use of this method is associated with difficulties that result from the influence of oxygen levels, the presence of inhibitors in the blood and problems with electrodes, among others. In addition, detection results in consumption of the analyte that may result in difficulties when measuring low glucose concentrations.

[0006] A rapidly advancing area of biosensor development is the use of fluorescently labeled periplasmic binding proteins (PBP's). As reported by Cass (*Anal Chem.* **1994**, 66, 3840-3847), a labeled maltose binding protein (MBP) was demonstrated to be a useful maltose sensor. In this work, MBP, which has no native cysteine residues, was mutated to provide a protein with a single cysteine residue at a position at 337 (S337C). This mutation position was within the maltose binding cleft and experienced a large environmental change upon maltose binding. Numerous fluorophores were studied, some either blocked ligand binding or interfered with the conformational change of the protein. Of those studied, N-((2-iodoacetoxy)ethyl)-N-methyl)amino-7-nitrobenzoxadiazole (IANBD) resulted in a substantial increase in fluorescence (160%) intensity upon maltose binding. This result is consistent with the location of the fluorophore changing from a hydrophilic or solvent exposed environment to a more hydrophobic environment, as would have been theoretically predicted for the closing of the binge upon maltose binding. However, this mutant protein and the associated reporter group do not bind diagnostically important sugars in mammalian bodily fluids. Cass also disclosed association of this protein with TiO_2 surfaces, however, the surface-bound protein suffered from reduced activity with time and required constant hydration (*Analytical Chemistry* **1998**, *70*(23), 5111-5113).

[0007] Hellinga, *et al.* (US 6,277,627), reports the engineering of a glucose biosensor by introducing a fluorescent transducer into a Galactose/Glucose Binding Protein (GGBP) mutated to contain a cysteine residue, taking advantage of the large conformation changes that occur upon glucose binding. Hellinga *et al* (US 6,277,627) disclose that the transmission of conformational changes in mutated GGBPs can be exploited to construct integrated signal transduction functions that convert a glucose binding event into a change in

fluorescence *via* an allosteric coupling mechanism. The fluorescent transduction functions are reported to interfere minimally with the intrinsic binding properties of the sugar binding pocket in GGBP.

[0008] In order to accurately determine glucose concentration in biological solutions such as blood, interstitial fluids, ocular solutions, perspiration, etc., it may be desirable to adjust the binding constant of the sensing molecule of a biosensor so as to match the physiological and/or pathological operating range of the biological solution of interest. Without the appropriate binding constant, a signal may be out of range for a particular physiological and/or pathological concentration. Additionally, biosensors may be configured using more than one protein, each with a different binding constant, to provide accurate measurements over a wide range of glucose concentrations as disclosed by Lakowicz (US 6,197,534).

[0009] Despite the usefulness of mutated GGBPs, few of these proteins have been designed and examined, either with or without reporter groups. Specific mutations of sites and/or attachment of certain reporter groups may act to modify a binding constant in an unpredictable way. Additionally, a biosensor containing reporter groups may have a desirable binding constant, but not result in an easily detectable signal upon analyte binding. One of the overriding factors that determines sensitivity of a particular reporter probe attached to a particular protein for the detection of a specific analyte is the nature of the specific interactions between the selected probe and amino acid residues of the protein. It is not currently possible to predict these interactions within proteins using computational methods, nor is it possible to employ rational design methodology to optimize the choice of reporter probes. In addition, it is not possible to predict the effect on either the binding constant, or the selectivity based on the position of any reporter group, or amino acid substitution in the protein (or *vice-versa*).

[0010] To develop reagentless, self-contained, and/or implantable and/or reusable biosensors using proteins, the transduction element must be in communication with a detection device to interrogate the signal to and from the transduction element. Typical methods include placing proteins within or onto the surface of optical fibers or planner waveguides using immobilization strategies. Such immobilization strategies include, but are not limited to, entrapment of the protein within semi-permeable membranes, organic

polymer matrices, or inorganic polymer matrices. The immobilization strategy used may ultimately determine the performance of the working biosensor. The prior art details numerous problems associated with the immobilization of biological molecules. For example, many proteins undergo irreversible conformational changes, denaturation, and loss of biochemical activity. Immobilized proteins can exist in a large number of possible orientations on any particular surface, for example, with some proteins oriented such that their active sites are exposed and others oriented such that their active sites are not exposed (and thus not able to undergo selective binding reactions with the analyte). Immobilized proteins are also subject to time-dependent denaturation, denaturation during immobilization, and leaching of the entrapped protein subsequent to immobilization. This results in problems including, for example, an inability to maintain calibration of the sensing device and signal drift. In general, binding proteins require orientational control to enable effective use, thus physical absorption and random or bulk covalent surface attachment or immobilization strategies as taught in the literature generally are not successful.

[0011] There have been several reports of encapsulating proteins and other biological systems into simple inorganic silicon matrices formed by a low temperature sol-gel processing methods (e.g. Brennan, J. D. Journal of Fluorescence **1999**, *9*(4), 295-312, and Flora, K.; Brennan, J. D. Analytical Chemistry **1998**, *70* (21), 4505-4513). Some sol-gel matrices are optically transparent, making them useful for the development of chemical and bio-chemical sensors that rely on optical transduction, for example absorption or fluorescence spectroscopic methods. However, in order to be functional, entrapped or immobilized binding proteins must remain able to undergo at least some analyte induced conformational change. Conformational motions of binding proteins may be substantially restricted in most sol-gel matrices taught in the literature. It has been reported that sol-gel entrapped proteins can exhibit dramatically altered binding constants, or binding constants that change over relatively short time periods or under varying environmental conditions. In addition, the function of protein entrapped in the sol-gel matrix has been reported to be time dependent, a characteristic that limits general applicability of sol-gels in biosensors for *in vitro* as well as *in vivo* use.

[0012] Therefore, there is a need in the art to design additional useful mutated proteins and mutated GGBP proteins generating detectable signals upon analyte binding for

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use as biosensors, and additionally there is a need for incorporation of these proteins into analyte-permeable matrices for interfacing to signal transmitting and receiving elements.

[0012a] Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.

SUMMARY OF THE INVENTION

[0012b] Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

[00013] The invention provides entrapped or encapsulated mutated binding proteins and mutated binding proteins having reporter groups attached thereto, for their use as in vivo or in vitro biosensors. Furthermore, the invention provides a glucose biosensor including (a) a mutated binding protein and at least one reporter group attached thereto such that said reporter group provides a detectable signal when said mutated binding protein is exposed to glucose and (b) a matrix permeable to analyte where the mutated glucose/galactose binding protein and the reporter group capable of encapsulation in the matrix.

[00014] The invention also provides compositions comprising a mixture including (a) at least one mutated glucose/galactose binding protein and at least one reporter group attached thereto and (b) a hydrogel, dialysis membrane, sol-gel, or combination thereof to provide for a matrix permeable to analyte wherein the mutated glucose/galactose binding protein and the reporter group are encapsulated in the matrix.

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[00015] The invention also provides a device including (a) a mutated maltose binding protein (MBP) and at least one reporter group attached thereto such that the reporter group provides a detectable signal when the mutated MBP is bound to maltose and wherein the MBP includes a cysteine present at position 337 and (b) a matrix permeable to maltose wherein the mutated MBP and the reporter group are encapsulated in the matrix.

[00016] The invention further provides a device and compositions thereof suitable for in vivo use including (a) a mutated glucose/galactose binding protein and at least one reporter group attached thereto such that the reporter group provides a detectable and reversible signal when the mutated glucose/galactose binding protein is exposed to varying glucose concentrations and (b) a matrix permeable to analyte wherein the mutated glucose/galactose binding protein and the reporter group are encapsulated in the matrix.

[0016a] In one aspect, the present invention provides a glucose biosensor comprising at least one mutated glucose/galactose binding protein (GGBP), and at least one luminescent reporter group covalently attached thereto, wherein the mutant GGBP has at least one amino acid substitution of a non-reactive amino acid with a reactive amino acid and said mutant GGBP is encapsulated in an analyte-permeable matrix comprising covalently crosslinked hydrogels or sol-gels augmented with organically modified silicates or a combination thereof.

[0016b] In a further aspect, the present invention provides a method for glucose detection comprising:

a) providing at least one mutated glucose/galactose binding protein having at least one amino acid substitution of a non-reactive amino acid with a reactive amino acid and at least one luminescent reporter group covalently attached thereto;

b) entrapping or encapsulating said mutated glucose/galactose binding protein in an analyte permeable matrix comprising covalently crosslinked hydrogels or sol-gels augmented with organically modified silicates or a combination thereof;

c) exposing said mutated glucose/galactose binding protein to varying glucose concentrations; and

d) detecting a signal from said luminescent reporter group.

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[0016c] In a further aspect, the present invention provides a composition comprising a mixture of:

a) a mutated glucose/galactose binding protein having at least one amino acid substitution selected from a cysteine at position 1, a serine at position 1, a cysteine at position 11, a cysteine at position 14, a cysteine at position 19, a cysteine at position 43, a cysteine at position 74, a cysteine at position 107, a cysteine at position 110, a cysteine at position 112, a cysteine at position 113, a cysteine at position 137, a cysteine at position 149, a cysteine at position 213, a cysteine at position 216, a cysteine at position 238, a cysteine at position 287, a cysteine at position 292, a cysteine at position 152, a cysteine at position 182, a cysteine at position 236 and a cysteine at position 296 corresponding to the amino acid positions of E. *coli* GGBP;

b) an analyte permeable matrix comprising covalently crosslinked hydrogels or sol-gels augmented with organically modified silicates or a combination thereof; and

c) at least one luminescent reporter group (luminescent label).

BRIEF DESCRIPTION OF THE DRAWINGS

[00017] FIG 1 illustrates the change in fluorescence response to glucose of A213C/L238C NBD amide GGBP H6 in solution.

[00018] FIG 2 illustrates signal enhancement of entrapped binding proteins in the absence and presence of analyte relative to solution.

[00019] FIG 3 illustrates an entrapped binding protein in the absence and presence of analyte relative to solution.

[00020] FIG 4 illustrates reversible signal from an entrapped binding protein from one embodiment of the present invention following exposure to solutions of glucose at the indicated concentrations.

DETAILED DESCRIPTION OF THE INVENTION

[00021] The term biosensor generally refers to a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds, usually by electrical, thermal or optical signals. As used herein a "biosensor" refers to a protein capable of binding to analyte that may be used to detect analyte or a change in analyte concentration by a detector means as herein described. More specifically, a biosensor of the invention includes at least one mutated glucose/galactose binding protein and at least one reporter group attached thereto and a matrix permeable to analyte wherein the mutated glucose/galactose binding protein and the reporter group capable of encapsulation in the matrix.

