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Review

Sensor and biosensor preparation, optimisation and applications of Prussian Blue modified electrodes

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Abstract

Being one of the most commonly used electrochemical mediators for analytical applications, Prussian Blue has found a wide use in the biosensor field during the last years. Its particular characteristic of catalysing hydrogen peroxide reduction has been applied in the construction of a large number of oxidase enzyme-based biosensors for clinical, environmental and food analysis.

By modifying an electrode surface with Prussian Blue, it is in fact possible to easily detect hydrogen peroxide at an applied potential around 0.0 V versus Ag/AgCl, thus making possible coupling with oxidase enzymes while also avoiding or reducing electrochemical interferences.

Papers dealing with glucose, lactate, cholesterol and galactose biosensors that are based on the use of Prussian Blue have recently appeared in the most important analytical chemistry journals.

Another recent trend is the use of a choline probe based on choline oxidase for pesticide determination to exploit the inhibition of acetylcholinesterase by these compounds.

In addition, the use of Prussian Blue in the development of biosensors for food analysis has captured the interest of many research groups and led to improved methods for the detection of glutamate, galactose, alcohol, fructosyl amine, formate, lysine and oxalate.

This review will focus on the biosensing aspects of Prussian Blue-based sensors giving a general overview of the advantages provided by such mediator as well as its drawbacks. A comprehensive bibliographic reference list is presented together with the most up to date research findings in this field and possible future applications. The commercial potential of sensors based on this mediator will also be discussed.
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Keywords: Prussian Blue; Modified electrodes; Review; Biosensor; Hexacyanoferrate

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1. Introduction

The most used configuration for the assembly of amperometric enzyme biosensors is that involving the use of an oxidase enzyme. This class of enzymes acts by oxidising the substrate and then returning to their original active state by transferring electrons to molecular oxygen, so that the final products of these enzymes are the oxidised form of the substrate and, as a side product, hydrogen peroxide. Both the measurement of oxygen consumption (Clark and Lyons, 1962) and hydrogen peroxide production (Updike and Hicks, 1967; Guilbault and Lubrano, 1973; Harrison et al., 1988) can provide information about the initial concentration of the enzyme substrate. Methods based on the measurement of hydrogen peroxide have been greatly preferred during recent years to those based on the reduction of oxygen. However, a great drawback in this approach is represented by the high overpotential needed for hydrogen peroxide oxidation (ca. 0.7 V versus Ag/AgCl) at which many electroactive substances (i.e. ascorbic acid, uric acid, etc.), which are usually present in real samples, could also be oxidised to give interfering signals.

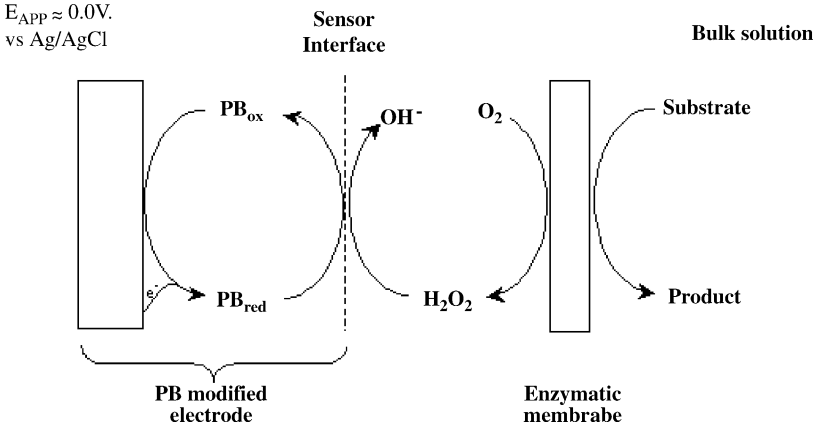
One of the most common ways to overcome this problem has been the use of another enzyme, namely horseradish peroxidase (HRP), a prototypical hemeprotein peroxidase, which catalyses the reduction of H_2O_2 and, due to its peculiar structure, allows the direct electron transfer between its active site and the electrode surface (Lindgren et al., 2000; Razola Serradilla et al., 2002; Csoregi et al., 1994; Ruzgas et al., 1996). This approach, although exhibiting good sensitivity and accuracy, suffers from some important shortcomings such as high cost, low stability and the limited binding of HRP to solid surfaces.

For this reason, in the last decade, electrochemical inorganic mediators (Chaubey and Malhorta, 2002; Zen et al., 2003a; Newman et al., 1995), which catalyse the oxidation or reduction of hydrogen peroxide, have been preferred to HRP and have been used for the assembling of oxidase-based biosensors. This results in a decrease of the applied potential and the consequent avoidance of many electrochemical interferences. In this perspective, hexacyanoferrates and in particular Prussian Blue (ferric hexacyanoferrate—PB) have found a large use (see Scheme 1) (Karyakin, 2001; Koncki, 2002; de Tacconi et al., 2003).

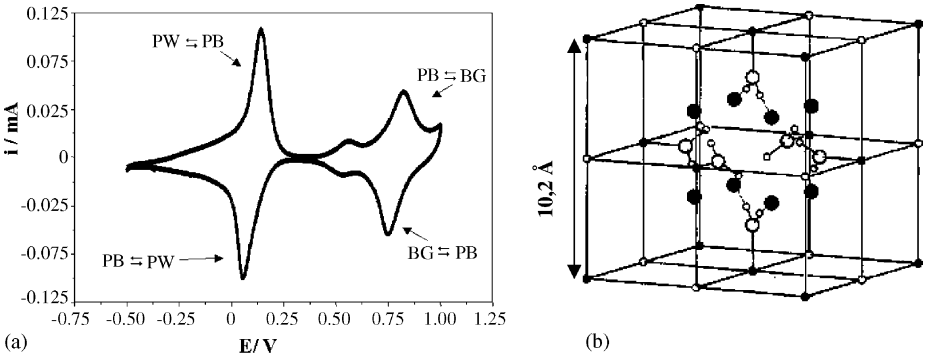
This review will deal with all the analytical aspects of Prussian Blue with a comprehensive and exhaustive bibliographic survey of the biosensor examples appearing in the literature and based on this mediator. The review intends to be a valuable tool for all the researchers who are interested in this field and intends to illustrate all possible application of PB in the biosensor field.

2. Chemical, physical and electrochemical properties of Prussian Blue

In 1978, Neff (1978) reported for the first time the electrochemical behaviour and the successful deposition of a thin layer of Prussian Blue on a platinum foil. The cyclic voltammetry of the modified electrode revealed the classic and today well-known form of the reversible reduction and oxidation of Prussian Blue (see Fig. 1a). It was only some years later that the electrochemistry of Prussian Blue was fully investigated (Ellis et al., 1981; Rajan and Neff, 1982; Itaya et al., 1982a,b, 1984a,b, 1986; Ohzuku et al., 1985). Itaya et al. addressed



Scheme 1. General overview for PB-based biosensor with an oxidase enzyme.



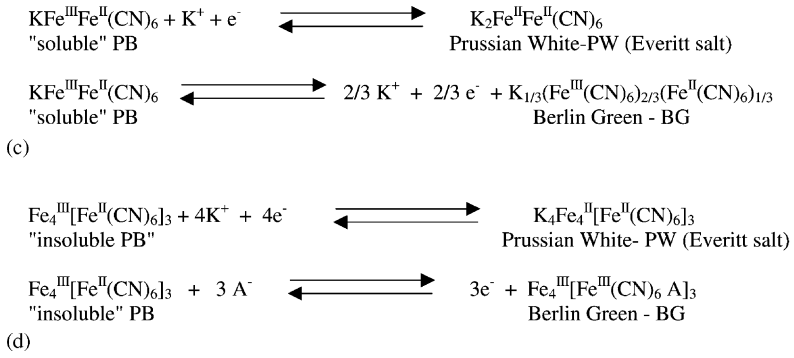


Fig. 1. (a) Cyclic voltammogram of a PB modified electrodes showing the reduction and oxidation peaks of Prussian Blue (Ricci et al., 2003c). Copyright Wiley/VCH. (b) Unit cell of Prussian Blue. Permission from Herren et al. (1980a). Copyright (1980) American Chemical Society. ORTEP plot of the unit cell of Prussian Blue displaying part of the hydrogen bond network. Cyanide ions are omitted for the sake of clarity and the radii of the atoms are chosen arbitrarily (•, Fe(III); ○ , Fe(II); ◯, oxygen; ◯ , D; ●; D_2O). (c) Redox processes for "soluble" Prussian Blue (Ellis et al., 1981). (d) Redox processes for "insoluble" Prussian Blue (Itaya et al., 1982a, 1986).

a new versatile method for the preparation of Prussian Blue modified electrodes based on a simple electrochemical reduction of a ferric–ferricyanide solution. The procedure was adopted with different electrode materials (SnO_2, platinum, gold, glassy carbon) and, under certain conditions, a high stability of the layer deposited through successive cycling was demonstrated (i.e. no degradation after 10^5 cycles). Itaya et al. (1984a) demonstrated the most important feature of Prussian Blue (in terms of analytical application). It was in fact shown that the reduced form of Prussian Blue (also called Prussian White) had a catalytic effect for the reduction of O_2 and hydrogen peroxide. Also, the oxidised form of Prussian Blue showed a catalytic activity for the oxidation of hydrogen peroxide. The catalytic effect of PB towards oxygen and hydrogen peroxide was ascribed to the peculiar structure of PB. The zeolitic nature of PB with a cubic unit cell of 10.2 Å and with channel diameters of about 3.2 Å allows the diffusion of low molecular weight molecules (such as O_2 and H_2O_2) through the crystal. For this reason, Itaya described PB as a three-dimensional catalyst. By taking into consideration the previous diffraction study of PB structure by Ludi et al. (Ludi and Gudel, 1973; Herren et al., 1980a), which indicated the presence of about eight uncoordinated water molecules in the unit cell of PB, Itaya hypothesized that such vacancies were responsible for the catalytic activity towards H_2O_2. When the molecule of hydrogen peroxide (or oxygen) penetrates the PB lattice structure, it will be located in the center of each vacancy being surrounded by four divalent high spin iron ions on average, which can bring about a catalytic reduction of H_2O_2 via a four electron reaction.

