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REVIEW

Biosensing applications of clay-modified electrodes: a review

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Abstract Two-dimensional layered inorganic solids, such as cationic clays and layered double hydroxides (LDHs), also defined as anionic clays, have open structures which are favourable for interactions with enzymes and which intercalate redox mediators. This review aims to show the interest in clays and LDHs as suitable host matrices likely to immobilize enzymes onto electrode surfaces for biosensing applications. It is meant to provide an overview of the various types of electrochemical biosensors that have been developed with these 2D layered materials, along with significant advances over the last several years. The different biosensor configurations and their specific transduction procedures are discussed.

Keywords Clays · Layered double hydroxides · Enzymes · Modified electrodes · Biosensors

Introduction

Biosensors are the subject of extensive research for the development of wide fields of application including medical diagnostics, environmental monitoring, and food analysis [1, 2]. They are analytical devices involving a biological recognition component (enzyme, antibody, or nucleic acid) in intimate contact with a transducer surface that converts the biological signal into a quantifiable electrical signal. Owing their simplicity of use, portability, and low cost, biosensors based on an electrochemical transduction constitute the main category; several amperometric biosensors are utilized commercially, for detection of glucose, for instance. Two aspects which are the most problematic in biosensor development are the immobilization of biomolecules in a suitable matrix and diffusional limitation of the analyte within this matrix. Consequently, research on new immobilization matrices for biomolecules has attracted the attention of the chemist community.

The use of inorganic host matrices is an alternative to organic polymers that are widely used for immobilizing biomolecules [3–6]. Compared to polymers, some inorganic matrices have many advantages, for example high chemical inertness and biocompatibility, a tuneable crystal lattice with adjustable crystal size and open pore structure, chemical and physical stability, and low production costs. Moreover, the inorganic partner in biohybrid materials is not only seen as a protective matrix for the preservation or increase of the bioactivity or lengthening of the lifetime of the biological material—it may contribute to novel multifunctionalities by a synergic effect at the nanoscale level of its own specific properties (electronic conductivity, magnetism, redox or acid-base properties, etc...) combined to the bioactive feature of the biomolecules. Amorphous silica matrices generated by a mild sol–gel procedure have been extensively used for fabrication of biosensors, as illustrated by the recent publication of several review articles [7–10]. Moreover, nano and macro-structuring of electrode surfaces with this type of material for applications in electroanalysis has recently been reported [11, 12].

On the other hand in the last few years, because of either fundamental scientific interest or wide and promising applications, research activity on biohybrids based on 2D layered materials has become a new field for research and technological developments, particularly in the domain of biosensors. This review aims to describe all important

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aspects and new developments of electrochemical biosensors based on clays and layered double hydroxides (LDHs).

Definitions of cationic and anionic clays

The stable immobilization of an enzyme on an electrode surface, with complete retention of its biological activity and good diffusional properties for substrates, is a crucial problem for biosensor development. Solids with a 2D structural arrangement have an open structure favourable for intercalation a large variety of organic molecules, macromolecules, and biomolecules. Therefore, clay minerals and related layered structures can be advantageously exploited to improve the analytical characteristics of biosensors. They are essentially based on three smectite clays (laponite (Lap), montmorillonite (Mont), and nontronite (Nont)), and on layered double hydroxides (LDHs) (Fig. 1). Only a few papers have reported biosensors based on layered Zr phosphate [13–18].

Smectite clays are 2:1 phyllosilicates with a layer structure consisting of an octahedral sheet sandwiched between two tetrahedral siliceous sheets. The octahedral sheet is predominantly occupied by trivalent cations (dioctahedral smectites: i.e. Mont, Nont, Lap) or divalent cations (trioctahedral smectites: i.e. saponite). In tetrahedral sheets the dominant cation is Si^{4+}; in octahedral sheets the cations are usually Al^{3+} or Mg^{2+}. Other cations such as iron can occupy either tetrahedral or octahedral sites. Isomorphic substitutions within octahedral and/or tetrahedral sheets lead to negative charges of the layers compensated by interlayer cations. Properties such as layer charge, charge location, nature of the interlayer cations affect smectite hydration ability and swelling behaviour [19]. Cationic clays can serve as matrices for electroactive ions because they are usually able to incorporate these ions by an ion-exchange process. The resulting clay-modified electrodes (CME) were be used for their electrocatalytic properties or analytical applications [20]. Moreover, adsorption of proteins on clay mineral surfaces plays a very important role in fields related to the agricultural and environmental sciences, but also in the development of biosensors [21].