[00022] The term "binding proteins" refers to proteins that interact with specific analytes in a manner capable of transducing or providing a detectable and/or reversible signal differentiable either from a signal in the absence of analyte, a signal in the presence of varying concentrations of analyte over time, or in a concentration-dependent manner, by means of the methods described. The transduction event includes continuous, programmed, and episodic means, including one-time or reusable applications. Reversible signal transduction may be instantaneous or time-dependent, provided a correlation with the presence or concentration of analyte is established. Binding proteins mutated in such a manner to effect transduction are preferred.

[00023] The term "galactose/glucose binding protein" or "GGBP" or "maltose binding protein" or "MBP" as used herein refers to a type of protein naturally found in the periplasmic compartment of bacteria. These proteins are naturally involved in chemotaxis and transport of small molecules (e.g., sugars, amino acids, and small peptides) into the

cytoplasm. For example, GGBP is a single chain protein consisting of two globular α/ß domains that are connected by three strands to form a hinge. The binding site is located in the cleft between the two domains. When glucose enters the binding site, GGBP undergoes a conformational change, centered at the hinge, which brings the two domains together and entraps glucose in the binding site. X-ray crystallographic structures have been determined for the closed form of GGBP from *E coli* (N. K. Vyas, M. N. Vyas, F. A. Quiocho *Science* **1988**, *242*, 1290-1295) and *S. Typhimurium* (S. L. Mowbray, R. D. Smith, L. B. Cole *Receptor* **1990**, *1*, 41-54) and are available from the Protein Data Bank (http://www.rcsb.org/pdb/) as 2GBP and 3GBP, respectively. The wild type *E. coli* GGBP DNA and amino acid sequence can be found at www.ncbi.nlm.nih.gov/entrez/ accession number D90885 (genomic clone) and accession number 230520 (amino acid sequence). Preferred GGBP is from *E. coli.*

[00024] "Mutated binding protein" (for example "mutated GGBP") as used herein refers to binding proteins from bacteria containing amino acid(s) which have been substituted for, deleted from, or added to the amino acid(s) present in naturally occurring protein. Preferably such substitutions, deletions or insertions involve fewer than 5 amino acid residues, more preferably one or two residues. Exemplary mutations of binding proteins include the addition or substitution of cysteine groups, non-naturally occurring amino acids (Turcatti, et at. *J Bio, Chem.* 1996 271, 33, 19991-19998) and replacement of substantially non-reactive amino acids with reactive amino acids to provide for the covalent attachment of electrochemical or photo-responsive reporter groups. By "reactive" amino acid is meant an amino acid that can be modified with a labeling agent analogous to the labeling of cysteine with a thiol reactive dye. Non-reactive amino acids include alanine, leucine, phenylalanine, and others, which possess side chains which cannot be readily modified once incorporated in a protein (see Greg T. Hermanson, Bioconjugate Techniques, Academic Press, 1996, San Diego, pp. 4-16 for classification of amino acid side chain reactivity).

[00025] Exemplary mutations of the GGBP protein include: a cysteine substituted for a lysine at position 11(K11C); a cysteine substituted for aspartic acid at position 14 (D14C); a cysteine substituted for valine at position 19 (V19C); a cysteine substituted for asparagine at position 43 (N43C); a cysteine substituted for a glycine at position 74 (G74C); a cysteine substituted for a tyrosine at position 107 (Y107C); a cysteine substituted for threonine at position 110 (T110C); a cysteine substituted for serine at position 112 (S112C); a double

mutant including a cysteine substituted for a serine at position 112 and serine substituted for an leucine at position 238 (S112C/L238S); a cysteine substituted for a lysine at position 113 (K113C); a cysteine substituted for a lysine at position 137 (K137C); a cysteine substituted for glutamic acid at position 149 (E149C); a double mutant including a cysteine substituted for an glutamic acid at position 149 and a serine substituted for leucine at position 238 (E149/L238S); a double mutant comprising a cysteine substituted for histidine at position 152 and a cysteine substituted for methionine at position 182 (H152C/M182C); a double mutant including a serine substituted for an alanine at position 213 and a cysteine substituted for a histidine at position 152 (H1 52C/A213S); a cysteine substituted for an methionine at position 182 (M182C); a cysteine substituted for an alanine at position 213 (A213C); a double mutant including a cysteine substituted for an alanine at position 213 and a cysteine substituted for a leucine at position 238 (A213C/L238C), a cysteine substituted for an methionine at position 216 (M216C); a cysteine substituted for aspartic acid at position 236 (D236C); a cysteine substituted for an leucine at position 23 8 (L238C); a cysteine substituted for a aspartic acid at position 287 (D287C); a cysteine substituted for an arginine at position 292 (R292C); a cysteine substituted for a valine at position 296 (V296C); a triple mutant including a cysteine substituted for an glutamic acid at position 149, an alanine substituted for a serine at position 213 and a serine substituted for leucine at position 238 (El49C/A213S/L238S); a triple mutant including a cysteine substituted for an glutamic acid at position 149, an arginine substituted for an alanine at position 213 and a serine substituted for leucine at position 238 (E149C/A213R/L238S); a quadruple mutant including a serine at position 1, a cysteine at position 149, an arginine at position 213 and a serine at position 238 (A1S/E149C/A213R/L238S); a quadruple mutant including a serine at position 1, a cysteine at position 149, a serine at position 213 and a serine at position 238 (A1S/E149C/A213S/L238S); and a quadruple mutant including a cysteine at position 149, a cysteine at position 182, a cysteine at position 213 and a serine at position 238 (E149C/M182C/A213C/L238S). Additional examples are listed in Table 2 hereinbelow. Amino acid residue numbers refer to the published sequence of *E. coli* having 309 residues, as detailed below, or the corresponding amino acid residue in any substantially homologous sequence from an alternative source (e.g., glucose/galactose binding proteins from *Citrobacter freundii* or *Salmonella typhimurium*, sequence accession numbers P23925 and P23905, respectively).

[00026] The entrapped or encapsulated mutated binding proteins of the present invention may be used in an *in vitro* or *in vivo* analyte assay which, for example, is capable of following the kinetics of biological reactions involving an analyte (e.g. glucose), as well as in clinical assays and food or beverage industrial testing. The concentration of the binding protein in the matrix is preferably less than the binding constant (K_d) of the protein with its analyte.

[00027] The mutation may serve one or more of several purposes. For example, a naturally occurring protein may be mutated in order to change the long-term stability of the protein; to conjugate the protein to a particular encapsulation matrix or polymer; to provide binding sites for detectable reporter groups; to adjust its binding constant with respect to a particular analyte; or any combination thereof.

[00028] In the instant invention, analyte and mutated protein act as binding partners. The term "associates" or "binds" as used herein refers to binding partners having a relative binding constant (K_d) sufficiently strong to allow detection of binding to the protein by a detection means. The K_d may be calculated as the concentration of free analyte at which half the protein is bound, or vice versa. When the analyte of interest is glucose, the K_d values for the binding partners are preferably between about 0.0001 mM to about 30 mM.

[00029] In the present invention, it has been shown that mutated GGBPs may be used to detect glucose binding by attaching thereto a reporter group that provides a detectable signal upon glucose binding. To "provide a detectable signal", as used herein, refers to the ability to recognize a change in a property of a reporter group in a manner that enables the detection of ligand-protein binding. For example, in one embodiment, the mutated GGBPs comprise a detectable reporter group whose detectable characteristics alter upon a change in protein conformation that occurs on glucose binding. In a preferred embodiment, the reporter group is a luminescent label that results in a mutated GGBP with an affinity for glucose that exhibits a detectable shift in luminescence characteristics on glucose binding. The change in the detectable characteristics may be due to an alteration in the environment of the label bound to the mutated GGBP.

[00030] The luminescent label may be a fluorescent label or a phosphorescent label. The use of fluorescent labels, which may be excited to fluoresce by exposure to certain wavelengths of light, is preferred.

[00031] In one embodiment, the reporter group is a fluorophore. As used herein, "fluorophore" refers to a molecule that absorbs energy and then emits light. Non-limiting examples of fluorophores useful as reporter groups in this invention include fluorescein, coumarins, rhodamines, 5-TMRIA (tetramethylrhodamine-5-iodoacetatnide), Quantum Red™, Texas Red™, Cy3, N-((2-iodoacetoxy)ethyl)-N-methyl)amino-7-nitrobenzoxadiazole (IANBD), 6-acryloyl-2-dimethylaminonaphthalene (acrylodan), pyrene, Lucifer Yellow, -Cy5, Dapoxyl® (2-bromoacetamidoethyl)sulfonamide, (*N*-(4,4-difluoro-1,3,5,7-tetrainethyl-4-bora-3a,4a-diaza- *s*-indacene- 2-yl)iodoacetamide (Bodipy507/545 IA), *N*-(4,4-difluoro-5,7-diphenyl- 4-bora- 3a,4a-diaza-s-indacene- 3-propionyl)-*N*-iodoacetylethylenediamine (BODIPY® 530/550 IA), 5- ((((2-iodoacetyl)amino)ethyl) amino)naphthalene-l-sulfonic acid (1,5-IAEDANS), and carboxy- X-rhodamine, 5/6-iodoacetamide (XRIA 5,6). Preferably, IANBD is used. Many detectable intrinsic properties of a fluorophore reporter group may be monitored to detect glucose binding. Some properties that may exhibit changes upon glucose binding include fluorescence lifetime, fluorescence intensity, fluorescence anisotropy or polarization, and spectral shifts of fluorescence emission. Changes in these fluorophore properties may be induced from changes in the fluorophore environment such as those resulting from changes in protein conformation. Environment-sensitive dyes such as IANBD are particularly useful in this respect. Other changes of fluorophore properties may result from interactions with the analyte itself or from interactions with a second reporter group, for example when FRET (fluorescence resonance energy transfer) is used to monitor changes in distance between two fluorophores.

[00032] Although the use of fluorescent labels is preferred, it is contemplated that other reporter groups may be used. For example, electrochemical reporter groups could be used wherein an alteration in the environment of the reporter gives rise to a change in the redox state thereof. Such a change may be detected, for example, by use of an electrode.

[00033] Furthermore, it is envisaged that other spectroscopically detectable labels, for example labels detectable by NMR (nuclear magnetic resonance), may be used.

[00034] The reporter group may be attached to the mutated protein or GGBPs by any conventional means known in the art. For example, the reporter group may be attached *via* amines or carboxyl residues on the protein. However, especially preferred is covalent coupling *via* thiol groups on cysteine residues. For example, for mutated GGBP, cysteines

located at position 11, position 14, position 19, position 43, position 74, position 107, position 110, position 112, position 113, position 137, position 149, position 152, position 213, position 216, position 238, position 287, and position 292 are preferred in the present invention.

[00035] Any thiol-reactive group known in the art may be used for attaching reporter groups such as fluorophores to a cysteine of an engineered or mutated protein. Iodoacetamide, bromoacetamide, or maleimide are well known thiol-reactive moieties that may be used for this purpose.

