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TOPICAL REVIEW

The intrinsic fluorescence of FAD and its application in analytical chemistry: a review

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Abstract

This review (with 106 references) mainly deals with the analytical applications of flavin-adenine dinucleotide (FAD) fluorescence. In the first section, the spectroscopic properties of this compound are reviewed at the light of his different acid–base, oxidation and structural forms; the chemical and spectroscopic properties of flavin mononucleotide (FMN) and other flavins will be also briefly discussed.

The second section discusses how the properties of FAD fluorescence changes in flavoenzymes (FvEs), again considering the different chemical and structural forms; the glucose oxidase (GOx) and the choline oxidase (ChOx) cases will be commented.

Since almost certainly the most reported analytical application of FAD fluorescence is as an auto-indicator in enzymatic methods catalysed by FvE oxidoreductases, it is important to know how the concentrations of the different forms of FAD changes along the reaction and, consequently, the fluorescence and the analytical signals. An approach to do this will be presented in section 3.

The fourth part of the paper compiles the analytical applications which have been reported until now based in these fluorescence properties. Finally, some suggestions about tentative future research are also given.

In the last decades, the research related to optical chemical (bio)sensors has undergone an exponential increase. The final purpose of this investigation is the development of efficient and autonomous systems capable of continuous monitoring (bio)chemical parameters in clinical, environmental, food or industrial samples [1–5].

To do that, the main target is the selection of the (bio)receptor which should have the appropriate selectivity, sensitivity, concentration range response. In addition, it should be also stable after integration in a solid support. Although the literature gives examples of (bio)receptors which are chemically adequate, there is a lack of efficient and autonomous systems capable of continuous monitoring the (bio)chemical parameter. This is because the (bio)receptor should also meet some additional requirements:

1. It is strongly advisable that the (bio)receptor has transducer capacity. For optical continuous monitoring systems this usually means that the receptor (or any of the samples components), should have molecular absorption or fluorescence properties that change during the recognition event. That is, they should be auto-indicated systems.
2. It is also highly desirable for the recognition event to be represented as a single reaction between (bio)receptor and the analyte, which simplifies the system and facilitates its autonomy.
3. It should be easily regenerated, or preferably auto-regenerated, after the reaction. Regeneration should be complete (100%), otherwise the bio(receptor) lifetime becomes reduced and the (bio)chemical systems quickly loses its calibration.

Using the analytical possibilities that the flavoenzymes (FvEs) offer they can circumvent the three above-mentioned drawbacks. As will be discussed along the paper, the FvEs are enzymes containing a flavin nucleotide (FMN or FAD) (figure 1) as a prosthetic group that show fluorescence (and absorption) properties which

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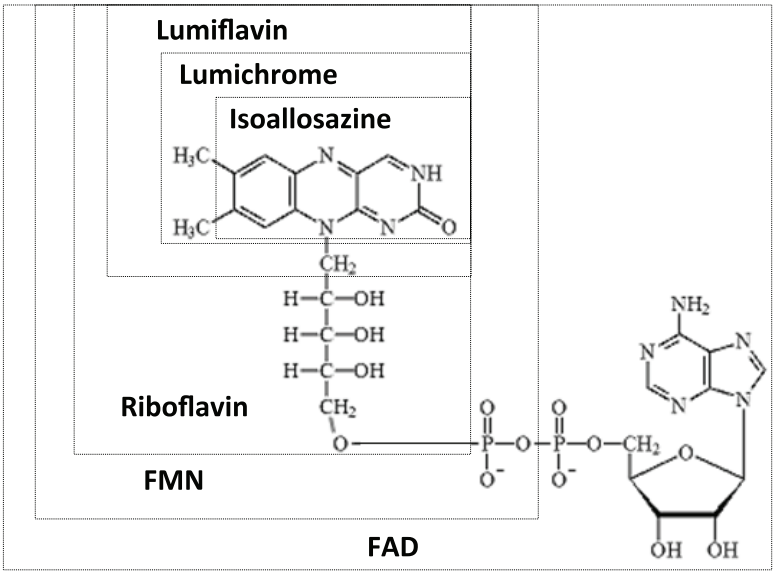

Figure 1. Structure and nomenclature of FAD and derivatives.

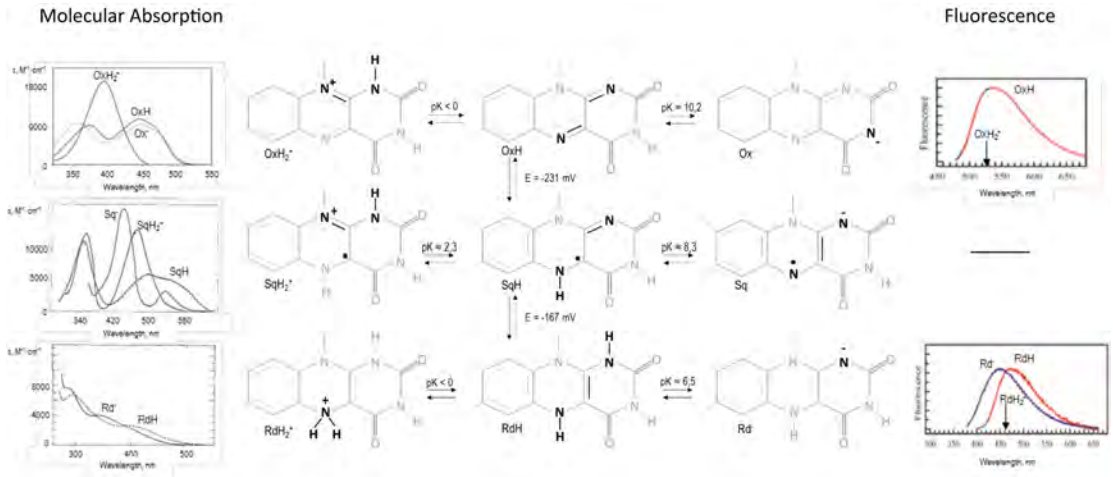

Figure 2. Representation of the different chemical species (redox and pH) of FAD, including their molecular absorption and fluorescence spectra. Atoms involved in these forms are in bold. Ox = oxidized, Sq = semiquinone, Red = reduced. (Reproduced with permission from figure 2B [7], copyright 2013; figure 1 [16], copyright 1974; figure 4 [9], copyright 1969 and figures 1(A) and (C) [13], copyright 2008 American Chemical Society.)

change during the enzymatic reaction; in addition, they are regenerated after the reaction by the oxygen in solution and thus avoid the necessity to couple any other dye or indicator to follow the enzymatic reaction. Then, they can be considered as label-free receptors.

In this paper we review the fluorescence properties of free FAD (and other flavins), how they change when the flavins take part of FvEs and the applications of FAD fluorescence, especially in analytical methods.

1. Spectroscopic properties of flavins

1.1. General ideas

The spectroscopic properties of all the components of the flavin family are similar because they have in common the isoalloxazine system which is responsible for these properties [6]. Due to this system, flavins have three different oxidation states: oxidized (quinone), half-reduced (semiquinone) and reduced (hydroquinone). Moreover, each oxidation state has three acid–base forms (cationic, neutral and anionic) depending on the pH [6]. Figure 2, shows the different oxidation states and the acid–base forms; the atoms involved in the redox and the acid–base processes are highlighted. This figure also shows the spectra characterizing the UV–vis molecular absorption and fluorescence of each species. It should be noted that the semiquinone state is unstable in solution [7] (only the absorption spectra for this species have been reported), while the reduced form needs a strongly reducing environment in order to be stabilized. Although all the forms have their chemical interest, the most important are those predominant at physiological pH values and in oxidized state. The following nomenclature will be used throughout the manuscript: subscripts 'ox', 'sq' and 'rd' will be used for oxidized, semiquinone and

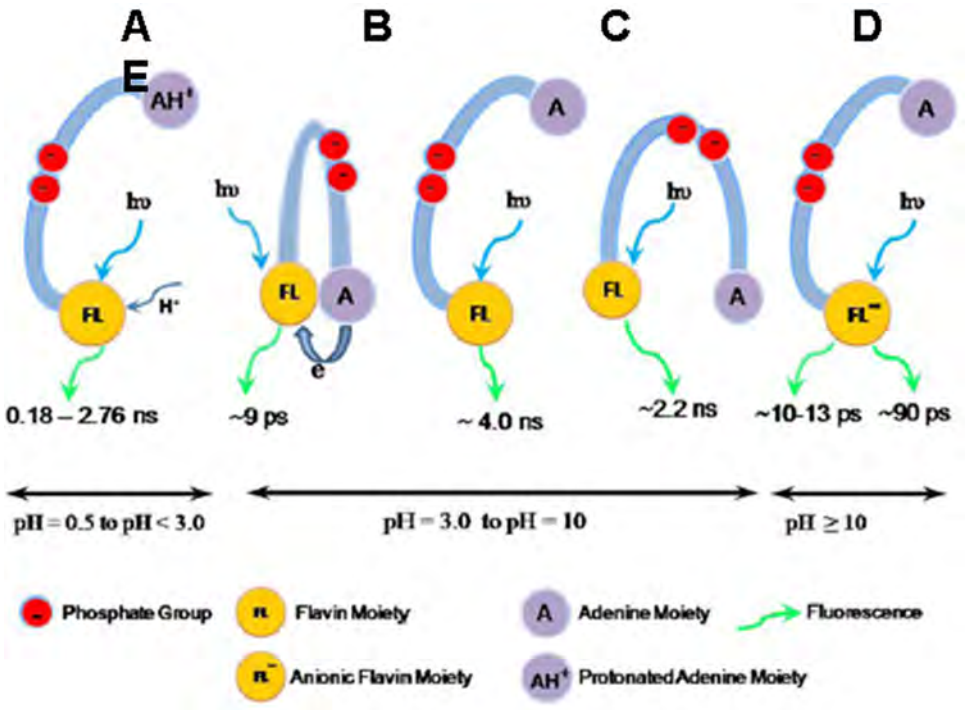

Figure 3. Schematic representation for various structures and dynamics of FAD at different pHs open form: (A), (C) and (E) stacked form: (B) Semi-stacked form: (D). (Reproduced from scheme 2 [10], copyright 2011 with permission from Elsevier.)