Although this hypothesis is rather suggestive, the mechanism of catalysis of PB towards H_2O_2 reduction is still not completely clear, probably due to the fact that, as with cross-linked organic polymers (Itaya et al., 1986), it is very difficult to characterize PB and there is still no complete agreement concerning its structure and its stoichiometric composition.

For many years, the structure of PB has been a subject of investigation in order to explain the electrochemical behaviour and its catalytic activity. Keggin and Miles (1936) first discussed the structure of PB on the basis of powder diffraction patterns. The authors distinguished between two different forms of PB, one called soluble and the other insoluble. These names, given by dye makers, do not refer to the real solubility in water but rather indicate the ease with which potassium ions peptize. The unsuitability of these terms has been much stressed and it was demonstrated that both the forms are highly insoluble ($K_{ps} = 10^{-40}$) (Ellis et al., 1981; Mortimer and Rosseinsky, 1984). According to Keggin and Miles, the soluble form of PB has a cubic structure in which iron(II) and iron(III) are located on a face centered cubic lattice, where iron(II) ions are surrounded by carbon atoms and iron(III) ions are surrounded octahedrically by nitrogen

atoms. The "insoluble" form differs from the "soluble" one by virtue of the excess of ferric ions which replace potassium ions in the interstitial sites. Following this study, a new approach for determining the structure of the insoluble form was performed by Ludi and co-workers (Ludi and Gudel, 1973; Herren et al., 1980a,b; Buser et al., 1977) which showed some differences from that proposed by Keggin and Miles (see also Fig. 1b). The structure was found to be more disordered with one-fourth of the ferrocyanide sites unoccupied. The presence of 14–16 water molecules per unit cell was demonstrated. A portion of the water molecules are filling empty nitrogen sites of the ferrocyanide vacancies, while the rest are occupying interstitial sites and represent uncoordinated water. Moreover, no iron(III) ions were found in the interstitial sites. This conclusion seems also to be confirmed by Mossbauer and infrared studies (Robin and Day, 1967; Duncan and Wrigley, 1963). However, it should also be stressed that this uncertainty concerning the PB structure has also to be attributed to the fact that Prussian Blue is a generic chemical designation for a complex material with variable stoichiometry (Itaya et al., 1986). The structure, depending on the materials and procedure used to crystallize PB, could contain co-precipitated ions, indefinite amounts of water, hydrolysed ferrocyanide and various vacancies in the crystal structure.

The uncertainty as to the structure of Prussian Blue has also been responsible for the uncertainty concerning the electrochemical reaction that takes place at the electrode surface. Initially, it was proposed (Ellis et al., 1981) that PB could be oxidised and reduced according to the following reactions:

$$\underset{\text{"soluble" PB}}{KFe^{III}Fe^{II}(CN)_6} + K^+ + e^- \rightleftarrows \underset{\text{Prussian White (Everitt salt)}}{K_2Fe^{II}Fe^{II}(CN)_6} \quad (1a)$$

$$\underset{\text{"soluble" PB}}{KFe^{III}Fe^{II}(CN)_6} \rightleftarrows \frac{2}{3}K^+ + \frac{2}{3}e^- + \underset{\text{Berlin Green (Prusssian Yellow)}}{K_{1/3}Fe^{III}(CN)_6)_{2/3}(Fe^{II}(CN)_6)_{1/3}} \quad (1b)$$

assuming that the "soluble" form of PB ($KFeFe(CN)_6$) was involved in the process.

A different reaction was instead proposed by Itaya et al. (1982a, 1986), who claimed the following scheme for the two reactions:

$$\underset{\text{"insoluble" PB}}{Fe_4{}^{III}[Fe^{II}(CN)_6]_3} + 4K^+ + 4e^- \rightleftarrows \underset{\text{Prussian White (Everitt salt)}}{K_4Fe_4{}^{II}[Fe^{II}(CN)_6]_3} \quad (2a)$$

$$\underset{\text{"insoluble" PB}}{Fe_4{}^{III}[Fe^{II}(CN)_6]_3} + 3A^- \rightleftarrows 3e^- + \underset{\text{Berlin Green (Prussian Yellow)}}{K_4Fe_4{}^{III}[Fe^{III}(CN)_6A]_3} \quad (2b)$$

where A is the anion supplied by the electrolyte.

These reactions were proposed because no potassium ions were found in the film of PB electrodeposited and thus, the "insoluble" form was taken to be the only possibility for the reaction. Since potassium ions appear to be involved in both the reduction and oxidation reactions, some uncertainty regarding the validity of one reaction or the other remains. By contrast, it is instead well documented and univocous the effect of cations on the PB structure. It has been demonstrated that the PB electrochemical activity is supported in the presence of K^+ ions (Garcia-Jareno et al., 1998a). Also, Rb^+, Cs^+ and $NH_4{}^+$ were found to allow the cyclic electrochemical reactivity of PB. Conversely, in the presence of Na^+, Li^+ and H^+, as of all group II cations, the activity of PB is blocked after very few cycles. This behaviour has been explained in terms of the hydrated ionic radii and the channel radius of the PB lattice. PB has in fact a channel radius of about 1.6 Å (Fig. 1b) which will easily accommodate K^+, Rb^+, Cs^+ and $NH_4{}^+$ whose hydrated molecules have radii of 1.25, 1.28, 1.19, 1.25 Å, respectively (Itaya et al., 1982a, 1986).

The electrochemical stability of the PB layer has also been a matter of investigation since its first use. Although it was demonstrated from the beginning that the storage stability of PB was quite high (better if in dark), it was clear that the pH and solution composition in which PB was tested were extremely important. The effect of pH on PB activity and stability is still a subject of interest for researchers involved in the characterisation of PB and will be addressed in more depth later, since it represents one of the major issues for the application of PB in biosensor field.

The findings of Itaya and Neff in the 1980s have had very important implications for PB catalytic activity and for its subsequent use in electroanalytical applications. They were the first to demonstrate the possibility of depositing PB layers onto different electrode materials (platinum, glassy carbon, SnO_2, TiO_2) and showed the interesting electrocatalytic activity relative to hydrogen peroxide reduction. These studies were the basis for the future applications of PB in biosensors as a means for amperometric detection of H_2O_2.

3. Prussian Blue modified electrodes as hydrogen peroxide sensors

3.1. General aspects

The problem of hydrogen peroxide amperometric detection is well known to all the researchers involved in the biosensor field, since hydrogen peroxide is the side product of the classic reaction catalysed by oxidase enzymes (Eq. (3)).

$$\text{Substrate} + O_2 \xrightarrow{\text{Oxidase enzyme}} \text{product (oxidised substrate)} + H_2O_2 \quad (3)$$

For any biosensor employing an oxidase enzyme as recognition element, the detection of hydrogen peroxide becomes of great importance, its concentration being directly proportional to the concentration of the enzymatic substrate (i.e. the analyte). However, direct hydrogen peroxide amperometric detection at conventional electrodes is only possible at ca.

0.6 V versus Ag/AgCl. At this potential, the presence of easily oxidisable compounds such as ascorbate, bilirubin, urate, etc. can easily interfere in the measurement, being oxidised at the electrode together with hydrogen peroxide (Scheller et al., 1987). Initially, the solution to this problem was the use of a protective layer made from a cut-off membrane which was able to eliminate the interferents (Palleschi et al., 1986). This, however, gave rise to other problems, namely diffusion barrier, long time preparation, etc. To overcome this problem, the use of mediators which were able either to reoxidise the enzyme active site or to reduce or oxidise one of the products of the enzymatic reaction was proposed. The optimisation of a sensitive, stable, reproducible and interference-free hydrogen peroxide mediator thus represents a major issue in the biosensor field. The development of such a layer would provide the perfect substrate for the immobilisation of an oxidase enzyme which would give the specificity for the analyte of interest. The subsequent coupling of a specific enzyme with an interference-free H_2O_2 sensor would make possible the assembly of a biosensor with the desired characteristics of sensitivity and selectivity.

