Abstract: This review presented recent development of novel kinds of inorganic layer-enzyme composite materials and their application to electrochemical biosensors. Typical preparation methods of the composite materials were described in detail. The biosensors constructed by the composite materials were discussed. The inorganic layer supports could protect the enzyme from the extreme media, thus the related biosensors offered great promise for industrial operations under extreme conditions, such as in harsh acidic solution.

Keywords: inorganic layer, enzyme, composite, biosensor

1. Introduction

Enzyme-based biosensors are very interesting because of their applications in various fields, such as clinical diagnoses, the food and medical industries, environment monitoring and bioassays [1-5]. Such devices combine the advantages of the inherent sensitivity and selectivity of enzymes to unique substrates. However, the electric communication between enzyme and the conventional electrode surface is poor due to the deeply buried electroactive center, unfavorable orientation and denaturation of the enzyme. Therefore, immobilization of enzyme is the key to solve the problems for constructing an enzyme-based biosensor. A lot of suitable supports and methods were applied to immobilize the enzymes on the electrode surfaces in order to facilitate the electron transfer between the enzymes and the electrodes, make the enzymes orientated as well as protect the enzymes from the outer media.
The supports are very important for the performance of an immobilized enzyme system. Usually, the commercially available immobilized enzymes made on the organic supports. However, inorganic supports offer many advantages for industrial uses over the organic counterparts due to their physical properties of high mechanical strength and thermal stability, resistance to organic solvents and microbial attacks, easy regeneration, and so forth. The typical inorganic materials with high surface areas were chosen as the supports, such as activated carbon [6], clays [7], porous glass [8] and sol-gel silica [9]. In recent years, porous silica [10], nanotubes [11-13], nanoparticles [13,14] and delaminated zeolites [16] were exploited as the supports. Mesoporous molecular sieves (MMS), in particular, attracted much more attention during these years in these fields [17,18], because of their unidirectional pore topologies and large surface areas. However, uptake of guest species was limited by their minimal channel cross section of the rigid pores, which restricts the application for immobilization of large molecules such as proteins.

Recently, a series of inorganic layered materials were found to be the competitive supports for immobilization of enzymes based on the following advantages. Firstly, they can be readily expanded to accommodate enzymes with varied dimensions because of their flexible interlayer distances [17]. Secondly, these materials may provide a support stable to extreme conditions of chemical, thermal and mechanical stresses, and resistance to microbial attacks. The protective environment of the supports can make the immobilized enzymes more stable than that of the free ones. Thirdly, these supports may be further tailored to obtain optimally biochemical functions or desired physical properties. Besides, the inorganic layered materials may also meet some other requirements for a protein support, such as large surface area, permeability, insolubility, hydrophilic character, and high rigidity. For example, Kumar and co-workers [20-23] reported the immobilization of enzymes by reassembly of the exfoliated $\alpha$-zirconium phosphates with guest molecules under mild conditions (pH = 7.2) in a short reaction time. Results showed that immobilized enzymes retained their activities, which even increased compared with that of the free ones in some cases. Our group has studied several kinds of inorganic layered materials for the immobilization of enzymes, such as layered magadiites [24], titanates [25-27] and niobates [28]. The immobilization of enzymes in the galleries of these layered supports possessed of enhanced activities in organic media or at high temperatures. The typical inorganic layered supports used to entrap enzymes are mainly listed in Table 1.

Among various inorganic layered supports used for enzyme-based biosensors, clays may be the pioneers and have still been attracting great attention [41-46]. The anionic clays of layered double hydroxides (LDH) with negative charge in their layers were also reported to be attractive materials for biosensor construction [35-37]. In addition, other inorganic layered materials, such as niobates [47], titanates [48], and $\alpha$-zirconium phosphate [49] were proved to be efficient supports for biosensor construction recently. These biosensors could be applied under the extreme conditions due to the protection of the inorganic layers. Here, we especially described the preparation methods and their application to biosensors to detect $\text{H}_2\text{O}_2$.  