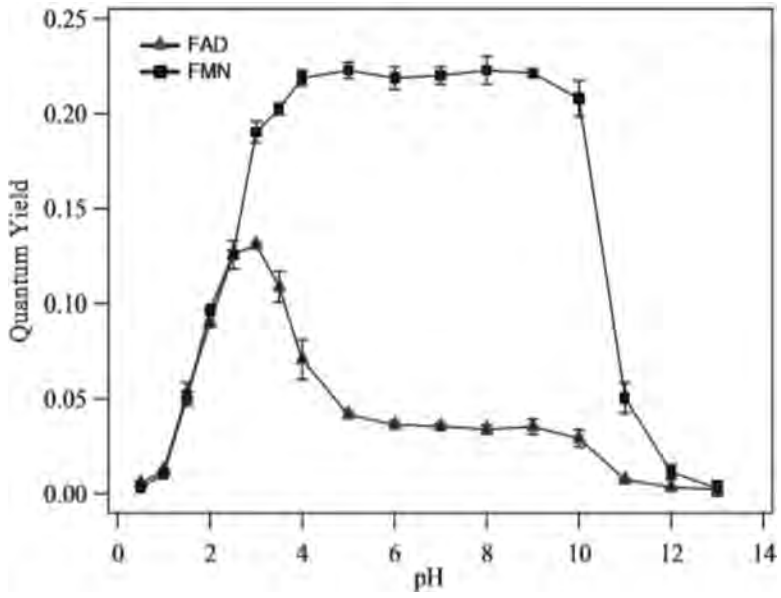

Figure 4. Fluorescence quantum yield of FAD and FMN in aqueous solution versus pH. (Reproduced from figure 5 [10], copyright 2011 with permission from Elsevier.)

reduced forms respectively: H_2Ox^+, HOx, Ox^- for acid, neutral and anionic oxidized forms (the same for 'rd' and 'sq' oxidation states).

Absorption spectra are influenced by both redox potential of the isoalloxazine system and by the pH [8–10], but are almost independent of the flavin skeleton (FAD, FMN, riboflavin) [11], because in the same oxidation state and acid–base form, they have similar absorption spectra; for example, riboflavin, lumiflavin, FMN and FAD present all bands at 450 and 370 nm with similar molar absorptivities (around 12 000 and 11 000 M^{-1} cm^{-1} respectively). The fluorescence of flavins appears mainly in the neutral form. The cationic form is practically non-fluorescent and the anionic form is weakly fluorescent [9, 10].

1.2. Oxidized forms

Although the molecular absorption properties of most flavins are very close to each other, the fluorescence properties are different, especially those of FAD

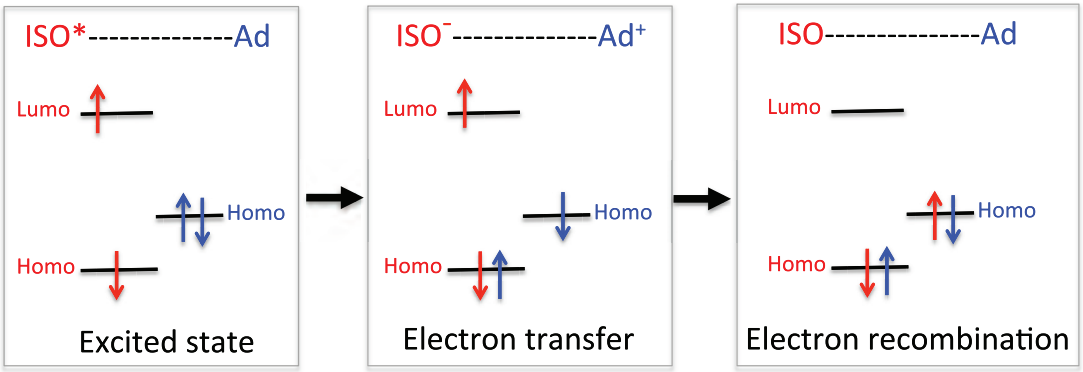

Figure 5. Illustration of reductive photo-induced electron transfer in aqueous FAD. In red, electrons which initially belonged to isoalloxazine (Iso); in blue, electrons which initially belonged to adenine (Ad). (Based on figure 13 [5], copyright 2003 with permission from Elsevier.)

(lumiflavin, riboflavin, FMN, FAD quantum yields are 0.29, 0.27, 0.22, 0.04 and average lifetimes are 5.2, 5.06, 4.70, 2.27 ns respectively) [8–10, 12, 13]; this is mainly due to the adenine ring. This ring may be folded over the isoalloxazine system, resulting in a less fluorescent conformation [8, 12, 14]. This is the so-called stacked conformation in opposition with the open conformation (figure 3).

In order to understand how these conformational changes affect the fluorescence changes in the oxidized state, the quantum yields and lifetimes of FAD_{ox} have been studied in solution at different pH values [8, 12] (figure 4). Below pH 1, the amount of the cationic form is considerable and therefore the fluorescence is weak. In the pH range of 1–2.5, the low fluorescence is explained by considering that the N5 pK in excited state is about 2.5. Up to this pH there are no differences between the FAD and other flavins. However, in the pH range from 3 to 10, the differences between FAD and flavins are important. In this pH interval the stacked conformation is predominant due to the dipolar attraction between the isoalloxazine system and the adenine system, and photo-induced electron transfer (ET) occurs from adenine to isoalloxazine; in the open conformation this process does not occur because the distance (ribityl bridge) between the electron donor (adenine) and the electron acceptor (isoalloxazine) is too large; figure 5 shows the schematic photo-induced ET in FAD. These interactions reduce the quantum yield of FAD_{ox}. Above pH 10, the deprotonation of N3, whose pK is 9.75, reduces the fluorescence of both FAD and flavins.

Lifetime studies of FAD_{ox} and FMN_{ox} permit giving additional conclusions about the prevalence of the FAD_{ox} conformers [8, 10] (figure 3). So, below pH 2.5, there is a single exponential decay with a progressive increase in lifetime from 0.18 to 2.76 ns. The lifetime is still lower than the neutral form of any flavin, because in this pH range protonation of N5 in the excited state occurs (pk 2.5). In the pH range from 3 to 9 there is a biexponential decay. The first component has a lifetime of 2.2 ns and the second between 3.5 and 4.0 ns. The longer life corresponds to an open FAD_{ox} conformer because FMN_{ox} has a similar value (the small differences are attributed to an extra phosphate group in FAD_{ox}). The smaller lifetime is caused by a semi-stacked form that interacts with the adenine but not optimally, since the closed form of FAD_{ox} has a much lower lifetime, 5–9 ps [15]. Above pH 10, there is a new component of 90 ps. This component is due to deprotonation of the FAD_{ox} (adenine ring), which is not observed in the other flavins.

Finally, FAD and FMN tend to form dimers in solution [16]. The absorption and fluorescence spectra of these dimers are similar to those of the corresponding monomers; however, they produce an additional lifetime component (about 30 ps) and their molar absorptivities are smaller.

1.3. Reduced forms

There are few studies about the fluorescence of the reduced state of flavins in solution. After borohydride addition, an emission spectrum of 3,4-dihydrolumiflavin was obtained with the excitation wavelength at 405 nm and the emission wavelength at 475 nm [17]. Other studies of modified flavins in reduced state have shown that it is necessary to work with rigid media at a low temperature in order to observe the fluorescence [18].

First emission spectra of $FADH_{rd}$ and FAD_{rd-} were measured by Kao *et al* [13]. The excitation wavelength was 360 nm and the emission wavelength 455 nm for anionic hydroquinone and 480 nm for neutral hydroquinone. In this study it was demonstrated that the fluorescence of the reduced state in solution was lesser than the fluorescence of reduced flavoproteins. In addition the deactivation of anionic reduced flavin (both those coming from FMN and FAD) was very fast. Upon 325 nm excitation, the fluorescence transient at 450 nm emission exhibits multiple decay dynamics in 5.0 ps (84%), 31 ps (13%) and 2.0 ns (3%).

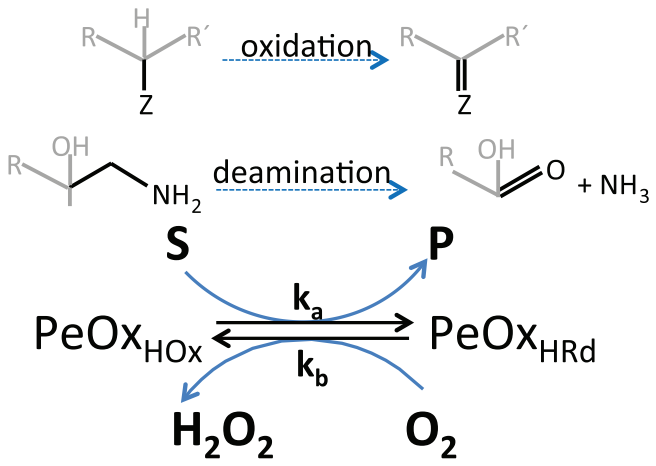

Figure 6. Simplified representation of the kinetic mechanism of pOx. FAD referes to oxidized enzyme and FAD · H_2 to reduced enzyme. The most interesting types of substrates (from the analytical point of view) and the corresponding products formed, are also indicated.