Following the initial findings by Itaya and Neff, the first attempt to study Prussian Blue as electrochemical mediator for hydrogen peroxide was made by Boyer et al. (1990) using a PB modified carbon paste electrode. Although they demonstrated good analytical performance (linear range between 3×10^{-2} and 3 mM at −0.4 V versus SCE), using this mediator, the experiment generated little interest until 1994 when Karyakin et al. (1994) made the claim for a PB modified electrode as a powerful tool for hydrogen peroxide detection at low applied potential. They demonstrated the possibility of the effective electrochemical deposition of a PB layer onto a glassy carbon electrode providing an efficient and selective catalytic activity towards hydrogen peroxide reduction (Karyakin et al., 1994, 1995) and they found that the catalytic activity of PB towards H_2O_2 was about 100 times greater than that of oxygen and that the rate constant for H_2O_2 reduction under those experimental conditions was $5 \times 10^2\ M^{-1}s^{-1}$. In the first instance, a potential of 180 mV versus Ag/AgCl was applied at the modified electrode with a resulting detection limit of about 1 μM and a linear range up to 0.01 M. After this, an optimised procedure for PB deposition and a more careful investigation of the hydrogen peroxide cathodic current at different potentials allowed the use of PB modified electrodes at an applied potential of 0.0 V versus Ag/AgCl with a sensitivity in the micromolar range (Karyakin et al., 1996). For the optimised conditions (thin layer of PB), the recalculated bimolecular rate constant for the reduction of H_2O_2 was $3 \times 10^3\ M^{-1}s^{-1}$, which is very similar to that measured for the peroxidase enzyme ($2 \times 10^4\ M^{-1}s^{-1}$) (Hasinoff and Dunford, 1970). This high catalytic activity and selectivity led Karyakin to consider PB as an "artificial peroxidase". The operating potential (i.e. 0.0 V versus Ag/AgCl) was low enough to avoid or greatly reduce the contribution from all the most common interferents (ascorbic acid, paracetamol, uric acid) (Karyakin et al., 1995, 1996), rendering the PB modified electrode selective for hydrogen peroxide. However, the low applied potential does not by itself explain the high selectivity of PB. This feature has again been accounted for in terms of the peculiar structure of PB which enables low molecular weight molecules to penetrate the cubic lattice and be reduced while excluding molecules with higher molecular weight. This is probably the main advantage of using PB as mediator for H_2O_2 reduction. The selectivity and activity achieved are comparable to those of a biological binding component but with all the advantages of an inorganic species (low cost, high stability at certain conditions, ease of electrode surface modification, no saturation effect for substrate).

For this reason, a great attention was devoted to PB modified electrodes and their use increased following the publication of these pioneering papers. In Table 1 are summarised the most significant results that were obtained for H_2O_2 detection by use of PB modified electrodes based on different electrode materials, modification procedures and principles of measurement. By now, PB modification has been exploited in conjunction with all the most commonly used electrode materials; glassy carbon (Karyakin et al., 1996, 1999, 2000; Karyakin and Karyakina, 1999; de Mattos et al., 2000b), graphite (Turner and Jaffari, 1995; Dostal et al., 1995; Chi and Dong, 1995; Deng et al., 1998), carbon paste (Weibenacher et al., 1992; Moscone et al., 2001; Zakharchuk et al., 1995; Garjonyte and Malinauskas, 1998), platinum (Garjonyte and Malinauskas, 1999a,b), and gold electrodes (de Mattos et al., 2003). It has also been applied to innovative electrode forms: screen printed electrodes (SPEs; O'Halloran et al., 2001; Ricci et al., 2003a; Zen et al., 2003b), ITO electrodes (Garcia-Jareno et al., 1998b, 1999) and carbon nanotubes-based carbon paste electrodes (Ricci et al., 2003d). In all these cases, the analytical behaviour towards H_2O_2 was highly satisfactory with a detection limit always in the micromolar range and with excellent characteristics relative to the most common electrochemical interferents.

Among the carbon-based electrode materials, also glassy carbon paste (Ricci et al., 2003c), carbon ceramic material (Wang et al., 2000) and carbon fiber cone nanoelectrodes (Zhang et al., 1999) have been used as substrate for PB modification. The self-assembling of PB hybrid films directly on cinder containing carbon paste electrodes has also been extensively reported (Zen et al., 2001; Kumar et al., 2004; Zen and Kumar, 2004). PB hybrid films have also been formed inside the natural iron-rich materials of nontronite (Zen et al., 2000). These materials contain a natural excess of Fe^{3+} ions in their structures which effectively assists hybrid Prussian Blue formation in the presence of electrochemical cycling in a solution of $Fe(CN)_6{}^{3-}$.

For hydrogen peroxide detection, glassy carbon has provided the highest sensitivity ($1\ A/(M\,cm^2)$) among the carbon-based electrodes (Karyakin et al., 1995), but in 2003, Gorton's group demonstrated the best sensitivity towards H_2O_2 for PB modified gold screen printed electrodes ($2\ A/(M\,cm^2)$) employed in a FIA experiment (de Mattos

Table 1
Examples of hydrogen peroxide sensors based on Prussian Blue modified electrodes

Type of sensor	Deposition method	Procedure of measurement	Applied potential (mV)	LOD (μM)	Linear range (μM)	Sensitivity (mA/(M cm^2))	Comments	Reference
Glassy carbon	Galvanostatic	Rotating disk electrode	+180	1.0	1.0–10^3	–	First H_2O_2 sensor based on PB	Karyakin et al. (1994)
Glassy carbon	Galvanostatic	Rotating disk electrode	+180	1.0	1.0–5×10^3	1000	–	Karyakin et al. (1995)
Basal pyrolitic graphite	Cycling of ($FeCl_3$ + $Fe(CN)_6$)	Stirred batch amperometry	−50	–	?–900	55*	First using very low potential	Chi and Dong (1995)
Glassy carbon	Galvanostatic	FIA	0	1.0	1.0–200	250*	–	Karyakin et al. (1996)
Carbon rod	Cycling of $K_3Fe(CN)_6$	Stirred batch amperometry	+450 vs. SCE	–	–	1000*	Patented procedure	Turner and Jaffari (1995)
Glassy carbon	Galvanostatic	FIA	−50	0.1	0.1–100	600	–	Karyakin and Karyakina (1999)
Platinum	Cycling of ($FeCl_3$ + $Fe(CN)_6$)	Steady-state amperometry	0	–	–	0.27	Very low stability of the PB layer	Garjonyte and Malinauskas (1999a)
Graphite screen printed electrodes	PB microparticles mixed with carbon ink	Stirred batch amperometry	0	0.4	0.4–100	137	–	O'Halloran et al. (2001)
Carbon paste	Chemical deposition on graphite powder	Stirred batch amperometry	0	0.25	0.25–200	45*	High pH stability	Moscone et al. (2001)
Graphite screen printed electrode	Chemical deposition on graphite/GC mixed with carbon ink	Stirred batch amperometry	−50	0.3	0.5–10^3	135	High pH stability	Ricci et al. (2003b)
Glassy carbon paste	Chemical deposition on glassy carbon particles	Stirred batch amperometry	−50	0.25	0.25–500	188	High pH stability	Ricci et al. (2003c)
Graphite screen printed electrode	Chemical deposition on graphite screen printed electrode	Stirred batch amperometry	−50	0.1	0.1–50	234	High pH stability	Ricci et al. (2003a)
Platinum screen printed electrode	Galvanostatic	FIA	−50	5.0	–	1000	–	de Mattos et al. (2003)
Gold screen printed electrodes	Galvanostatic	FIA	−50	–	–	2000	Highest sensitivity ever found for H_2O_2	de Mattos et al. (2003)
Nanoelectrode array	Galvanostatic	FIA	+50	0.01	0.01–10^4	60	Lowest detection limit for H_2O_2	Karyakin et al. (2004)

Sensitivities values recognised by * have been calculated by the authors from the experimental data found in literature. Applied potential is intended vs. Ag/AgCl except when specified.

et al., 2003). In the case of nanoelectrode arrays on an inert glassy carbon electrode (Karyakin et al., 2004), the LOD achieved was 10^{-8} M with an extended linearity up to 10^{-2} M. To our knowledge, this represents the lowest detection limit for H_2O_2 and the greatest linear range ever found for PB-based sensors.

Screen printed electrodes have played an important role in the field of sensors in recent years (Collier et al., 1998; Rippeth et al., 1997; White et al., 1996). In particular, they have found a large use in the construction of hexacyanoferrate (Wang and Zhang, 1999) and especially PB-based H_2O_2 probes. An important advantage of screen printed electrodes is that they are inexpensive, simple to prepare, versatile and suitable for the mass-production of disposable electrodes (Albareda-Silvert et al., 2000; Hart et al., 2004).

An early advance came when Guilbault and co-workers (O'Halloran et al., 2001; Pravda et al., 2002) showed the possibility of including PB microparticles in the ink used for the printing of the working electrode of a SPE. The LOD achieved for these SPEs was 0.4 μM with a linear range up to 100 μM. In 2003, our group proposed the use of a simple and reproducible chemical deposition for the modification of a SPE with PB (Ricci et al., 2003a). The electrodes showed excellent analytical parameters for H_2O_2 detection (LOD = 0.1 μM, linear range up to 50 μM) with an increased stability at alkaline pH.

