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REVIEW

Reagentless fluorescent biosensors based on proteins for continuous monitoring systems

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Abstract There is a lack of commercially available efficient and autonomous systems capable of continuous monitoring of (bio)chemical data for clinical, environmental, food, or industrial samples. The weakest link in the design of these systems is the (bio)chemical receptor (bCR). The bCR should have transducer ability, the recognition event should be a single reaction, and the bCR should be easily regenerated. Transport proteins and enzymes are well placed as bCR for optical continuous monitoring systems (OCMS). In this paper we review quantitative aspects and the main transducer strategies which have been developed for transport proteins, using periplasmic binding proteins (linking an environmentally sensitive fluorophore or FRET between two fluorophores) and concanavalin A (competitive reversible assays) as representative examples. Efficient immobilization systems and implementation in OCMS are also reviewed. Some kinds of enzymes can fulfil the necessary requirements to be appropriate bCR. Strategies using flavoenzymes chemically modified with fluorophores can be successfully implemented in OCMS and they are, in our opinion, the most appropriate option.

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Introduction

During the last two or three decades there has been an exponential increase in research related to chemical sensors in general and optical sensors in particular. This is evident from publications on organic and inorganic chemistry (new synthetic products), biotechnology (genetically modified biomolecules), nanotechnology (transduction mechanisms), or materials engineering (new materials) in which chemical sensors have been identified as possible targets for application of the developed research. This large volume of work contrasts with the limited appearance of important developments that have affected the quality of life of ordinary people. It is true that some significant advances in single-use (or a very limited number of uses) sensors have emerged; for example, an extensive catalogue of immunoreagents, in a greater variety of sensors, is commercially available (surface resonance plasmon, SRP, being one of the most versatile). However, there is a lack of efficient and autonomous systems capable of continuous monitoring of (bio)chemical data in clinical, environmental, food, or industrial samples.

Commercially available monitoring optical [1] sensors are mainly used for gas or water samples and lack a (bio) chemical receptor (bCR), i.e. almost all their selectivity depends on sample composition. This is because, although the design of continuous monitoring systems requires many components (electronics, optics, mass transport, and computational) to be implemented in the same device, the heart of the system is the recognition element and, to date,

research has not provided appropriate bCRs. The bibliography gives examples of bCRs which are conceptually brilliant (because they are new scientific developments) but do not seem sufficiently robust to be implemented in continuous monitoring systems. In addition to having the appropriate selectivity, sensitivity, and concentration range response, and being stable after integration in a solid support, bCR should meet some additional requirements:

1 It is strongly advisable that the bCR has transducer capacity. For optical continuous monitoring systems (OCMS), this usually means that the receptor should have molecular absorption or fluorescence properties which change during the recognition event. These kinds of bCR are called reagentless sensors. Alternatively, the bCR can be based on the transducer capacity of any of the sample components. For example, our group developed a sensor for determination of glucose in blood using glucose oxidase (GOx); the analytical signal used was the endogenous haemoglobin absorbance change observed during the GOx enzymatic reaction [2].
2 It is also highly desirable for the recognition event to be represented as a single reaction between the bCR and the analyte:

$$\text{bCR} + \text{analyte} \leftrightarrow \text{product} \quad (1)$$

and that the reaction should not be dependent on other chemical conditions (for example, pH). This has several advantages:

- 2A The OCMS does not require additional reagents, which simplifies the system and facilitates its autonomy. Also in this case the OCMS can use (bio) chemicals which can be taken from the endogenous sample components. Very well known examples are the O_2 in enzymatic reactions involving oxidases, and the Ca(II) required in binding proteins which are present in many biological fluids.
- 2B It is more likely that the measured analytical signal (absorbance, fluorescence) versus analyte concentration will be linear, which reduces quantification uncertainty and facilitates calibration.

3 It should be easily regenerated, or preferably autoregenerated, after the reaction. This property is indispensable because it conditions the autonomy of the OCMS. Regeneration should be complete (100%), otherwise the bCR lifetime becomes reduced and the OCMS quickly loses its calibration.

Researchers have gradually found new groups of molecules with recognition ability and which can potentially be used as bCR in optical sensors. In our opinion, proteins are the natural receptor which best fit the requirements of OCMS indicated above. Within this category, immune proteins have excellent selectivity; however, because of their high affinity they need regeneration conditions which are not compatible with the OCMS work operation. This means that transport proteins and enzymes are more satisfactory than immune proteins, mainly for the small organic molecules or metabolites on which we mainly focus in this paper.

Reagentless optical biosensors based on transport proteins

General considerations

Transport proteins (TP) are biomolecules which link to small molecules (ligands, L) and move them to different locations in the living body. The binding reaction is often selective, fits with Eq. (1), and is fully reversible, so TP seem to be excellent candidates for OCMS. However, the transduction ability of the binding event largely depends on:

1. Their optical properties. When a TP interacts with the ligand, the refractive index, mass, and optical activity of the bCR change and they can be used as a basis for sensors; however, this alternative often does not fulfil the basic requirements of selectivity (without sample pretreatment) and sensitivity which are necessary in OCMS. Very few TP belong to the so called photoproteins [3] (i.e. proteins having specific spectroscopic properties in the visible zone), and they only have the spectroscopic properties which are common to all proteins—UV molecular absorption (because of the amino acids), UV fluorescence (largely because of tryptophan residues) [4, 5], circular dichroism (related to α and β structures), IR absorption (the amine bands), and Raman. The only intrinsic spectroscopic property of TP which has so far been demonstrated to be useful for detection is molecular fluorescence (autofluorescence). However, in many cases this autofluorescence does not result in sufficient sensitivity or selectivity for use in a real situation. This problem can be overcome by attaching an appropriate fluorophore to the protein.
2. Their switching ability. It may be thought that the binding event will produce conformational changes in the TP and, as a consequence, a change in their intrinsic or induced spectroscopic properties (protein switches). However, in many cases complex formation follows a “lock and key” event [6] and the TP does not have transduction ability; this condition greatly reduces the number of proteins available for OCMS. The question that logically arises is whether it is possible to know in advance when this will occur for a given protein. In recent years various computational tools (developed to study the degree of internal

order of a protein in different situations) have been proposed that allow such predictions [7], but this subject is beyond the scope of this review.

Periplasmic binding proteins

Research has led to the discovery of several TP families which behave as protein switches. For sensor application, one of the most intensively studied is that of the periplasmic binding protein (PBP) from *E. coli*. These proteins (Fig. 1) consist of two domains connected by a hinge region; the ligand binds the PBP at the interface between the two domains (the binding pocket). In the absence of the ligand, the PBP adopts an open conformation and when the ligand interacts the PBP has a closed conformation.

PBPs have been identified for many potential analytes, including, mainly, carbohydrates, amino acids, and inorganic compounds (anions and metal ions). In addition, other PBPs different from that of *E. coli* are known, in particular those derived from thermophilic bacteria (which are able to work at higher temperatures, even up to 100 °C) and eukaryotes (even derived from humans) [8]. The selectivity of these proteins is generally good, but not complete (all of are able to transport more than one type of ligand).

Quantitative aspects

Because many PBPs only have one binding site, the PBP–L complex stoichiometry is 1:1:

$$\mathrm{PBP} + \mathrm{L} \quad \rightleftarrows \quad \mathrm{PBP-L} \qquad \mathrm{K_D} = \frac{[\mathrm{L}][\mathrm{PBP}]}{[\mathrm{PBP-L}]} \tag{2}$$

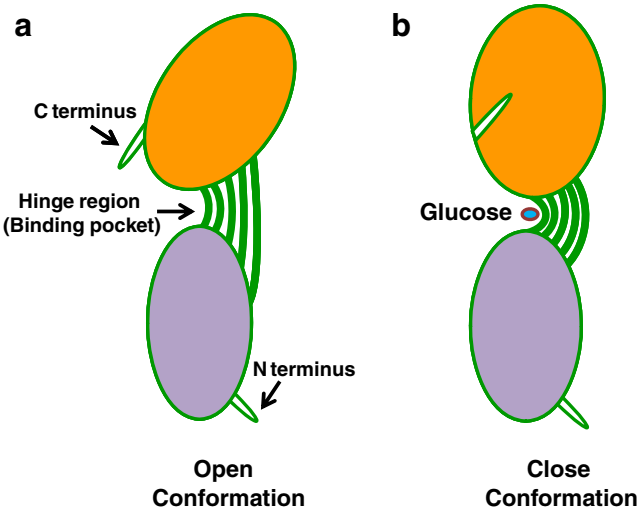


Fig. 1 Schematic representation of GBP: **a** open conformation; **b** closed conformation

where K_D is the binding constant (in reality, it is an unbinding constant). Several transducer strategies can be used for ligand quantification but, in general, all are based on the fact that PBP and PBP–L have different fluorescence (if K_{PBP} and K_{PBP-L} are the corresponding fluorescence constants; generally $K_{BPB-L} > K_{BPB}$). Denoting the fluorescence intensity observed after ligand (analyte) addition by F and that due to the free PBP by F_0, ΔF_{rel} is defined as:

$$\Delta \mathrm{F_{rel}} = 100\left(\frac{\mathrm{F}-\mathrm{F_0}}{\mathrm{F_0}}\right) = 100\left(\frac{\mathrm{K_{BPB-L}}-\mathrm{K_{BPB}}}{\mathrm{K_{BPB}}}\right)\left(\frac{[\mathrm{L}]}{[\mathrm{L}]+\mathrm{K_D}}\right) \tag{3}$$