2. Flavin groups in FvEs: spectroscopic properties

2.1. Types of FvEs

Flavoproteins and, consequently FvEs, refers to a very huge protein family containing flavin groups [19, 20]; according to a survey by Nacheroux *et al* published in 2011 [21], 276 flavoproteins were fully characterized and information from another 98 (unclassified or incompletely classified) were also been compiled from data obtained after reviewing the three most popular protein databases [22]: BRENDA, Protein Data Bank and Enzyme Structures Database. FvEs are involved in various biological processes [23], but most of these entries corresponded to oxidoreductases (91%), while smaller fractions corresponded to transferases (4.3%), lyases (2.9%), isomerases (1.4%) and ligases (0.4%) [20]. The majority of them contained FAD (about, 75%) and the remainder contained FMN; no flavoproteins containing just riboflavin were described.

The flavin group can be covalently attached to the flavoprotein (only about a 10% of all oxidoreductases) or tightly (but non covalently) attached [24, 25]. In the first case, flavin is always linked in the position 8α, and in some cases the enzyme is linked by two sites in positions 8α and 6α. In the second case, flavin usually interacts across the phosphate group to the FvE.

The redox transformations that FvEs catalyze are typically oxidations, and according to the stoichiometry and the mechanism of the enzymatic reaction, oxidoreductases are usually split into two categories: monooxygenases (MOx) and pure oxidase (pOx). pOx are, by far, the most important for biotechnological and analytical applications, probably due to the fact that they only require O_2 as co-substrate for the real substrate oxidation. In biotechnology they present potential interest in pharmaceutical, fine-chemical and food industries. Regarding to analytical chemistry, there are several kinds commercially available for clinical and biological interesting substrates (analytes), and many of those enzymes are stable or increase their stability after immobilization, so they are easier to implement in analytical sensors. This review is especially devoted to pOx, but MOx will also be briefly commented.

2.2. Chemical and structural aspects of pure oxidases

pOx contain generally FAD as cofactor. The whole enzymatic process they catalyse consists of two steps [25, 26]. In the first, often called the 'reductive half reaction', the FAD_{ox} becomes reduced to FAD_{rd} after oxidizing the substrate (S) to the final product (P). In the second, the 're-oxidative half reaction', the FAD_{rd} is oxidized back to FAD_{ox}, usually by the O_2 present in the medium (figure 6).

Different types of substrates can suffer oxidation reactions mediated by pOx the most important being oxidative deamination and the oxidation of –ZH groups (Z = O, N, S) [27]. To the first group belong, for example, (D or L)-amino-acid oxidase (DAAO, LAAO) or monoamine oxidase (MAO-A, MAO-B) and, to the second group belong enzymes such as glucose oxidase (GOx). choline oxidase (ChOx), alcohol oxidase (AlOx), cholesterol oxidase (COx) or lactate oxidase (LOx).

According to literature [25], this type of oxidoreductases can be classified in several families, the more relevant in the context of this review being the glucose-methanol-choline oxidoreductase family (GMC) and the amino oxidase family (AO). This classification arises from the structural similarities between members of the same family. For example, enzymes belonging to GMC share [25, 28] a conserved N-terminal domain where FAD is binding to the protein (by the phosphate of the ADP), a less conserved C-terminal domain where the substrate binds, a histidine critically involved in both half-reactions, a similar molecular mechanism for the reaction (the transfer of the hydride ion from the substrate to the FAD) and the same position for the substrate linking to FAD (N5). Taking all of these coincidences into account, a similar environment for FAD

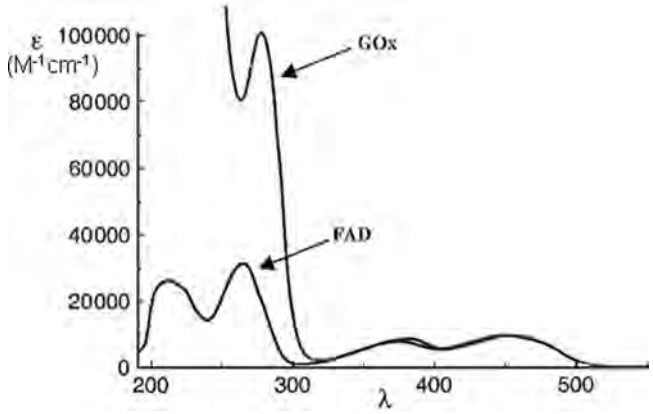

Figure 7. Molecular absorption spectra obtained, expressed as molar absorptivity, for GOx ($4.0 * 10^{-5}$ M, *A. niger*) and FAD ($8.0 * 10^{-5}$ M). As each GOx contains two FAD molecules, the total concentration of FAD in both solutions is the same.

could be expected in the GMC family and also a similar behaviour of their fluorescence during the corresponding enzymatic reactions, but in fact this does not happen.

2.3. Chemical properties of FAD in pure oxidases

The most important differences between the properties of flavins when they are free or when they are taking part of FvEs in general, or pOx in particular, can be summarised as follows:

1. Their normal potential values appreciably change. For example, the standard reduction potential (E_0) of the redox pair FAD_{ox}/FAD_{rd} could shift between −400 to 60 mV depending on the FvEs [29]; as with free FAD, E_0 in FvEs depend on the pH [30, 31]. The consequence is that the semiquinonic forms of the flavins, which are almost non-existent for free flavins are stabilized in some proteins so that their spectroscopic properties can be observed [32]. At physiological pH neutral and anionic semiquinone can be found, the pK being around 8.5.
2. Normally, the acid–base pK values of the oxidized or the reduced forms do not change appreciably (less than 1 pK values), but exceptions have been described [18]. Obviously, many FvE are not stable at extreme pH values. When proteins are brought to these values they denaturalize, so the properties observed correspond to these of the free forms. Considering the pK values given in figure 2, this is especially important for the oxidized forms; only the neutral oxidized species are stable. Similarly, only the basic and neutral reduced forms are stable. Regarding the semiquinonic forms, in some cases (such as glucose oxidase, GOx), the pK is about 8,5, but in other proteins the pK shifts to higher or lower values, so only one of the species is stabilized in the whole range of pH [33].
3. The information about the spectroscopic properties, especially fluorescence, of FvE is scattered throughout the literature. It is very difficult to find a complete description of the fluorescence of the different redox and acid–base forms corresponding to the same protein (we were not able to find one example). Consequently, and although spectra will be given here, it is not clear if there are representative spectra of each redox or acid–base forms for FvEs.
4. In addition to the conventional redox or acid–base forms previously described, FvE can also produce additional chemical states to those of the three redox forms indicated; these are the so-called charge-transfer states (CTS) resulting from intermediate electronic distribution. These states appear especially in oxidase FvE and are a consequence of complexes between the active centre of the enzymes and the substrates.

Let us review the information about the properties of the specific oxidation states of these proteins.

2.4. Spectroscopic properties of FAD in pure oxidases

2.4.1. Oxidized form

The molecular absorption properties of flavins do not greatly change when they form part of a FvE. For example, figure 7 shows the molecular absorption spectra of both glucose oxidase (GOx_{Hox}) and a free FAD_{Hox} concentration equivalent to that present in GOx. As can be seen, neither its maximum wavelength nor its molar absorptivity greatly changes. Fluorescence is a different story. In general, slight changes in the fluorescence wavelength are observed in different flavins, but the fluorescence intensity changes. There are two main reasons:

(1) It becomes quenched by the environment surrounding the flavin, especially by specific groups of some aminoacids. In several studies

carried out at femtosecond scale on FvE, it has been observed that quenching is mainly due to ET from tryptophan or tyrosine to flavins, leading to its half-reduction reaction [34]. However, this quenching greatly changes from one FvE to another. For example, GOx fluorescence is greatly quenched by ET from two tryptophan and two tyrosine units close to the flavin [35] and FAD_{Hox} in ChOx gives a fluorescence intensity about 50 more intense than that of FAD_{Hox} in GOx [36].

(2) Some authors have attributed this high quenching effect not only to the aminoacids, but also to the prevalence of the stacked or the semi-stacked forms (commented above) instead of the open form when FAD is part of a protein [37]. According to this, the structure of the protein should play a very important role in this quenching/folding effect.

Fluorescence lifetimes of FAD in FvEs contain similar components and values that those indicated in section 1.2 [13] for the free flavin, excepting that due to the dimer species.

As has been indicated above, many biochemical studies have been performed in order to obtain the type of linking between the FAD and the pOx skeleton (covalent or not), about the structure of the proteins (structural motif) and how the FAD is located in it (FvE families). One could guess that all of these structural reasons will affect on the predominant FAD form in the protein (stacked or open), and the rigidity/planarity of the isoalloxazine system, and consequently the fluorescence quantum yield. For example, the covalent link could restrict the FAD fold, so the open form could be the predominant, and the fluorescence quantum yield should be higher in these proteins; ChOx (covalent link) and GOx (non-covalent link), are consistent with this hypothesis. However, as far as we know, studies trying to establish correlations between FAD_{ox} fluorescence and structural considerations of pOx have yet not been presented.