As noted, a primary advantage of Prussian Blue is its very high electronic transfer rate which makes the electrodes modified with such a mediator very rapid and versatile for many electrochemical methods. It is for this reason that Prussian Blue modified electrodes have been used with almost all the amperometric techniques and procedures: FIA (Karyakin et al., 1996, 2004; Karyakin and Karyakina, 1999; de Mattos et al., 2003), continuous flow systems (Ricci et al., in press) such as batch mode (Ricci et al., 2003a; Chi and Dong, 1995; Turner and Jaffari, 1995; O'Halloran et al., 2001; Moscone et al., 2001) and chronoamperometry (Lupu et al., 2004). These various approaches have always provided very high analytical performances with respect to hydrogen peroxide detection.

Despite the advantages reported, initially all the PB modified sensors were affected by a marked instability at alkaline pH which limited their use to neutral or acidic solutions.

3.2. pH stability of Prussian Blue

Typical experiments to evaluate the stability of PB have included the use of cyclic voltammetry and were based on calculation of the decrease of the peak currents of PB after several cycles under differing conditions. Neff and Itaya always claimed a high stability of the Prussian Blue (10^5 cycles at 100 mV/s (Itaya et al., 1982a)) layer, but it was in 1992 that the negative effect of slightly alkaline pH on PB stability was reported (Stilwell et al., 1992). While for a pH range between 2 and 3, cycle lifetimes in excess of 10^4 were achieved, long-term film cycling stability in neutral solution was never established. The PB layer was reported to be disrupted after a few scans at neutral pH and a very low stability was observed with alkaline pH. The reason for this behaviour is probably to be ascribed to the strong interaction between ferric ions and hydroxyl ions (OH^-) which forms $Fe(OH)_3$ at pH higher than 6.4 (Feldman and Murray, 1987), thus leading to the destruction of the Fe–CN–Fe bond, hence solubilising PB (Karyakin and Karyakina, 1999). Its leakage from the electrode surface results in a decreased signal. For many years, this unstability has represented the main drawback to the use of Prussian Blue modified electrodes, especially when they are coupled with an enzyme having its optimum pH in the basic range (Garjonyte and Malinauskas, 1999a; Malinauskas et al., 2004).

The pH stability seems however to be dependent also on the different modes of deposition of the PB layer. As already pointed out, the structure of Prussian Blue is still a matter of investigation, and it is likely that the structure itself is directly related to the procedure used to form the PB layer. If the pH unstability is only due to the reaction between OH^- ions and ferric ions, it is possible that a very slight difference in the three-dimensional structure of PB could lead to a different availability of the ferric ions for reaction with OH^-, thus accounting for a different effect of alkaline pH on the stability.

An increased stability of the Prussian Blue at alkaline pH was observed by our group after adopting a chemical deposition method for the modification of the electrode surface with Prussian Blue (Moscone et al., 2001; Ricci et al., 2003a). The greatly increased stability of PB layer made possible the practical application of these sensors even at alkaline pH (no loss of signal for H_2O_2 after 50 h at pH 7.4 (Ricci et al., 2003a)) and with the coupling to an oxidase enzyme (choline oxidase) having an optimum pH of 8.0. Chemical deposition has been shown (Moscone et al., 2001; Ricci et al., 2003a,b) to be a useful alternative method to the most used electrochemical approach for PB surface modification which was commonly used since Itaya works and optimised by Karyakin. It is based on the spontaneous reaction between ferric chloride and potassium ferricyanide and resulted in a very stable PB layer. It has to be noted that the increased operational stability at alkaline pH is still not well understood. As already hypothesized, this effect is likely related to the structure of deposited PB which is, as previously reported (Itaya et al., 1986), highly dependent on the procedure adopted for PB deposition. It could be that the chemical procedure allows the deposition of a form of PB with a structure more similar to the "soluble" PB which, as demonstrated by Garcia-Jareno et al. (1996), is more stable than the insoluble one. This hypothesis finds some confirmation from the initial works reported by Neff and Itaya on the deposition of PB. First, Neff (1978) reported the use of a chemical deposition similar to that applied by our group, and proposed that only the "soluble" form of PB was deposited on the electrode surface and was involved in the redox reactions (Ellis et al., 1981). It was in 1982 that Itaya, using an electrochemical

procedure for PB deposition, demonstrated that no potassium ions were found in the PB film and then that only the "insoluble" form of PB was deposited (Itaya et al., 1982a,b,c). On the basis of these observations, Itaya concluded that the electrochemical procedure led to the deposition of the "insoluble" form of PB which has been reported to be less stable.

Karyakin and Garjonyte, who also reported a low pH stability for the PB layer, always adopted an electrochemical procedure based on the Itaya procedure and followed by voltammetry cycles. According to Itaya's study, this would result in the formation of the "insoluble" form of Prussian Blue and would account for the low stability of the PB layer formed. However, this conclusion seems to be contradicted by other studies which have reported that, even in the case of the electrochemical deposition, the cycling of potential in a potassium chloride solution for a few scans (made by Karyakin in the electrochemical procedure) causes the transformation of the PB structure from the "insoluble" form to the "soluble" one (Garcia-Jareno et al., 1996; Mortimer and Rosseinsky, 1983; Roig et al., 1994). All of this points back to the fact that the designation PB is a generic term which denotes a complex material with variable stoichiometry and thus, it is difficult to prove that one has a specific structure. The passage from the "insoluble" form to the "soluble" one which occurs with the repetitive cycling following the electrochemical procedure is known to involve the loss of one-quarter of the high spin iron Fe(III) and the occupancy of the interstitial sites by potassium ions (Mortimer and Rosseinsky, 1983). Although this, it is difficult to believe that cyclic voltammetry in potassium chloride solution would provide the conversion of the PB present in the deeper layers near the electrode surface. Probably, only the most superficial layer, in direct contact with the solution, will be able to be converted to the "soluble" form and the bulk will be represented by the "insoluble" one which is responsible for the limited stability at alkaline pH.

Another hypothesis is that the chemical procedure results in a large excess of deposited PB which acts as a PB reservoir. This would minimise the effect of the leakage due to the hydrolysis of ferric ions at alkaline pH. Although these hypotheses have not been confirmed by any experimental proof, it is clear that the chemical deposition is directly responsible for the stability obtained. It has in fact been demonstrated that the same procedure applied to many other electrode materials (i.e. carbon paste (Moscone et al., 2001); glassy carbon (Ricci et al., 2003e); carbon nanotube (Ricci et al., 2003d)) has always resulted in a high stability of the PB layer at alkaline pH, a result which has not yet been reported elsewhere with other materials or deposition procedures.

Other methods, ranging from the use of protective polymers to the use of additives in the deposition buffer, have also been proposed to increase the operational stability of PB.

In recent years, conducting and non-conducting polymers have attracted attention relative to the biosensors development both as matrix for enzyme and/or mediator entrapment and also for their role in electron transfer and in the protection of the protein and mediator molecules (Gerard et al., 2002; Cosnier, 1999; Lewis et al., 1999). A first report on the possibility of electropolymerisation on top of a PB film appeared in 1994 (Karyakin and Chaplin, 1994), followed by an electrochemical study of the PB layer protected by a polyaniline (PAn) membrane (Nakayama et al., 1997). Following this, Garjonyte and Malinauskas proposed the use of poly(*o*-phenylendiamine) (i.e. poly-1,2-diaminobenzene) for the immobilisation of glucose oxidase (GOx) onto PB modified electrodes (Garjonyte and Malinauskas, 1999b). It was in 2002 that the use of the same non-conducting polymers was reported to improve the performance of a PB layer by providing a better stability and greater sensitivity towards hydrogen peroxide. PB-poly(1-2DAB) showed improved stability in continuous flow conditions (1 mL/min) for more than 20 h (pH 6.0) and exhibited a detection limit for H_2O_2 comparable to that obtained without the use of polymers (Lukachova et al., 2002, 2003). Also, the use of poly(vinylpyrrolidone) was reported to protect PB nanoparticles and offers an enhanced solubility in organic solvents (Uemura and Kitagawa, 2003). Electropolymerisation of *o*-aminophenol (Pan et al., 2004b) and poly[4,4′-bis(butylsulphanyl)-2,2′-bithiophene] (Lupu et al., 2002) on a PB modified platinum electrode resulted in an enhanced reproducibility and stability of the sensor produced.

In addition, an ionic conductor such as Nafion has provided a powerful tool for improvement in performance of PB-based biosensors. The presence of Nafion not only allows the transport of counterions, necessary for electroneutrality, from the solution to the inner PB film (Garcia-Jareno et al., 1996), but also provides a higher stability of the PB film (Chi and Dong, 1995; Karyakin et al., 1995, 1996).

Some additives have also been proposed to improve the stability of PB film. Lin and Shih (1999) showed that tetrabutylammonium toluene 4-sulfonate (TTS) present in the working solution helped stabilise hexacyanoferrate modified electrodes. After this study, it was demonstrated that TTS used both in the carrier stream of a FIA experiment or in the buffer used for the electrodeposition of PB markedly improved the stability of the PB layer (de Mattos et al., 2003; Haghighi et al., 2004).