ΔF_{rel} is usually chosen instead of F because it does not depend on the fluorimeter instrumental conditions, thus the data obtained in the laboratory (with cuvettes) and with the analyser are easily compared. According to this equation, ΔF_{rel} increases (when $K_{BPB-L} > K_{BPB}$) with ligand concentration until a maximum value is obtained when all the PBP is converted to PBP–L; in this case:

$$\Delta \mathrm{F_{rel,max}} = 100\left(\frac{\mathrm{K_{BPB-L}}-\mathrm{K_{BPB}}}{\mathrm{K_{BPB}}}\right) \tag{4}$$

and thus

$$\Delta \mathrm{F_{rel}} = \Delta \mathrm{F_{rel,max}}\left(\frac{[\mathrm{L}]}{[\mathrm{L}]+\mathrm{K_D}}\right) \tag{5}$$

Equation (5) has often been used in the bibliography for quantitative purposes. However, it has a very limited utility because [L] instead of $[L]_0$ appears; in reality these are related by:

$$[\mathrm{L}] = \frac{\left([\mathrm{L}]_0 - \mathrm{K_D} - [\mathrm{PBP}]_0\right) + \sqrt{\left([\mathrm{PBP}]_0 - [\mathrm{L}]_0 + \mathrm{K_D}\right)^2 + 4\mathrm{K_D}[\mathrm{L}]_0}}{2} \tag{6}$$

so the real quantitative equation for PBP-based sensors will be obtained after substituting Eq. (6) in Eq. (5), and is much more complex than expected. There are some quantitative aspects to emphasise (theoretical data are presented in the electronic supplementary material):

1. The calibration lines are sigmoid. ΔF_{rel} changes with analyte (ligand) concentration over a range of 3 or more orders of magnitude. Interpolation into a sigmoid or into a non-linear calibration curve results in high uncertainty in the concentration obtained; this is a very important aspect of OCMS but is scarcely taken into account in the literature. Because the central zone of the sigmoid can be approximated to a straight line, use of this zone smoothes the problem. This reduces the concentration range in which the method can be applied but reduces the uncertainty. In any case, in many published papers the confidence intervals for the calibration points are

very high, indicating that the measurement uncertainty is also high.

2. The analyte concentration corresponds to the midpoint of the sigmoid ($[L]_{0,1/2}$), i.e. when $\Delta F_{rel} = \Delta F_{rel,max}/2$. At this point half of the protein is free and the other half is binding the ligand and then:

$$[L]_{0,1/2} = K_D + \frac{[BPB]_0}{2} \quad (7)$$

This equation indicates that the centre of the calibration line (which fits with the centre of the linear response range) depends on the K_D value and the protein concentration used for the calibration study. This enables the linear response range to be tuned by changing either K_D or protein concentration.

3. The slope of the central zone of the calibration line also gives the sensitivity of the method. K_D, $[PBP]_0$, and $\Delta F_{rel,max}$ all affect the slope. On the one hand, when $[PBP]_0$ increases or K_D decreases, the slope of the line increases but simultaneously the linear response range becomes shorter; this means that appropriate selection of K_D and/or $[PBP]_0$ gives more sensitivity at the expense of a shorter concentration range. On the other hand, according to Eq. (5) $\Delta F_{rel,max}$ affects the slope (sensitivity) but leaves the linear response unchanged; obviously, the higher $\Delta F_{rel,max}$ the greater the sensitivity.

Finally, when PBP are to be implemented in sensors, fluctuations in the working of the sensor must be corrected. To do this, it is very helpful to measure at two wavelengths: the analytical (which changes with analyte concentration) and the reference (which does not depend or barely depends on analyte concentration). In this case, the quotient of both fluorescence measurements is used as the analytical data.

Transduction mechanisms

The most intensively investigated PBPs for analytical applications are those for maltose (maltose binding protein, MBP), which is used as a model for other applications, and glucose (glucose/galactose binding protein, GBP), because of its relevance to the development of sensors for diabetes monitoring. A review of the literature relating to GBP and MBP enables us to understand how PBPs are used for biosensors, and the analytical performance of these biosensors; the conclusions reached can be extended to all the PBPs and many TPs.

GBP [9] and MBP [10] from *E. coli* native forms (or wild type, WT) bind their respective ligands with high affinity; the K_D values for GBP/G and MBP/M are 0.2 μmol L^{-1} and 3.5 μmol L^{-1}, respectively. Because both proteins contain tryptophan residues, they autofluoresce. After linking to the corresponding ligand, the fluorescence changes slightly, in a sigmoidal manner: in GBP there is an increase ($\Delta F_{rel,max}$= 30%) and in MBP a decrease ($\Delta F_{rel,max}$=−20%). Their corresponding lifetimes and anisotropies also change but to a lesser extent than the fluorescence intensity.

When a PBP is being used to design a sensor, three main problems can occur. The first is binding selectivity; in reality all of the PBP binds to different ligands. For, example, the K_D for GBP/galactose (0.4 μmol L^{-1}) is similar to that for glucose. This problem has been overcome by using mutated GBP (for example, D14N [9, 11]).

The second problem is the K_D value. As has been indicated above, the K_D value is related to the ligand concentration which can be determined [10]. For example, the glucose concentration in blood, serum or interstitial fluids is in the millimolar range, four orders of magnitude higher than the GBP K_D value. This problem can be addressed in four different ways:

1. changing the GBP source, for example to *Thermotoga maritima* [12];
2. increasing the GBP concentration—surprisingly, this alternative is not usually mentioned by authors and GBP is used as obtained;
3. GBP immobilization—procedures based on entrapment or inclusion into membranes tend to increase K_D values by up to three, or even more, orders of magnitude [13]; this is extremely important when an OCMS is to be designed and is frequently the best option; and
4. GBP mutation—by changing specific amino acids, the K_D can be modified. This strategy is based on the fact that the affinity of the PBP for the ligand is proportional to the sum of the energy difference between the open and closed forms of the protein with and without ligand [8].

The third problem involves the reporting ability. PBP autofluorescence is analytically poor. On the one hand, the fluorescence appears in the UV region, which is very prone to spectral interferences; on the other hand the intrinsic $\Delta F_{rel,max}$ values are low. To improve these properties, researchers link PBPs with fluorophores for which the fluorescence changes after binding. Two main methods are used:

1. Linking an environmentally sensitive fluorophore (ESF) in a specific zone of the PBP in which the recognition event causes an important conformational change. Fluorophores can be linked to different protein reactive groups, mainly $–NH_2$ terminal groups. Because proteins have many of these terminal groups it is very difficult to link the fluorophore to a specific site. More selectivity can be obtained by using the –SH groups of the PBP cysteines as linkers. Because GBP and MBP do not have endogenous cysteine groups in their structures,

researchers have prepared mutated proteins by changing a specific amino acid for a cysteine. In this respect, one of the most interesting papers was published by Hellinga's group [14]. They used ten different PBPs of known structure and eight different fluorophores. Cysteines were introduced in several selected positions and the fluorescence behaviour of each fluorophore was studied. The authors defined three different zones in the PBPs where the fluorophores could sense the recognition event (Fig. 2a): the endosteric zone (in the ligand binding zone), the peristeric zone (near the binding zone; specific positions were chosen by visual inspection); and the allosteric zone (far from the recognition zone but greatly affected by the recognition event; specific positions were chosen by computational methods). Table 5 in Ref. [14] gives a complete list containing more than 300 PBP/mutant/fluorophore combinations; depending on the specific mutation and the fluorophore, the $\Delta F_{rel,max}$ (both the value and the sign) and the K_D change. For the GBP it seems that the H152C mutation could give GBP mutants with high $\Delta F_{rel,max}$ (up to 1,500%) and the W183C mutation gives an appropriate K_D (approx. 5 mmol L^{-1}) for glucose determination in blood. Other published results seem to confirm these hypotheses. It is very difficult [15] to know how an environment surrounding a fluorophore in a specific site can change its fluorescence, and then to make predictions about how the fluorescence of a fluorophore can be modified after the binding event. We have recently published a model explaining how the environment surrounding a fluorophore could change its quantum yield; this can aid understanding of this complex issue [16].

2. Fluorescence resonance energy transfer (FRET) between two fluorophores, located at different sites of the protein. For GBP and MBP, this alternative is actually more complicated and gives lower $\Delta F_{rel,max}$ (rarely reaching 50%) than ESF, so it seems appropriate only when ESF cannot be applied [17]. However, FRET has provided the best performance for other PBPs (for example, ribose BP) [18] and enables alternative strategies for PBP immobilization. In addition, it has been suggested that the high $\Delta F_{rel,max}$ for ESF corresponds to tests carried out in solution and that these values greatly diminish after immobilization (compared with FRET [19]), so in fact FRET can be competitive in OMS. Three alternatives procedures have been proposed for FRET in PBP.