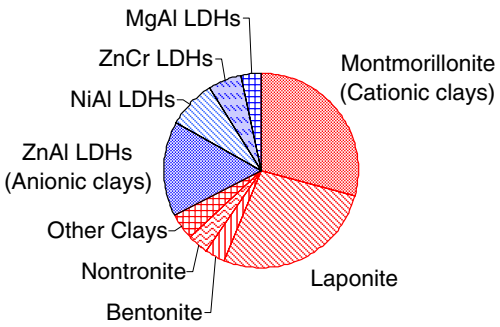

Fig. 1 Clay types used for biosensor development, on the basis of the literature published between 1990 and 2009. *Red parts* correspond to cationic clays and *blue parts* to the anionic clays (LDHs)

Layered double hydroxides (LDHs), also called anionic clays, display unique physical and chemical properties surprisingly close to the properties of cationic clays. LDHs have a layered structure built on stacking of positive layers $[MI^{II}_{1-x}M^{III}(OH)_2]^{x+}$, separated by interlamellar domains constituted of anions and water molecules $[X_{x/q}.nH_2O]^{x-}$. The increasing interest in LDHs as host matrix arises from their versatile properties in terms of chemical composition of both layers and interlayers, their high and tuneable layer charge density, and their anion-exchange capacity. For example, organic electroactive molecules bearing an anionic group such as anthraquinone mono and disulfonate (AQS) [22], 2,2′-azinobis 3-ethylbenzothiazoline-6-sulfonate (ABTS) [23], ferrocene (Fc) derivatives [24, 25], and nitroxide [26] can be intercalated into the interlayer domain of LDHs and remain electroactive. Moreover, these intrinsic LDHs properties make them very favourable for confinement of negatively charged biomolecules such as amino acids, DNA, nucleoside monophosphates, and enzymes with isoelectric points varying over a large pH range [27, 28].

Enzyme immobilization strategies

The use of a cationic clay (laponite) as a matrix for immobilization of enzymes improved the analytical characteristics and the long-term stability of biosensors compared with the corresponding biosensors simply obtained by chemical cross linking of glucose oxidase (GOx) or polyphenol oxidase (PPO, tyrosinase) on the electrode surface [29, 30]. Similarly, incorporation of laponite particles within an electrogenerated polypyrrole matrix strongly improved the analytical performance of amperometric biosensors based on PPO and cholesterol oxidase (CO) [31, 32]. Figure 2 illustrates this by comparison of catechol calibration curves obtained in chronoamperometry at –0.2 V/Ag-AgCl with four PPO biosensors. These biosensors were based on immobilization of PPO within different matrices: an electrogenerated polypyrrole ammonium film, a laponite-polypyrrole ammonium composite, the pure laponite, and a ZnAl–LDHs, coated on glassy carbon electrodes. The same comparison was made with GOx biosensors [33]. In all cases, the 2D inorganic matrices (laponite or LDHs) gave the best analytical characteristics.

The entrapment of biomolecules in clays constitutes an inexpensive, fast, and easy method for preparation of such bioelectrodes. The procedure consists in the deposition of an aliquot of a colloid enzyme/clay aqueous mixture on to the electrode surface and subsequent evaporation of water.

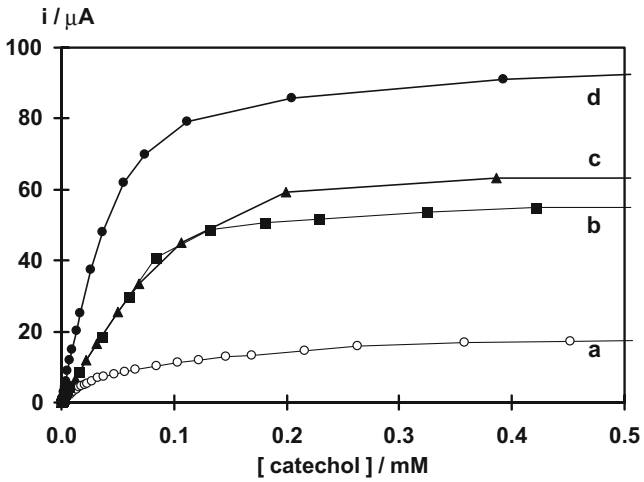

Fig. 2 Comparison of catechol calibration plots obtained in chronoamperometry at –0.2 V/Ag-AgCl at glassy carbon electrodes modified with (**a**) PPO/polypyrrole ammonium (80 μg, 30 nmol), (**b**) PPO/polypyrrole ammonium + laponite (40 μg, 30 nmol + 40 μg), (**c**) PPO/laponite (25:25 μg), (**d**) PPO/ZnAl-LDHs (25:25 μg)

However, the resulting biosensor may suffer from poor lifetime because of enzyme leaching in the electrolyte solution. To overcome this problem, enzyme cross linking agents, for example glutaraldehyde (GA) [34], bovine serum albumin (BSA) + GA [35], poly(methyl methacrylate) (PMMA), or poly(*o*-phenylenediamine) (PPD) [36], have been added to the enzyme/clay coating films. This method of deposition offers the possibility of entrapping a large and precisely known amount of enzyme into the clay film and can be adapted for classical electrodes (Pt, glassy carbon electrode (GC), and ITO transparent electrodes) but also for smaller devices such as interdigitated microelectrodes [37] or pH-sensitive field-effect transistors (pH-FETs) [38].

A clay sandwich method has also been used to immobilize GOx [39, 40] and hydrogenase (Hase) [41, 42]. Coche-Guérente et al. adopted a polycationic organosilasesquioxane laponite (Lap-PS) organo-clay for their biosensor development [43–45]. Good knowledge of the structure of the enzyme–octamer–laponite material enables them to propose an enzymatic kinetic model for the PPO amplification process. More recently, Mbouguen et al. have also immobilized GOx and PPO using organoclay prepared by grafting either γ-aminopropyltriethylsilane (APTS) or *N*-trimethoxysilyl propyl-*N*,*N*,*N*-trimethylammonium (TMPA) [46, 47]. Covalent bonding of GOx with smectite-APTS was performed using GA as coupling agent. PPO was entrapped by electrostatic interactions in smectite-TMPA.