[00036] Fluorophores that operate at long excitation and emission wavelengths (for example, about 600 nm or greater exitation or emission wavelengths) are preferred when the molecular sensor is to be used *in vivo*, for example, incorporated into an implantable biosensor device (the skin being opaque below 600 nm). Presently, there are few environmentally sensitive probes available in this region of the spectrum and perhaps none with thiol-reactive functional groups. However, thiol-reactive derivatives of Cy-5 can be prepared, for example, as taught by H. J. Gruber, et al, *Bioconjugate Chem.*, (2000), 11, 161-166. Conjugates containing these fluorophores, for example, attached at various cysteine groups contained in mutated GGBPs, can be screened to identify which results in the largest change in fluorescence upon glucose binding.

[00037] Mutated GGBPs useful in the present invention may be engineered or mutated to have a histidine tag on the protein N-terminus, C-terminus, or both. Histidine fusion proteins are widely used in the molecular biology field to aid in the purification of proteins. Exemplary tagging systems produce proteins with a tag containing about six histidines and preferably such tagging does not compromise the binding activity of the mutated GGBP.

[00038] As used herein, "matrix" refers to an essentially three-dimensional environment capable of immobilizing, entrapping or encapsulating at least one binding protein for the purpose of measuring a detectable signal from a ligand-protein interaction. The relationship between the constituents of the matrix and the binding protein include, but are not limited to, covalent, ionic, and Van derWals interactions and combinations thereof. The spatial relationship between the matrix and binding protein includes heterogeneous and homogeneous distribution within and/or upon any or all of the matrix volume. The matrix

may be comprised of organic, inorganic, glass, metal, plastic, or combinations thereof. The matrix provides for the binding protein transducing element configuration which may, for example, be incorporated at the distal end of a fiber or other small minimally invasive probe to be inserted within the tissue of a patient, to enable an episodic, continuous, or programmed reading to the patient. Information from the transducing element to the patient may be provided, for example, by telemetry, visual, audio, or other means known in the art, for example, as taught in US 5,517,313, US 5,910,661, US 5,894,351, and US 5,342,789 as well as in Beach, R.D., et al. *IEEE Transactions on Instrumentation and Measurement* (1999) **48**, 6, p. 1239-1245. Information includes electrical, mechanical, and actinic, radiation suitable for deriving analyte concentration or change in concentration, as is suitable.

[00039] In one aspect of the present invention, the biosensor is used for analyte sensing *in vivo*. In this aspect, the biosensor is encapsulated into a matrix that may then be used as an implantable device. The "matrix" may be any desirable form or shape including one or more of disk, cylinder, patch, nanoparticle, microsphere, porous polymer, open cell foam, providing it is permeable to analyte. The matrix additionally prevents leaching of the biosensor. The matrix permits light from optical sources or any other interrogating light to or from the reporter group to pass through the biosensor. When used in an *in vivo* application, the biosensor will be exposed to a substantially physiological range of analyte and determination or detection of a change in analyte concentration would be desired, whereas the determination or detection includes continuous, programmed, and episodic detection means. Thus, the envisaged *in vivo* biosensor of the present invention comprises at least one mutated binding protein in an analyte permeable entrapping or encapsulating matrix such that the mutated binding protein provides a detectable and reversible signal when the mutated binding protein is exposed to varying analyte concentrations, and the detectable and reversible signal can be related to the concentration of the analyte. The implantable biosensors may, in some embodiments, be implanted into or below the skin of a mammal's epidermal-dermal junction to interact with the interstitial fluid, tissue, or other biological fluids. Information from the implant to the patient may be provided, for example, by telemetry, visual, audio, or, other means known in the art, as previously stated.

[00040] Preferably, the matrix is prepared from biocompatible materials or incorporates materials capable of minimizing adverse reactions with the body. Adverse

reactions for implants include, inflammation, protein fouling, tissue necrosis, immune response and leaching of toxic - materials. Such materials or treatments are well known and practiced in the art, for example, as taught by Quinn, C. P.; Pathak, C. P.; Heller, A.; Hubbell, I. A. *Biomaterials* 1995, *16*(5), 3 89- 396, and Quinn, C. A. P.; Connor, R. E.; Heller, A. *Biomaterials* 1997,*18*(24),1665-1670.

[00041] The biosensor may be encapsulated into a matrix derived substantially from a hydrogel. The polymer portion of the hydrogel may contain functionality that is suitable for hydrogen bonding or covalent coupling (e.g. hydroxyl groups, amino groups, ether linkages, carboxylic acids and esters and the like) to either the protein or reporter group.

[00042] Numerous hydrogels may be used in the present invention. The hydrogels may be, for example, polysaccharides such as agarose, dextran, carrageenan, alginic acid, starch, cellulose, or derivatives of these such as, e.g., carboxymethyl derivatives, or water-swellable organic polymers such as, e.g., polyvinyl alcohol, polyacrylic acid, polyacrylamide, polyethylene glycol, copolymers of styrene and maleic anhydride, copolymers of vinyl ether and maleic anhydride and derivatives thereof. Derivatives providing for covalently crosslinked networks are preferred. Synthesis and biomedical and pharmaceutical applications of hydrogels based on, comprising polypeptides, have been described by a number of researchers. (See, e.g. "Biosensors Fundamentals and Applications", edited by A. D. F. Turner, I. Karube and G. S. Wilson; published from Oxford University Press, in 1988). An exemplary hydrogel matrix derived from a water-soluble, UV crosslinkable polymer comprises poly(vinyl alcohol),N-methyl-4(4'-formylstyryl)pyridinium methosulphate acetal (CAS Reg. No. [107845-59-0]) available from PolyScience Warrington, PA.

[00043] In one embodiment of the encapsulation process, one or more hydrogels in water is added to the mutated binding protein in an aqueous buffer solution having a pH in the range of about 4 to about 10 depending on the protein. Subsequent curing of the matrix, for example, crosslinking, provides physical form. Using this technique and a conventional fabrication process (e.g. block casting, reverse emulsion polymerization, screen or contact printing, fluid-bed coating and dip or spin coating) one can obtain matrices in various configurations (e.g. granulates, nanoparticles, microparticles, monoliths, and thick and thin films) suitable for *in vitro* and *in vivo* use.

[00044] The matrix may, in one embodiment, be comprised of modified sol-gels. Modified sol-gels includes at least partial cured (or gelled) preparations comprised of permeable metal oxide glass structures containing in addition to the sol-gel precursor materials, preferably one or more organic components which hydrolytically condense along with the sol-gel precursor such that the resultant sol-gel matrix imparts properties suitable for, by example, implantation. Suitable properties include low volume shrinkage over time, resistance to cracking and other physical defects, maintenance of protein function, and compatibility with the protein and/or reporter group, and compatibility with the animal or subject to which it may be implanted. Suitable organic materials include polyols such as glycerol, ethylene glycol, propylene glycol, polyethylene glycol, and the like, for example, as taught by Gill and Ballesteros *Journal of the American Chemical Society* **1998**, *120*(34), 8587-8598. It is understood that those skilled in the art can appreciate the attributes described are generally not predictable for a given protein/sol-gel/reporter group combination, thus optimization of sol-gel precursor, organic component and protein solution materials may be expected for any given binding protein-reporter pair. It has been found by the applicants that such optimization may provide for unexpected enhanced signal, shifted binding constants; improved physical performance attributes of the matrix, and combinations thereof relative to that of other matrices or aqueous solutions thereof. Optimization of performance attributes of the protein-reporter pair and functional performance attributes of the encapsulating matrix may be achieved, for example, by way of combinatorial methods or other statistical, based design methods known in the art.

[00045] Sol-gel matrices useful for the present invention include material prepared by conventional, well-known sol-gel methods and include inorganic material, organic material or mixed organic/inorganic material. The materials used to produce the sol-gel can include, but are not limited to, aluminates, aluminosilicates and titanates. These materials may be augmented with the organically modified silicates, (ormosils) and functionalized siloxanes, to provide an avenue for imparting and manipulating hydrophilicity and hydrophobicity, ionic charge, covalent attachment of protein, and the like. As used herein the term "hydrolytically condensable siloxane" refers to sol-gel precursors having a total of four substituents, at least one, preferably two, and most preferably three or four of the substituents being alkoxy substituents covalently bound to silicone through oxygen and mixtures thereof. In the case of three, two, and one alkoxy substituent precursors, at least one of the remaining substituents preferably is covalently bound to silicone through carbon,

and the remaining substitutent(s) contains an organic functionality selected from alkyl, aryl, amine, amide, thiol, cyano, carboxyl, ester, olefinic, epoxy, silyl, nitro, and halogen.

[00046] In one embodiment of the encapsulation process, one or more of hydrolytically condensable siloxane is hydrolyzed in water, either spontaneously or under acid or base catalysis to form derivatives with an organic polyol component present in a molar amount relative to the hydrolytically condensable siloxane up to about 10:1 to 1:10, preferably to about 5:1 to 1:5, and most preferably to about 1:1. To this mixture, prior to final gellation, is added the mutated binding protein in an aqueous buffer solution having a pH in the range of about 4 to about 10 depending on the protein. At least partial condensation reactions give rise to the final matrices.

[00047] In another embodiment, the hydrolytically condensable siloxane hydrolyzed in water, either spontaneously or under acid or base catalysis to form derivatives with the organic polyol, is mixed with a water-soluble polymer component. Suitable water-soluble polymers include polyvinyl alcohol (PVA), poly-(maleic acid co-olefin) sodium salt (PMSA), poly-(vinylsulfonic acid) sodium salt (PVSA), and polyvinyl, pyrollidone (PVP). Poly-(maleic; acid co-olefin) includes copolymers of maleic anhydride with styrene, vinyl ether, and C1-C8 olefins and salts thereof, for example, sodium, potassium, ammonium, tetraalkylammonium, and the like. Preferably, the water-soluble polymer component is from 0 to about 30% by weight of the sol-gel composition.

[00048] In another embodiment, the hydrolytically condensable siloxane hydrolyzed in water, either spontaneously or under acid or base catalysis to form derivatives with the organic polyol, is mixed with one or more functionalized silicone additives (FSA) in amounts from 0 to about 0.6% mole ratios to hydrolytically condensable siloxane. Exemplary FSA's include *alkyl* derivatives: for example, methyltrimethoysilane (MTMOS): *amine* derivatives: for example, 3-aminopropyl triethoxysilane (ATEOS); and *bis* silane derivatives: for example, (bis(3-methyldimethoxysilil)propyl) polypropylene oxide (BIS).