A. PBP is fused with two green fluorescence protein (GFP) mutants having efficient FRET. The pioneering work developed by Ye and Schultz [20] using GFP and yellow fluorescence protein (YFP) already indicated that GFP was not sufficiently suitable because of the excitation wavelength. Subsequently, the enhanced cyan fluorescence protein (ECFP) and enhanced yellow fluorescence protein (EYFP) seemed more appropriate [21]. During the initial studies of this alternative, GFP were located at the ends of the two GBP lobes. As a consequence of recognition, the GBP closed but the terminus end became separated and $r_{FRET} = F_{acceptor}/F_{donor}$ decreased. This was the case for GBP and other PBPs belonging to the so-called class II (Fig. 2b, c); in class I members, for example MBP, the FRET increased after recognition. This alternative gives a reporting system with rather low sensitivity ($\Delta F_{rel,max}$ approx. 25–30%) and little change in K_D compared with WT. In-depth studies

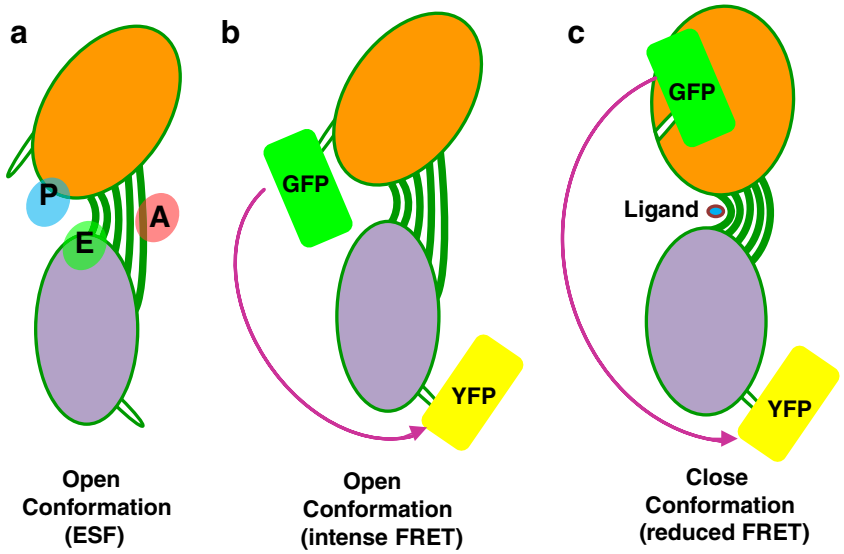


Fig. 2 Schematic representation of ESF and FRET PBP reporting mechanism: **a** fluorophore location in ESF; *A* = allosteric; *P* = persiteric; *E* = endosteric; **b** location of fluorophores in FRET, open conformation; **c** location of fluorophores in FRET, closed conformation

into a more suitable location of GFP mutants have been carried out by Pickup et al. [22]; they indicated that locating both ECFP and EYFP in the same GBP lobe led to an increase of up to 70% in $\Delta F_{rel,max}$.

B. Two fluorophores are used instead of GFP mutants. Here the problem to solve is that each protein molecule binds one molecule of each fluorophore and always in the same relative positions. Several options have been developed and the reader is referred to these for a more detailed explanation. In essence, a conventional mutation including a cysteine is carried out and the first fluorophore is linked. The second fluorophore can be linked using cleaver alternatives based on the Cys2His2–Zn finger domain [18] or transglutaminase conjugation of a primary amine (previously linked to a fluorophore) to a glutamine-containing tag (previously introduced in the GBP) [17]. In these cases, the K_D values obtained for PBPs immobilized and in solution were similar and $\Delta F_{rel,max}$ for the immobilized PBPs were slightly higher.

C. Two molecules of the same fluorophore which are able to form excimers. Many fluorophores are able to form excimers (excited state dimers). In some cases excimer formation causes quenching of monomer fluorescence; in other cases, new energy levels appear which produce a new fluorescence band. Because dimer formation largely depends on fluorophore distance, excimer formation in a PBP can work similarly to FRET. Dattelbaum [19] et al. tested this option. When two tetramethyl rhodamines were attached to a GBP mutant (containing two cysteines at the ends of each lobe), the GBP had a K_D similar to that for the native GBP and the $\Delta F_{rel,max}$ was approximately 40%.

Recently a new reporting mechanism based on bioluminescence has been proposed. Each GBP lobe is fused with half of a luciferase [11] or aqueorin [23]. In the absence of glucose, the enzymes have no activity. When GBP is linked to glucose, both enzyme halves fuse and the biochemiluminescent activity is recovered. The system is really interesting but the indicating reaction needs different reagents (Ca(II) and ...) which complicates its implementation in an OCMS.

In addition to GBP and MBP, studies have been carried out using other PBP, to demonstrate their utility as bCR. In most cases, ESF has been used as the transduction mechanism and different mutated PBP have been assayed to change the ligand K_D; examples are glutamine-binding protein (for glutamine [24, 25]), ribose-binding protein (for ribose [26]), or phosphonate-binding protein (for organophosphonate acids [27]).

Immobilization procedures

After choosing the mutant transduction mechanism, the next stage of obtaining the sensor is immobilization in an appropriate support. A variety of methods are used for protein immobilization, but from the perspective of the protein to sample interaction, two main groups can be identified:

1. protein coating on a solid surface, and
2. protein entrapment (with or without crosslinking) by a polymeric support.

Very few studies have been published about PBP (GBP) immobilization in solid supports, and the most important have appeared recently.

As is well known, protein coating reduces analyte mass-transport effects (so the sensor is mathematically easier to design) and reduces the response time, but it is more prone to physical and chemical interferences. Thus, this option is recommended for discontinuous and/or laboratory sensors, when it is possible to apply sample pretreatment before determination (for example, when the PBP is to be immobilized on to well-plates), and it is also being considered for protein array construction. To date, only proof of concept studies have been reported. Direct linkage can cause protein denaturation and even interference with conformational movements [28] (increases in K_D); however, indirect linkage (by using a bridge or a spacer) only slightly affects the protein properties, then the K_D remains practically unchanged, compared with methods developed in solution, and the signal stabilization time is short. Hellinga's group [12] studied the GBP (from *Thermotoga maritima*) and immobilization of the ribose binding protein (RBP) on surfaces by use of three different immobilization strategies: streptavidin (surface)–biotin (PBP), sulfhydryl (PBP)–maleimide (surface), and Hys_2Cys_2Zn–finger domain (PBP)–maleimide (surface); the reporter method was based on ESF. The reaction times were of the order of seconds, the K_D values were generally of the same order of magnitude as for WT GBP (or RBP), and the $\Delta F_{rel,max}$ moved generally between twofold and half of that for the WT protein. As an alternative to chemical or biochemical linkage, strong adsorption has also been tested [29]. In this context, an interesting approach has been proposed [28] which consists in linking glutamine BP (GlBP) to the polypeptide E12 (by means of a spacer), which adheres to hydrophobic surfaces (for example well-plate materials); the K_D does not change. Immobilization is very easy and the PBP is practically unaffected, so this is also a promising strategy for protein microarray preparation.

Surface coating enables the development of new alternatives in transduction schemes based on FRET. One of the most popular was developed by Medintz et al [30] (Fig. 3),

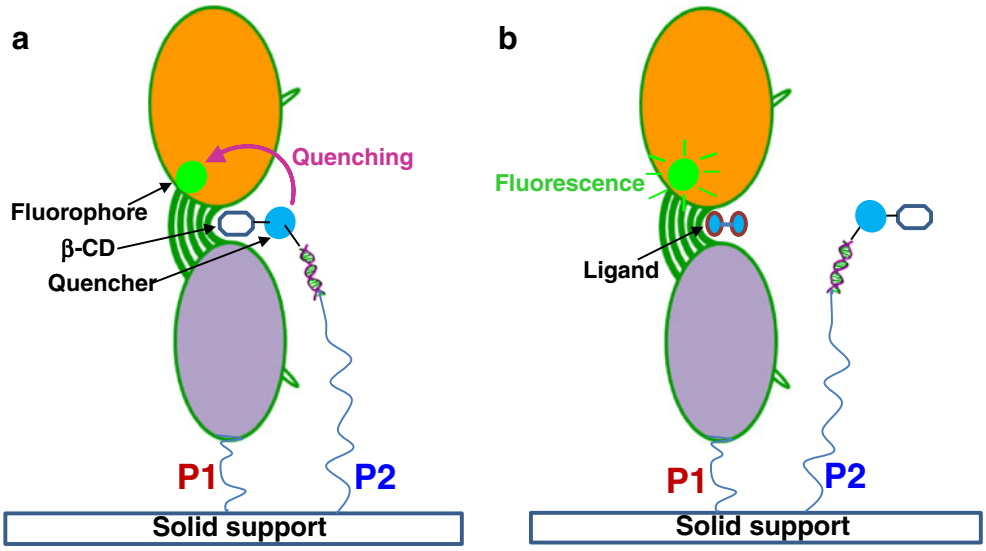


Fig. 3 Schematic representation of full sensor based on PBP: **a** in the absence of analyte no fluorescence is observed, because of quenching; **b** when the analyte is present, quenching disappears

who developed an MPB sensor based on a competitive assay between maltose and β-cyclodextrin maltose analogue (β-CDm), which also has affinity for the MBP. In brief, the system consists of two "arms" (P1 and P2) attached to the same solid support via a neutravidin–biotin link; the arms are very close to each other. P1 consists of the MBP bonded to a fluorophore; P2 consists of the β-CDm bonded to a quencher (or another fluorophore) and tethered to the attaching biotin by a DNA modulator. In the absence of maltose, the β-CDm binds to the MBP, and the fluorescence is quenched (either FRET or quenching FRET is produced). Maltose is able to displace the β-CD to the MBP and the quenching becomes disrupted. Depending on the dyes and the tether, the K_D (from 2 to 600 μmol L^{-1}) and $\Delta F_{rel,max}$ (from 10 to 200) for MBP can be properly tuned. This system has been coated on microtitre well surfaces and the system is reversible at least 8–10 times (before photobleaching occurs). This methodology can also work on glass as the supporting surface and with quantum dots as the dye-1 [31].