Cosnier's group developed several biosensors based on laponite-electrogenerated polymer composites. These biosensor configurations combine the mechanical stability of the inorganic matrix with a possible electrocatalytic effect of redox polymers, i.e. poly(pyrrole–viologen), poly(azure B), and polymethylene blue (PMB) [48–50].

Enzymes were immobilized within LDHs mainly by the adsorption method using GA as stabilizing agent. Urease has been also trapped within a ZnAl-LDHs matrix by delamination–restacking or coprecipation methods [51, 52] (Fig. 3). The first stepwise immobilization process consists in the preparation of a colloidal solution of LDHs intercalated with a surfactant (ZnAl-dodecylsulfate) in organic medium. This delaminated LDHs phase was mixed with an aqueous solution of urease, resulting in restacking of the LDHs layers with entrapment of the enzyme (Fig. 3a) [51]. On the other hand, LDHs can be prepared under very mild conditions by the coprecipitation method using aqueous metal salt solution at various pH and at low temperature [53]. The immobilization of enzymes can also be realized in one step by inclusion of the biomolecule in the precipitation medium. For instance, different ZnAl-urease LDHs were obtained from a mixed aqueous solution of $ZnCl_2$ and $AlCl_3$ with various Zn/Al molar ratios (R=2, 3, 4) which was introduced at a constant flow into a reactor containing urease aqueous solution (Fig. 3b). The pH was maintained constant at 8.0 during the coprecipitation process by addition NaOH solution. After aging for 24 h, Enz–LDHs biohybrids were recovered by centrifugation [52].

The strong association between the enzyme and the LDHs layers obtained under these conditions makes the use of GA unnecessary. Similarly, GA can be avoided by using chitosan–laponite or chitosan–ZnAl-LDHs composites to immobilize enzymes [54–57].

The "soft chemistry" coprecipitation route, described above for the preparation of Enz–LDHs biohybrids, is a very tuneable process for preservation of the enzyme. Indeed, this method enables choice of variable conditions (pH, temperature, reagent nature, and concentration) avoiding structural change of the enzyme and denaturation of its catalytic activity. The efficient immobilization of alkaline phosphatase (AlP) in MgAl-LDHs was also realized by this coprecipitation method [58, 59]. The role of the metal cations of LDHs layers on the enzyme–matrix interactions and consequently on the enzyme stability was shown. It seemed that the basic nature of MgAl-LDHs improved the stability of immobilized AlP with regard to pH variation.

The enzyme grafting method with LDHs pillared by glutamate ions (Glut) proposed by Ren et al. [60] has also been used to immobilize GOx for amperometric biosensor development [27]. The immobilization of GOx was performed by covalent grafting in the interlayer LDHs galleries using Glut-GA-Enz bonds. Finally, a more sophisticated method was developed by Tonelli's group for immobilization of GOx during the electrodeposition of NiAl-LDHs [61–64]. Scanning electrochemical microscopy (SECM) has been used to probe local enzymatic activity and to obtain topographic images of the GOx/NiAl-LDHs-modified elec-

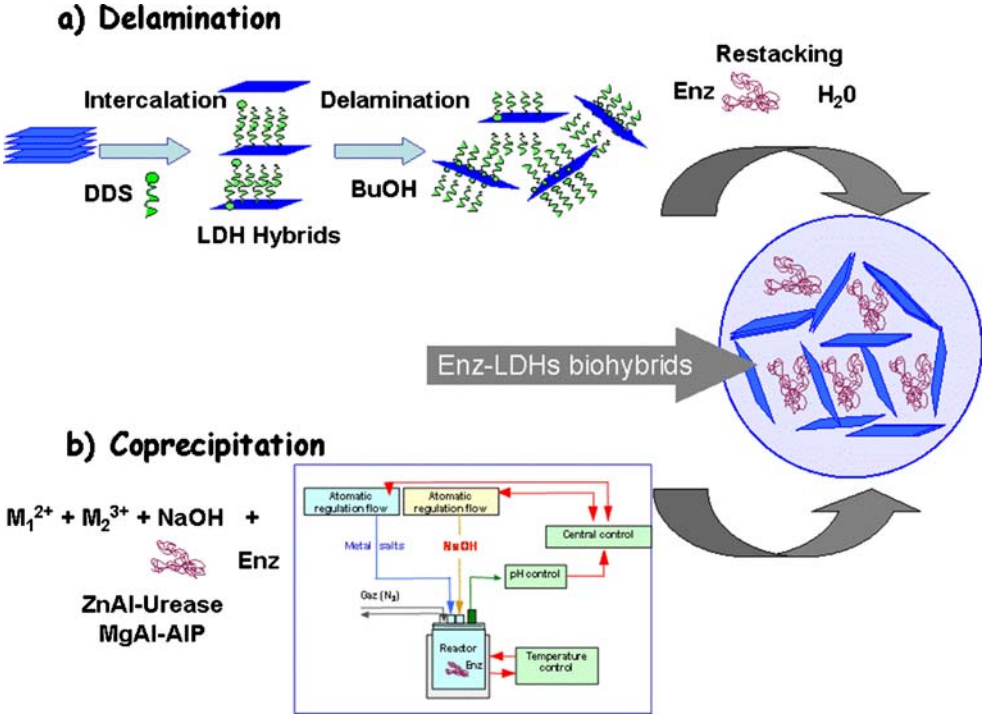

Fig. 3 Preparation procedures of enzyme–LDHs biohybrids: (**a**) delamination–restacking of ZnAl-dodecylsulfate (DDS) in the presence of enzyme, (**b**) schematic diagram of the automatic reactor used for coprecipitation of LDHs in the presence of enzyme

trodes [65]. Unfortunately in both of the last immobilization methods, the amount of entrapped enzymes was considerably reduced compared with the adsorption method (Table 1).