[00049] In another embodiment, both the water-soluble polymer component and the functionalized silicone additive are mixed together with the hydrolytically condensable siloxane hydrolyzed in water, either spontaneously, or under acid or base catalysis to form derivatives with the organic polyol, to provide for a matrix suitable for entrapment or encapsulation the binding protein. Using the aforementioned sol-gel technique and a

conventional fabrication process (e.g. block casting, reverse emulsion polymerization, screen or contact printing, fluid bed coating and dip or spin coating) one can obtain aerogel- or xerogel- matrices in various configurations (e.g. granulates, nanoparticles, microparticles, monoliths, and thick and thin films) suitable for use *in vitro* and *in vivo*.

[00050] In another embodiment the matrix may be formed from dialysis membranes. The dialysis membranes can be constructed to physically encapsulate or entrap the protein. Covalent attachment to the membrane is considered within the scope of the as described embodiment. The membrane should be chosen based on molecular weight cut-off such that analytes of interest can readily permeate the membrane whilst high molecular weight materials would be restricted from entering, or in the case of the mutated binding proteins, leaving the membrane matrix. The molecular weight cut-off required would be such as to meet the afore-mentioned requirement and is within the skill of one familiar with this art. Typically, membranes having molecular weight cut-off between about 1000 to about 25,000 Daltons are suitable. Using this technique, matrices in various configurations and shapes suitable for use *in vitro* and *in vivo* can be prepared.

[00051] It is also contemplated that matrices containing the binding protein and reporter group be combinations of one or more hydrogel, sol-gel, and dialysis membranes. For example, a protein entrapped or encapsulated within, a hydrogel or sol gel can be placed within a dialysis membrane of a suitable shape and size as well provide for implantation within a subject, or to manipulate mass-transport properties or permeability to the analytes of the matrix.

[00052] The matrix entrapped or encapsulated binding protein biosensors of this invention are capable of measuring or detecting micromolar (10^{-6} molar) to molar analyte concentrations without reagent consumption. In some embodiments, their sensitivity to analyte may enable the biosensors, to be used to measure the low analyte concentrations known to be present in low volume samples of interstitial fluid. The implantable biosensors may, in some embodiments, be implanted into or below the skin of a mammal's epidermal-dermal junction to interact with the interstitial fluid, tissue, or other biological fluids. The binding protein biosensors of the present invention provide for the means to monitor analyte continuously, episodically, or "on-demand" as would be appropriate to the user or to the treatment of a condition.

[00053] In other embodiments, sensitivity of the biosensors to analyte (for example glucose) is such that they may be used to test blood analyte levels or the concentration of analyte in a biological solution or other solution may be determined. As used herein, a "biological solution" includes, but is not limited to, blood, perspiration, and/or ocular or interstitial fluid, and combinations thereof.

EXAMPLES

[00054] The following examples illustrate certain preferred embodiments of the instant invention, but are not intended to be illustrative of all embodiments. Labeled mutated maltose binding protein S337C MBP with fluorophore reporter probe NBD used herein in accordance with the procedure set forth by Cass, A. et al. (*Anal. Chem.* **1994,** *66,* 3840-3847). Fluorescence emission spectra of mutated, labeled protein was measured using an SLM Aminco fluorimeter (Ontario, Canada) with slit settings of 8 and 4 for excitation and settings of 5 and 5 on the MC250 emission monochromator to compare the ligand-binding performance of the entrapped fluorophore-labeled proteins in various matrices to the performance of the same proteins in solution. The initial fluorescence emission intensity is defined as I_0. The relative ratio of the emission intensity maxima in the presence of the protein's respective ligand (I_f) to the ligand's absence (I_o) is defined as ΔF.

[00055] Binding constants were determined by titration of increasing concentrations of glucose into a protein solution with mixing following each addition of glucose. Slit settings were the same as listed above. The K_d was determined from the following relationships as adapted from Pisarchick and Thompson (1990):

$$F = \frac{F_{inf} + F_0 - F_{inf}}{1 + x/Kd} \qquad (1)$$

where F is fluorescence intensity, F_{inf} is fluorescence at infinity, F_0 is fluorescence at zero glucose, and x is the free concentration of glucose ($[Glc]_{free}$) as determined by the relationship:

$$[GLc]_{free} = \frac{[GLC]_{tot} - [Prot]_{tot} - Kd + \sqrt{([Glc]_{tot} - [Prot]_{tot} - Kd)2 + 4 * [Glc]_{tot} * Kd}}{2}$$

where $[Glc]_{tot}$ and $[Pro]_{tot}$ are the total concentrations of glucose and protein, respectively.

[00056] EXAMPLE 1: This example describes the method for the expression and purification of mutant proteins without histidine tags. GGBP is encoded by the MglB-1 gene in *E. coli*. This protein was altered by introducing the amino acid cysteine at various positions through site directed mutagenesis of the MglB-1 gene. These proteins were then expressed in *E. coli* and purified.

[00057] Cassette mutagenesis of MglB-1 was accomplished as follows. The wild-type MglB-1 gene was cloned into a pTZ18R vector (Dr. Anthony Cass, Imperial College, London, England). Mutant plasmids were generated from this parent plasmid using cassette mutagenesis producing randomized amino acid sequences, essentially as described by Kunkel (1991) and cloned in *E. coli* JM109 (Promega Life Science, Madison, WI). Mutant plasmids were identified by sequencing. The mutant protein was induced in JM109 and purified as described below. An *E. coli* JM109 colony containing the mutant plasmid was grown overnight at 37°C with shaking (220 rpm) in LB broth containing 50 μg/mL ampicillin (LB/Amp). The overnight growth was diluted 1:100 in 1 L fresh LB/Amp and was incubated at 37°C with shaking until the OD_{600} of the culture was 0.3-0.5. Expression of the mutant was induced by the addition of 1mM IPTG (Life Technologies, Gaithersburg, MD) final concentration with continued incubation and shaking at 37°C for 4-6 hours. The cells were harvested by centrifugation (10,000 x g, 10 min, 4°C).

[00058] The mutant protein was harvested by osmotic shock and was purified by column chromatography. The cell pellet was resuspended in a sucrose buffer (30 mM Tris-HCL pH 8.0, 20% sucrose, 1mM EDTA), incubated at room temperature for 10 min, and then centrifuged (4000 x g, 15 min, 4°C). The supernatant was poured off and kept on ice. The cell pellet was resuspended, and 10 mL ice cold, sterile deionized H_20 was repeated, and the suspension was incubated on ice and centrifuged. The remaining supernatant was pooled with the other collected supernatants and was centrifuged once again (12,000 x g, 10 min,

4°C). The pooled shockate was filtered through a 0.8 μm and then a 0.45 μm filter. Streptomycin sulfate (Sigma Chemical Co., St. Louis. MO), 5% w/v, was added to the shockate and was stirred once for 30 min followed by centrifugation (12,000 x g, 10 min, 4°C). The shockate was then concentrate using the Amicon Centriprep 10 (10,000 MWCO) filters (Charlotte, NC) and dialyzed overnight against 5 mM Tris-HCl pH 8.0, 1 mM $MgCl_2$. The dialyzed shockate was centrifuged (12,000 x g, 30 min, 4°C). The resulting supernatant was added to a pre-equilibrated DEAE Fast Flow Sepharose column (Amersham Pharmacia Biotech, Piscataway, NJ) at 0.5 mL/min. The column was washed with 5-10 column volumes. A linear gradient from 0-0.2 M NaCl was applied to the column and fractions were collected. The mutant protein containing fractions were identified by SDS-PAGE with Coomassie Brilliant Blue staining (MW approx. 32 kDa). The fractions were pooled and dialyzed overnight (4°C) against phosphate buffered saline (PBS) or 10 mM ammonium bicarbonate (pH 7.4), concentrated using Amicon Centriprep 10 filters, and stored at 4°C or -20°C with glycerol. The ammonium bicarbonate dialyzed protein was lyophilized.

[00059] EXAMPLE 2. This example describes the expression and purification of mutant GGBPs containing Histidine Tags. GGBP mutants were engineered either by site-directed mutagenesis or by cassette mutagenesis. Site-directed mutagenesis (QuikChange, Stratagene, La Jolla, CA) was performed to alter individual amino acids in the pQE70 vector by replacing one amino acid with another, *specifically* chosen amino acid. The cassette mutagenesis method (Kunkel 1991) was performed to *randomize* amino acids in a specified region of the GGBP gene. The mutated cassettes were then subcloned into the pQE70 expression vector. The pGGBP-His plasmid contained the GGBP gene cloned into the pQE70 expression vector (Qiagen, Valencia, CA). This construct places six histidine residues on the C-terminus of the GGBP gene. *E. coli* strain SG13009 was used to overexpress mutant GGBP-His following standard procedures (Qiagen). After overexpression of a 250 mL culture, the cells were collected by centrifugation (6000 rpm) and resuspended in 25 mL bugbuster (Novagen, Madison, WI). Lysozyme (25 mg was added to the lysate and the mixture was gently mixed at room temperature (RT) for 30 min. Clear lysate was produced by centrifugation (6000 rpm) and to this, 0.5 ml imidizole (1M) and 3 ml of Ni-NTA beads (Qiagen) was added. After 30 minutes of gently mixing at RT, the mixture was centrifuged (6000 rpm) and the lysate removed. The beads were washed 25 ml of solution (lM NaCl, 10 mM tris, pH 8.0) and recentrifuged. The mutant GGBP-His was eluted from the beads by adding 5 mL solution (160 mM imidazole, 1 M NaCl, 10 mM

Tris, pH 8.0) and mixing for 15 min. The protein solution was immediately filtered through a Centriplus YM-100 filter (Amicon, Charlotte, NC) and then concentrated to 1-3 mg/ml using a Centriplus YM-10 filter. The protein was dialyzed overnight against 2 L of storage solution (1 M NaCl, 10 mM Tris, 50 mM $NaPO_4$, pH 8.0)

[00060] EXAMPLE 3. This example describes generically the labeling of binding protein with reporter probe. An aliquot of mutant GGBP containing cysteine (4.0 nmol) in PBS was treated with 2 mM dithiothreitol (5 μL, 10 nmol) for 30 min. A stock solution of N,N'-dimethyl-N- (iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine (IANBD amide, 0.5 mg) was prepared in DMSO (100 μL, 11.9 mM) and 3.36 μL (40 nmol) was added to the protein. The reaction proceeded at room temperature for 4 h on a Dynal rotamix in the dark. The labeled protein was purified by gel filtration on a NAP-5 column (Amersham Pharmacia). The labeling ratios were determined using an estimated extinction coefficient (50 mM^{-1} cm^{-1}) for GGBP that was calculated in GeneWorks 2.45 (IntelliGenetics), ε_{478} (IANBD amide) =25$mM^{-1}cm^{-1}$), and a measurement of O.D. for a standard solution of IANBD amide at 280 nm and 478 nm. The dye concentration in the protein was calculated as $C_{dye} = A_{478}/\varepsilon_{478}$. The absorbance of protein at 280 nm was calculated as $A_{prot(280)}=A_{total(280)}-A_{dye(280)}$, where $A_{dye(280)} = A_{478} \times (A_{280}/A_{478})_{dye\ std}$. The concentration of protein was then $C_{prot(280)} = A_{prot(280)}/\varepsilon_{280}$. FIG 1 illustrates the change in fluorescence response to glucose concentration of a representative example, A213C/L238C NBD amide GGBP H_6 in solution. Table 1 summarizes the change in fluorescence of various GGBP mutants labeled with reporter groups, including reporting groups having either excitations or emission maximum of at least 600 nanometers. Table 2 summarizes the change in fluorescence, and determined K_d values of mutations of one, two, three, and four amino acid substitutions. This data clearly shows mutations of the GGBP labeled with a reporter group can provide desirable attributes as glucose biosensors. The data shows the mutation-reporter group relationship for the samples tested.