For OCMS, entrapment is more appropriate. In an attempt to combine the best of both systems, an interesting approach consists in immobilizing the GBP into dialysis tubing and placing this in a luminometer flow-cell; the pore diameter of the tube enables glucose perfusion without protein leaching [20]. This system has been recently tested as a sensor for continuous monitoring [32, 33]. The reporting was FRET using two GFP mutants and the K_D of the mutant GBP was tuned to glucose blood levels; however, all assays were performed using glucose solutions. The results obtained indicate that the sensor could be stable for three weeks (calibration tests were carried out on days 3, 7, and 21 and no leaching was observed during this time period).

As has been indicated above, ESF are more sensitive and more suitable for OCMR. However, two main factors have slowed progress in obtaining OCMR [34]:

1. the difficulty of reproducing the conformational changes necessary for signal transduction in entrapped systems; and
2. the endogenous polarity of the sensor matrix which masks the effect of the analyte on the fluorophore.

New ideas have been proposed to overcome (at least partially) these problems and several PBP entrapped systems have been proposed. Dattelbaum [34] has prepared a sol–gel-based MBP; the most important novelty here is bioconjugation with poly(ethylene glycol) (PEG) which promotes the retention of water molecules around the protein and reduces steric effects between the sol–gel matrix and MBP. As far as we are aware, this sensor has not been tested on real samples; neither have lifetime studies been carried out. Very recently [35] a sensor based on GBP immobilization in a methacrylate hydrogel matrix has been built by researchers at BD Technologies. This is in fact the first pre-clinical device for subcutaneous glucose sensing based on GBP, and consists of a needle containing an optical fibre with the methacrylate–GBP gel fitted at its end. The reporting mechanism is ESF (using acrylodan as the fluorophore, which is very usual in GPB studies) and measurements at two wavelengths enable correction of the sensor fluctuations. Although K_D, $\Delta F_{rel,max}$, and glucose concentration ranges of application are not reported, very interesting in-vitro and in-vivo assays are described. The in-vitro assays included an interference study (the most common metabolites and antibiotics present in serum samples), validation of glucose determination (using well-plate configuration) in 100 serum samples (13.2% mean percentage error), and continuous glucose measurement for three days

(approx. 860 measurements). Two kinds of in-vivo assay were carried out:

1. in a one-day trial, the fibre-optic sensor was inserted under the skin of a Yorkshire swine and measurements (3 min each) were performed for 6–12 h; and
2. in multi-day trials, the sensor was also inserted subcutaneously for one week, but glucose measurements were carried out during glucose excursion conducted every 2–3 days.

In both assays the results were compared with those obtained by measuring glucose in blood with a clinical analyzer; promising results were obtained.

Other transport proteins

There are, in fact, indexes of more than 500 different families of TP in living organisms on earth [36]. Analytically, TP for many important biochemical metabolites (neurotransmitters, carbohydrates, lipids, hormones, odorants) have been identified and isolated and some are even commercially available. Many TP autofluoresce because of amino acids and in some cases this fluorescence changes after binding. This property has mainly been used to obtain structural, functional or biochemical information; very few studies have been conducted with the specific purpose of designing analytical methods or sensors [4, 5].

If the GBP and MBP are typical of the strategies developed for PBP, then concanavalin A (ConA) can be regarded as the equivalent for the other transport proteins. ConA is a lectin (a protein family which transports carbohydrates) which has high affinity for glucose (only mannose can be regarded as a serious interference). As with GBP, the main interest in this protein is for the design of sensors for monitoring glucose in biological fluids. However, there are interesting differences between GBP and ConA both in their analytical properties and the way in which research has been approached:

1 Mathematically, Eqs. (5) and (6) could be applied; K_D values for ConA/glucose binding are approximately 2 mmol L^{-1}, which better fit the concentration range to be determined in blood or interstitial fluid. However, ConA is a tetramer which can bind four glucose molecules. Although experimental results suggest a practical stoichiometry of 1:1 [37], this could complicate both biosensor design and the shape of the calibration lines.
2 Although it is very usual to prepare GBP mutants which fit with sensor requirements, ConA modifications are usually based on chemical procedures. Fluorophores are chemically attached without previous ConA mutation. In addition, because ConA is commercially available this protein is more suitable for the chemist's work.
3 Many of the papers about ConA include assays with the immobilized protein. As a consequence, it is frequent to find papers proposing a global solution for the whole sensor (or OCMS) design.
4 ConA is not a molecular switch, so glucose binding does not cause a structural change; this complicates the design of the detection procedure.

Methods for optical transducing of the ConA/glucose binding event are mainly based on a competitive (but reversible) assay. The idea is similar to homogeneous competitive immunoassays. The glucose analyte competes with a reagent for ConA; both the competitive reagent (CR) and the ConA are chemically modified with fluorophores (F_1 and F_2) and this competition is monitored by FRET or quenching FRET. The whole system is immobilized in an appropriate support. Two main types of competitive methods have been proposed.

1 By far the most studied alternative is that proposed by Schultz et al in 1982 [38]. They used a dextran (mannose-containing polymer) as the CR; the whole system was enveloped in a glucose-permeable membrane (dialysis membrane) acting as the biosensor solid support. Several modifications have subsequently been proposed, mainly consisting in testing other fluorophores or moving the fluorophores through the chemical system, but with very few relevant improvements [39, 40].

   In addition to ConA concentration [41], the most important condition affecting the sensor figures of merit is the "free volume" between dextran and ConA; the higher the free volume the higher the $\Delta F_{rel,max}$ of the sensor. For this reason, a very interesting improvement consisted in transforming the homogeneous assay into a heterogeneous one [42]. The dextran was located in the sensor as little beads (Sephadex, approx. 20 μm) leaving free volume through which the ConA-F_1 could move. In the absence of glucose, ConA-F_1 was bonded to the dextran polymer; because this polymer also has two colorants immobilized (absorbing at the excitation and fluorescence F_1 wavelengths, respectively) a very low fluorescence background was detected from the sensor. In the presence of glucose, ConA was released from the dextran and reacted with the analyte, remaining in the free volume of the sensor. The inner filter then disappeared and the fluorescence increased. With this system the $\Delta F_{rel,max}$ was approximately 200%.

   The system was tested as an in-vitro OCMS [43]. Alexa 647, which fluoresces at approximately 670 nm, was the F_1; this fluorophore avoided inner filter effects and autofluorescence because of the skin (obviously the fluorescence blocking colorant was also changed) but the $\Delta F_{rel,max}$ diminished. Measurements were performed during four months and problems were detected which

should also be considered when working with other transport proteins:

A. ConA inactivation because of agglomeration of protein dimers and trimers; this inactivation is delayed in the presence of glucose, so should not actually be an important problem for OCMS;
B. the sensor signal drops, probably because of ConA leakage from the sensor capsule;
C. the response time is too long (approx. 400 min from each measurement to the next), which can be minimized by proper design of the sensor envelope [37]; and
D. the human toxicity (in several ways) of Con A is the subject of controversy [44].

These problems can also occur with other transport proteins when OCMS are being used to monitor human fluids.

2 The second method is similar to that previously described for GBP. The CR is a β-CD, F_1 and F_2 are a quantum dot and rhodamine, respectively, and the whole system is immobilized in a PEG matrix [45]. In fact, this idea has given rise to a new family of sensors known as Sencils. The whole system is attached at the end of an optical fibre which is chronically implanted under the skin; the PEG matrix enables rapid sensor response without physical interference from fibroblasts. The authors indicate that because the affinity of ConA for β-CD is lower than that for dextran, better fluorescence signals can be obtained for the same glucose concentration range. From the results, it can be deduced that $\Delta F_{rel,max}$ was approximately 180% with a noise level of approximately 20%. Again, alternative fluorophores have been used with the same CR [46].

In addition to competitive assays, other methods have been proposed on the basis of different phenomena. One of the most interesting was that described by Hamachi et al [47]. The idea is elegant and quite simple. It consists in using an environmentally sensitive fluorophore. In the absence of glucose, the fluorophore is located in the glucose binding pocket of ConA, which is highly hydrophobic; addition of glucose moves the fluorophore out of the binding pocket, which is highly hydrophilic (water solution) and its fluorescence intensity changes. The authors were able to develop a synthetic procedure enabling the fluorophore to be linked to ConA in a suitable location. These authors proposed another different transducer mechanism based on the very well known fact that the fluorescence of a fluorophore linked to a boronic acid (F–BA) is activated when the glucose is bound to the F–BA (because the photoinduced electron transfer which quenches the F fluorescence disappears) [48]. To date these methods have only been assayed as proof of concept and no analytical performance has been described.

As has been indicated, these schemes can be translated to other TP. However, as far as we are aware, very few analytical studies have been published, and those which have been published generally refer to laboratory methods. Methods based on folate binding protein (for folate determination using the tryptophan fluorescence) [49], odorant binding protein (for determination of polycyclic aromatic compounds by use of the fluorescence of tryptophan) [50], or ADP (using protein ParM and FRET) [51] can be regarded as representative examples.

Reagentless optical biosensors based on enzymes

General aspects; apoenzymes

At first glance, enzymes seem less attractive than TP for sensors and OCMS. This is because the recognition event is followed by a reaction, and several problems appear:

1. the analyte will be consumed;
2. more important, a second reagent (which is also consumed) is necessary; and
3. in some cases the reaction product formed can affect the enzyme or can be poisonous for the system.