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Table 1. Enzymes immobilized in different kinds of inorganic layered supports.

| Layered supports         | Proteins                                                                 |Refs.|
|--------------------------|--------------------------------------------------------------------------|--|--|
| α-zirconium phosphate    | protamine, lysozyme, cytochrome c, horseradish peroxidase                |  20-23 |
|                          | myoglobin, lysozyme, hemoglobin, chymotrypsin, glucose oxidase           |    | |
| Magadiite                | hemoglobin, myoglobin                                                    |  24 |
| Titanate                 | hemoglobin, myoglobin                                                    |  25-27 |
| Niobate                  | hemoglobin                                                               |  28 |
| γ-zirconium phosphate    | hemoglobin                                                               |  29 |
| γ–titanium phosphate     | papain, lipase, peroxidase, α-amylase, glucoamylase, glucose oxidase, catalase |  30 |
| Manganese oxide          | hemoglobin, myoglobin                                                    |  31 |
| Phyllosilicates          | hemoglobin, myoglobin, glucose oxidase                                   |  32,33 |
| Layered double hydroxide | penicillin G acylase, urease, laccase, horseradish peroxidase            |  34-37 |
| MnPS₃                    | lysozyme                                                                |  38 |
| Spongy Co₃O₄             | Hemoglobin                                                              |  39 |
| Clay                     | α-chymotrypsin, catalase, polyphenol oxidase, horseradish peroxidase etc. |  40-43 |

2. Preparation methods

In general, the strength of in-plane bonds (often covalent bonds) of the layered materials is much stronger than that between the layers (often electrostatic or van der Waals bonds), and small guest species may reversibly insert into a lamellar structure with maintenance of the structural features of the supports [50]. Usually, it is difficult to directly insert large molecules, such as proteins, into their interlayer regions. One effective method is to pre-intercalate small molecules in the galleries, which can expand the interlayer distance and weaken the interaction between the layers. And then the proteins may be intercalated into the enlarged galleries (Scheme 1A). Another method is to exfoliate the support materials into single layers. The immobilized protein composite materials may be formed by the self-assembly of the single layers and protein molecules (Scheme 1B). Coprecipitation method is also useful in some cases based on the synergetic effect between the hosts and guests.
Scheme 1. Immobilization of proteins in the galleries of inorganic layered supports by (A) pre-intercalation and (B) exfoliation-reassembly methods.

2.1. Pre-intercalation method

Layered polysilicate magadiite is a natural crystalline hydrated sodium silicate (Na$_2$Si$_{14}$O$_{29}$•$n$H$_2$O), which is compatible with organism and can be easily synthesized. Its surface silanol group (Si-OH) may react with many organic compounds to form pre-intercalated precursors, which can then be used as intermediates for proteins immobilization [24]. As illustrated in Scheme 2, Na-magadiite synthesized under hydrothermal condition can react with HCl to obtain H-magadiite through ion exchange. The interlayer distances of Na- and H-magadiite materials were 1.56 nm and 1.16 nm, respectively. Neither Na- nor H-magadiite material was able to be directly intercalated by proteins. In order to expand the interlayer spacing and weaken the interaction between layers, tetrabutylammonium hydroxide (TBAOH) was intercalated in the galleries to form TBA-magadiite and its basal spacing expanded to 2.56 nm. This TBA-magadiite can readily react with hemoglobin (Hb) or myoglobin (Mb) to form Hb- or Mb-magadiite. The expansions of interlayer spacing from 2.56 nm to 6.82 nm and 4.32 nm were resulted from the insertion of Hb (5.3 nm × 5.4 nm × 6.5 nm) and Mb (4.5 nm × 3.5 nm × 2.5 nm) into the galleries of magadiite.
Scheme 2. Syntheses of Mb and Hb intercalated magadiite composite materials: (a) Mb and Hb directly intercalated into Na-magadiite; (b) ion exchange with H⁺; (c) Mb and Hb directly intercalated into H-magadiite; (d) TBA⁺ pre-intercalated into magadiite; and (e) Mb and Hb directly intercalated into TBA-magadiite. [24].