2.4.2. *Semiquinone forms*

The molecular absorption spectra of semiquinones have been known for a long time. Massey and Palmer published in 1966 [38] the absorption spectra of the neutral and anionic semiquinone forms of FAD of GOx which they obtained after GOx irradiation in an anaerobic medium and in the presence of EDTA; these findings were later confirmed by other authors [39, 40]. The mechanism through which the semiquinone species is formed using EDTA has been extensively studied. For GOx and many other FvEs, the process takes place by a direct ET from EDTA to the excited state of the oxidized form. For some enzymes having several FAD units, the process follows a two-step mechanism: first, one flavin in the excited-state is reduced by EDTA; second, a reduced and an oxidized flavin molecules comproportionate to the semiquinone form [38, 41]. There are other procedures for obtaining the semiquinone species, but the EDTA method and that using dithionite, are usually described as the most efficient [42]. The molecular absorption spectra of many other semiquinone FvEs have been reported [43–45] and they essentially fit with that of GOx, so they should be considered sufficiently representative.

Regarding the fluorescence, recently Zhong *et al* [13, 46] have done an exhaustive study about this subject. They used cryptochrome and photolyase (FAD containing), and flavodoxine (FMN containing) as FvE models and were able to obtain some spectroscopic properties of these species.

Figure 8(A) shows the excitation and fluorescence spectrum of the neutral semiquinone of flavodoxine. The fluorescence spectrum shows a broad maximum at about 700 nm, and the excitation maxima are consistent with those found for GOx. The fluorescence decay is mono-exponential with a lifetime of about 230 ps. The authors indicated that the spectrum for the neutral semiquinone of photolyase was similar to this. This figure also shows the spectroscopic properties for the anionic semiquinone. In this case, the excitation spectrum also fits with that obtained for GOx, and the fluorescence spectrum shows a maximum at 510 nm. The fluorescence decay curve is tri-exponential with lifetimes of 2.2 (41%), 30 (35%) and 530 (24%) ps.

Spectra for the semiquinone forms of other FvEs have scarcely been shown. Gadda *et al* [47] obtained the fluorescence spectra for the anionic semiquinone of FAD in ChOx showing a fluorescence maxima at 452 nm, which does not exactly fit with that shown in figure 8(A).

2.4.3. *Reduced form*

As occurs with the fluorescence of reduced flavins in solution, contradictory results are found in scientific literature. In general it is assumed that the reduced forms present low fluorescence. However, very few specific studies aimed at obtaining reliable results can be found. The most celebrated papers were published by Ghisla, Massey *et al* in the 1970s [18, 41, 48, 49]. They showed that the absorption spectra of reduced FvEs suffer a slight batochromic displacement of about (5–20 nm) compared to the free reduced flavin, indicating a change in the planarity of their structure. Figure 8(B) shows a representative excitation and fluorescence spectra of the reduced-anionic form of the Photolyase.

Fluorescence lifetimes of the reduced forms in FvEs are longer than those indicated in section 1.3 [13] for the free reduced flavin.

Structural studies have also been carried out to examine how the planarity of the isoalloxazine system is modified during the corresponding enzymatic reaction. As will be shown later, many oxidase reactions follow a molecular mechanism involving the formation of –NH intermedia in the N5 atom. The angles defined by the N10–N5–hydrogen-bond donor and N10–N5–C

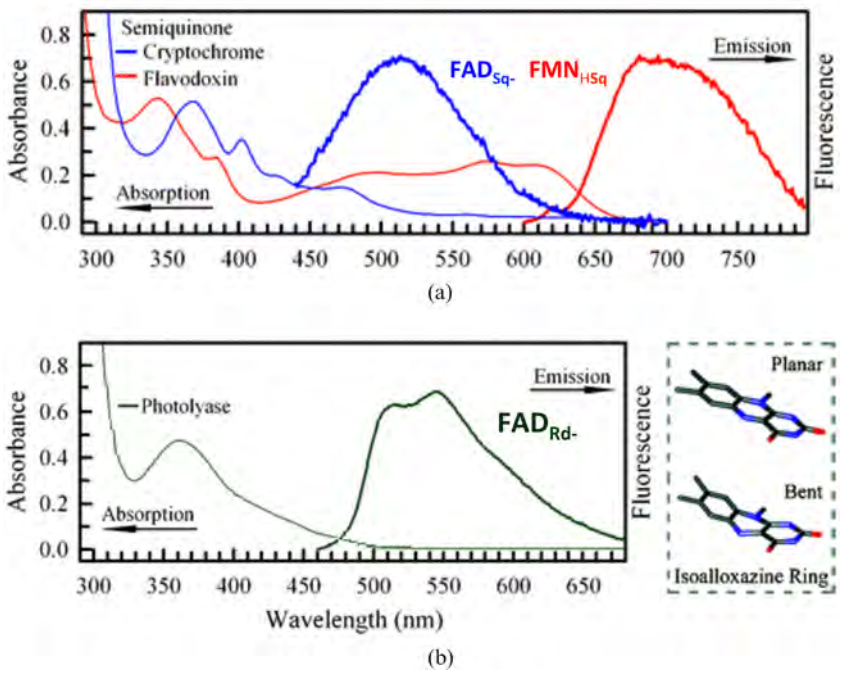

Figure 8. (A): Absorption and fluorescence spectra corresponding to the semiquinone forms of flavins. The cryptochrome spectra (blue) corresponds to the anionic semiquinone of FAD (FAD_{Sq-}); the Flavodoxin spectra correspond to the neutral semiquinone of FMN (FMN_{HSq}). (Reproduced with permission from figure 1 [13], copyright 2008 American Chemical Society.) (B): Absorption and fluorescence spectra corresponding to the reduced form of flavin. The Photoyase spectra corresponds to the anionic reduced form of FAD (FAD_{Rd-}). (Reproduced with permission from figure 1 [13], copyright 2008 American Chemical Society.)

atoms show how this planarity is affected and a bent form appears (figure 8(B)). Fraaije and Mattevie [29], compiling data for 11 FlP, showed that their corresponding angles ranged from 116° to 170°; this planarity should be related to the fluorescence quantum yield of this form, but we have not found references dealing with this. Studies also showed that the reduced FvE are in general less fluorescent than the corresponding oxidized forms, but with maxima at similar wavelengths; similar results were obtained for FAD and FMN. Interestingly, these authors described the case of lactate oxidase which shows higher fluorescence intensity in the reduced form than in the oxidized. As far as we know, no more examples of this behaviour have been described.

2.4.4. Other states

Flavins are able to generate inter- or intra-molecular charge-transfer compounds (CTC). Since they do not belong, formally, to any specific oxidation state, the absorption properties of these compounds are usually different to those of oxidized, reduced or semiquinone [33, 50–52], but we have not found information about fluorescence of these species.

2.5. Monooxygenase FvEs

MOx sub-classes are also a very huge group of FvEs, containing FAD or FMN. As a difference of pOx, they use O_2 but these act by transferring only one oxygen atom to the substrate, the other being liberated mainly as water. MOx catalyses the oxidation reaction of xenobiotic substrates (i.e. substances which are not present in the living organism, such as drugs or antibiotics) [53]. In order to do this, MOx requires a second substrate (external monooxygenase) to transfer the other O atom, usually NADPH or NADH. The need for this second substrate complicates the analytical method so these enzymatic reactions are not popular from the analytical point of view.

An exception to this behaviour is the lactate monoxygenase, whose mechanism is quite unusual [53–55]. This enzyme catalyses the oxidation of lactate by O_2, following a similar route excepting that the second substrate necessary for the process is the pyruvate, a product of the reaction (internal monoxygenase). This simplifies the mechanism and made the reaction very useful for developing analytical methodology. In addition, the reduced form presents a higher fluorescence than the oxidized

3. Specific studies of enzymes: GOx and ChOx

3.1. Glucose oxidase

GOx is, perhaps, the most frequently used enzyme for analytical purposes, either in electrochemical and optical methods, in both batch determinations and biosensors [56–58]. This enzyme is able to catalyse the oxidation of different monosaccharides (glucose, mannose, galactose, xylose) by O_2 according to the general pOx given in figure 6. The full mechanism of the reaction with substrates fits with the substituted-enzyme mechanism (ping-pong) according to figure 9. In this mechanism it has been taken into account that in an open system the O_2 is supplied continuously by the

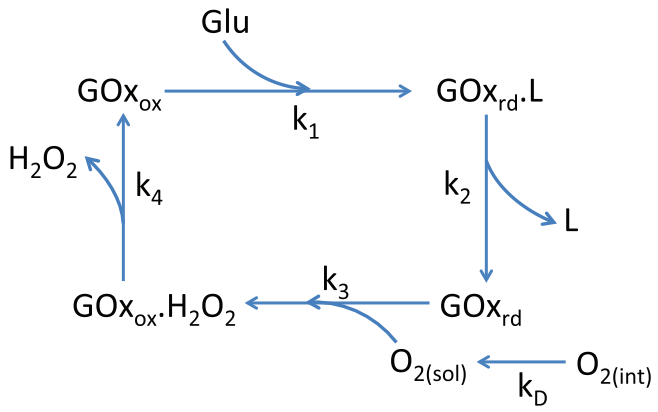

Figure 9. Mechanism of the enzymatic oxidation of glucose (Glu) to gluconolactone (L) with O_2 catalyzed by GOx. Kinetic constants of the reaction are: $k_1 = 1.2 * 10^4\,M^{-1}\,s^{-1}$ mM, $k_2 = 3.3 * 10^3\,s^{-1}$, $k_3 = 1.2 * 10^6\,M^{-1}\,s^{-1}$, $k_4 = 720\,s^{-1}$. The O_2 diffusion constant $k_D = 10^{-4}\,M^{-1}\,s^{-1}$.

environment surrounding the chemical system (K_D is the diffusion constant).