TABLE 1
Percent Change in Fluorescence for GGBP Mutants[1]

Dye	Excitation / emission (nm)	S112C	M182C	A123C	A213C His_6	M216C
IANBD amide	470 / 550	0	4	3	51	7
IANBD ester	470 / 550					
IAEDANS	336 / 490	-7	-8	0		-9
Bodipy 530/550 IA	530 / 550	7	-10	33		4
XRIA 5,6	575 / 600	-21	-19	-38		-15
Lucifer Yellow IA	426 / 530			-14		-3
Bodipy 507/545 IA	507 / 545			25		-3
Cy5	640 / 660	2	0	11		-7
Texas Red-maleimide	580 / 610				-13	
Dapoxyl	375 / 580	15	7	12		2

[1] F from 0 to 1 mM glucose at 0.5uM[dye]. Unless otherwise indicated all mutants were without histidenc tags.

Table 2. Summary of GGBP-H6 NBD Mutations

IDENTIFICATION	Solution Δ F(%)[1]	Kd(mM)[2]	Dye/Prot	Sol-Gel Δ F(%)	Kd(mM)
wild type	intrinsic	0.0002			
A1C					
A1S					
A1S,E149C,A213R,L238S	+21[3]		0.31		
A1S,E149C,A213S,L238S	+480	0.37	0.9		
K11C	10		1.8		
D14C	1		1.5	21	
V19C	-56	0.0001	0.38	-0.99	
N43C	40	0.0002	0.28		
G74C	-3	0.0009	1.43		
Y107C	-30	0.001	0.93		
T110C	-9				
S112C	220	0.05	1.15		
S112C,L238S	6		1.5		
K113C	15		0.65		
K137C	-5	0.00004	1.17		
E149C	300	0.0002	0.96	57	
E149C,A213C	+110		0.70		
E149C,A213R	660	1	1.1		
E149C,A213S	240[4]	0.0023	1.1		
E149C,A213T	350	1	0.6		
E149C,A213L	280	0.1	1.1		
E149C,A213Y	280	0.1	1.1		
E149C,A213C,L238C	+39[3]		1.08		
E149C,A213S,K223N					
E149C,K223N	260	0.003	0.7		
E149C,L238C	260	5	1.6		
E149C,L238S	660[4]	0.08	1.36		
E149C,K223N,N256R					
E149C,N256S	1		0.93		
E149C,N256R	200	742[6]	0.9		
E149C,M182C,A213C,L238S	200	216[6]	3.2		
E149C,A213S,L238S	480	0.47	0.76		
E149C,A213R,L238S	500	12	1.1		
H152C	210	0.07	1.3	317	0.36
H152C,A213S	100	0.16			
H152C,A213R	-3		1.2		
H152C,K223N	200	0.003	1		
H152C,M182C					
M182C	11				
A213C	50	0.124	0.68		
A213C,L238C	24, 67[3]	6	1.4	70	1
A213C,L255C	-5		0.98		
M216C	67	0.008	0.91		
D236C	+2[3]		0.43		
L238C	-6, +3[3]	.003 (SPR)	1.3		
D287C	4		1.1		
R292C	-34	0.0008	1.5		
V296C	-10	0.00001[5]	1.08		

[1] ΔF from 0 to 1 mM Glc at 0.5 mM [dye]

[2] K_d measured at 0.1 mM [dye]

[3] ΔF when measured from 0 to 100 mM Glc

[4] ΔF when measured from 0 to 10 mM Glc

[5] Estimated; Sigma Plot calc. did not converge

[6] Estimated; curve did not reach saturation

[00061] EXAMPLE 4. This example describes the immobilization of a biosensor of the instant invention using glycerol modified silicate condensate (GMSC). The additions of

glycerol modified silicate condensate (GMSC). The additions of glycerol directly followed the initial tetraethoxyorthosilicate (TEOS) or tetramethoxyorthosilicate (TMOS) acid hydrolysis. A range of hydrolysis times, pH levels, reagent addition order, and TEOS:glycerol ratios were evaluated to determine the optimal conditions for beginning the glyceration reaction. Preferred conditions were found using an interval of 10 to 30 minutes between hydrolysis and glycerol addition, a pH range of between 0.5 and 1, and a 1:1 mole ratio of TEOS to glycerol. The following describes a modified procedure of Gill and Ballesteros for a TEOS-based glycerol modified silicate condensate (GMSC) preparation using the following ratios of reagents: TEOS or TMOS:1; H_2O:1, Methanol:4, Glycerol:1. TEOS or TMOS in methanol was added to a flask and cooled to 0°C over ice. Next 0.6M HCl was added drop-wise to the solution. After 20 minutes of stirring, glycerol was added dropwise. The reaction was warmed slowly over 1-2 hours to 20 - 25°C. Following this the reaction vessel was heated further and maintained at a temperature range of 60 - 70°C under nitrogen for between 36 and 42 hours. The optimal time was 40 hours. Incomplete glyceration was indicated by an observable phase separation for reactions stopped before 36 hours. Reactions maintained beyond 42 hours produced GMSC sol-gel monoliths with greatly reduced physical properties, for example, increased brittleness. Following the 40 hours reaction at 60 - 70°C, the solution volume was reduced by rotary evaporation until it was viscous and transparent, at which point methanol was added to the solution in a 4:1 ratio by weight. This GMSC solution proved to be stable and provided consistent results for several months when stored at freezer temperature. When the GMSC solution was to be used methanol was removed by rotary evaporation and distilled water was added in a 1:1 ratio by weight to the GMSC reagent to catalyze the final hydrolysis/gelation. Monoliths, thin films, and powders were created with this procedure using an appropriate container to function as a mold. The GMSC sol-gel monoliths were not brittle and had shrinkage of about 8% after curing at 4°C at 50% relative humidity for 2 weeks (% shrinkage was the average of changes in diameter and length measured with a microcaliper and compared to original mold dimensions). Electron microscopy (SEM) further illustrated the significant improvements in surface fracturing between monoliths created with TEOS hydrolysis and the monoliths created through the GMSC procedure described above. This set of experiments demonstrates how sol-gels with improved physical characteristics can be produced in accordance with the methods taught in the instant invention.

[00062] EXAMPLE 5. This example describes further optimization of physical properties by GMSC sol-gels in which glycerol has been partly substituted with either ethylene glycol (EG) or polyethylene glycol (PEG). Ethylene glycol (EG) was evaluated as a substitute for glycerol in mixtures where the ratio of glycerol and EG was varied but the mole ratio of total glycerol and EG was maintained constant relative to other reagents. Sol-gel monoliths were prepared by the procedure described in the preceding example, cured for two weeks at 4°C and 50% relative humidity and their % shrinkage was determined as shown in Table 3. Percent shrinkage is defined as the average of the decrease in length and diameter verses original dimensions. Monoliths used for determination of shrinkage had no protein/fluorophore present. For F measurements, the samples listed in Table 1 were prepared containing H152 GGBP-H6 NBD (from Example 3) as described below.

Table 3. Average % shrinkage and ΔF of sol-gel Matrix after 2 weeks.

Sol-gel Matrix	Average % shrinkage	F (10mM Glucose)
1. Solution (H152 GGBP-H6 NBD 0.8-1.2uM	Not Applicable	1.53
2. TEOS	35.95 +/- 0.24	1.39
3. GMSC-TEAO	8.01 +/- 0.19	1.57
4. GMSC-TEOS 15wt% PMSA, 0.145Mol% MTMOS	3.99 +/- 0.27	-
5. 1% EG/GMSC-TEOS	3.10 +/- 0.17	1.53
6. 5% EG/GMSC-TEOS	2.48 +/- 0.15	1.47
7. 10% EG/GMSC-TEOS	-	1.37
8. 20% EG/GMSC-TEOS	-	1.34

[00063] The 1% and 5% EG/GMSC sol-gels (entries 5 and 6 respectively in Table 3) were found to have significantly less % shrinkage than either the plain TEOS sol-gels or GMSC modified TEOS sol-gels (entries 2 and 3 respectively in above Table 3). Polyethylene Glycol (PEG) was also evaluated qualitatively as a partial substitute for glycerol in similar proportions in GMSC sol-gels and produced monoliths with favorable surface properties and rubber-like flexibility. In summary, partial substitution of either ethylene glycol (EG) or polyethylene glycol (PEG) for glycerol in GMSC sol-gels provides improvements in physical properties, for example, minimized shrinkage and reduced surface fracturing. These sol-gels matrices containing binding protein were found to possess performance equal to or better than that of protein in solution.