Glucose biosensors

Amperometric detection of glucose dominated both analytical and fundamental studies, mainly because of the physiological importance of this analyte, the stability of glucose oxidase, and the diversity of sensing methods applied [66]. Therefore, glucose oxidase (GOx) was often used as a model enzyme to test different configurations of biosensors. GOx biosensors based on clays and LDHs are summarized in Table 1. Different concepts of transduction processes are reported. The oxidation of glucose is carried out by the flavin adenine dinucleotide (FAD) component of GOx which is converted into $FADH_2$. The latter is re-oxidized to FAD by dioxygen (a cofactor) with the production of hydrogen peroxide (H_2O_2) or under anaerobic conditions by redox mediators.

In the first examples, the transduction step corresponded to electro-oxidation at the underlying Pt electrode of the enzymatically generated hydrogen peroxide following the reactions:

$$\text{Glucose} + O_2 \xrightarrow{\text{GOx}} \text{Gluconolactone} + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{Pt}} O_2 + 2H^+ + 2e^-$$

Because of the high polarizing voltage applied (E_{app} ≈0.6 – 0.8 V), interferents such as ascorbic acid and uric acid, which are commonly present in biological fluids, can also be oxidized, leading to non-specific signals. Several attempts have been made to overcome this problem. Poyard et al. used clay–semipermeable polymer composite electrodes to reduce the permeability to organic interfering compounds [67, 68]. Another possibility consists of reducing the electrode potential using redox mediators. Indeed, hydrogen peroxide can be reduced by redox mediators immobilized within clay-modified electrodes (CME) [69–71]. Such a concept was applied to glucose biosensors based on cationic clays, using cationic methyl viologen (MV^{2+}) [39], ruthenium complexes [36, 72] associated with structural iron cations of the clays, or TiO_2 underlying films [29]. Recently, Colombari et al. have also exploited the mediated oxidation of H_2O_2 by ferrocene anions ($FcSO_3$ and $FcCO_2$) in their GOx/MgAl-LDHs biosensors [73].

Similarly, bienzymatic configurations, composed of GOx and horseradish peroxidase (HRP) associated with redox mediators immobilized at CME clays or LDHs, have also been applied to the detection of glucose at 0.0 V [74, 75]. The resulting bienzymatic configurations seemed to be very sensitive to glucose and the low applied potential prevented any possible oxidation of ascorbate and urate.

Under anaerobic conditions, the redox mediators (i.e. tetrathiofulvalene (TTF), dopamine, or ferrocene derivatives were used as oxidizing agents of $FADH_2$ instead of O_2 [25, 35, 40, 76]. Therefore, the amperometric determination of glucose concentration consists in measurement of the current due to the regeneration of the reduced form of the mediators. These biosensor configurations enabled the detection of glucose at lower potential, with dramatically reduced