In the case of glucose (Glu), some authors have also considered that the first step is an equilibrium, but the kinetics as a whole is not substantially affected by this. The kinetic constants for the Glu reaction have been measured several times [59–61]; the values given in figure 9 caption can be considered representatives.

From these data it is deduced that the kinetic of the whole process is driven by the k_1; for substrates different from Glu the k_1 values are much lower (about 3000 times lower) [57, 58, 60], so the interferences caused by other monosaccharides on Glu can usually be neglected. GOx can be obtained from different sources [57, 58], however most companies offer GOx from *Aspergillus niger*. No substantial differences in the kinetic mechanism have been established between GOx coming from different organism, but the relative values of the constants are different each other [22].

To know how the fluorescence of the enzyme changes during the enzymatic reaction and how this can be related to the Glu concentration, the differential equation system describing the evolution of the four species appearing in figure 9 have to be set out and solved. Unfortunately, it is not possible to get an analytical solution of this system, but using a powerful computational package such as Mathematica™ or others [62, 63] a numerical solution can be obtained. The following conclusions are derived from this numerical solution:

1. The concentrations of the intermediates $GOx_{rd} \cdot L$ and $GOx_{ox} \cdot H_2O_2$ can be neglected, so the mechanism of the reaction can be simplified a two-step reaction as shown in figure 6, where:

$$k_a = k_1 = 1.2 * 10^4\,M^{-1}\,s^{-1}$$
$$k_b = k_3 = 1.2 * 10^6\,M^{-1}\,s^{-1}. \quad (1)$$

Therefore, only the fluorescence coming from GOx_{ox} and GOx_{rd} species need to be taken into account along the reaction.

2. Considering the kinetic constants, only when the $[Glu]_0 > [O_2]_0$ the concentration of GOx_{ox} changes along the reaction, i.e. Glu concentrations lower than that of O_2 cannot be measured using GOx fluorescence.

When the differential equation system is solved for different Glu concentrations fulfilling that $[Glu]_0 > [O_2]_0$, results such as those given in figure 10 are observed. Considering that the GOx_{ox} presents both a higher molar absorptivity and quantum yield than GOx_{rd}, these figures also represent how the fluorescence intensity will change during the enzymatic reaction. As can be seen, when the glucose is added, an instantaneous small decrease in the $[GOx_{ox}]$ is observed (an initial equilibrium between GOx_{ox}/GOx_{rd} is established); after a given time (t_a) the $[GOx_{ox}]$ begins to decrease appreciably until the whole the enzyme is in the reduced form. From here, the reaction continues and when the whole glucose is consumed the GOx_{ox} is again fully recovered (not shown in figure 10).

From the analytical applications point of view two parameters can be related to the Glu concentration:

(1) The concentration of the oxidized form just after the Glu addition ($[GOx_{ox}]_{t=0}$):

$$[GOx_{ox}]_{t=0} = [GOx]_0 \frac{k_b[O_2]_0}{k_a[Glu]_0 + k_b[O_2]_0}. \quad (2)$$

(2) The time required for the $[GOx_{ox}]$ starts to decrease (t_a) [64]:

$$t_a = \frac{1}{k_a\,[GOx]} \ln\left[\frac{[Glu]_0}{[Glu]_0 - [O_2]_0}\right] \quad (3a)$$

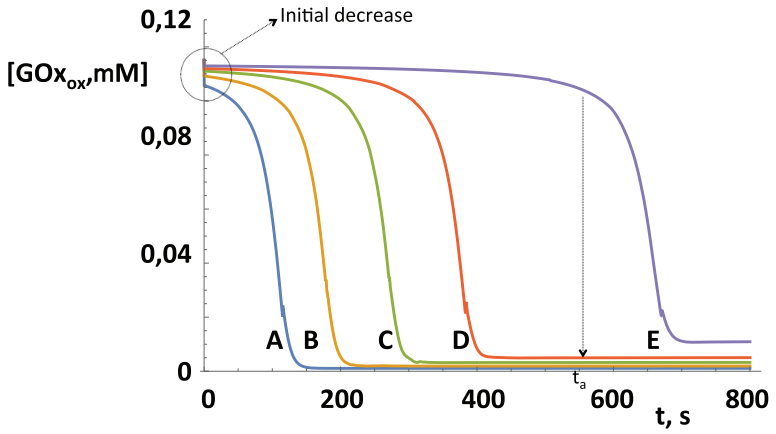

Figure 10. Simulated calculation of the concentration of the [GOx] during the enzymatic reaction for different glucose concentrations. Conditions used: $[GOx]_0 = 1.15 * 10^{-7}$ M. Glucose concentrations (mM): $A = 2.0$; $B = 1.2$; $C = 0.80$; $D = 0.60$; $E = 0.40$. Rate constants as indicated in figure 9. The circle shows the instantaneous small decrease in the [GOx$_{ox}$] for the different glucose concentrations. The t_a value for the E solution is also shown.

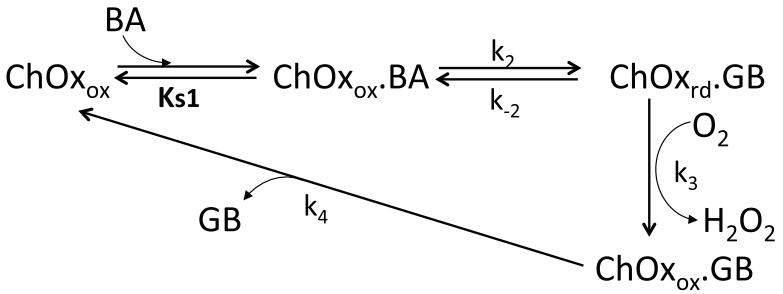

Figure 11. Mechanism of the enzymatic oxidation of betaine aldehyde (BA) to glycine betayne (GB) with O_2 catalyzed by ChOx. Constants of the reaction are: $K_{s1} = 0.45$ mM, $k_2 = 135\ s^{-1}$, $k_{-2} \approx$ slow, $k_3 = 53.4\ mM^{-1}\ s^{-1}$, $k_4 \approx$ very fast. The O_2 diffusion constant $k_D = 10^{-4}\ M^{-1}\ s^{-1}$.

$$t_a = \frac{[O_2]_0}{k_a\,[GOx]\,[Glu]_0}. \qquad (3b)$$

Equation (3*b*) is a simplified version of (3*a*), which can be used when $[Glu]_0 > 10^{-3}$ M [65].

In the next section, the analytical applications of the intrinsic properties of GOx for glucose determination will be reviewed. Methods based on molecular absorption fit well with the proposed model. For those based on fluorescence, most authors observed that the intensity increases (instead of decreasing) when the enzyme is in the reduced form, which is the opposite behaviour to that expected.

In order to explain this behaviour we have made additional assays. First, we observed that the concentration of the GOx necessary to observe fluorescence has to be very high because of its low quantum yield; this would produce an inner filter effect when fluorescence is being measured. Second, when the enzyme is in the reduced form, its molecular absorption spectrum (obtained using a spectrophotometer) agrees with the expected; however the fluorescence excitation spectrum corresponds to that of the oxidized form and the intensity is slightly higher.

These results can be accounted for considering that the enzyme is photooxided by the excitation light of the fluorimeter. Obviously this process will only be produced in the volume of the cell which is being illuminated, but in the rest of the solution the enzyme will be in the reduced form. In addition, the increase in the fluorescence intensity can be account for considering the inner filter effect caused for the higher concentration used; when the enzyme is reduced due to the enzymatic reaction, the inner filter effect decrease and the fluorescence of the photooxided GOx increases.

3.2. Choline oxidase

ChOx is also a very well-known enzyme which catalyses choline (Ch) oxidation in two steps. First, Ch is oxidized to betaine aldehyde (BA) and later to glycine betaine (GB); both steps fulfil the general scheme given in figure 6. One O_2 molecule is involved in each step so two O_2 molecules are consumed in the whole reaction. This reaction is very important from the analytical point of view, not only for BA or Ch determination but also for lipids-containing Ch determination (phosphatidylcholine, phosphorylcholine, choline phosphate). In this case the ChOx reaction has to be coupled with a hydrolytic enzymatic reaction.

Gadda *et al* have studied this reaction in depth [66, 67]. For BA they obtained a model as shown in figure 11. Despite ChOx belongs to the GMC family, the

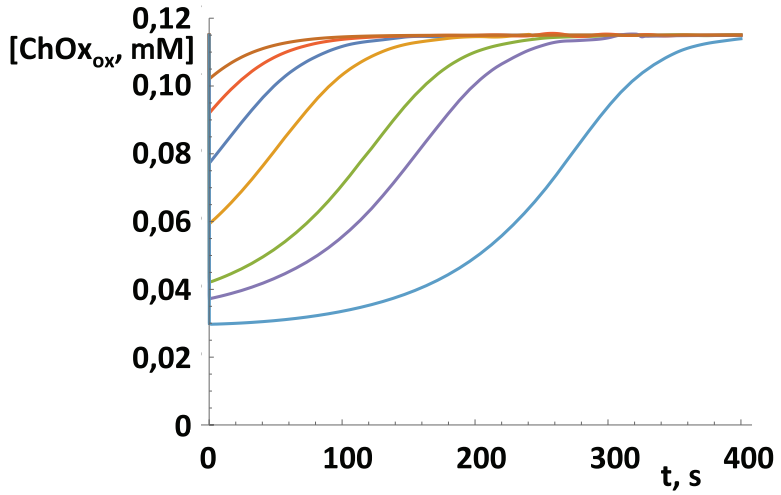

Figure 12. Simulated calculation of the concentration of the [ChOx] during the enzymatic reaction for different BA concentrations. Conditions used: $[ChOx]_0 = 1.15 * 10^{-7}$ M, $K_D = 10^{-4}$ s^{-1}. Betaine Aldehyde (mM): $A = 0.005$; $B = 0.01$; $C = 0.02$; $D = 0.04$; $E = 0.08$; $F = 0.1$; $G = 0.15$. Rate constants as indicated in figure.

kinetic of the reaction does not follow a conventional ping-pong mechanism, as that of GOx. The intermediate ChOx forms appearing during the reaction are $ChOx_{ox} \cdot GB$, $ChOx_{rd} \cdot GB$ and $ChOx_{ox} \cdot GB$.