[00064] EXAMPLE 6. Entrapment Of Binding Proteins In GMSC Sol-Gels Containing Functionalized Silicone Additives (FSA) and Polymers. This example describes

the addition of polymer and organic polyol additives to optimize the GMSC sol-gels for entrapping binding proteins to both maintain and enhance their spectral properties upon ligand binding. The binding proteins were labeled with a fluorophore (as described Example 3). The protein solutions were added during the final hydrolysis/gelling step described previously to produce final concentrations of 2-4 μM protein within the sol-gel. The polymer additives and functionalized silicone additives (FSA's) were obtained from Sigma-Aldrich Chemicals (St. Louis, MO). Polymer additives were evaluated in amounts between 0 to about 30wt. FSA's were evaluated as additives to the GMSC sol-gels in amounts from 0 to about 0.6% mole ratio. Thus, rotary evaporation of the GMSC reagent to remove methanol from its storage solution was followed by reconstitution in water in a 1:1 ratio by weight. To a 400 μL aliquot of this mixture, 800 μL of buffer (HEPES, PBS or Tris) with a premixed water-soluble polymer additive was added along with any FSA-modified GMSC. A mutated binding protein in solution was then added, and after thorough mixing, 100 μL of the mixture was dispensed into a 96 well microplate (Falcon white flat bottom plates, product # 35-3941, BD Labware, NJ). The sol-gel containing microplates were cured 12-18 hours at 4 °C and 50% relative humidity. GMSC-BIS was prepared by the same procedure as the TEOS-based GMSC, but with substitution of (Bis(3-methyldimethoxysily)propyl) polypropylene oxide for TEOS. GMSC-MTMOS and GMSC-ATEOS were prepared similarly except that the hydrolysis was carried out with either 10% of the amount of acid, or no acid in the hydrolysis step, respectively, compared to the TEOS-based GMSC procedure. Fluorescence emission was measured with a Varian Cary Eclipse scanning fluorometer with microwell plate adapter (Varian Instruments, Victoria, Australia). Excitation was at 475 nm and emission recorded from 500 to 600 nm, typically monitoring emission maximum peak fluorescence. Slit widths were 5 nm for excitation and 10 nm for emission. Individual I_o determinations were made for each well and 100 μL of a ligand solution (1 mM maltose in the case of S337C MBP) was added and I_f readings were obtained, from which $\triangle F$ values were calculated. The modified sol-gel entrapped proteins exhibited greater initial fluorescence (I_o) in the absence of ligand when compared to equivalent concentrations of the same protein in solution. Figure 2 shows the fluorescence emission before and after glucose addition for GGBP H152 His6 NBD in the H152 optimized sol-gel and in solution. The I_o spectra for each experiment was normalized to a maxima of 1.0. The figure shows about 2-3-fold enhancement of $\triangle F$ obtained for the optimized sol-gel matrices containing binding protein when exposed to analyte in comparison to protein in solution. Thus, after

optimization of the sol-gel formulations for each protein, an enhancement of ΔF was observed. It should be noted that emission maximum may be shifted for sol-gel entrapped protein-reporter group samples as compared to solution. In addition, these modified sol-gel matrices provide improved physical properties as shown in entry 4 of Table 3. Table 4 shows an approximate range of components of formulations giving improved response for each of the individual proteins evaluated.

[00065] Table 4. Optimized sol-gel formulations for H152C GGBP His6-NBD, A213C/L238C GGBP His6-NBD, and S337C-MBP-NBD.

	H 152 C GGBP-NBD		**A213C/L238C GGBP-NBD**		**S337C MBP-NBD**	
		Range		Range		Range
Polymer additive	PMSA	14 - 16%wt	PMSA	4 - 5 % wt	PMSA	14 - 16 % wt
FSA additive	Alkyl	0.13 - 0.16mol%	Alkyl	0.01 - 0.03mol%	Amine	0.01 - 0.03mol%
Buffer	Tris		PBS		PBS	
pH Range		7.3 - 7.5		7.4 - 7.7		7.4 - 7.7
K_d (mM) [solution value]	0.36 [0.07]		2.2 [6]		-	
ΔF (enhancement vs. solution) [sugar challenge]	2.93x [10mM]		2.36x [100mM]		2.53x [0.1mM]	

(GGBP=glucose/galatose binding protein; MBP=maltose binding protein; NBD=N9acetoxy)ethyl)-N-methyl)amino-7-nitrobenzoxadiazole.)

[00066] The formulation optimization experiments described above used Design-Expert 6.0.5 (Stat-Ease, Inc., Minneapolis, MN) to design several Design of Experiments (DOE's). Among other variables in the formulation that were optimized in each DOE were buffer type (HEPES, PBS and Tris) and pH (from 6.6 to 7.8). Surprisingly, the optimal formulation constituents and concentration ranges were quite different for each protein. In all cases, however, substantial performance improvements were obtained for the optimized formulations in comparison to either solution performance or performance in unmodified sol-gels.

[00067] Example 7. This example describes the entrapment of GGBP H152C in UV cross-linked hydrogel matrix and the effect of the matrix on the fluorescence change and binding affinity. In this experiment SbQ-PVA from Polysciences Inc. was added 100 ul of PBS buffer and mixed for one hour to mix in a rotary mixer. 80 ul of this solution was then

mixed with 20 ul of labeled protein. Final protein concentration was spectroscopically determined to be 0.15 mg/ml. After mixing, aliquots were dispensed into 96-well plates and dried in a chamber maintained at 20% humidity for 12h followed by curing with UV light. Wells containing protein encapsulated in matrix were challenged with 2µl of 10mM glucose and compared to protein solution without matrix having equivalent protein loading. Figure 3 shows the ability of the mutated protein matrix to respond to the analyte in a manner, and with a sensitivity, equivalent to that obtained in solution. The K_d of the entrapped protein was comparable to that obtained in solution.

[00068] EXAMPLE 8. This example describes the immobilization of a biosensor of the instant invention into a dialysis membrane matrix and ability of the matrix to provide reversible and continuous readings. Using a Varian Eclipse fluorimeter with a fiber optic attachment, GGBP L238C/A213C protein (2 µM in PBS buffer) entrapped with a dialysis membrane having a molecular cut-off of 3500 Daltons affixed to the distal end of the fiber. Solutions were prepared containing PBS buffer, 2mM, and 20mM glucose in PBS buffer. With the probe in PBS solution, readings were recorded at 0.02 second intervals of the emission wavelength 521 nm, followed by insertion of the fiber into the glucose solutions. Replacement of the fiber into buffer-only solution resulted in the return of initial signal. Figure 4 depicts multiple cycles alternating between buffer and glucose solutions demonstrating the reversibility of the biosensor entrapped within a permeable matrix within physiological range. Similar results were observed with sol-gel entrapped samples demonstrating applicability for continuous use.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-

1. A glucose biosensor comprising at least one mutated glucose/galactose binding protein (GGBP), and at least one luminescent reporter group (luminescent label) covalently attached thereto, wherein the mutant GGBP has at least one amino acid substitution of a non-reactive amino acid with a reactive amino acid and said mutant GGBP is encapsulated in an analyte-permeable matrix comprising covalently crosslinked hydrogels or sol-gels augmented with organically modified silicates or a combination thereof.

2. The biosensor of claim 1, wherein said at least one luminescent reporter group is capable of providing a detectable and reversible signal in response to binding to said mutant GGBP.

3. The biosensor of claim 1, wherein the luminescent reporter group is a fluorescent label or a phosphorescent label.

4. The biosensor of any one of claims 1 to 3, wherein said amino acid substitution is selected from a cysteine at position 1, a serine at position 1, a cysteine at position 11, a cysteine at position 14, a cysteine at position 19, a cysteine at position 43, a cysteine at position 74, a cysteine at position 107, a cysteine at position 110, a cysteine at position 112, a cysteine at position 113, a cysteine at position 137, a cysteine at position 149, a cysteine at position 213, a cysteine at position 216, a cysteine at position 238, a cysteine at position 287, a cysteine at position 292, a cysteine at position 152, a cysteine at position 182, a cysteine at position 236 and a cysteine at position 296 corresponding to the amino acid positions of E. *coli* GGBP.

5. The biosensor of claim 4, wherein said amino acid substitution is selected from a cysteine at position 1, a serine at position 1, a cysteine at position 11, a cysteine at position 14, a cysteine at position 19, a cysteine at position 43, a cysteine at position 74, a cysteine at position 107, a cysteine at position 110, a cysteine at position 112, a cysteine at position 113, a cysteine at position 137, a cysteine at position 149, a cysteine at position 213, a cysteine at position 216, a cysteine at position 238, a cysteine at position 287, a cysteine at position 292, a cysteine at position 152, a cysteine at position 182, a cysteine at position 236 and a cysteine at position 296 of E. *coli* GGBP.

6. The biosensor of claim 4 comprising at least two amino acid substitutions selected from a cysteine at position 112 and a serine at position 238, a cysteine at position 149 and a serine at position 238, a cysteine at position 152, and a cysteine at position 182, a cysteine at position 152 and a serine at position 213, a cysteine at position 213 and a cysteine at position 238, a cysteine at position 149 and an arginine at position 213, a cysteine at position 149 and a cysteine at position 213, a cysteine at position 149 and a threonine at position 213, a cysteine at position 149 and a leucine at position 213, a cysteine at position 149 and a tyrosine at position 213, a cysteine at position 149 and an asparagine at position 223, a cysteine at position 149 and a cysteine at position 238, a cysteine at position 149 and a serine at position 256, a cysteine at position 149 and an arginine at position 256, a cysteine at position 152 and an arginine at position 213, a cysteine at position 152 and an asparagine at position 223, a cysteine at position 213 and a cysteine at position 255 of E. *coli* GGBP.

7. The biosensor of claim 4 comprising at least three amino acid substitutions selected from a cysteine at position 149, a serine at position 213 and a serine at position 238; a cysteine at position 149, an arginine at position 213 and a serine at position 238; a cysteine at position 149, a cysteine at position 213 and a cysteine at position 238; a cysteine at position 149, a serine at position 213 and an asparagine at position 223; and a cysteine at position 149, an asparagines at position 223 and an arginine at position 256 of E. *coli* GGBP.

8 The biosensor of claim 4 comprising at least four amino acid substitutions selected from a serine at position 1, a cysteine at position 149, an arginine at position 213 and a serine at position 238; a serine at position 1, a cysteine at position 149, a serine at position 213 and a serine at position 238; and a cysteine at position 149, a cysteine at position 182, a cysteine at position 213 and a serine at position 238 of E. *coli* GGBP.

9. The biosensor of any one of claims 4 to 8, wherein said binding protein additionally comprises at least one histidine tag.

10. The biosensor of claim 1, wherein

(i) said luminescent label has an excitation wavelength of more than about 600 nanometers; or

(ii) said luminescent label has an emission wavelength of more than about 600 nanometers; or

(iii) said luminescent label is covalently coupled to said at least one glucose/galactose binding protein by reaction with a member selected from fluorescein, coumarins, rhodamines, 5-TMRIA (tetramethylrhodamine-5- iodoacetamide), R-phycoerythrin coupled to (9-(2(or4)-(N-(2-maleimdylethyl)-sulfonamidyl)-4(or2)-sulfophenyl)-2,3,6,7,12,13,16,17-octahydro-(1H,5H,11H,15H-xantheno(2,3,4-ij:5,6,7-i'j')diquinolizin-18-ium salt), (9-(2(or4)-N-(2-maleimdylethyl)sulfonamidyl)-4(or2)-sulfophenyl)-2,3,6,7,12,12,16,17-octahydro-(1H,5H,11H,15H-xantheno(2,3,4-ij:5,6,7-i'j')diquinolizin-18-ium salt), 2-(5-(1-(6-(N-(2-maleimdylethyl)-amino)-6-oxohexyl)-1,3-dihydro-3,3-dimethyl-5-sulfo-2H-indol-2-ylidene)-1,3-propyldienyl)-1-ethyl-3,3-dimethyl-5-sulfo-3H-indolium salt, N-((2-iodo-acetoxy)ethyl)-N-rmethyl)amino-7-nitrobenzoxadiazole (IANBD), 6-acryloyl-2- ditmethylaminonaphthalene (acrylodan), pyrene, Lucifer Yellow, 2-(5-(1-(6-(N-(2-maleimdylethyl)-amino)-6-oxohexyl)-1,3-dihydro-3,3-dimethyl-5-sulfo-2H-indol-2-ylidene)-1,3-pentadienyl)-1-ethyl-3-3-dimethyl-5-sulfo-3H-indolium salt, (2-bromoacetamidoethyl)sulfonamide): 4-5-(4-dimethylaminophenyl)oxazol-2-yl)phenyl-N-(2-bromoacetamidoethyl)sulfonamide, (N-(4,4 -difluoro-1,3 ,5,7- tetramethyl-4-bora-3a,4a-diaza-s-indacene-2-yliodoacetamide (BODIPY® 507/545 IA), N-(4,4-difluoro-5,7-dipheny1-4-bora-3a,4a-diaza-s-indacene-3- propiony1)-N-iodoacetylethylenediamine (BODIPY® 530/550 IA), 5-((((2- iodacetyl)amino)ethyl)amino)naphthalene-l-sulfonic acid (1,5-IAEDANS), and carboxy-X-rhodamine, 5/6-iodoacetamide (XRIA 5,6).