Apoenzymes could be an interesting solution to these problems. These proteins preserve, in general, the binding ability of the corresponding enzymes, but with different theoretical analytical possibilities. Again, many of the studies involving apoenzymes refer to glucose, mainly apoGOx (obtained after FAD removal by chemical procedures). Compared with ConA, apoGOx seems to be more stable to agglutination [52], and in comparison with GBP, chemical fluorophore attachment can be used for inducing reporting ability. It has been reported that apoGOx tryptophan fluorescence is slightly modified after binding to glucose and this can be used for glucose sensing [53]; however, our results did not agree with this finding. Procedures for reporting the binding event include the strategies previously indicated, for example competitive binding assays with dextran and FRET [52, 54], furnishing analytical figures of merit similar to those previously described; it has recently been demonstrated that binding could also be detected by using the NIR fluorescence of carbon nanotubes [55]. More in-depth studies of apoenzymes are needed to properly evaluate their ability as optical biosensors.

The three above-mentioned drawbacks of enzymes can, in fact, be circumvented by using the analytical possibilities of these proteins offer. The first problem is not very important because the real quantity of analyte consumed is negligible. The second problem can be minimized if the second reagent is obtained from the medium surrounding the analyte; this suggests that the

hydrolysis and oxidation reactions involving O_2 as oxidant are the most appropriate.

$$\text{Analyte} + H_2O \leftarrow \text{hydrolytic enzyme} \rightarrow \text{Products} \quad (8)$$

$$\text{Analyte} + O_2 \leftarrow \text{Oxidase enzyme} \rightarrow \text{Product} + H_2O_2 (\text{or } H_2O) \quad (9)$$

The third problem occurs mainly in oxidase-type reactions when H_2O_2 is the reaction product; this problem can also be minimized by adding a second enzyme, usually catalase (Cat), which results in disproportionation of H_2O_2.

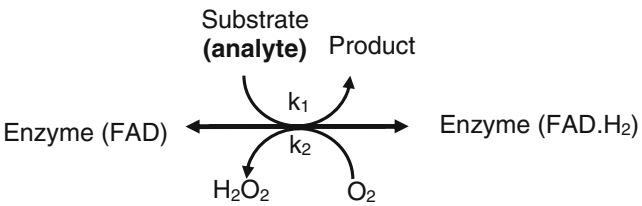
A further advantage of enzymes over TP is reporting ability. Some covalently linked cofactors have optical properties and during the enzymatic reaction these optical properties (or improvements obtained after chemical modification) change so they can be used as a reporter. Unfortunately, this is not, in fact, a general property of enzymes [56]. The most interesting are metalloenzymes and flavoenzymes, which are involved in oxidation enzymatic reactions [12]; the first have molecular absorption properties so will not be considered in this paper.

Hydrolytic enzymes do not have these properties, and alternatives similar to those described for TP can be used. A very interesting example is the hydrolytic enzyme β-lactamase I, which hydrolyzes β-lactam antibiotics. By appropriate mutation of the active centre with cysteine and later attachment of a fluorophore, a sensor is obtained whose fluorescence changes as a consequence of the hydrolytic reaction. This sensor is able to detect penicillin G or cefuroxime at 0.05 μmol L^{-1} levels [57, 58], but immobilization on a solid support has not yet been tested.

Flavoenzymes

Generation of the analytical signal

Flavoenzymes catalyze oxidative reactions in accordance with Eq. (8). Kinetically, flavoenzyme reactions follow a substituted-enzyme mechanism (ping pong mechanism) [59] which can be simplified to:



(10)

i.e., the substrate (the analyte to be determined) reduces FAD to FAD.H_2 and O_2 regenerates FAD. In addition to the usual UV fluorescence because of tryptophan, flavoenzymes also have intrinsic optical properties because of the flavin groups. As is well known, the optical properties (molecular absorption and fluorescence) of flavin depend on its oxidation state (FAD, FAD.H_2) and whether it is free (in solution) or linked to the protein [60–62]. Briefly, FAD has stronger absorption than FAD.H_2 in the UV and visible regions; these properties barely change when flavin is free or linked to a protein. In addition, FAD solutions fluoresce in the visible region, but FAD.H_2 is nearly non fluorescent. In proteins, the fluorescence is highly dependent on the protein itself, but as a general rule it is much less intense than when flavins are free.

Optical biosensors have been designed on the basis of the optical properties of FAD and the FAD.H_2. During the enzymatic reaction, changes in fluorescence and absorbance are observed which can be empirically related to the substrate (analyte) concentration. However, if a robust method is to be developed, the mechanism causing the signal changes during the reaction and the signal variation over time require a deeper explanation than is the case with transport proteins. To achieve this, it is necessary to know how the FAD/FAD.H_2 concentrations change during the enzymatic reactions, which largely depends on the k_1/k_2 value (i.e. the K_M value). Let us consider two flavoenzyme types.

Type A The oxidation reaction is faster than the regeneration reaction (K_M is low). Then, just after addition of the analyte, the FAD of the GOx is reduced to FAD.H_2, which is the predominant species in the medium. Only when the analyte is fully consumed is the initial FAD concentration recovered and, as a consequence, the enzyme regenerated. Figure 4a indicates how the reduced form of the GOx (represented as GOx.H_2) changes during the reaction using different analyte concentrations. This is observed, for example, for reactions of cholesterol oxidase (COx) [63] and choline oxidase (ChOx) [64].

Type B The rate of regeneration is faster than that of oxidation (K_M is high). In this case, after addition of the analyte, FAD is still the predominant species in the medium. It is necessary that [analyte] > $[O_2]_0$; so when the O_2 concentration becomes very small (because of the reaction), regeneration becomes slower than the oxidation and GOx.H_2 will be the predominant species. Because O_2 is supplied by the surrounding environment, the FAD concentration will finally be recovered and the enzyme regenerated. Figure 4b shows how the GOx.H_2 changes during the reaction using different analyte concentrations. Glucose oxidase (GOx) [65] behaves in this way.

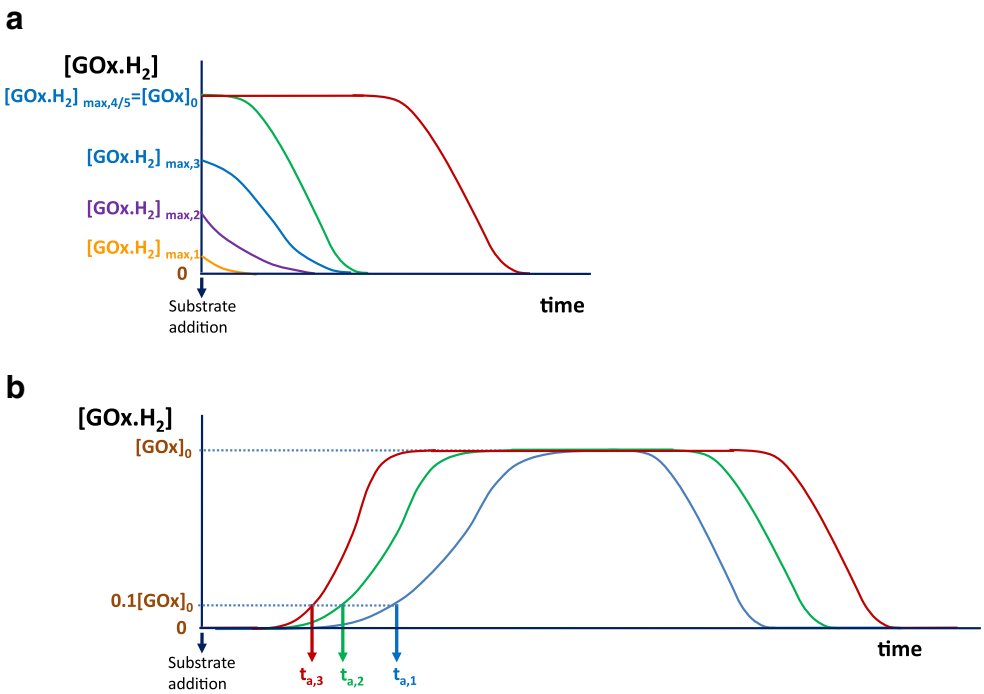


Fig. 4 [GOx.H_2] versus time plot observed during flavoenzyme enzymatic reactions: **a** type A enzymatic reaction; analyte (substrate) concentration increases in the sequence 1–2–3–4–5; **b** type B enzymatic reaction; analyte concentration increases in the sequence 1–2–3

From Fig. 4 it can be deduced that different factors affect substrate concentration. Depending on these, both the analyte concentration range which can be determined and the time necessary for quantification change. When fast measurements are necessary, the maximum GOx.H_2 (see below) for type A flavoenzymes, or the time (t_a) necessary for GOx.H_2 to begin increasing can be used. In both cases, the concentration range which can be determined is limited to the saturation. However, when the analysis time is not critical, the area of the [FAD.H_2] versus time plot (or the area in a given time) enables the response range to be extended almost without theoretical limit. This is very important, because analytical methods based on this will provide longer response ranges than those based on TP.

Sensors based on GOx fluorescence

After the pioneering work of Trettnak and Wolfbeis [66], optical sensors or OCMS based on FAD fluorescence of GOx began to be used for glucose determination. The FAD fluorescence of GOx is very weak so it is necessary to use a very high enzyme concentration (as high as 4,000 U mL^{-1} or higher). Although sensors have been developed using this option, in addition to the very high GOx consumption, several problems are detected:

1. the fluorescence can be affected by autoabsorption, which gives anomalous signals; as a consequence, the analytical response is very difficult to model and the design is weakened;
2. immobilization of the GOx in solid matrix becomes unstable, so GOx is easily leached from the sensors; and
3. in general, a short response range is obtained (lower than 1 order of magnitude).