By applying the same methodology than as with GOx, the simulation of the variation of the enzyme form concentrations during the reaction, gives the following conclusions:

1. The concentrations of the intermediates $ChOx_{ox} \cdot Ba$ and $ChOx_{ox} \cdot GB$ are negligible. Again the mechanism of the reaction can be simplified to a two-step reaction, similar to that given in figure 9 but replacing the reduced form by $ChOx_{rd} \cdot GB$; it is important to consider that, from the spectroscopic point of view the spectral properties of this species and that of the pure reduced form ($ChOx_{rd}$) are expected to be nearly the same. For this case, it can be demonstrated that:

$$K_a = \frac{k_7}{k_{s1}} = 3.0 * 10^5\,\mathrm{M^{-1}\,s^{-1}} \qquad (4)$$
$$k_b = k_9 = 5.3 * 10^4\,\mathrm{M^{-1}\,s^{-1}}.$$

2. According to the kinetic constants the re-oxidative half-reaction is now faster, so it is not necessary to use BA concentrations higher than that of the O_2 for observing concentration changes in the oxidized and reduced forms.

After solving the differential equation system for different BA concentrations ($[BA]_0 < [O_2]_0$), the $[ChOx_{ox}]$ variation during the enzymatic reaction such as those given in figure 12 are observed. Again, the molar absorptivity and quantum yield of the oxidized form are higher than those of the reduced form, so the fluorescence intensity changes similarly to that of $[ChOx_{ox}]$. The enzyme concentration at the beginning of the reaction ($[ChOx_{ox}]_{t=0}$) also follows the equation (2), so the fluorescence intensity measured in this moment can be used as the analytical parameter.

We have also studied the spectroscopic properties of choline oxidase (ChOx, from both *Alcaligenes sp* and *Arthrobacter globiformis)* [36] during the enzymatic oxidation reaction of both, BA and Ch to GB, and also we find a remarkable behaviour of the ChOx fluorescence. Figure 13(A) shows the 3D spectra obtained with ChOx at pH = 6 before the reaction (FAD oxidized) and figure 13(B) shows the 3D spectra after the addition of a very high excess of BA (or Ch), when the enzyme is in the reduced form. As can be seen, the spectroscopic properties of both, the oxidized and the reduced forms of ChOx fit very well with those of the free FAD; especially the fluorescence of the reduced form is clearly observable. In both cases the absorption spectra fit, with the fluorescence excitation.

When the ChOx solution is brought to the optimum pH of the reaction (pH = 9), the 3D spectrum before the BA addition (figure 13(C)), indicates that both excitation maxima had suffered a hypochromic shift of about 50 nm (maximum at about 400 nm), without changes in the position of the fluorescence maxima. However the molecular absorption spectra still maintain the conventional maxima of the free FAD_{ox}. After the BA addition the 3D spectra was similar to that observed at pH = 6 (figure 15(C)). The same results were observed working at pH 8 and 10, which are more appropriate for the reaction than pH 6. When BA is added to the solution, the fluorescence intensity at 400 nm suffers a sharp decrease such as expected from the simulation given in figure 12.

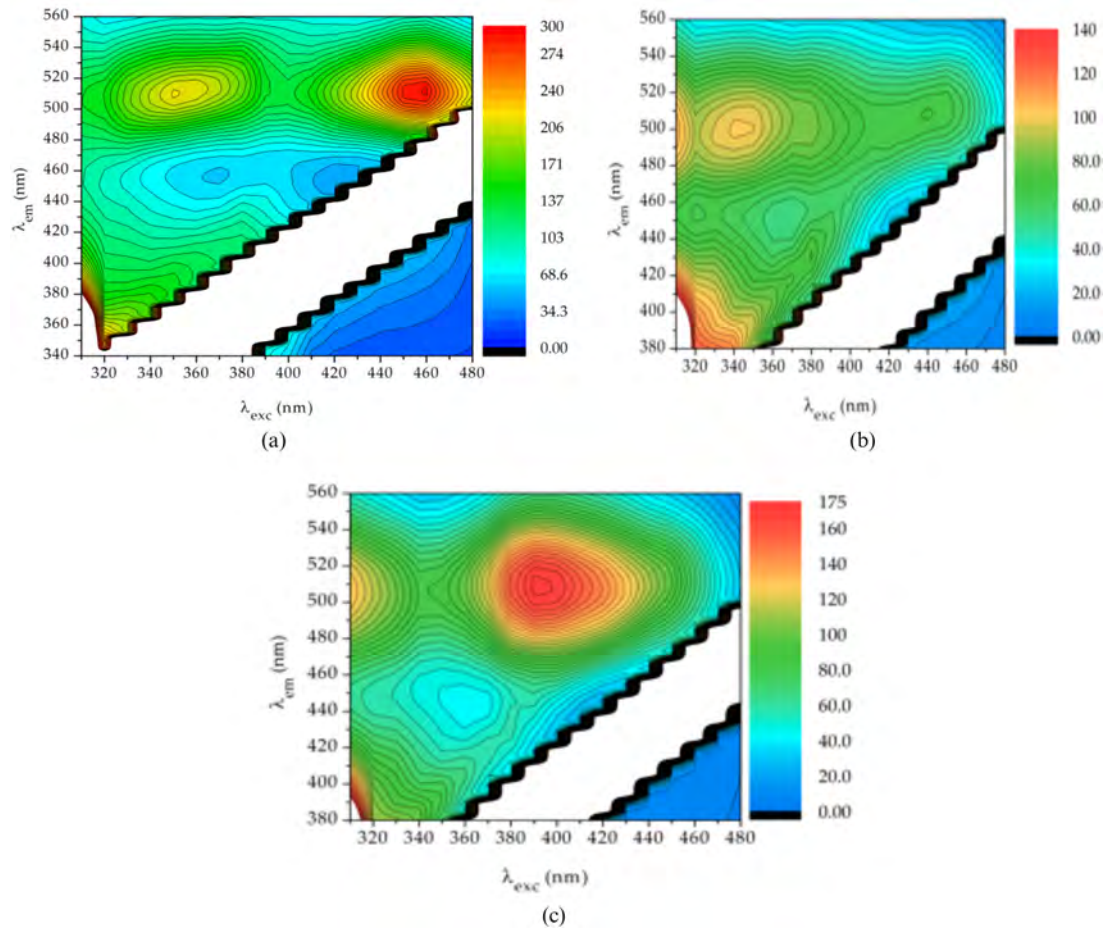

Figure 13. 3 D fluorescence contour plot obtained for ChOx (10 U ml^{-1}). Intensity increases in the sequence blue → green → yellow → red. White colour refers to Rayleigh and tryptophan bands. (A) pH = 6 before the reaction; (B) pH = 6 and 9 after Ch or BA addition; (C) pH = 9 before the reaction.

Since no photoreduction could be observed in the spectra, we accounted for these results as being a consequence of a modification in the polarity of the active centre linked to the capability of the enzyme for the reaction. However, we have no definitive evidences.

We have tried several hypotheses in order to explain these spectra, but we do not yet have any specific evidence about any of them.

3.3. Model generalization and further applications

It would be expected that all the pOx reactions involving substrates (S) fulfilling that the re-oxidative half-reaction is faster than the reductive half-reaction will have a similar behaviour to that shown for GOx. Accordingly, all the pOx reactions fulfilling that the reoxidative half-reaction is faster than the reductive half-reaction will have a similar behaviour to that shown for ChOx. The kinetic mechanism or the rate constants of different pOx enzymes as MAO [68], DAAO [69] or LOx [70] with specific substrates have been reported, so it could be possible to approximately predict the fluorescence profile and the analytical possibilities of the methods based on this fluorescence.

4. Analytical applications based on FAD properties of FvEs

4.1. Autofluorescence imaging

Previously to discuss the FAD fluorescence application in optical biosensors, we will briefly refer to another interesting application of fluorescence of flavoproteins.

It is well known that Autofluorescence, i.e. the detection of the autogenous fluorescence of biological tissues, is gaining position as an imaging technique [71]. This fluorescence is emitted by molecules such as collagen, porphyrins, retinol, riboflavin or melanin but it is especially important that due to NADPH and flavin coenzymes. Most cell autofluorescence originates from mitochondria and lysosomes.

Autofluorescence may serve as a useful non-invasive diagnostic indicator [72] because it can be used as an intrinsic marker of biological processes in which the above mentioned molecules are involved (avoiding the possible toxicity of exogenous markers).