11. The biosensor of claim 1, wherein said analyte is glucose or galactose.

12. The biosensor of claim 1, wherein said analyte-permeable matrix comprises a covalently crosslinked hydrogel, preferably said covalently crosslinked hydrogel is selected from polypeptides, polysaccharides, polysaccharide derivatives, polyvinyl alcohols, polyacrylic acids, polyacrylamide, polyethylene glycols, copolymers of styrene and maleic anhydride, copolymers of olefin and maleic anhydride, and copolymers of vinyl ether and maleic anhydride, most preferably crosslinked hydrogel is a polyvinyl alcohol comprising poly(vinyl alcohol) and N-methyl-4(41-formylstyryl)phyridinium acetal salts.

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13. The biosensor of claim 1, wherein said analyte-permeable matrix comprises a sol-gel augmented with organically modified silicates, preferably said analyte-permeable matrix is a glycerol modified condensate (GMSC)-derived sol gel.

14. The biosensor of any one of claims 1 to 13, wherein the GGBP encapsulated in the matrix is contained within an additional matrix, preferably said additional matrix is a hydrogel, a sol-gel or a dialysis membrane.

15. The biosensor of any one of claims 1 to 14 which is an implantable device.

16. The biosensor of any one of claims 1 to 15 for use as a diagnostic device.

17. A method for glucose detection comprising:

a) providing at least one mutated glucose/galactose binding protein having at least one amino acid substitution of a non-reactive amino acid with a reactive amino acid and at least one luminescent reporter group covalently attached thereto;

b) entrapping or encapsulating said mutated glucose/galactose binding protein in an analyte permeable matrix comprising covalently crosslinked hydrogels or sol-gels augmented with organically modified silicates or a combination thereof;

c) exposing said mutated glucose/galactose binding protein to varying glucose concentrations; and

d) detecting a signal from said luminescent reporter group.

18. The method of claim 17:

(i) additionally comprising the step of exposing said luminescent reporter group to an energy source capable of exciting said reporter group to emit said signal; or

(ii) wherein said detecting comprises reversible signal detection corresponding to said varying glucose concentrations; or

(iii) wherein said detecting is continuous, programmed, episodic, or combinations thereof.

19. The method of claim 17 or 18, wherein said glucose/galactose binding protein is as defined in any one of claims 4 to 10.

20. A composition comprising a mixture of:

a) a mutated glucose/galactose binding protein having at least one amino acid substitution selected from a cysteine at position 1, a serine at position 1, a cysteine at position 11, a cysteine at position 14, a cysteine at position 19, a cysteine at position 43, a cysteine at position 74, a cysteine at position 107, a cysteine at position 110, a cysteine at position 112, a cysteine at position 113, a cysteine at position 137, a cysteine at position 149, a cysteine at position 213, a cysteine at position 216, a cysteine at position 238, a cysteine at position 287, a cysteine at position 292, a cysteine at position 152, a cysteine at position 182, a cysteine at position 236 and a cysteine at position 296 corresponding to the amino acid positions of E. *coli* GGBP;

b) an analyte permeable matrix comprising covalently crosslinked hydrogels or sol-gels augmented with organically modified silicates or a combination thereof; and

c) at least one luminescent reporter group (luminescent label).

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21. The composition of claim 20, wherein said binding protein comprises at least one combination of amino acid substitutions selected from a cysteine at position 112 and a serine at position 238; a cysteine at position 149 and a serine at position 238; a cysteine at position 152 and a cysteine at position 182; a cysteine at position 152 and a serine at position 213; a cysteine at position 213 and a cysteine at position 238; a cysteine at position 149 and an arginine at position 213; a cysteine at position 149 and a cysteine at position 213; a cysteine at position 149 and a threonine at position 213; a cysteine at position 149 and a leucine at position 213; a cysteine at position 149 and a tyrosine at position 213; a cysteine at position 149 and an asparagine at position 223; a cysteine at position 149 and a cysteine at position 238; a cysteine at position 149 and a serine at position 256; a cysteine at position 149 and an arginine at position 256; a cysteine at position 152 and an arginine at position 213; a cysteine at position 152 and an asparagine at position 223; a cysteine at position 213 and a cysteine at position 255; a cysteine at position 149, a serine at position 213 and a serine at position 238; a cysteine at position 149, an arginine at position 213 and a serine at position 238; a cysteine at position 149, a cysteine at position 213 and a cysteine at position 238; a cysteine at position 149, a serine at position 213 and an asparagine at position 223; a cysteine at position 149, an asparagine at position 223 and an arginine at position 256; a serine at position 1, a cysteine at position 149, an arginine at position 213 and a serine at position 238; a serine at position 1, a cysteine at position 149, a serine at position 213 and a serine at position 238; and a cysteine at position 149, a cysteine at position 182, a cysteine at position 213 and a serine at position 238 corresponding to the amino acid positions of E. *coli* GGBP.

22. The composition of claim 20 or 21, wherein said mutated glucose/galactose binding protein additionally comprises at least one histidine tag.

23. The composition of claim 20 or 21, wherein:

(i) said luminescent label has an excitation wavelength of more than about 600 nanometers, or

(ii) said luminescent label has an emission wavelength of more than about 600 nanometers, or

(iii) said luminescent label is covalently coupled to said at least one glucose/galactose binding protein by reaction with a member selected from fluorescein,

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coumarins, rhodamines, 5-TMRIA (tetramethylrhodamine-5- iodoacetamide), R-phycoerythrin coupled to (9-(2(or4)-(N-(2-maleimdylethyl)-sulfonamidyl)-4(or2)-sulfophenyl)-2,3,6,7,12,13,16,17-octahydro-(1H,5H,11H,15H-xantheno(2,3,4-ij:5,6,7-i'j')diquinolizin-18-ium salt), (9-(2(or4)-N-(2-maleimdylethyl)sulfonamidyl)-4(or2)-sulfophenyl)-2,3,6,7,12,12,16,17-octahydro-(1H,5H,11H,15H-xantheno(2,3,4-ij:5,6,7-i'j')diquinolizin-18-ium salt), 2-(5-(1-(6-(N-(2-maleimdylethyl)-amino)-6-oxohexyl)-1,3-dihydro-3,3-dimethyl-5-sulfo-2H-indol-2-ylidene)-1,3-propyldienyl)-1-ethyl-3,3-dimethyl-5-sulfo-3H-indolium salt, N-((2-iodo-acetoxy)ethyl)-N-methyl)amino-7-nitrobenzoxadiazole (IANBD), 6-acryloyl-2- ditmethylaminonaphthalene (acrylodan), pyrene, Lucifer Yellow, 2-(5-(1-(6-(N-(2-maleimdylethyl)-amino)-6-oxohexyl)-1,3-dihydro-3,3-dimethyl-5-sulfo-2H-indol-2-ylidene)-1,3-pentadienyl)-1-ethyl-3-3-dimethyl-5-sulfo-3H-indolium salt, (2-bromoacetamidoethyl)sulfonamide): 4-5-(4-dimethylaminophenyl)oxazol-2-yl)phenyl-N-(2-bromoacetamidoethyl)sulfonamide, (N-(4,4 -difluoro-1,3 ,5,7- tetramethyl-4-bora-3a,4a-diaza-s-indacene-2-yliodoacetamide (BODIPY® 507/545 IA), N-(4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-s-indacene-3- propionyl)-N-iodoacetylethylenediamine (BODIPY® 530/550 IA), 5-((((2- iodacetyl)amino)ethyl)amino)naphthalene-l-sulfonic acid (1,5-IAEDANS), and carboxy-X-rhodamine, 5/6-iodoacetamide (XRIA 5,6).

24. The composition of claim 20, wherein said analyte permeable matrix is defined as in any one of claims 12 to 13.

FIG. 1

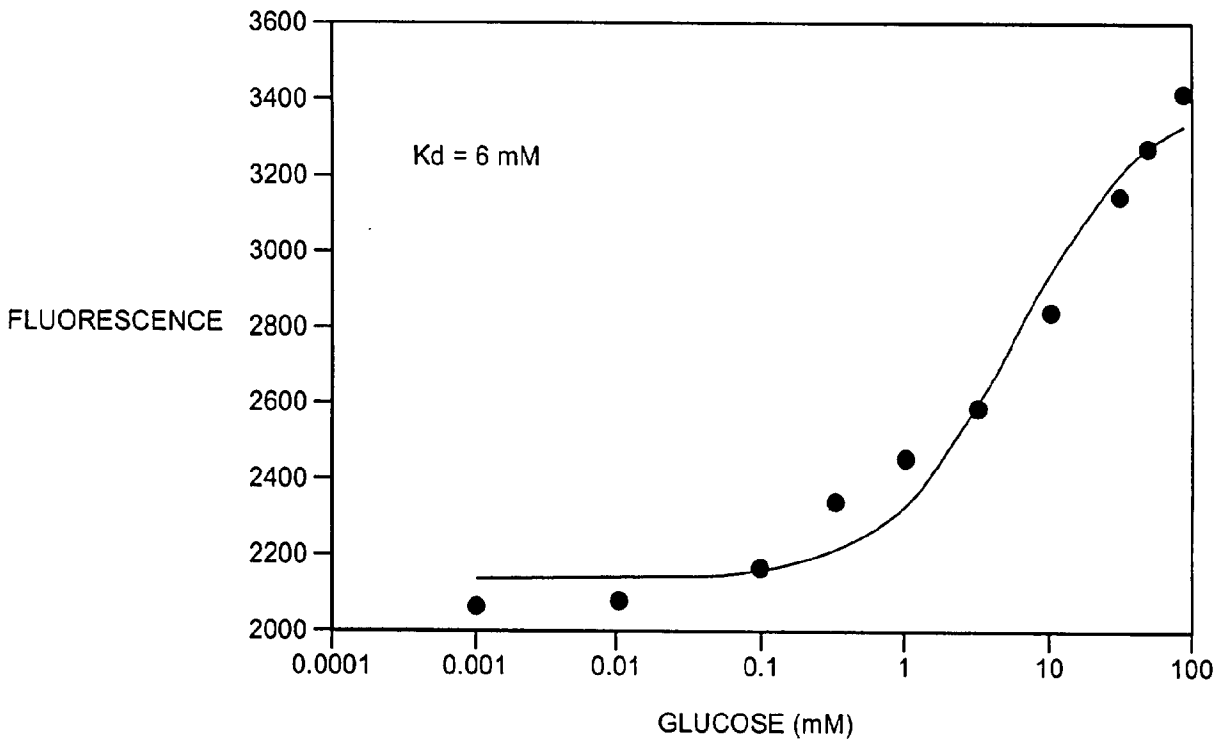

3600
3400
3200
3000
2800
2600
2400
2200
2000
FLUORESCENCE
Kd = 6 mM
0.0001
0.001
0.01
0.1
1
10
100
GLUCOSE (mM)