In an attempt to overcome these problems, we designed two methods based on energy-transfer processes. The first involves the intrinsic tryptophan residues and FAD [65]. Figure 5 shows the fluorescence spectrum of GOx because of trytophan and the excitation spectra of GOx because of FAD and FAD.H_2. As can be seen, the FAD and FAD.H_2 absorption spectrum overlaps the fluorescence spectra of tryptophan, so energy transfer from tryptophan to flavin readily occurs in the enzyme. Because FAD.H_2 absorbs less than FAD at both wavelengths, when FAD becomes reduced to FAD.H_2 the energy transfer diminishes and the trytophan fluorescence increases. This explains why the GOx fluorescence intensity at the tryptophan wavelengths changes during the reaction giving plots similar to those for [FAD.H_2] in Fig. 4b.

A kinetic and mathematical study of this process enables us to relate t_a (Fig. 4b) to glucose concentration according to the equation:

$$t_a = \frac{1}{k_1 [GOx]_0} \ln\left(\frac{[Glucose]_0}{[Glucose]_0 - [O_2]_0}\right) \quad (11)$$

when the analyte concentration ranges from 0.5 to 20 mmol L^{-1} with an RSD of approximately 2%; however, when the signal area is used the linear range can be largely extended. This model has recently been used by other

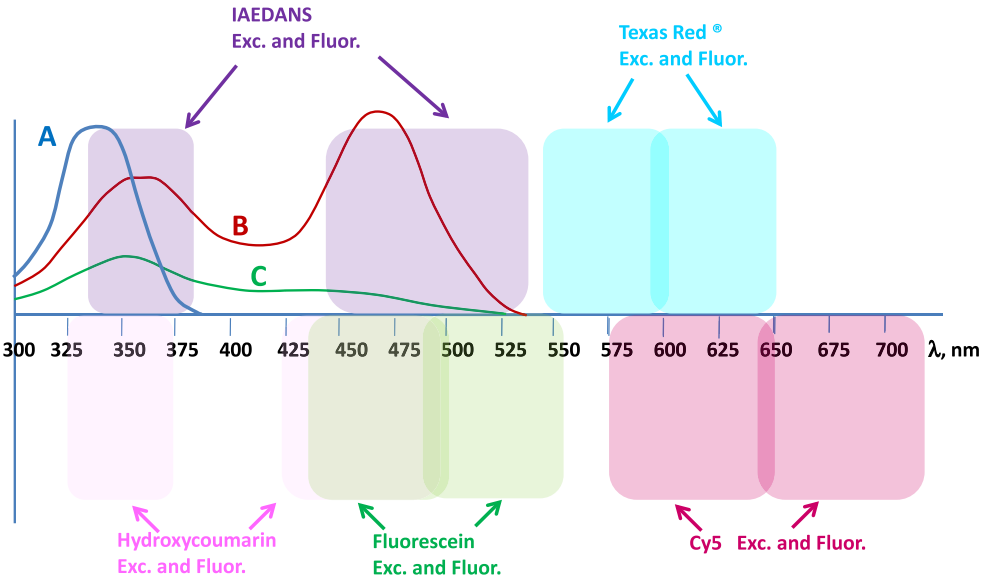


Fig. 5 Wavelength range of excitation and fluorescence of different attached fluorophores compared with: *A* (*blue line*) the tryptophan fluorescence spectrum in proteins; *B* (*red line*) FAD absorption spectrum in protein; *C* (*green line*) FAD. H_2 absorption spectrum in protein

authors [67]. For low glucose concentration this expression can be simplified to [68]:

$$t_a = \frac{[O_2]_0}{k_1 [GOx]_0 [Glucose]_0} \quad (12)$$

which enables the fluorescence anisotropy to be used for quantification but reduces the linear response range (0.5 to 5 mmol L^{-1}) and increases the RSD (up to approx. 4%). In our view, Eq. (12) is very important for sensor or method design because it explains how O_2 and GOx concentrations affect method sensitivity and, as a consequence, the glucose concentration response range.

Although the GOx concentration needed is <5 U mL^{-1}, which is approximately 1,000 times lower than that used for FAD fluorescence measurements, and the total fluorescence change $\Delta F_{rel,max}$ is 40% (FAD fluorescence changes are approximately 10%), an important drawback of this method is that the excitation and the fluorescence appear in the UV region. This makes the method very prone to interference and greatly complicates sensor device development because most of the solid matrix absorbs in this zone. Despite these problems, this methodology has been recently implemented in gelatine sensors [67].

The second approach is based on chemical modification of GOx with a fluorophore whose fluorescence is also affected during the enzymatic reaction. In theory this can be achieved if flavin spectra overlap those of the attached fluorophore. Tests performed [69–71] after attachment of hydroxycoumarin, IAEDANS, fluorescein (FS), and pyrene (which change their fluorescence during the reaction) or Texas Red and Cy5 (which are not affected by energy transfer) confirm this hypothesis (Fig. 5). The fluorescence changes during the reaction in a similar way to that of tryptophan (so Eqs. (11) and (12) are applicable), and the kinetics of the enzymatic reaction are hardly affected (the k_1 value is slightly different), the only difference being the total $\Delta F_{rel,max}$.

Several matrices have been tested in order to obtain the appropriate stability for OCMS [72] and a mathematical model has been developed relating the intensity to sensor composition [73]. It has been claimed that sol–gel encapsulation is a good method for GOx fluorescence measurement [74, 75]; in our experience, however [76], it does not seem to be fully appropriate. We have observed that, although GOx-FS (GOx linked to FS) seems initially to be immobilized in the sol–gel, the GOx-FS subsequently begins to be leached from the matrix and in less than two weeks the sol–gel loses more than 50% of the initial GOx-FS. Entrapment in polyacrylamide gel (PPA) has proved to be the best option. GOx-FS was incorporated during PAA formation and the enzyme could be used for at least three months (more than 350 measurements). This enabled us to design an optical sensor consisting of a flow cell which can be implemented in commercial fluorimeters [77]. Working in FIA mode, and because of the kinetics of the reaction (Fig. 4b), there is a phase out between addition of glucose to the sample and the signal beginning to appear (t_a); this enables spectral interferences to be eliminated.

Despite the advantages, OCMS based on GOx-FS has several problems:

1. Sensor response depends on O_2 concentration which must be known or at least controlled. This is, in fact, also a problem with sensors based on GOx, not only fluorimetric but also amperometric. Because O_2 is consumed in the GOx reaction, a second optical system measuring the O_2 concentration should be implemented in the sensor to make the appropriate correction. Alternatively, using GOx from another

organism following a Type A mechanism could solve the problem.

2. The measurement wavelength is approximately 500 nm, and for some clinical applications it would be better to shift this to higher values. We are now testing a ruthenium chelate as a fluorophore. Because its fluorescence also responds to O_2 quenching, it might be better used for solving both problems.
3. The minimum glucose concentration which can be determined depends on the O_2 concentration in the sample.
4. The chemical procedure used for fluorophore attachment to GOx can be improved. We have estimated that approximately eight FS molecules are attached to each GOx molecule, but we have not investigated the exact location of these molecules in the GOx. If this were known, the total fluorescence change during the reaction could be increased and the background fluorescence signal could be minimized.

Other flavoproteins

We have also developed methods with flavoproteins following the type A behaviour. As indicated above, in this case the fluorescence obtained immediately after substrate addition (maximum FAD.H_2 concentration), F_{max}, was used, and we were able to develop a mathematical model relating this signal to analyte concentration [64]:

$$\frac{F_0}{F_{max}-F_0}=\frac{1}{\Delta F_{rel,max}}\left(1+\frac{k_2}{k_1}\frac{[O_2]_0}{[Analyte]_0}\right) \quad (13)$$

which is fulfilled by both alternatives previously explained (tryptophan and attached fluorophore-based methods); F_0 is the fluorescence intensity before analyte addition. Unlike the type B methods, in Type A it is not necessary for the O_2 to be fully consumed before obtaining the signal; it is, moreover, better that the analyte concentration remains < $[O_2]_0$ for faster regeneration, so lower analyte concentrations (compared with type B) can be determined. For example, choline [64] and cholesterol [63] have been determined in the range from 0.5 to 50 μmol L^{-1} and from 0.013 to 0.2 mmol L^{-1}, respectively.

One of the most interesting applications of the flavoenzyme fluorescence (tryptophan or attached fluorophore)-based method is the simultaneous determination of species in the same measurement. Two different methodologies have been developed.

1 Determination of two substrates with flavoenzymes belonging to different enzyme kinetic types, for example glucose and choline. In the combined analytical signal obtained, the time at which the maximum appears and the maximum intensity enable determination of glucose and choline, respectively [78].

2 Speciation studies. Several organic metabolites are present in biological samples in the free and esterified forms, for example cholesterol (free and esterified), choline (free), phosphate ester, phosphatidylcholine, etc. Because the corresponding hydrolytic enzymes do not have autoindicating properties (with neither tryptophan nor fluorophore attached), the free and the esterified forms can be simultaneously determined when the hydrolytic and oxidative reactions are carried out simultaneously and the hydrolytic reaction is slower than the oxidation reaction. For example, Fig. 6 shows the intensity versus time representation obtained when a mixture containing free and esterified cholesterol is submitted to this procedure. Because cholesterol oxidase belongs to the type A flavoenzymes, the initial intensity increment (F_1) is because of the free cholesterol; the deesterification reaction then begins and the fluorescence intensity increases, because of deesterification to the free cholesterol. From the maximum intensity the total cholesterol (free + esterified) can be obtained, enabling speciation to be performed.