3.3. PB deposition procedures

In Table 2 are shown all the most commonly used procedures adopted for PB deposition on various electrode surfaces.

Almost all the procedures adopted for PB deposition are based on an electrochemical step which employs a constant applied potential (galvanostatic) in a solution of ferricyanide and ferric chloride (Karyakin et al., 1996, 1998, 2000; Garjonyte and Malinauskas, 1999a, 2000a,b) or potential cycling in the same solutions (Garjonyte and Malinauskas, 1999a). The galvanostatic strategy is usually followed by (1) a series of cyclic voltammetry (15–25) which enables a sort of activation of the PB layer and (2) by a heating step (100 °C for 1–1.5 h) for the stabilisation of the same layer.

Table 2
Most commonly used methods adopted for Prussian Blue deposition

Deposition method	Brief description	Electrodes used	Reference
Electrochemical galvanostatic	Applied constant potential for a fixed period in $K_3Fe(CN)_6 + Fe(Cl)_3$ solution (for Karyakin followed by cyclic voltammetry in buffer and 1 h at 100 °C)	Pt, Au, graphite-based electrodes, Al, SnO_2, TiO_2	Karyakin et al. (1995, 1996, 1998), Itaya et al. (1982a,b, 1984a,b, 1999)
Electrochemical cycling 1	Cyclic voltammetry in a $K_3Fe(CN)_6 + Fe(Cl)_3$ solution	Pt, basal pyrolitic graphite	Chi and Dong (1995), Garjonyte and Malinauskas (1999a)
Electrochemical cycling 2	Cyclic voltammetry in a $K_3Fe(CN)_6$ solution	Carbon rods	Turner and Jaffari (1995), Jaffari and Turner (1997)
Chemical 1	Spontaneous reaction between $K_3Fe(CN)_6 + Fe(Cl)_3$ in presence of KCl and HCl	Pt, Au, graphite-based electrodes, Al, SnO_2, TiO_2	Neff (1978)
Chemical 2	Spontaneous reaction between $K_3Fe(CN)_6 + Fe(Cl)_3$ in presence of KCl and HCl; followed by 1.5 h at 100 °C	Graphite-based electrodes	Moscone et al. (2001), Ricci et al. (2003a,b,c)

See references given for further details.

The chemical deposition procedure has already been discussed in the previous section. This procedure, which does not involve long electrochemical steps, can also be followed by a 100 °C heating for 1.5 h in order to stabilise the PB layer formed. It should be noted that among the other procedures (mostly electrochemical), a chemical procedure based on the simple chemical reaction between ferric chloride and ferricyanide seems to be much easier to perform and more promising for a mass production of PB modified sensors (for example, with SPE).

Another approach, which is quite different from the others and has been also patented, was adopted by Jaffari and Turner and consists of a simple voltammetric cycling of a potassium hexacyanoferrate solution (Jaffari and Turner, 1997; Jaffari and Pickup, 1996) in order to form a PB layer on the surface of a graphite disk electrode. In this case, the authors claimed a good stability for the "PB analogue" layer (20 h of continuous function at pH 7.4) which, to our knowledge, is one of the best ever achieved, relative to those reported and obtained with the chemical deposition. This sensor was reported to detect H_2O_2 at +450 mV versus SCE by measuring the oxidation current instead of the usual H_2O_2 reduction measured with other PB-based sensors.

Various applications involving the use of PB microparticles has also been proposed. Guilbault and co-workers reported the inclusion of microparticles in the ink of the working electrode of a SPE. In 2003, a new procedure for the preparation of ultrathin layers of Prussian Blue nanoclusters created in a ferricyanide solution at negative potentials was proposed. PB formed as a consequence of the dissociation of ferricyanide which, being very slow, could be easily controlled and led to a compact and defect-free ultrathin PB layer (Zhang et al., 2003). Finally, the fabrication of highly ordered Prussian Blue nanowire arrays has been reported by use of an electrodeposition procedure employing anodic aluminium oxide films (Zhou et al., 2002).

Independently of the nature of the electrode material and the procedure of deposition used and despite the problem related to low stability at alkaline pH, it has been clear from the beginning that PB had great potential as a solution for the main problem relating to the electrochemical detection of H_2O_2 and for the construction of sensitive and interference-free biosensors based on oxidase enzymes.

4. Prussian Blue-based biosensors

4.1. General aspects

The histogram shown in Fig. 2 represents the trend in papers published concerning the use of PB-based biosensors. As can be seen, there has been a steady increase in the application of PB in the biosensor field starting from the report that it could be used to obtain a sensitive and selective probe for H_2O_2 detection (Karyakin et al., 1994). In Table 3, we summarise the most important examples of PB-based biosensors. Over and beyond the large use of GOx, a series of other oxidases enzymes have also been exploited. The list includes: lactate oxidase (Chi and Dong, 1995; Garjonyte et al., 2001; Ricci et al., 2003c; Garjonyte and Malinauskas, 2003), cholesterol oxidase (Li et al., 2003; Vidal et al., 2004), amino-acid oxidase (Chi and Dong, 1995), ethanol oxidase (Karyakin et al., 1996), glutamate oxidase (Wang et al., 2003; Karyakin and Karyakina, 1999), lysine oxidase (Ricci et al., 2003b) and oxalate oxidase (Fiorito and Cordoba de Torresi, 2004). More than 10 different enzymes have been coupled with PB, and in all cases, good performance in terms of detection limit and interference level was obtained. The biosensors were suitable for different amperometric procedures. Flow injection analysis, batch stirred amperometry, steady-state amperometry were all successfully used for the detection of the enzymatic substrate. The use of bi- and tri-enzymatic system for acetylcholine (Ricci et al., 2003a; Ivanov et al., 2003) and sucrose (Haghighi et al., 2004) has also been reported.

Due to its high stability and the importance of its natural substrate, glucose oxidase has always represented the model enzyme to test a particular kind of sensor, and evaluated the possibility of its use in the biosensor field. Also, in the case of PB modification, this was the route chosen and GOx was thus the first enzyme immobilised on the surface of a PB modi-

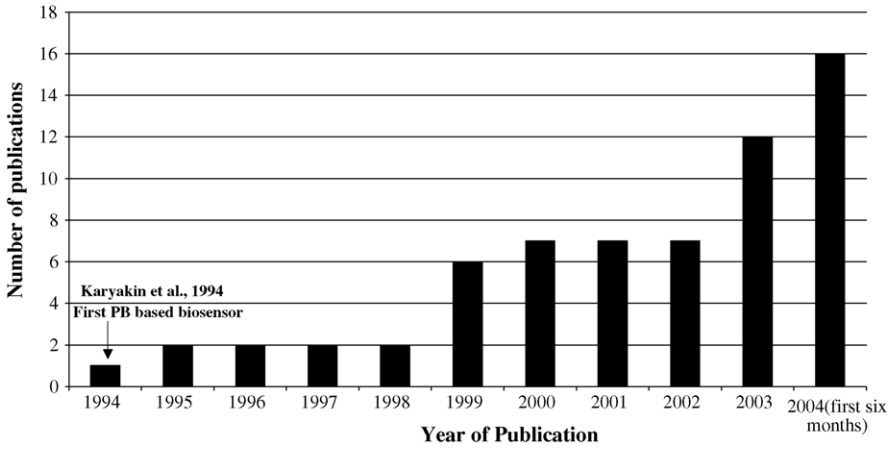


Fig. 2. Histogram plot of the papers concerning Prussian Blue-based biosensors (updated till June 2004).

fied carbon electrode (Jaffari and Turner, 1994; Turner and Jaffari, 1995; Karyakin et al., 1994, 1995). It was also routinely reported when a new procedure or a different electrode material was used to perform H_2O_2 measurement. It has also to be considered that the use of GOx has been probably encouraged by the fact that this enzyme has a wide pH range of activity and thus could easily be used at neutral pH values which do not affect PB stability.

4.2. Glucose biosensors

It is well known that the first example of a glucose biosensor based on the use of a PB modified electrode was reported by Karyakin et al. (1994) who also made a successive optimisation in 1995 (Karyakin et al., 1995). The biosensor was claimed to have a low detection limit (10^{-6} M) and a wide linear range (up to 5 mM). In the same year (Chi and Dong, 1995), new examples of biosensors based on PB appeared in literature. The enzyme was in this case co-immobilised with the mediator. High analytical performances were observed (LOD 2×10^{-6} M, linear range 10^{-5} to 10^{-3} M) and no signal was found for 2×10^{-3} M of ascorbic acid and uric acid which were tested as standard interferents.

In 1996 and 1997 (Jaffari and Pickup, 1996; Jaffari and Turner, 1997), there were two articles describing the analytical performances of an amperometric biosensor for the determination of blood glucose using a PB modified graphite electrode which had first appeared in a UK patent application by Jaffari and Turner (1994) that then extended to an international patent (Turner and Jaffari, 1995). The biosensor was reported to give a detection limit of 5×10^{-6} M and a linear range up to 4.5×10^{-3} M. The same biosensors also showed a high stability and no interferent signals from ascorbic acid, uric acid or 4-acetamidophenol (Jaffari and Pickup, 1996; Jaffari and Turner, 1997). When tested with plasma, it showed a good stability although with a lower sensitivity (Jaffari and Pickup, 1996).