Table 1 Comparison of the analytical performance of GOx/Clay biosensors

Clay	GOx (μg cm^{-2} (U mg^{-1}))	CME configuration	Electrode	E_{app} (V)	S (mA mol^{-1}L cm^{-2})	Ref.
Laponite	337 (115)	Enz-GA-clay	Pt/O_2	0.53	82.3	[34]
Laponite	505 (115)	Enz-clay-GA/Nafion	Pt/O_2	0.6	130	[67]
Laponite	– (115)	Enz-clay-PVPS composite	Pt/O_2	0.6	30	[68]
Laponite-PS	114 (115)	Enz-Organoclay	Pt/O_2	0.53	27	[43]
Clay Baba (Cameroon)	–	Grafted Organoclay	Pt/O_2	0.6	–	[46]
Laponite	318 (108)	Enz-Chitosan-clay	Pt/O_2	0.6	33.9	[54]
ZnCr-Cl	204 (218)	Enz-GA-LDHs	Pt/O_2	0.6	19	[75]
ZnAl-Cl	204 (218)	Enz-GA-LDHs	Pt/O_2	0.7	55	[33]
ZnAl-Cl	1400 (108)	Enz-GA-LDHs	Pt/O_2	0.6	35	[130]
ZnAl-Cl	1051 (108)	Enz-Chitosan-LDHs	Pt/O_2	0.6	62.6	[54]
NiAl-NO_3	46 (179)	Electrodep LDHs	Pt/O_2	0.45	6.2	[63]
NiAl-NO_3	75 (179)	Enz-GA-LDHs	Pt/O_2	0.45	6.5	[61]
ZnAl-Glut-GA	0.76 (218)	Grafted LDHs	Pt/O_2	0.6	0.21	[27]
Nontronite	–	Clay-sandwich conf	MV^{2+}-clay/GC/O_2	–0.75	52	[39]
Laponite	198 (115)	Enz-GA-clay	TiO_2/O_2	–0.15	3.33	[29]
Montmorillonite	192 (137)	Enz-BSA-GA-clay	RP-clay/ITO/O_2	–	0.00388	[36]
MgAl-$FcSO_3$	107 (179)	Enz-GA-clay	Fc-LDHs/GC/O_2	0.5	2.1	[73]
MgAl-$FcCO_2$	107 (179)	Enz-GA-clay	Fc-LDHs/GC/O_2	0.4	4.8	[73]
Laponite	428 (115)	Enz-clay-polymer composite	HRP-DHB-clay/GC/O_2	0.0	14.95	[74]
ZnCr-ABTS	102 (218)	Enz-GA-clay	HRP-ABTS-LDHs/GC/O_2	0.0	23	[75]
Montmorillonite	7143(150)	Enz-BSA-GA-clay	TTF-clay/GC/N_2	0.14	10.7	[35]
Nontronite	–	Clay-sandwich configuration	Dopamine-clay/GC/N_2	0.6	60.3	[40]
FcMeOH/ZnAl-Cl	– (108)	Enz-GA-clay	Fc-LDHs/Pt/N_2	0.25	60	[76]
ZnCr-$FcPSO_3$	816 (269)	Enz-GA-clay	Fc-LDHs/GC/N_2	0.5	68	[25]
Laponite	400 (180)	Enz-GA-clay /polymer bilayer	μelectrode/O_2	–	57	[37]
Montmorillonite	192 (137)	Enz-GA-clay	Luminol-clay/ITO/O_2	0.8	–	[77]
Montmorillonite	192 (137)	Enz-GA-clay	$Ru(bpy)_3^{3+}$-clay/ITO/O_2	0.9	–	[78]
Mont K10	– (137)	Enz-GA-Clay	Amplex red-clay/ITO/O_2	–	–	[79]

Abbreviations: ABTS, 2,2′-azinobis 3-ethylbenzothiazoline-6-sulfonate; BSA, bovine serum albumin; Enz, enzyme; Fc, ferrocene derivative; GA, glutaraldehyde; GC, glassy carbon electrode; GOx, glucose oxidase; Glut, glutamate; ITO, indium tin oxide; Lap-PS, organosilasesquioxane pillared laponite; Mont, montmorillonite; Pt, platinum electrode; PVPS, poly(4-vinylpyridine-co-styrene); RP, ruthenium purple ($Fe_4[Ru(CN)_6]_3$)

interference from easily oxidisable interferents. However other biosensors, working under aerobic conditions, generally have better storage stability.

The immobilization of GOx on microelectrode arrays and their use for successful amperometric and conductimetric detection of glucose illustrates well the potential of enzyme/clay coating for the fabrication of conductimetric microbiosensors [37]. In addition to the previously described amperometric biosensors, it should be noted that peculiar GOx/clay electrodes were constructed for detection of glucose by means of electrochemiluminescence [77, 78]. Finally, a glucose sensor was fabricated on the basis of a Mont K10, amplex, GOx bioelectrode. The iron species contained in Mont K10 promotes the generation of superoxide anion radical from H_2O_2 that can be characterized by a fluorescence assay using amplex red [79].

Results summarized in Table 1 show that first or second (mediated) generations of GOx biosensors based on CME have excellent analytical characteristics compared with other glucose biosensors based on inorganic matrices such as sol–gel [54, 76] or polymers [33] A further challenge would be improvement of direct electron transport between the FAD redox centre of the enzyme and the electrode surface, by means of adequate adsorption of the biomolecule on the clay platelets, to obtain a third-generation GOx biosensor [66].

Phenol biosensors

PPO catalyses the metabolic conversion of monophenols and diphenols to *o*-quinone. In particular, catechol is reported to

be the best substrate for this enzyme. Enzymatically generated *o*-quinones can be detected via their electrochemical reduction at –0.2 V. Different configurations of PPO biosensors based on CME have been reported, giving rise to very sensitive (subnanomolar) determination of phenol derivatives (Table 2). In particular, rutin, a flavonoid, has been detected at a PPO/laponite electrode [80].

A major drawback inherent to these PPO biosensors is, generally, their limited lifetime because of electrode fouling induced by polymerization of unstable *o*-quinone derivatives, products of the enzymatic reaction. Azure B, polyazure B, or polymethylene blue (PMB), immobilized within laponite, acted as redox mediators and enabled the mediated reduction of *o*-quinone preventing any electrode fouling [30, 49, 81]. Consequently, the operational stability of the PPO/laponite electrodes was greatly enhanced. In the case of the PPO-TMPA-clay film, the best performance was also obtained with a three-layer configuration made of an underlayer of ferrocene, as a redox mediator, which was covered with the PPO-clay layer and finally overcoated with an enzyme-free TMPA-clay to prevent enzyme leaching in the electrolyte solution [47].

With sensitivities for catechol ranging between 2800 and 7800 mA mol^{-1} L cm^{-2} and detection limits in the nanomolar range (Table 2), these PPO-CME have performance much better than those achieved, for instance, with inorganic materials (ZnO, silica, alumina, or $CaCO_3$) [82–85] or conductive polymers [86, 87] (LoD$\geq 10^{-8}$mol L^{-1}).