Concluding remarks

Several kinds of protein can be used as bCR for OCMS. PBP have switching molecule ability and their quantitative analytical bases are well known. They have been extensively studied, but the strategies developed require mutant preparation for fitting the selectivity, the sensitivity, and the linear response range, and for introducing a cysteine in a suitable location before chemical modification with a fluorophore. PBP for different ligands have been found, but the question to solve is what occurs with those ligands for which a molecular switch cannot be found. Strategies

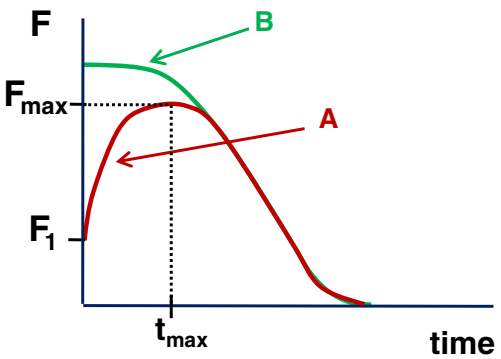


Fig. 6 Variation of cholesterol oxidase (chemically modified with FS) fluorescence intensity (F) during the enzymatic reaction: *A* obtained with a sample containing free ($[Ch]_{A,0}$) and esterified cholesterol ($[Ch\text{-}Es]_{A,0}$); *B* obtained with a sample containing free cholesterol only ($[Ch]_{B,0}=[Ch]_{A,0}+[Ch\text{-}Es]_{A,0}$)

directed to drastically redesigning the ligand-binding sites using a PBP as a scaffold for building the mutant BP [8, 79, 80] may be a solution. However, despite the good prospects and apparent success, the predicted conformational changes after binding do not fully fit with the conformational changes shown by the crystal structure [81], so this methodology is the subject of controversy [82]. Alternatively, synthetic peptides are opening up a new route for obtaining bCR for OCMS [51, 83].

Other TP can also be used. In theory, TP for many ligands exist but they have not usually been analytically tested so very few are part of the available range of bCR for OCMS. The extensive work done with ConA has revealed strategies which can be implemented in other TP for equipping them with reporting ability; in addition, the quantitative study performed with PBP can also be applied to other TP to predict analytical utility.

Flavoenzymes are also a valuable option. Their analytical behaviour has been mathematically modelled, so it is possible to predict analytical properties for new members of this family. Nowadays it is possible to find commercially available flavoenzymes for several analytes (substrates). However, the number of available flavoenzymes is still low, this being its most important disadvantage. Fluorophores can be easily attached to improve their reporting ability, and they are stable in PAA and other solid supports. Type B flavoproteins are not appropriate for determining analyte concentrations which are below the O_2 concentration in the sample; this problem can be partially overcome by combining them with HRP or other heme-proteins. In our opinion, when a bCR for a given analyte is to be chosen, flavoproteins could be an appropriate starting point.

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References

1. Marks RS, Lowe CR, Cullen DD, Weetall HH, Karube I (2008) Handbook of Biosensors and Biochips. Vol I and II. Wiley, Cambridge, UK
2. Sanz V, de Marcos S, Galban J (2009) A blood-assisted optical biosensor for automatic glucose determination. Talanta 78:846
3. Daunert S, Deo SK (2006) Photoproteins in Bioanalysis. Wiley-CH, Weinhein, Germany
4. Permyakov EA (1993) Luminescent Spectroscopy of Proteins. CRC Press, Unites States
5. Lakowicz J (1992) Topics in Fluorescence Spectroscopy. Volume 3: Biochemical Applications. Plenum Press, New York
6. Stratton MM, Loh SN (2011) Converting a protein into a switch for biosensing and functional regulation. Protein Sci 20:19
7. Uversky VN, Dunker AK (2010) Understanding protein non-folding. Biochim Biophys Acta Protein Proteomics 1804:1231
8. Dwyer M, Hellinga H (2004) Periplasmic binding proteins: a versatile superfamily for protein engineering. Curr Opin Struct Biol 14:495
9. Sakaguchi-Mikami A, Taneoka A, Yamoto R, Sode K, Ferri S (2008) Engineering of ligand specificity of periplasmic binding protein for glucose sensing. Biotechnol Lett 30:1453
10. Gilardi G, Mei G, Rosato N, Agró AF, Cass AEG (1997) Spectroscopic properties of an engineered maltose binding protein RID B-4523-2010. Protein Eng 10:479
11. Taneoka A, Sakaguchi-Mikami A, Yamazaki T, Sode K, Tsuwaga W (2009) The construction of a glucose-sensing luciferase. Biosens Bioelectron 25:76
12. Tian Y, Cuneo MJ, Changela A, Hocker B, Beese LS, Hellinga HW (2007) Structure-based design of robust glucose biosensors using a Thermotoga maritima periplasmic glucose-binding protein RID G-4993-2010. Protein Sci 16:2240
13. Geitmann M, Retra K, de Kloe GE, Homan E, Smit AB, de Esch IJP, Danieldson UH (2010) Interaction kinetic and structural dynamic analysis of ligand binding to acetylcholine-binding protein. Biochemistry (NY) 49:8143
14. De Lorimier R, Smith J, Dwyer M, Looger LL, Soli KM, Paavola CD, Rizk SS, Sadigov S, Conrad DW, Loew L, Hellinga HW (2002) Construction of a fluorescent biosensor family. Protein Sci 11:2655
15. Khan F, Saxl TE, Pickup JC (2010) Fluorescence intensity- and lifetime-based glucose sensing using an engineered high-K(d) mutant of glucose/galactose-binding protein. Anal Biochem 399:39
16. Galban J, Mateos E, Cebolla V, Dominguez A, Delgado-Camón A, de Marcos S, Sanz-Vicente I, Sanz V (2009) The environmental effect on the fluorescence intensity in solution. An analytical model. Analyst 134:2286
17. Khan F, Gnudi L, Pickup JC (2008) Fluorescence-based sensing of glucose using engineered glucose/galactose-binding protein: a comparison of fluorescence resonance energy transfer and environmentally sensitive dye labelling strategies. Biochem Biophys Res Commun 365:102
18. De Lorimier RM, Tian Y, Hellinga HW (2006) Binding and signaling of surface-immobilized reagentless fluorescent biosensors derived from periplasmic binding proteins. Protein Sci 15:1936
19. Der BS, Dattelbaum JD (2008) Construction of a reagentless glucose biosensor using molecular exciton luminescence. Anal Biochem 375:132
20. Ye K, Schultz J (2003) Genetic engineering of an allosterically based glucose indicator protein for continuous glucose monitoring by fluorescence resonance energy transfer. Anal Chem 75:3451
21. Garrett JR, Wu X, Jin S, Ye K (2008) pH-insensitive glucose indicators. Biotechnol Prog 24:1085
22. Deuschle K, Okumoto S, Fehr M, Looger LL, Kozhukh L, Frommer WB (2005) Construction and optimization of a family of genetically encoded metabolite sensors by semirational protein engineering RID A-8256-2008. Protein Sci 14:2304
23. Rowe L, Dikici E, Daunert S (2009) Engineering bioluminescent proteins: expanding their analytical potential. Anal Chem 81:8662
24. Lam H, Kostov Y, Rao G, Tolosa L (2008) Low-cost optical lifetime assisted ratiometric glutamine sensor based on glutamine binding protein. Anal Biochem 383:61
25. Dattelbaum J, Lakowicz J (2001) Optical determination of glutamine using a genetically engineered protein. Anal Biochem 291:89
26. Vercillo NC, Herald KJ, Fox JM, Der BS, Dattelbaum JD (2007) Analysis of ligand binding to a ribose biosensor using site-directed mutagenesis and fluorescence spectroscopy. Protein Sci 16:362
27. Rizk S, Cuneo M, Hellinga H (2006) Identification of cognate ligands for the Escherichia coli phnD protein product and engineering of a reagentless fluorescent biosensor for phosphonates. Protein Sci 15:1745