Glucose biosensors based on PB have been successfully applied to blood and serum samples. In one case (Deng et al., 1998), the serum samples obtained from healthy and diabetic persons were diluted 1/50 in potassium phosphate buffer and a good agreement was found with results obtained with reference method. In a paper published in 2003 (Wang et al., 2003), Chitosan/Prussian Blue-based biosensors are applied to glucose detection in whole blood samples. Ten microliters of sample was dropped onto the probe's sensitive area and the output values obtained at an applied potential of 50 mV versus Ag/AgCl were compared with those obtained with a spectrophotometric method. Results from 100 samples were in excellent agreement, with a correlation coefficient of 0.9903 (Fig. 3). This trial demonstrates the suitability of the mediator in avoiding interference. This was the first case in which a glucose biosensor based on PB has been used with whole blood samples without any dilution step. The good results, which are also attributable to the use of a double-functional Chitosan membrane which immobilised the enzyme and prevented the penetration of interferents, are highly encouraging for the possible future application of such a mediator in clinical applications.

The possibility of measuring glucose in a whole blood sample is in fact very important for diabetes treatment and PB-based glucose biosensors seem to be suitable for the assembly of finger stick sensors. To this final objective are pointed the majority of the attempts to modify screen printed electrodes with a PB layer.

During recent years, the screen printing (thick film) technology applied to sensor and biosensor construction has been considerably improved and a large number of papers and recently some reviews have appeared in the literature (Hart and Wring, 1997; Albareda-Silvert et al., 2000; Hart et al., 2004). Screen printed electrodes are in fact inexpensive, simple to prepare, rapid and versatile and this technology also appears to be the most economical means for large-scale production and for the assembling of spot test for clinical and environmental analysis.

The first report in this context appeared in 1999 and detailed the mixing of a PB analogue (cupric hexacyanoferrate) together with GOD into the carbon ink used to print the work-

Table 3
Prussian Blue-based biosensors

Analyte	Enzyme	Procedure of measurement	Working pH	LOD (μM)	Linear range (μM)	Sensitivity (mA/(M cm^2))	Analyte/H_2O_2 (%)	Real sample	Comments	Reference
Glucose	GOx	Rotating disk electrode amperometry	6.0	1.0	1.0–10^3	–	–	–	First biosensor based on PB	Karyakin et al. (1994)
		Rotating disk electrode amperometry	6.0	1.0	1.0–10^3	180	18	–	–	Karyakin et al. (1995)
		Stirred batch amperometry	6.4	2.0	10–3 10^3	0.3*	0.5	–	No interferences for 2 mM of ascorbic acid and uric acid Low stability at pH 7.4	Chi and Dong (1995)
		FIA	5.5	2.0	2.0–5 × 10^3	20	8	–	–	Karyakin et al. (1996)
		Steady-state amperometry	7.4	–	?–1.5 × 10^3	0.05	7	–	High stability No interference for ascorbic acid, uric acid, 4-acetamidophenol	Turner and Jaffari, (1995)
		Steady-state amperometry	7.4	–	0–3.8 × 10^3	130*	7	–	Same procedure as patent (Turner and Jaffari, 1995) No interference for ascorbic acid, uric acid, 4-acetamidophenol	Jaffari and Pickup (1996)
		Steady-state amperometry	6.5	5.0	5.0–4.5 × 10^3	1.14	–	Serum samples	No interference for 1/50 dilution	Deng et al. (1998)
		Steady-state amperometry	6.4	100	100–2 × 10^4	0.23*	–	–	nL samples used No interference from ascorbate	Zhang et al. (1999)
		Stirred batch amperometry	7.4	220	220–3 × 10^3	3.21	2	–	Good stability at pH 7.4	O'Halloran et al. (2001)
		FIA	5.5	2.5	2.5–5.0 × 10^3	20	5	–	Different forms of GOx tested	de Mattos et al. (2001)
		FIA	5.5	0.5	0.5–2.5 × 10^3	0.36	–	–	10 h stability at pH 5.5	de Mattos et al. (2000a)
		Stirred batch amperometry	6.0	100	100–2 × 10^4	2.3	5	–	High stability at alkaline pH First example of optimum alkaline pH enzyme	Moscone et al. (2001)
		Steady-state amperometry	6.5	60	100–6 × 10^3	3.1	–	–	Planar platinum microelectrode used	Zhu et al. (2002)
		FIA	6.0	0.1	0.1–100	50	–	–	Best glucose detection limit	Karyakin et al. (2002)
		Steady-state amperometry	6.86	30	100–10^4	3.1*	–	Serum sample	100 serum samples gave a good correlation (R.S.D.% = 0.998) with spectrophotometric measurements	Wang et al. (2003)
		Stirred batch amperometry	6.0	5.0	5.0–500	14	10	–	Operational stability > 48 h (pH 6)	Ricci et al. (2003b)
		Stirred batch amperometry	6.0	30	50–5 × 10^3	3.7	3	Beverage samples	Recovery values ca. 95% Dilution factor 1/400	Ricci et al. (2003b)
		Stirred batch amperometry	6	50	50–800	5.2	3	–	–	Ricci et al. (2003c)
		Stirred batch amperometry	7.4	2.0	2.0–100	63.0	26	–	–	Ricci et al. (2003a)
		Stirred batch amperometry	7.4	70	100–5 × 10^4	3.3	6	–	First carbon nanotube PB-based sensor	Ricci et al. (2003d)
		FIA	6.0	10	10–10^3	2.5	–	Urine Serum Beverages Drugs Wines	Dilution for urine 1/2 Dilution for blood 1/20 Good agreement with spectrophotometric methods	Derwinska et al. (2003)
		Stirred batch amperometry	6.5	20	20–4.75 × 10^3	16.0*	–	Serum	Dilution factor 1/50 No interference for ascorbic ac. 0.2 mM Average recovery = 97%	Li et al. (2004)

Table 3 (*Continued*)

Analyte	Enzyme	Procedure of measurement	Working pH	LOD (μM)	Linear range (μM)	Sensitivity (mA/(M cm^2))	Analyte/H_2O_2 (%)	Real sample	Comments	Reference
		Chronoamperometry	7.0	1.0	$1.0–5 \times 10^3$	1.4*	–	Red and white wine	Dilution factor 1/50 Average recovery = 85%	Lupu et al. (2004)
		Stirred batch amperometry	7.0	200	$200–6 \times 10^3$	16.0	–	–	ITO electrode used Enzyme immobilised with LbL films	Ferreira et al. (2004)
		Stirred batch amperometry	7.0	10	$10–5 \times 10^3$*	24	–	–	Use of poly(*o*-aminophenol) film to improve stability	Pan et al. (2004a)
		FIA	6.0	1.0	$1.0–10^3$	60	–	Red and white wine	No matrix effect; dilution factor 1/1000 Good correlation with spectrophotometric method	Ulasova et al. (2003)
		Continuous flow amperometry	7.4	25	$25–10^3$	54	25	Dialysed human serum	High operative stability (50–60 h) No matrix effect with dialysed sample Possible use for glucose continuous monitoring	Ricci et al. (in press)
Acetylcholine (pesticide detection)	ChOx + AchE	Stirred batch amperometry	7.8	4.0	5.0–100	7.0	–	Red and white grape juices	Minimal matrix effect No dilution required	Ivanov et al. (2003)
Acetylcholine	ChOx + AchE	Stirred batch amperometry	8.0	1.0	5.0–100	103.0	44	–	–	Ricci et al. (2003a)
Cholesterol	ChoOx	Stirred batch amperometry	6.8	1.0	1.0–80	–	–	Serum	Recovery values ca. 97% Serum diluted 1/10	Li et al. (2003)
			–	8.0	–	8.5	–	–	–	Vidal et al. (2004)
Choline	ChOx	Stirred batch amperometry	8.0	20	$20–2 \times 10^3$	3.5	8	–	High stability at alkaline pH First example of optimum alkaline pH enzyme	Moscone et al. (2001)
		Stirred batch amperometry	8.0	0.5	0.5–100	110.0	47	–	–	Ricci et al. (2003a)
Ethanol	AlOx	FIA	7.5	100	$100–10^4$	1.0*	0.4	–	–	Karyakin et al. (1996)
Galactose	GaOx	Steady-state amperometry	6.86	60	$100–10^4$	1.6*	–	Serum sample	100 serum samples gave a good correlation (R.S.D.% = 0.998) with spectrophotometric measurements	Wang et al. (2003)
Glutamate	GlOx	Steady-state amperometry	6.86	60	$100–10^4$	2.0*	–	–	–	Wang et al. (2003)
		FIA	6.0	0.1	0.1–100	100*	17	–	–	Karyakin and Karyakina (1999)
Lactate	LOx	Stirred batch amperometry	6.4	30	$70–1.4 \times 10^4$	0.3 *	0.5	–	No interferences for 2 mM of ascorbic acid and uric acid Low stability at pH 7.4	Chi and Dong (1995)
		FIA	5.5	1.0	1.0–800	28*	–	–	–	Garjonyte et al. (2001)
		Stirred batch amperometry	7.0	5.0	5.0–60	33.0	17	–	–	Ricci et al. (2003c)
Lysine	LyOx	Stirred batch amperometry	8.0	5.0	6.0–700	52	38	–	Good stability at pH 8.0	Ricci et al. (2003b)
		Stirred batch amperometry	8.0	2.5	2.5–50	68.9	36	–	–	Ricci et al. (2003c)
Oxalate	OxaOx	Stirred batch amperometry	3.8	50	$80–4.5 \times 10^2$	131.3	–	Serum	High operational stability (100 measurements) 2% interference for ascorbic acid (1 mM)	Fiorito and Cordoba de Torresi (2004)
Sucrose	GOx + Mut + Inv	FIA	6.5	4.5	4.0–800	–	–	–	Increased operational stability with TTS	Haghighi et al. (2004)
Xanthine	XOx	Stirred batch amperometry	7.4	1.0	1.0–20	32*	–	–	Gold used as working electrode	Liu et al. (2004)