Moreover, a comparative study of the properties of PPO biosensors based on immobilization of the enzyme within the cationic clay laponite and an anionic LDHs has shown that the PPO/ZnAl-LDHs biosensor had remarkable properties such as a high sensitivity for catechol (7800 mA mol^{-1} L cm^{-2}), very low detection limit (1.7 nmol L^{-1}) and good storage stability [88]. The same feature was observed when using a composite PPO/chitosan-LDHs electrode instead of PPO/chitosan-laponite films [56, 57]. The attractive analytical characteristics of LDHs-based biosensors were certainly because of the high permeability and the structure and charge of the LDHs particles which are highly compatible with PPO.

Biosensors based on heme proteins and enzymes

Enzymatic detection of hydrogen peroxide has been reported either at mediated horseradish peroxidase (HRP) CME or by direct enzyme regeneration at CME (third-generation biosensors). In the former case, the redox mediators, methylene green (MG), poly-3,4-dihydroxybenzaldehyde (DHB), or ABTS were co-immobilized within the clay matrices and played the role of electron shuttle between the redox centre of HRP and the electrode [35, 74, 75]. In particular, synthetic redox active ZnCr-ABTS LDHs seemed to be an efficient immobilization matrix for HRP wiring. This biosensor attained a better detection limit (10 nmol L^{-1}) for H_2O_2 compared with those obtained with other HRP biosensors based on clays or composite sol–gel matrices [75].

During the last ten years, increasing attention has been paid to the study of the direct electron transfer between proteins and electrodes [89]. Clay-modified electrodes have been found to facilitate the heterogeneous electron transfer process between heme proteins and the underlying electrode surface. It should be noted that these proteins usually have an isoelectric point around 7.0. The cationic clays may hold, by electrostatic interactions, the proteins in a "productive" orientation, facilitating a fast heterogeneous redox reaction with the electrode. Proteins in clay films generally retained their native structure without modification of their heme Fe(III)/(II) electroactive group.

Table 2 Characteristics of PPO biosensors

Analyte	Mediator redox	Clay	CME configuration	LoD (mol L^{-1})	Ref.
Phenol	–	Laponite	Enz-clay-polypyrrole composite	–	[31]
Catechol	–	Lap-PS	Enz-clay	5×10^{-10}	[44]
Phenol	–	Lap-PS	Enz-clay	5×10^{-10}	[45]
Catechol	–	Clay Baba (Cameroon)	Enz-TMPA-clay	1×10^{-7}	[46]
Catechol	Ferrocene	Clay Baba (Cameroon)	Enz-TMPA-clay	9×10^{-9}	[47]
Phenols	Azure B	Laponite	Enz-clay-GA	$1–17\times10^{-9}$	[30]
Phenols	Polyazure B	Laponite	Enz-clay-GA/polymer bilayer	$0.2–4\times10^{-9}$	[49]
Catechol	Polymethylene blue	Laponite	Enz-clay	5×10^{-7}	[81]
Phenols	–	Laponite ZnAl-LDHs	Enz-clay-GA	7×10^{-10}	[88]
Phenols	–	Laponite	Enz-Chitosan-clay	$5–22\times10^{-9}$	[57]
Phenols	–	ZnAl-LDHs	Enz-Chitosan-clay	$4–7\times10^{-10}$	[56]
Rutin	–	Laponite	Enz-GA-clay	1×10^{-6}	[80]

Abbreviations: La-PS, organosilasesquioxane pillared laponite; TMPA, *N*-trimethoxysilyl propyl-*N*,*N*,*N*-trimethylammonium

Scheller's group was the first to report on the direct electron behaviour of small metalloproteins (cytochrome c) at Mont-CME [90]. In 2000, Sallez et al. reported a detailed study on interactions between c-type cytochromes and clays [91]. Different types of clays or analogous synthetic minerals were used: montmorillonite, kaolinite, and goethite according to the global charge density of their layers. They confirm the role of electrostatic and hydrophobic interactions in the electrochemical promotion of c-type cytochrome systems. In the case of strong adsorption of these proteins on montmorillonite, a denaturation occurs and, consequently, the metal activity is lost. This study was completed with coupled quartz crystal microbalance and voltammetry measurements to characterize the process of incorporation of cytochrome C_3 in kaolinite and montmorillonite films [92] and to follow the building up layer by layer (LBL) of $\{C_3/Mont\}_n$ assemblies [93]. Recently, electron transfer of cytochrome c was also reported at attalpugite (a fibrous clay) CME [94].

Between 1999 and 2002, Rusling's group reported on the physical characterisation of interactions between hemoglobin (Hb), myoglobin (Mb), or horseradish peroxidase (HRP) and bentonite or hybrid surfactant-bentonite [95–97]. The electrocatalytic behaviour of the resulting heme proteins CME was also studied. Mb-CME and HRP-CME were also prepared with Mont by spontaneous adsorption [98], by LBL assemblies [99], or by entrapment in a composite Mont–Chitosan–Gold nanoparticle coating [99] or ionic liquid–clay [100]. Very recently, direct electron transfer of HRP was also reported at NiAl-LDHs [101].

Electron transfer of cytochromes P450, another large family of enzymes containing a heme group, was recently reviewed [102], in particular direct electron transfer of cytochromes P450 (CYP101 and CYP2B4) was also reported at Mont CME [103, 104].