In this respect, it is possible to use the fluorescence of intracellular coenzymes, such as NADH or FAD, as intrinsic biomarkers for metabolic activities and mitochondrial anomalies [73]. For example, the redox pair FAD/FADH$_2$ is associated with respiration in eukary-

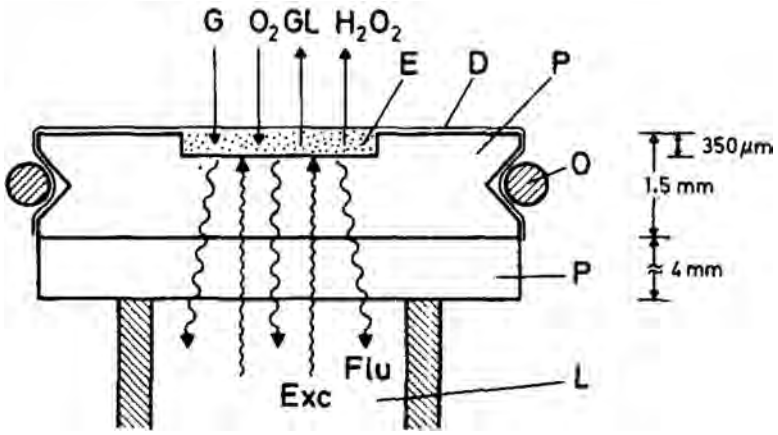

Figure 14. Cross-section through the sensing platelet of the fibre-optic glucose sensor. P, plexiglas; D, dialysing membrane; E, enzyme solution; O, O-ring; L, light guide. Arrows indicate the diffusion process involved (G, glucose; L, gluconolactore). The directions of the exciting light (Exc) and fluorescence (Flu) are also shown. The platelet has a o.d. 20 mm; diameter of cavity, 4 mm. (Reproduced from figure 1 [78], copyright 1989 with permission from Elsevier.)

otic cells [74] and the real-time monitoring of mitochondrial NADH and FAD provides a ratiometric measure of energy respiration as well as the redox state of a cell under certain physiological conditions [75]. The applications are diverse. Mitochondrial dysfunction and oxidative stress have been associated with the majority of neurodegenerative diseases. The concentrations of intracellular NADH and flavin are good biomarkers of mitochondrial dysfunction. As a result, these coenzymes have a great potential as diagnostics of neurodegenerative diseases [76]. Skala *et al* [77] monitored the cellular NADH and FAD autofluorescence lifetime to identify metabolic fingerprints of living cells at the earliest stages of cancer development.

Flavoprotein fluorescence imaging (FFI) is becoming a valuable imaging tool in neuroscience research (flavoprotein autofluorescence is enhanced during neuronal activity) representing an improvement over conventional hemodynamic signals [78].

4.2. Substrates determination: batch methods and biosensors

4.2.1. Direct FAD fluorescence

There are several commercial FAD-enzymes responsible for enzymatic reactions which are interesting from the analytical point of view; however, very few analytical methodologies have been developed based on the intrinsic spectroscopic properties (mainly fluorescence) of the FAD.

Determination of glucose

The FAD-GOx-fluorescence has been widely reported as a tool to characterise the enzyme [79–82] or even its specificity depending on the redox mediator used [82]. Nevertheless, the first use of the FAD-fluorescence for analytical purposes can be found in the seminal paper by Trettnak and Wolfbeis [83]. The authors developed an optical sensor for Glu based on the changes in the fluorescence of the GOx ($\lambda_{ex} = 450$ nm, $\lambda_{em} = 520$ nm) during the reaction. Until that time it had been thought [18] that GOx was a non-fluorescent enzyme in the visible region both in the oxidized and in the reduced state. This idea could probably be justified because lower concentrations of GOx had been used than in the studies of Trettnak–Wolfbeis. The device (figure 14) consisted of a Plexiglas disk with a cavity in its centre in which a solution of the enzyme was placed. The hole was covered with a dialyzing membrane, which was not permeable for the enzyme but allowed the substrates (Glu and oxygen) to diffuse into the reaction cavity. Although there was a linear response to Glu, the range was very short (1.5–2 mM) and the response time was relatively high (2–30 min). They managed to extend the analytical range (i.e. 2.5–10 mM in the case of Glu) by working in kinetic mode and no loss of fluorescence activity was shown over a period of 8 h.

Later, Chudobová *et al* [84] developed a fibre optic biosensor based on the absorption changes of immobilized GOx on a nylon net (with no change in activity being observed after 215 analyses). The decrease in the GOx absorbance at 490 nm gave rise to a dynamic range from 2 to 10 mM of Glu. They have also used the variation of the FAD-GOx-fluorescence during the reaction.

More recently, Esposito *et al* [85–88] improved the stability and increased the linearity of the methodology by entrapping GOx in a gelatine membrane or by covalent bonding to an agarose membrane [85]. They observed reported a dynamic range up to 8 mM of Glu in the case of the gelatine and up to 5 mM of Glu for the agarose membrane. By entrapping the GOx in a sol-gel matrix the authors were able to couple the enzyme to the end of a fibre optic and follow the changes in the visible region, both for the steady-state [86] and for the time course [87, 88] of the GOx fluorescence. In this case the method showed a linear range, respectively, from 0.2 to 10 mM [86] and from 0.4 to 5 mM [87] of Glu and a high catalytic stability with a decrease of about 2% after 60 d. None of the above methods has been applied to real samples. Sol-gel immobilization of GOx for glucose reagentless biosensing has also been carried out by

other authors but much shorter lifetimes of the sensor (especially due to leaching) have been reported [89, 90].

As can be seen the linear response obtained began at high Glu concentrations, actually higher than the oxygen initially present in solution. This result can be accounted for in the light of the theoretical model described in section 3.1.

Accordingly, because the analytical figures of merit of the GOx fluorescence or absorption methodology based on FAD did not represent an improvement over other auto indicating systems [64] and the mechanics through the changes on the fluorescence are not clear, it have not been found more analytical-determinations of Glu based on these properties.

At this point could be interesting to evaluate these results with those described in the previous section. The decrease in the absorbance of the FAD-GOx with the concentration of Glu reported by Chudobová [79] is in accordance with the expected changes due to the oxidised and the reduced form of FAD. The decrease in the GOx fluorescence is justified by the prevalence of the less fluorescent GOxred (working with low concentrations of GOx). The results obtained by other researchers (fluorescence increases during the reaction) can be justified by the combination of fotooxidation/inner filter effect there commented. Moreover, it can be also understood why the linear response obtained began at Glu concentrations higher than the oxygen initially presents in solution.

Accordingly, because the analytical figures of merit of the GOx fluorescence or absorption methodology based on FAD did not represent an improvement over other auto indicating systems [59] and the mechanics through the changes on the fluorescence are not clear, it have not been found more analytical-determinations of Glu based on these properties.

Other substrates

Using a similar device to that described for Glu, Trettnak and Wolfbeis also developed [91] a lactate biosensor using lactate monoxygenase; in this case, the changes in the fluorescence of the flavin-cofactor FMN were followed. The analytical evaluation of the biosensor gave similar characteristics to that of Glu.

As has been indicated above, we have use the FAD fluorescence of ChOx for the determination of Ch and BA, obtaining longer linear response range than for Glu [36]. In this case, the fluorescence of the FAD, as expected, decreased during the enzymatic reaction and the evolution of the fluorescence spectrum during the enzymatic reaction indicated that the reaction takes place in two consecutive, but partially overlapping, steps and each of them follows a different mechanism. The chemical system can be used to determine the Ch in the presence of BA as interference in the range from 5×10^{-6} to 5×10^{-5} M (univariate and multivariate calibration) and the total Ch and BA concentration in the range from 5×10^{-6} to 5×10^{-4} M (multivariate calibration) with mean errors under 10%. A semiquantitative determination of the BA concentration can be obtained by difference. Ch has been successfully determined in an infant milk sample.

By coupling two enzymatic reactions, this methodology has also been used to determine other substrates containing Ch such as the phospholipid phosphorylcholine (ChoP) [92]. ChoP was first hydrolysed by the enzyme alkaline phosphatase (AP) and the Ch formed was then submitted to a reaction with ChOx. Measuring this intrinsic fluorescence, a linear response from 5.2×10^{-7} to 1.0×10^{-5} M was obtained for ChP. This methodology has also made possible the sequential determination of Ch and ChoP in milk powder samples using only one aliquot of the mixture, with good results.

4.2.2. Intrinsic and extrinsic fluorophores FAD-subrogated

The analytical use of FAD spectroscopic properties also has limitations, the most important being the low fluorescence quantum yield of FAD and the photophysical effects, especially when the fluorescence of GOx (and pOx behave similarly) are measured. These facts either limit the sensitivity of the methods or, more importantly, greatly hinder the interpretation of the results and the reliable design of analytical methods.

These problems can be softened by measuring other fluorescence signals coming from the enzyme and also related to the concentration of the oxidized and reduced forms of FAD. Two alternative procedures have been proposed based on intrinsic or chemically linked (extrinsic) fluorophores of the enzymes.

Intrinsic fluorescence

It is well-known that all enzymes have fluorescence in the UV region due to aminoacids, especially tryptophan. Both, the excitation and the fluorescence spectra of this aminoacid overlap with the absorption spectra of the FAD. The flavins can thus produce an inner filter effect on the tryptophan fluorescence, which is higher when the enzyme is in the oxidized form (GOx_{ox}) than in the reduced form (GOx_{rd}). The fluorescence intensity of tryptophan changes during the reaction, emulating the concentration of the reduced form, which are exactly the opposite to those shown in figures 10 or 12. Consequently, the analytical model given in equations (2) and (3) are fulfilled. Thus, our group proposed the Glu [64] determination based on the changes of the UV-Fl of GOx during the enzymatic reaction. The method allows the determination of Glu over the range 5×10^{-4} to 2×10^{-2} M with a RDS of 2.1%, and has been used to determine Glu in blood. The same methodology has been studied by other authors [86, 93].