FIG. 2

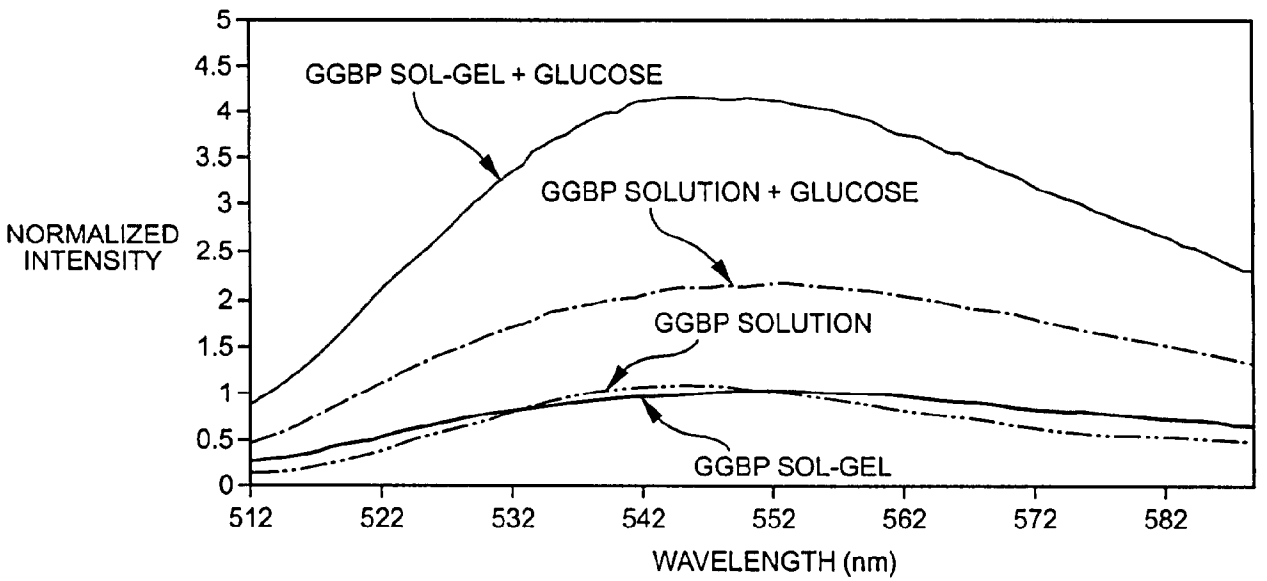

NORMALIZED INTENSITY
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
GGBP SOL-GEL + GLUCOSE
GGBP SOLUTION + GLUCOSE
GGBP SOLUTION
GGBP SOL-GEL
512
522
532
542
552
562
572
582
WAVELENGTH (nm)

FIG. 3

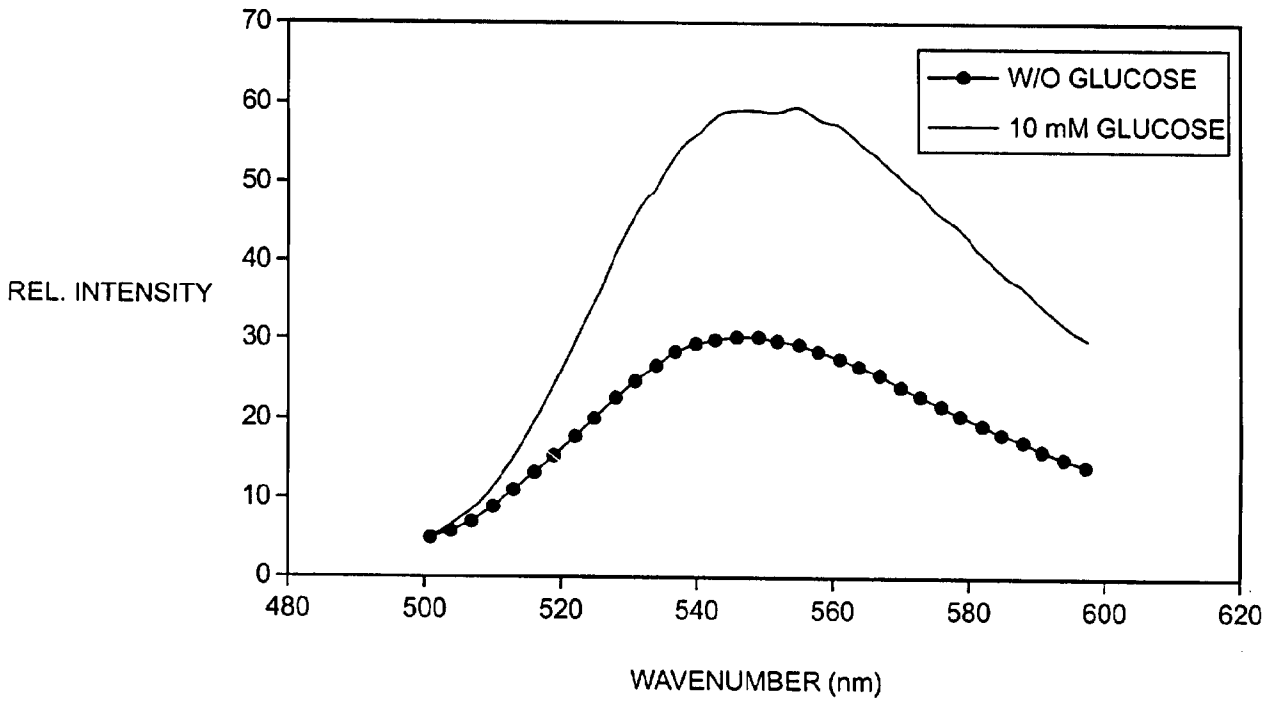
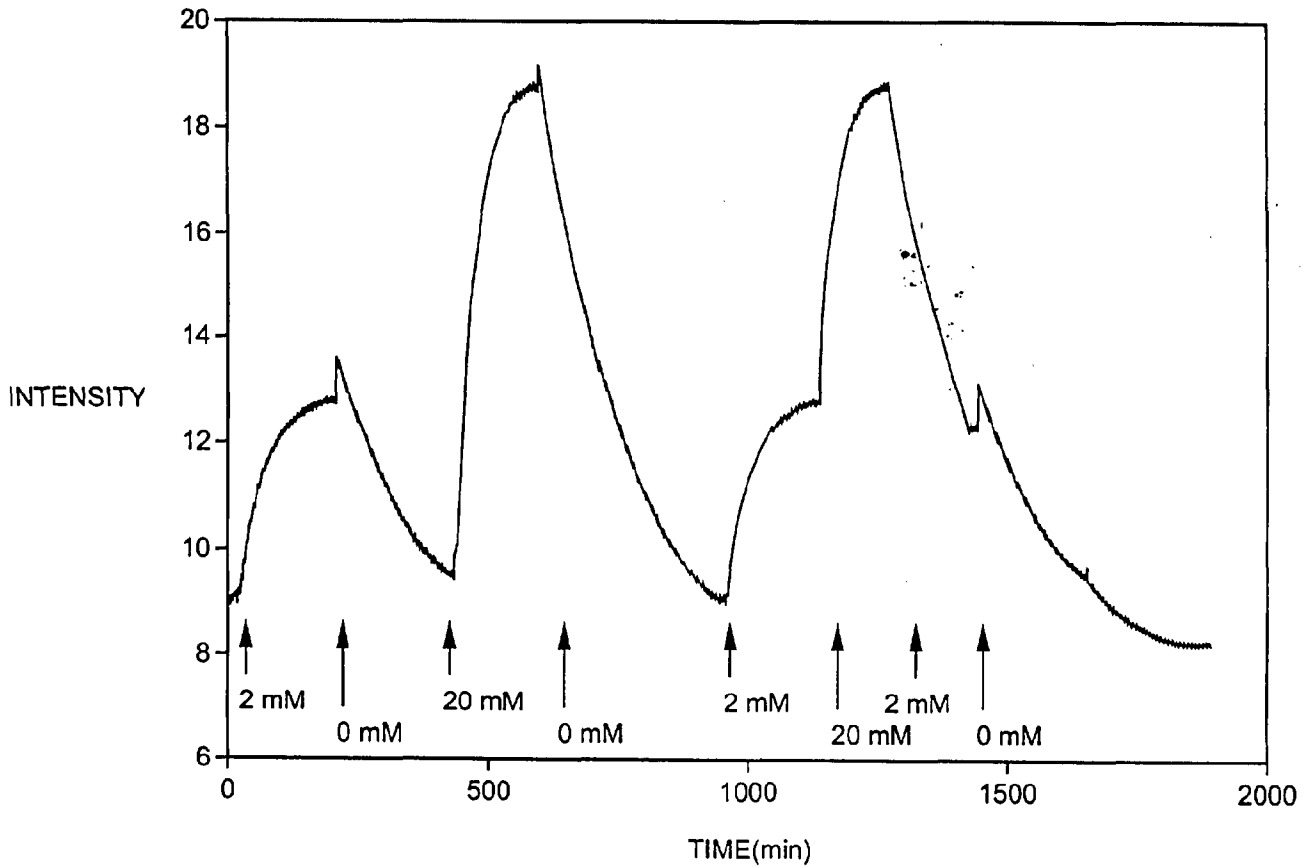

70
60
50
40
30
20
10
0
REL. INTENSITY
480
500
520
540
560
580
600
620
WAVENUMBER (nm)
W/O GLUCOSE
10 mM GLUCOSE

FIG. 4


INTENSITY
20
18
16
14
12
10
8
6
0
500
1000
1500
2000
TIME(min)
2 mM
0 mM
20 mM
0 mM
2 mM
20 mM
2 mM
0 mM