All the biosensors listed have been obtained with the enzyme immobilised on the electrode surface. Sensitivities values recognised by * have been calculated by the authors from the experimental data found in literature. Analyte/H_2O_2 (%) represents the ratio of the sensitivity for glucose and the one for hydrogen peroxide, and has been calculated by the authors where possible. *Abbreviations*: GOx, glucose oxidase; ChOx, choline oxidase; ChoOx, cholesterol oxidase; AlOx, alcohol oxidase; GaOx, galactose oxidase; GlOx, glutamate oxidase; LOx, lactate oxidase; LyOx, lysine oxidase; OxaOx, oxalate oxidase; XOx, xanthine oxidase; AchE, acetylcholinesterase; Mut, mutarotase; Inv, invertase.

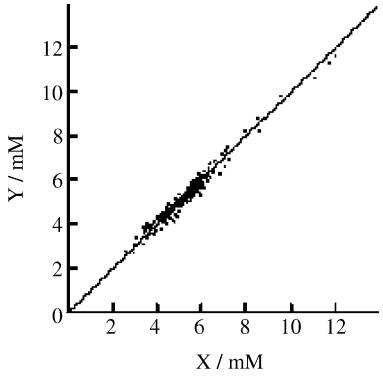


Fig. 3. Correlation and regression line for glucose measurements of 100 human blood samples assayed by spectrophotometry against the glucose sensors based on Prussian Blue. *X*, data assayed by the sensor; *Y*, data assayed by spectrophotometry ($R^2 = 0.9903$). Permission from Wang et al. (2003). Copyright (2003) Institute of Physics Publishing.

ing electrode. The resulting glucose biosensor showed good selectivity with regard to the common electrochemical interferences, but at physiological pH the signal decreased by 10% after 2 h. In 2001, Guilbault and co-workers (O'Halloran et al., 2001; Pravda et al., 2002) reported on a PB sensor obtained with the bulk modification of the carbon ink by PB microparticles. The PB–SPEs showed low detection limits (0.4 μmol L^{-1}) and a sensitivity of 137 mA mM cm^{-2} for H_2O_2 and, when modified as a glucose biosensor, a linear range up to 3 mM of glucose was observed with no interference. However, pH stability experiments, performed with the PB modified SPEs, revealed again a decrease of the H_2O_2 amperometric signal (50% of the initial activity after 4 h of continuous use) at pH values above 7. In 2003, our group reported a chemical deposition of a PB layer onto the surface of a SPE (Ricci et al., 2003a) which created a very stable (even at basic pH) sensor which could be coupled with glucose and choline oxidase. The resulting biosensors were very satisfactory in terms of sensitivity (LOD: 2×10^{-6} M for glucose, 5×10^{-7} M for choline), storage and operational stability.

Biosensors based on PB modified gold and platinum SPEs were also demonstrated by de Mattos et al. (2003). Both the sensors were first modified via an electrochemical deposition with PB and then GOx was immobilised by use of a Nafion membrane. The biosensors were reported to have good stability in a FIA system and the signal due to glucose remained constant for 10 h of continuous injections of a 1 mM glucose standard solution (injection frequency 1 min^{-1}, pH 5.5).

Recently, a paper by our group reported on the assembly of a screen printed glucose biosensor with excellent operative and storage stability (Ricci et al., in press). These probes, obtained by depositing PB on the electrode surface via chemical deposition and immobilising GOx with a cross-linking method, were tested over 50–60 h in a continuous flow mode (10 μL/min). A 0.5 mM concentration of glucose was continuously flowed into a biosensor wall-jet cell and the current due to the hydrogen peroxide reduction was continuously monitored. After 50–60 h, the drift of the signal observed was around 30%. This high stability suggests the possibility of using such biosensors in conjunction with a microdialysis probe for a continuous monitoring of glucose for clinical purposes. This was, to our knowledge, the first attempt at constructing a planar mediator (PB)-based biosensor for continuous glucose monitoring coupled with a microdialysis probe and a portable instrument. Preliminary results obtained with dialysed samples of human serum gave encouraging results demonstrating a low matrix effect and no drift of the signal for more than 30 h. Clinical tests are now in progress to evaluate the possibility of using such biosensors for continuous monitoring in patients.

Also, with other approaches, the use of PB for blood serum glucose testing was always found particularly advantageous (Derwinska et al., 2003; Li et al., 2004). In the first case, measurement of glucose under flow-injection analysis conditions was performed in various real clinical samples such as urine and blood serum that had been 2 and 20 times, respectively, diluted before determinations. The comparison with reference methods also gave very good agreement in this case and demonstrated the practical applicability of the biosensor based on PB for the determination of glucose in blood and urine samples (Derwinska et al., 2003). In the second example (Li et al., 2004), a new approach which involved the use of PB modified glassy carbon electrodes with a silica sol–gel outer layer was proposed. In this paper, the attempt to develop a biosensor combining the merits of the sol–gel technique for enzyme immobilisation and PB modified electrode was proposed. Serum sample assayed with these glucose biosensors (dilution factor 1/50) showed good agreement with spectrophotometric reference methods. The biosensor exhibited reasonable selectivity and produced satisfactory results with an average recovery of 101% and a R.S.D. of 5.9%.

All these examples, as already noted, illustrate the wide number of clinical applications of Prussian Blue-based glucose biosensors, ranging from the simple disposable spot test up to the continuous glucose monitoring.

The use of glucose oxidase-based PB biosensors was also reported in an immunoassay system. The polyclonal antibody used in the sandwich assay was labelled with glucose oxidase and its concentration, which was directly proportional to the concentration of the antigen (i.e. α-fetoprotein), was ultimately measured by the use of PB modified screen printed electrodes (Guan et al., 2004).

Glucose measurement with PB-based biosensors was also tested in other real samples ranging from wines (Lupu et al., 2004; Ulasova et al., 2003; Derwinska et al., 2003) to beverages (Ricci et al., 2003b) or glucose containing drugs (Derwinska et al., 2003). In particular, with red and white wines, it was demonstrated that with a minimum dilution of 1/20, any interferences from phenols or oxidable compounds, which always affect amperometric measurement in these matrices,

are eliminated and high recovery values could be achieved (Derwinska et al., 2003; Lupu et al., 2004).

4.3. Other biosensors

Many other oxidase enzymes have been coupled to PB modified electrodes and tested with different real matrices. Serum samples, for example, were analysed for cholesterol, oxalate and galactose using PB-based biosensors. In the case of cholesterol (Li et al., 2003), the biosensor was obtained with the use of a sol–gel matrix membrane to immobilise the enzyme. The detection limit for cholesterol was about 10^{-7} M and most of the interfering compounds were reported to not affect the determination. Dissociated cholesterol was also determined by an amperometric method in serum samples. A dilution of 1/10 and a heating step were required in order to solubilise the analyte in aqueous solution. The results showed recovery values in the range of 96.5–104% with a R.S.D. of ca. 2% ($n = 5$).

Similar results were found with the same analyte in synthetic and control blood serum samples (Vidal et al., 2004). In this case, instead of a sol–gel matrix, a polypyrrole layer electropolymerised onto the PB modified Pt electrode was used to immobilise the enzyme cholesterol oxidase. A Detection limit of 8 μM was found with a maximum sensitivity of 8.5 mA/(M cm^2). In addition, a considerable improvement of the PB stability was obtained through the addition of a layer of Nafion on the elctrode surface.

Galactose biosensors were also used for 100 whole blood samples (Wang et al., 2003) and even in this case, an excellent correlation with the spectrophotometric reference method was found. Recently, the use of a multilayer-organic oxalate biosensor has also been presented (Fiorito and Cordoba de Torresi, 2004). The multilayer is obtained by covering the PB film deposited on a glassy carbon electrode with a self-doped polyalinine (SPAN) layer. Oxalate oxidase was then immobilised with a cross-linking agent and a limit of detection of ca. 0.08 mM with a linear range up to 0.45 mM was observed. The SPAN layer was reported to act as protective barrier for the PB film so that high operative stability was achieved, enabling more than 100 determinations without degradation or loss of activity.