Following a similar idea, we determined Ch in serum [94] using the fluorescence of ChOx. From the figures 10 and 12 is clear that GOx and ChOx fluorescence change according to a different scheme during the reaction; at the beginning of the reaction, the concentration of the oxidized form of ChOx is minimum

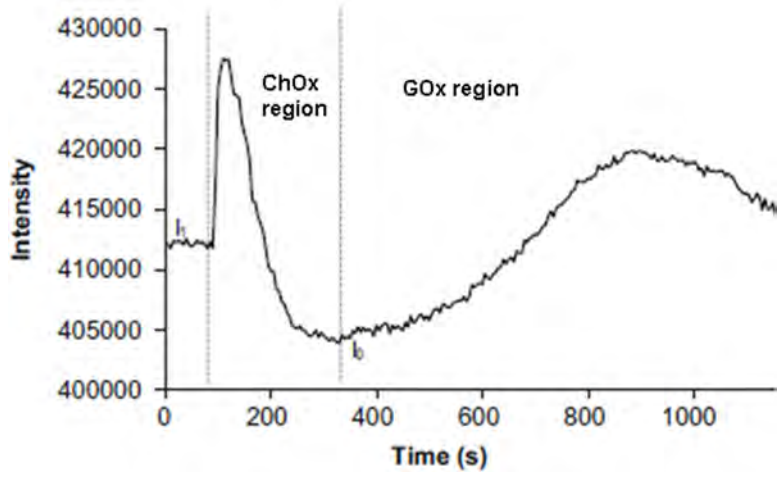

Figure 15. Fluorescence intensity variation at 340 nm (excitation at 286 nm) of a solution containing ChOx (1 U ml^{-1}), GOx (2 U ml^{-1}), Glu (0.55 mM) and Ch (5.5 μM). (Reproduced from figure 3 [86], copyright 2009 with permission of Springer.)

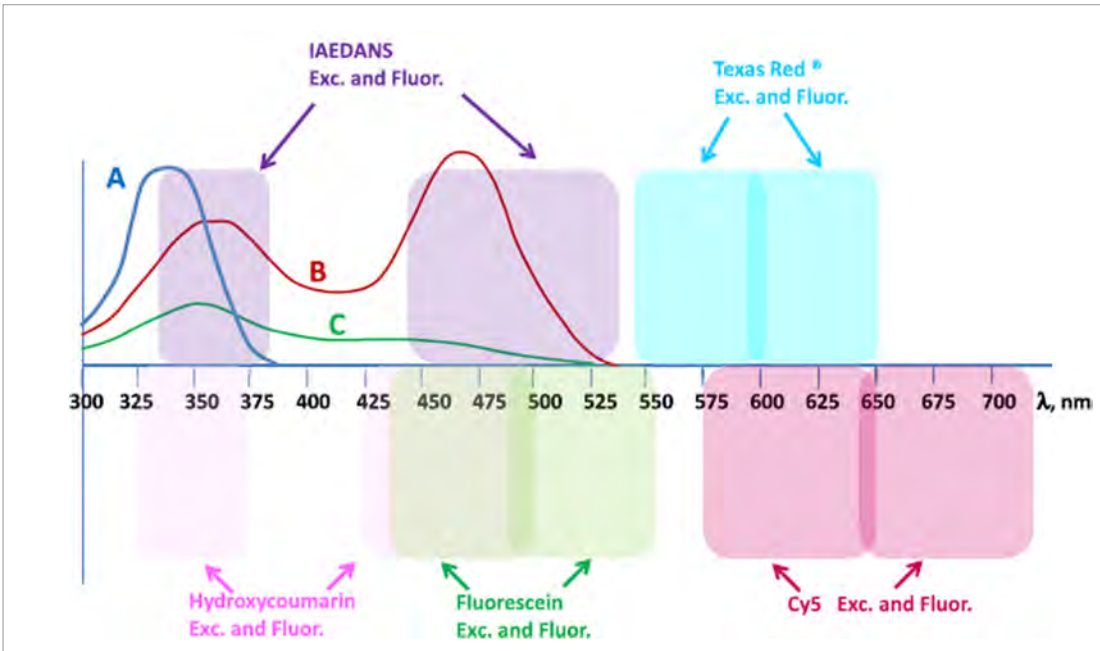

Figure 16. Wavelength range of excitation and fluorescence of different attached fluorophores compared with: (A) (blue line) the tryptophan fluorescence spectrum in porteins; (B) (red line) oxidized FAD absorption spectrum in protein; (C) (green line) reduced FAD absorption spectrum in protein. (Reproduced from figure 5 [3], copyright 2012 with permission of Springer.)

but the concentration of the oxidized GOx concentration is maximum; by properly combining these effects (figure 15) it has also been possible to perform the simultaneous determination of Glu and Ch [95].

The inner filter effect on tryptophan fluorescence has also been used in enzymatic reactions not involving FvEs [3, 4, 96].

Extrinsic fluorescence

In this approach, the pOx is chemically modified with a fluorophore whose spectroscopic properties overlap with those of the flavin which produces again an inner filter effect. Depending on the properties of the fluorophore, the filter effect can be produced during the fluorophore absorption, the fluorophore fluorescence or even both. We first demonstrated the viability of this approach using GOx linked to different fluorophores [3, 4] (figure 16) whose spectra overlap (hydroxycoumarin, IAEDANS or fluorescein (FS)), or not (Texas Red™ o Cy5) with those of the pOx; only in the first cases were changes in the fluorescence intensity of the linked fluorophore observed during the enzymatic reaction. Using this methodology, biosensors for Glu determination have been tested after immobilizing GOx chemically modified with fluorescein (GOx-FS) or pyrene [97, 98] in several supports as polyacrylamide [99, 100], sol-gel [90], or many others [101]. Other substrates as total cholesterol [102] and Ch [94] have been determined in blood or serum samples using COx-FS and ChOx-FS respectively (λ_{ex} = 490 nm, λ_{em} = 520 nm). In the case of total cholesterol determination, the use of derivatized COx-FS makes

it possible to simplify the methodology normally used in this type of determination (the indicator reaction is avoided and the number of reagents reduced), with the added advantage that the analytical signal is independent of the concentrations of oxygen and COx.

This methodology gives a better analytical performance than that of tryptophan-based, especially because it avoids the spectral interferences of the sample which more frequently appear in the UV region.

4.2.3. Combination of FAD spectroscopic properties and nanomaterials

Nanobiosensors based on FAD spectroscopic properties can also be designed. The nanomaterial may either be used as a support for the nanobiosensor (which could be based on the FAD fluorescence) or act as an extrinsic fluorophore. There are very few bibliographic references describing this possibility [103]. Del Barrio *et al* [104] have described the combination of upconversion materials (UCM) with GOx for the design of a fluorometric nanobiosensor for the continuous monitoring of Glu. The sensor combines the fluorescence of the GOx-FS and the luminescent properties of the upconverting luminescent nanoparticles (UCLNPs). Both the chemically modified enzyme and the UCLNPs are immobilized in a polyacrylamide film as a physical support. The system is excited with NIR radiation, which is absorbed by the nanoparticles, and their upconverted luminescence is used to excite GOx-FS, which undergoes a change in the fluorescence intensity during the enzymatic reaction with Glu. The sensor has sufficient stability and covers the physiological range of Glu levels in blood. The system responds linearly to Glu concentrations in the range from 3.3 to 16.6 mM in flow injection analysis mode. The excitation of the UCLNPs with NIR light is a major advantage, since fluorescence background from the matrix is minimized.

5. Future trends

The intrinsic fluorescence of FAD in FvEs is a powerful tool for analytical purposes, both in batch methods and optical biosensors, especially because: (1) a second substrate is not necessary (O_2 is provided by the medium) and, (2) the enzyme is able to repeat the oxidative cycles many times (continuous monitoring). Taking this into account, future research could involve the following topics:

1. New analytes. Up to date, FAD fluorescence applications have been reduced to very few analytes, especially Glu. In our opinion, it may be worth to explore the applicability of this fluorescence to other analytes as biogenic amines (MAO-A, MAO-B), aminoacids (DAAO), free and esterified cholesterol (ChOx) or phospholipids (ChOx). The methodology outlined in section 3 could be applied to know how the fluorescence will change along the reaction and to evaluate the concentration range of the analyte in which the method could be applied.
2. To taking advantage of this fluorescence, in depth studies to systematize the effect of the protein skeleton in FAD fluorescence would be of great interest.
3. Non-invasive monitoring and nanomaterials. Fluorescence has demonstrated its capability as a non-invasive technique. FvE can be used as recognition elements in implantable biosensors, using the FAD intrinsic fluorescence properties or subrogating them to extrinsic fluorophores. In this sense nanomaterials (quantum dots, Au nanoclusters), should be thoroughly tested as potential extrinsic fluorophores. Promising results have already been reported; for example, it has been demonstrated that FAD is able to interact with the plasmon of the Ag nanoparticles [105]. In addition, the interaction of enzymes with nanomaterials could improve their quantum yield; for example, when GOx is linked to graphene oxide the enzyme structure is slightly modified leaving the FAD more accessible and increasing its fluorescence [106].

Acknowledgments

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