Studies of direct electron transfer of Hb at CME are most often reported in the literature. Different types of Hb-CME were prepared by using: pure Mont [105, 106], bentonite [96, 97], or attapulgite [107], or with composite clay coatings, for example didodecyldimethylammonium-bentonite, polystyrenesulfonate–Mont [108], poly(vinyl alcohol)–Mont [109], chitosan–laponite [110], Pt colloids–bentonite [105], chitosan–Au–Mont [111], ionic liquid–bentonite [112], and ZnAl–dodecylsulfate LDHs [113]. In all cases, Hb displayed a well characterized quasi-reversible cyclic voltammogram, governed by a surface process with a formal potential $-0.30 \leq E^{\circ\prime} \leq -0.36$ V.

The possible role of structural iron in clays in promoting direct electron transfer of Hb was also investigated [71, 114]. Clays containing different amounts of iron situated in octahedral or tetrahedral sites have been used to immobilize Hb on glassy carbon electrodes. The electrostatic interactions between positively charged Hb and negatively charged layers of the cationic clays can be modulated by the nature and the charge density of the layers. Moreover, iron-rich clays (i.e. nontronite) can significantly enhance direct electron transfer between the heme protein and the underlying electrode, structural iron atoms playing the role of electron relay.

These CME allowing direct electron transfer of heme proteins or enzymes (Mb, Hb, cytochrome c, cytochrome P450 and HRP) have been applied as third-generation biosensors for the determination of H_2O_2, nitrite, O_2, NO, and trichloroacetic acid (TCA) [90, 95–97, 105–110, 112, 113, 115].

On the other hand, Hb also plays a major role in hemoglobinopathies and in anaemias, and analytical methods for its detection have been developed based on spectrophotometric measurements or on electrochemical methods at modified electrodes. Scheller et al. reported for the first time use of a clay-modified electrode for determination of Hb in solution [116]. We have also determined Hb concentration in solution at Nont-CME [114].

Amperometric biosensors based on other enzymes

Several other enzymes have been used for the development of biosensors, in particular using laponite or LDHs as immobilization matrices. These clays were chosen as immobilization matrices to enhance biosensor sensitivity and stability and/or to immobilize different redox mediators.

The incorporation of laponite nanoparticles within the cholesterol oxidase (CO)–amphiphilic polypyrrole film enhanced the sensitivity and the stability of the biosensor [32]. The same feature was observed for the bienzyme electrode based on flavin reductase (Fre) and lactate dehydrogenase (LaDH) immobilized in the same laponite–polypyrrole ammonium composite [117]. The observed improvement of sensitivity and stability can be ascribed to the high hydrophilic properties of the laponite that can counterbalance the hydrophobic character of the organic polymer.

A biosensor for phosphate detection has been fabricated by co-immobilization in laponite of three enzymes with complementary activities: maltose phosphorylase (MP), mutarotase (MR), and GOx; the amperometric detection corresponded to H_2O_2 oxidation at a Pt electrode [118]. In 2000, Hu et al. developed a hypoxanthine sensor based on xanthine oxidase (XO) immobilized within a polyaniline film [119]. Detection is based on oxygen consumed by the enzymatic reaction measured at a Mont-MV^{2+} carbon paste-modified electrode. Recently, Shan et al. reported a new xanthine biosensor based on immobilization of XO in LDHs; amperometric detection was performed at a Pt electrode [120]. In the former case the detection limit (LoD) of xanthine was 0.8 μmol L^{-1} and in the latter case the LoD was 0.1 μmol

L^{-1}. Furthermore, a novel third-generation biosensor based on immobilization of XO in laponite was also applied to the detection of xanthine (LoD=1×10^{-8}mol L^{-1}) [121]. Interestingly, the direct electron transfer observed at –0.370 V corresponded to the XO-FAD/$FADH_2$ redox process.

Cosnier and Le Lous presented lactate and ethanol biosensor configurations based on entrapment of LaDH and alcohol dehydrogenase (AlDH) within laponite gel containing an electropolymerised mediator [48]. The electrocatalytic properties of polymethylene blue (PMB) was used to regenerate the enzyme co-factor (nicotinamide adenine dinucleotide, NAD^+) at 0.0 V. When diaphorase was present in the clay film, the rate of oxidation of NADH was increased 10^2–10^4 times [122]. This may be explained by the possible role of PMB in the electron transfer between this enzyme and the electrode.

A hydrogen biosensor was developed by Qian et al. in which hydrogenase (Hase) was immobilized between two layers of Mont–polybutylviologen (PBV) [41, 42]. The immobilized PBV efficiently enhanced the electron transfer between the electrode, Hase, and the MV^{2+} in solution. Similarly, the electropolymerised pyrrole–viologen derivatives in laponite successfully carried out the electrical wiring of the entrapped nitrate reductase (NaR) [50].

LDHs are very favourable host structures for immobilization of enzymes with isoelectric points varying over a large pH range. Behind previously cited GOx, PPO and HRP biosensors, LDHs have also been used as immobilization matrix for urease [51, 52], acid or alkaline phosphatase (AcP and AlP) [58, 59, 123], laccase (Lac) [124], and nitrite reductase (NiR) [125]. In both last cases, redox mediators (ABTS or AQS) played the role of electron shuttles between the electrode and the enzymes, improving the electrochemical transduction step and achieving detection of dissolved oxygen in a dynamic concentration range of 6×10^{-8}–4×10^{-6}mol L^{-1} at the Lac/ZnCr-ABTS CME and a limit of detection (LoD) for nitrite of 4 μmol L^{-1} at NiR/ZnCr-AQS CME.