A choline biosensor was first reported by Moscone et al. (2001) and represents the first example of a PB-based biosensor operating at alkaline pH (pH 8) without loss of activity. Graphite powder was modified with PB chemically synthesised ‘in situ’. After the addition of mineral oil and choline oxidase, a highly active and very stable PB modified carbon paste biosensor was produced.

This work opened up new possibilities for the application of PB to enzymes having pH optimum in the basic range. Lysine (pH 8) was measured with glassy carbon paste electrodes (Ricci et al., 2003c) and also choline (pH 8) together with acetylcholinesterase (pH 8) was immobilised on PB modified screen printed electrodes as a valuable tool for pesticide measurement (Ricci et al., 2003a; Ivanov et al., 2003).

4.4. Bi- and tri-enzyme biosensors

Bi- and also tri-enzyme systems based on the use pf PB have also been reported in recent years. Of particular importance is the use of choline oxidase and cholinesterase for the final detection of pesticide (Ricci et al., 2003a; Ivanov et al., 2003). In the latter case, the two enzymes were co-immobilised via a glutaraldehyde cross-linking method on PB modified SPEs and the resulting biosensor was tested for detection of anticholinesterase targeted pesticides in spiked grape juices. Carbofuran and chloropyrifos-methyl, chosen as standard pesticides, were detected at concentrations as low as 10^{-6} and 10^{-5} g/L, respectively, and a minimal matrix effect was observed. In this case, no dilution of the sample is performed and so both the results of sensitivity and interference effect were extremely encouraging and indicated sufficient reliability for pesticide detection in real samples.

The only tri-enzyme system based on the use of PB modified electrodes was reported by the Gorton group (Haghighi et al., 2004). The co-immobilisation of three enzymes, invertase, mutarotase and glucose oxidase, was performed to obtain a sucrose sensor. Analytical performances of the biosensor (LOD for sucrose 4 μM, linear range 4–800 μM) were significantly better than most of the previously reported sucrose sensors based on different mediators. Also, an increase in the operational and storage stability of the PB layer was noted after a conditioning of the electrodes in a buffer containing 0.05 M TTS during the preparation of the PB films.

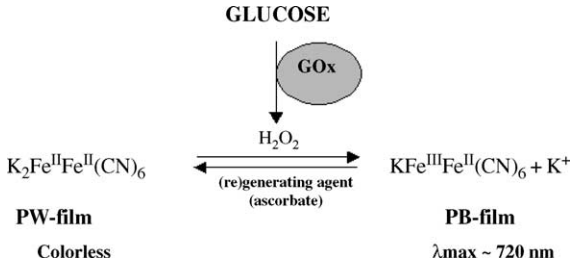
5. Other uses of Prussian Blue

5.1. Optical biosensors based on Prussian Blue

A different application of PB film was proposed and adopted for the first time by Koncki and Wolfbeis (1998b) who exploited the possibility of using PB as an optical transducer. It has already been noted that the changes in the oxidation state of PB are accompanied by a colour change which is also denoted by the common names given to the compounds (i.e. Prussian Blue and Prussian White). The detection of hydrogen peroxide (but also other oxidants) is performed by relating the change of the PB film absorbance to the concentration of the analyte. Also, pH changes could be measured with a PB film following the reversible hydrolysis of PB that occurs with increasing pH (Eq. (4)) (Koncki and Wolfbeis, 1998a; Guo et al., 1999).

$$Fe_4^{III}[Fe^{II}(CN)_6]_3 + 3H_2O \rightleftarrows Fe^{III}(OH)_3 + 3Fe^{III}Fe^{II}(CN)_6^- + 3H^+ \quad (4)$$

The Prussian Blue film was deposited via a non-electrochemical method onto non-conducting materials such as plastic and glass. Biosensors were based on a final measurement of hydrogen peroxide (Koncki et al., 2000, 2001) (as product of oxidase enzymes) or pH changes (Koncki and



Scheme 2. Scheme of optical system adopted for glucose detection. The enzyme (GOx) is deposited onto a PW film (colourless) which is easily oxidised in presence of hydrogen peroxide to PB giving a strong change in absorbance (λ_{max} = 720 nm). Reproduced by permission of the Royal Society of Chemistry (Koncki et al., 2001).

Wolfbeis, 1998a, 1999; Radomska et al., 2001). When urease was used as biosensing element, the hydrolysis of urea increases the pH of the solution (in the surroundings of the sensor), thus leading to a decrease in the absorbance of the pH-sensitive film (Koncki et al., 2001; Lenarczuk et al., 2001a). Urea concentration was then correlated with the different absorption values obtained.

Another glucose biosensor was obtained with the immobilisation of GOx onto a PW layer, a colourless film which could be easily oxidised by the hydrogen peroxide generated by the enzyme, to give a change in absorbance at 720 nm (Scheme 2). The biosensor gave a linear response in the range between 0.1 and 1.0 mM of glucose, which is much lower than that reported for electrochemical methods but still useful in the case of some real samples. When tested with fruit juices, red and white wines, the results were in a good agreement with reference method (Koncki et al., 2001). The same optical method was applied in a FIA system giving better sensitivity (LOD 0.05 mM for glucose) and a very good correlation with reference methods when tested with urine and serum samples from healthy and diabetic patients (Lenarczuk et al., 2001b).

5.2. Prussian Blue as mediator for other substances

Prussian Blue's use in analytical chemistry applications has not been limited to the assembling of oxidase-based biosensors. Recently, some articles have appeared in the literature reporting how PB acts as an electrocatalytic mediator for the reduction or oxidation of compounds other than hydrogen peroxide. The first attempts in this direction were reported by Ogura et al. (1994) who proposed the use of PB modified polyaniline electrodes to detect CO_2. Also, the use of Prussian Blue coupled with a conducting polymer (polypyrrole) was reported to have a catalytic action towards the oxidation of the redox protein cytochrome C and this system was applied in FIA systems (Lu et al., 1998). In 2001, Prussian White, the reduced form of Prussian Blue, was reported to act as electrocatalyst towards NO reduction at potentials lower than −0.4 V (Pan et al., 2001). Recently, Prussian Blue modified ITO electrodes were also demonstrated to catalyse the oxidation of morphine; when applied for morphine detection in amperometric mode, a linear range between 0.09 and 1.0 mM together with a sensitivity of 16.8 mA/(M cm^2) was found (Ho et al., 2004). Prussian Blue has also been applied as a mediator for NADH amperometric detection in the assembling of a formate biosensor (Zhao et al., 2002).

One of the most interesting alternative uses of PB was first reported by Hou and Wang (1991) showing the catalytic activity of PB towards the oxidation of some thiols such as cysteine, *N*-acetylcysteine and glutathione. The same catalytic action was then applied by Wilkins et al. (2000) for the detection of thiocholine and it was subsequently used as a means for pesticide detection (thiocholine is the product of acetylcholinesterase when acetylthiocholine is used as substrate). An extensive study in this area has been recently carried out by our group trying to elucidate the mechanism of catalysis which is still not clear (Ricci et al., 2004). Many thiocompounds were investigated and their differing reactivity was discussed. Thiocholine and cysteamine were found to give the best results in terms of LOD (i.e. 5×10^{-6} and 10^{-6} M respectively) and linear range (5×10^{-6} to 5×10^{-4} and 10^{-6} to 10^{-4} M, respectively) at an applied potential of 200 mV. Two applications were also proposed, one involving the measurement of thiocholine as a mean for pesticide detection and the other being an electrochemical alternative to Ellman's test for total thiol estimation (Ricci et al., 2004).

6. Conclusions and future perspective in the use of Prussian Blue

This review gives a clear picture of the increase in research interest concerning Prussian Blue in recent years. The increasing use of PB and the optimisation of modification procedures as well as elucidation of its action will provide a powerful stimulus to researchers who work in the biosensor field and who are interested in H_2O_2 amperometric detection. This review has dealt with general aspects and physical and chemical properties of Prussian Blue and its application in the field of hydrogen peroxide sensor and electrochemical biosensors have been covered. Prussian Blue applications, different from the classic hydrogen peroxide amperometric measurement, have been also reported.

Some limitations on the use of Prussian Blue such as the low stability in particular conditions of basic pH have been fully investigated and reviewed bringing to the conclusion that these drawbacks could be overcome by adopting the right modification procedure and electrode material.

From this review, it can be concluded that the use of Prussian Blue brings to outstanding hydrogen peroxide sensors with selectivity, sensitivity and stability never reported for other H_2O_2 mediator-based probes.

The future use of PB will be then very promising for the assembly of oxidase-based biosensors which could have a practical application in clinical, food and environmental analysis.

In particular, the ease of some modification procedures coupled with low cost electrodes will be suitable for the construction of probes to be used "in situ" or by non-skilled personnel. Also, the overcoming of some major drawback achieved in some of the cited papers will be extremely important for the increase in the use of Prussian Blue modified electrode and for its future use in commercial biosensors development.

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