28. Wada A, Mie M, Aizawa M, Lahoud P, Cass AEG, Kobatake E (2003) Design and construction of glutamine binding proteins with a self-adhering capability to unmodified hydrophobic surfaces as reagentless fluorescence sensing devices. J Am Chem Soc 125:16228
29. Topoglidis E, Cass A, Gilardi G, Sadeghi S, Beaumont N, Durrant JR (1998) Protein adsorption on nanocrystalline TiO2 films: an immobilization strategy for bioanalytical devices RID B-4523-2010. Anal Chem 70:5111
30. Medintz I, Mauro J (2004) Use of a cyanine dye as a reporter probe in reagentless maltose sensors based on E-coli maltose binding protein. Anal Lett 37:191
31. Sapsford K, Medintz I, Golden J, Deschamps JR, Uyeda HT, Matoussi H (2004) Surface-immobilized self-assembled protein-based quantum dot nanoassemblies. Langmuir 20:7720
32. Jin S, Veetil JV, Garrett JR, Ye K (2011) Construction of a panel of glucose indicator proteins for continuous glucose monitoring. Biosens Bioelectron 26:3427
33. Veetil JV, Jin S, Ye K (2010) A glucose sensor protein for continuous glucose monitoring. Biosens Bioelectron 26:1650
34. Dattelbaum AM, Baker GA, Fox JM, Srinivas I, Dattelbaum JD (2009) PEGylation of a maltose biosensor promotes enhanced signal response when immobilized in a silica sol-gel. Bioconjug Chem 20:2381
35. Weidemaier K, Lastovich A, Keith S, Pitner JB, Sistare M, Jacobson R, Kurisho D (2011) Multi-day pre-clinical demonstration of glucose/galactose binding protein-based fibre optic sensor. Biosens Bioelectron 26:4117
36. Transporter Classification Database: www.tcdb.org (2011), Division of Biological Sciences, University of California San Diego, USA. Accessed 08/20 2011
37. Clark H, Barbari T, Rao G (1999) Modeling the response time of an in vivo glucose affinity sensor. Biotechnol Prog 15:259
38. Meadows D, Schultz J (1988) Fibre-optic biosensors based on fluorescence energy-transfer. Talanta 35:145
39. Ballerstadt R, Schultz J (1997) Competitive-binding assay method based on fluorescence quenching of ligands held in close proximity by a multivalent receptor. Anal Chim Acta 345:203
40. McCartney L, Pickup J, Rolinski O, Olaf J, Birch DJA (2001) Near-infrared fluorescence lifetime assay for serum glucose based on allophycocyanin-labeled concanavalin A. Anal Biochem 292:216
41. Barone P, Parker R, Strano M (2005) In vivo fluorescence detection of glucose using a single-walled carbon nanotube optical sensor: design, fluorophore properties, advantages, and disadvantages. Anal Chem 77:7556
42. Ballerstadt R, Schultz J (2000) A fluorescence affinity hollow fibre sensor for continuous transdermal glucose monitoring. Anal Chem 72:4185
43. Ballerstadt R, Polak A, Beuhler A, Frye J (2004) In vitro long-term performance study of a near-infrared fluorescence affinity sensor for glucose monitoring. Biosens Bioelectron 19:905
44. Ballerstadt R, Evans C, McNichols R, Gowda A (2006) Concanavalin A for in vivo glucose sensing: a biotoxicity review. Biosens Bioelectron 22:275
45. Liao K, Hogen-Esch T, Richmond FJ, Marcu L, Clifton W, Loeb GE (2008) Percutaneous fibre-optic sensor for chronic glucose monitoring in vivo. Biosens Bioelectron 23:1458
46. Tang B, Cao L, Xu K, Zhuo L, Li Q, Yu L (2008) A new nanobiosensor for glucose with high sensitivity and selectivity in serum based on fluorescence resonance energy transfer (FRET) between CdTe quantum dots and an nanoparticles. Chem Eur J 14:3637
47. Tolosa L, Szmacinski H, Rao G, Lakowicz JR (1997) Lifetime-based sensing of glucose using energy transfer with a long lifetime donor. Anal Biochem 250:102
48. Nakata E, Nagase T, Shinkai S, Hamachi I (2004) Coupling a natural receptor protein with an artificial receptor to afford a semisynthetic fluorescent biosensor. J Am Chem Soc 126:490
49. Kaarsholm N, Kolstrup A, Danielsen S, Holm J, Hansen SI (1993) Ligand-induced conformation change in folate binding-protein. Chem Biol Pteridines Folates 338:753
50. Wei Y, Brandazza A, Pelosi P (2008) Binding of polycyclic aromatic hydrocarbons to mutants of odorant-binding protein: a first step towards biosensors for environmental monitoring. Biochim Biophys Acta Protein Proteomics 1784:666
51. Choulier L, Enander K (2010) Environmentally sensitive fluorescent sensors based on synthetic peptides. Sensors 10:3126
52. Chaudhary A, McShane MJ, Srivastava R (2010) Glucose response of dissolved-core alginate microspheres: towards a continuous glucose biosensor. Analyst 135:2620
53. D'Auria S, Herman P, Rossi M, Lakowicz JR (1999) The fluorescence emission of the apo-glucose oxidase from Aspergillus niger as probe to estimate glucose concentrations RID A-2626-2008. Biochem Biophys Res Commun 263:550
54. Chinnayelka S, McShane M (2005) Microcapsule biosensors using competitive binding resonance energy transfer assays based on apoenzymes RID D-3489-2009. Anal Chem 77:5501
55. Barone PW, Yoon H, Ortiz-Garcia R, Zhang J, Ahn J, Kim J, Strano MS (2009) Modulation of single-walled carbon nanotube photoluminescence by hydrogel swelling. Acs Nano 3:3869
56. Galban J, Andreu Y, Sierra J, de Marcos S, Castillo JR (2001) Intrinsic fluorescence of enzymes and fluorescence of chemically modified enzymes for analytical purposes: a review. Luminescence 16:199
57. Chan P, So P, Ma D, Zhao Y, Lai TS, Chung WH, Chan KC, Yiu KF, Chan HW, Siu FM, Tsang CW, Leung YC, Wong KY (2008) Fluorophore-labeled beta-lactamase as a biosensor for beta-lactam antibiotics: a study of the biosensing process. J Am Chem Soc 130:6351
58. Chan P, Liu H, Chen YW, Chan KC, Tsang CW, Leung YC, Wong KY (2004) Rational design of a novel fluorescent biosensor for beta-lactam antibiotics from a class A beta-lactamase. J Am Chem Soc 126:4074
59. Cornish-Bowden A (2004) Fundamentals of Enzyme Kinetics. Portland Press Ltd., London, UK
60. Kao Y, Saxena C, He TF, Guo L, Wang L, Sancar A, Zhong D (2008) Ultrafast dynamics of flavins in five redox states. J Am Chem Soc 130:13132
61. Massey V (2000) The chemical and biological versatility of riboflavin. Biochem Soc Trans 28:283
62. Silva E, Edwards A (2006) Flavins. Photochemistry and Photobiology Comprensive Series in Photochemistry and Photobiology. RSC Publishing, Cambrigde, UK
63. Galban J, Sierra J, López-Sebastian J, Castillo JR (2000) Direct fluorometric determination of total cholesterol in serum using derivatized cholesterol oxidase. Appl Spectrosc 54:1157
64. Galban J, Sanchez-Monreal O, Andreu Y, de Marcos S, Castillo JR (2004) Choline determination based on the intrinsic and the extrinsic (chemically modified) fluorescence of choline oxidase. Anal Biochem 334:207
65. Sierra J, Galban J, Castillo J (1997) Determination of glucose in blood based on the intrinsic fluorescence of glucose oxidase. Anal Chem 69:1471
66. Trettnak W, Wolfbeis O (1989) Fully reversible fibre-optic glucose biosensor based on the intrinsic fluorescence of glucose-oxidase. Anal Chim Acta 221:195
67. De Luca P, Lepore M, Portaccio M, Esposito R, Rossi S, Bencivenga U, Mita DG (2007) Glucose determination by means of steady-state and time-course UV fluorescence in free or immobilized glucose oxidase. Sensors 7:2612
68. Sanz-Vicente I, Castillo J, Galban J (2005) Fluorescence anisotropy: application in quantitative enzymatic determinations. Talanta 65:946
69. Sierra J, Galban J, de Marcos S, Castillo JR (1998) Fluorimetric-enzymatic determination of glucose based on labelled glucose oxidase. Anal Chim Acta 368:97

70. Sierra J, Galban J, De Marcos S, Castillo JR (2000) Direct determination of glucose in serum by fluorimetry using a labeled enzyme. Anal Chim Acta 414:33
71. Tatsu Y, Yamamura S (2002) Fluorescence measurement of glucose by pyrene-modified oxidase. J Mol Catal B Enzym 17:203
72. de Marcos S, Sanz V, Andreu Y, Galban J (2006) Comparative study of polymeric supports as the base of immobilisation of chemically modified enzymes. Microchim Acta 153:163
73. Galban J, Delgado-Camon A, Sanz V, Sanz-Vicente I, de Marcos S (2008) A theoretical approach for designing fluorescent reagentless biosensors: the optical model. Anal Chim Acta 615:148
74. Esposito R, Della Ventura B, De Nicola S, Altucci C, Velotta R, Mita DG, Lepore M (2011) Glucose sensing by time-resolved fluorescence of sol-gel immobilized glucose oxidase. Sensors 11:3483
75. Portaccio M, Lepore M, Della Ventura B, Stoilova O, Manolova N, Rashkov I, Mita DG (2009) Fibre-optic glucose biosensor based on glucose oxidase immobilised in a silica gel matrix. J Sol Gel Sci Technol 50:437
76. de Marcos S, Galindo J, Sierra J, Galban J, Castillo JR (1999) An optical glucose biosensor based on derived glucose oxidase immobilised onto a sol-gel matrix. Sensors Actuators B Chem 57:227
77. Sanz V, Galban J, de Marcos S, Castillo JR (2003) Fluorometric sensors based on chemically modified enzymes - glucose determination in drinks. Talanta 60:415
78. Sanz-Vicente I, Romero JJ, de Marcos S, Galban J (2009) Simultaneous determination of glucose and choline based on the intrinsic fluorescence of the enzymes. J Fluoresc 19:583
79. Brun MA, Tan K, Nakata E, Hinner MJ, Johnsson K (2009) Semisynthetic fluorescent sensor proteins based on self-labeling protein tags. J Am Chem Soc 131:5873
80. Mathonet P, Fastrez J (2004) Engineering of non-natural receptors. Curr Opin Struct Biol 14:505
81. Tainaka K, Sakaguchi R, Hayashi H, Nakano S, Liew FF, Morii T (2010) Design strategies of fluorescent biosensors based on biological macromolecular receptors. Sensors 10:1355
82. Schreier B, Stumpp C, Wiesner S, Hocker B (2009) Computational design of ligand binding is not a solved problem. Proc Natl Acad Sci U S A 106:18491
83. Siegrist J, Kazarian T, Ensor C, Smita J, Madou M, Wang P, Daunert S (2010) Continuous glucose sensor using novel genetically engineered binding polypeptides towards in vivo applications. Sensors Actuators B Chem 149:51