Inhibition biosensors

A large percentage of environmental pollutants are known to act as enzyme inhibitors, resulting in the development of sensors based on measurement of this property. Several years ago, no work was described in the literature using clay entrapped enzymes for inhibition biosensors applications. We have developed this concept, for the first time, in particular for cyanide determination at PPO and HRP biosensors based on immobilization in an LDHs matrix [126, 127]. It should be noted that the PPO/ZnAl-LDHs biosensor is more sensitive to cyanide traces (0.1 nmol L^{-1}) than conventional procedures based on spectroscopic or chromatographic methods. Enzyme immobilization in this anionic exchanging material seems to cause an increase in cyanide inhibition effects because of anion accumulation in the LDHs matrix.

An original amperometric biosensor based on the simultaneous entrapment of AcP and PPO into LDHs was developed for the specific detection of arsenate(V). The functioning principle of the bienzyme electrode consisted in successive hydrolysis of phenyl phosphate into phenol by AcP then oxidation of phenol into *o*-quinone by PPO. The detection of arsenate(V) was based on its inhibitory effect on AcP activity in the hydrolysis of phenyl phosphate to phenol, the detection limit being 2 nmol L^{-1} or 0.15 ppb [123].

A reagentless inhibitor biosensor was developed using Lac/ZnCr-ABTS CME [124]. The detection principle of inhibitors was based on their modulation effect on the enzyme activity which was measured by the mediated reduction of O_2. Such devices provided very low detection limits for azide (5.5 nmol L^{-1}), fluoride (6.9 nmol L^{-1}), and cyanide (6.2 nmol L^{-1}).

Finally, a new concept of enzyme inhibition-based biosensor was developed with two enzymes (HRP and catalase) separately entrapped into LDHs [128]. It displays a signal increase instead of a decrease in the presence of an inhibitor. Both enzymes catalyzed the decomposition of H_2O_2—HRP its reduction and catalase its breakdown into oxygen and water. Nitrite was selected as a specific inhibitor of catalase. In the presence of H_2O_2, addition of nitrite blocked H_2O_2 consumption by catalase, inducing thus an increase in the amperometric signal of the H_2O_2 reduction at 0 V by the wired HRP. It should be noted that all these analyzed inhibitors (cyanide, arsenate, azide, fluoride, and nitrite) are negatively charged and can be accumulated within the LDHs matrix, which probably enhanced the biosensor sensitivities.

Feasibility of amperometric sucrose and mercury biosensors based on the immobilization of invertase (Inv), GOx, and mutarotase (MR) entrapped in laponite has also been reported [129]. Mercury concentration was measured by inhibiting the invertase activity. Biosensor inhibition by inorganic and organic mercury was evaluated and the study of interference from other metal ions revealed good selectivity towards mercury(II) in the concentration range 1×10^{-8}–1×10^{-6}mol L^{-1}.

Urease biosensors

Urea biosensors have been developed by the immobilization of urease within the two different kinds of clay matrix:

laponite and ZnAl-LDHs. The response of the biomembrane to urea can be recorded by measurement of local pH changes, because of the reaction:

$$NH_2CONH_2 + H^+ + 2H_2O \xrightarrow{\text{urease}} 2NH_4^+ + HCO_3^-$$

The transduction process was performed by conductimetric measurement with interdigitated microelectrodes [37], by potentiometric measurements with pH-FETs [38], or by capacitance measurements on an insulated semiconductor structure [51, 52]. The first examples were developed by adsorption of urease on the laponite or ZnAl-LDHs [37, 38]. Other biosensors were based on urease-LDHs biohybrids obtained either by a stepwise exchange reaction with a ZnAl-DDS delaminated suspension [51] or by the coprecipitation method [52] (Fig. 3). Sensitivity and maximum potential response (V_{max}) increase according the sequence: adsorption < coprecipitation < delamination. Indeed biosensor characteristics depend on the activity of immobilized enzyme related to the biocompatibility of the matrix and to steric hindrance in the coating limiting diffusion of the substrate to the enzyme active site.

Conclusion

The number of publications on biosensors based on clays and LDHs as immobilization matrices is limited in comparison with that reported for entrapment of enzymes in sol–gel made host matrices. However since the last review on the analytical applications of clay-modified electrodes, published in 2004, more than sixty publications have reported applications of CME in biosensor development, showing the interest of the chemist community in this research domain. Moreover in comparison with cationic clays, layered double hydroxides have become increasingly important, with 33% of the research work on this subject. The great success of this type of biosensor lies in their ability to immobilize a large variety of enzymes within an open bidimensional structure favourable to analyte diffusion. The possible co-immobilization of adequate redox mediators in the interlayer domains of the 2D materials enables mediated detection of the substrates or regeneration of the enzyme active site. In conclusion, the results presented in this review confirm that the synthesis of new biohybrid materials based on organoclays and LDHs with specific textural and electrochemical properties and their handling as thin films or as so-called biological inks will certainly open new routes for biosensor development.

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