Layered α-zirconium phosphate (abbreviated as α-ZrP) is another kind of layered ion-exchange materials capable of binding various metal ions and organic cations in the interlayer regions [51]. Direct intercalating proteins in the galleries is also difficult because of the high layer charge densities. ε-aminocaproic acid can be inserted into the galleries of α-ZrP by contacting α-ZrP with an aqueous solution of ε-aminocaproic acid for 7 days. The basal spacing expands from 0.66 nm of α-ZrP to 1.52 nm of ε-aminocaproic acid-ZrP composite materials. Because the van der Waals length of ε-aminocaproic acid is about 1 nm, the observed expansion is more consistent with incorporation of a single layer of guest amino acid, oriented off-perpendicular with respect to the phosphate sheets with...
the tilt angle of approximately 59°. Intercalation of lysozyme in α-ZrP can be obtained by reacting ε-aminocaproic acid-ZrP composite material with solution of lysozyme at pH 3 for 17 days. The interlayer expansion due to the presence of lysozyme molecules in α-ZrP can be calculated by subtracting the layer thickness of α-ZrP from the basal spacing of the composite material: 2.55 nm - 0.66 nm = 1.89 nm, which accords with the dimensions of lysozyme, reported as 2.97 nm × 1.8 nm × 1.84 nm.

2.2. Exfoliation-reassembly method

Recent development of soft-chemical techniques, such as exfoliation, has brought about a new class of nanomaterials, e.g. nanosheets. The two-dimensional nanosheets exhibit some unique properties different from those of bulk materials because of their high anisotropy in dimensions. These nanosheets can be used as building blocks to construct functional composite nanomaterials. Lepidocrocite-type titanate H$_{x}$Ti$_{2-x/4}$O$_{4}$H$_{2}$O (x ~ 0.7, □ = vacancy) and perovskite-type niobate HCa$_{2}$Nb$_{3}$O$_{10}$ were delaminated into unilamellar colloidal nanosheets. The colloidal nanosheets are negatively charged and can be reassembled by adding cations. The amino acid side chains of proteins can vary from negatively charged to positively charged characteristic by lowering pH of the media below the isoelectric point. Thus, the positively charged protein molecules may reassemble the negatively charged nanosheets to form layered composite materials [25-28]. When heightening pH of the solution above the isoelectric point, the negatively charged proteins can be released from the layered structure through electrostatic repulsion. Scheme 3 illustrates the immobilization process taking Hb as an example. The layered structure can be intercalated by TBAOH, which leads to delamination of the solid into colloidal nanosheets. Hb solution was added to the colloidal suspension, the pH value had been adjusted to 8.0 before by adding dilute acid. Because the isoelectric point of Hb is at 6.8~7.0, the protein molecules were negatively charged at pH 8.0 and repulsed the negatively charged nanosheets, forming a stable colloidal mixture. Further lowering the pH of the colloidal mixture to 6.5 by adding dilute acid, the protein molecules switched to positive charge and reassembled the nanosheets to form layered composite materials (abbreviated as Hb-Ti$_{2-x/4}$O$_{4}$ and Hb-Ca$_{2}$Nb$_{3}$O$_{10}$). Increasing the concentration of Hb brought about different protein arrangement in the interlayer regions from single layer to bilayer proteins. When heightening the pH of the solution to 8.0 by adding dilute TBAOH solution, Hb can be released from the layered composite material through electrostatic repulsion.

Exfoliation-reassembly is an ideal choice to entrap proteins at the galleries of layered materials. One reason is that the nanosheets can entrap proteins under mild conditions, such as in neutral solution and at room temperature. Another reason is that the exfoliated nanosheets provide large surface areas for protein binding and decrease the diffusion distance for the substrate to access the immobilization enzymes. Furthermore, the exfoliation-reassembly process is reversible via simple pH adjustment.
Scheme 3. Syntheses of protein inorganic layered supports through exfoliation-reassembly method. (a) exfoliation by TBAOH intercalation, (b) tuning the pH of the colloidal nanosheets to 8 by adding dilute acid, (c) mixing the protein solution with the colloidal nanosheets, (d) reassembly by lowering the pH of the solution, and (e) re-exfoliation by adding TBAOH solution.

Another typical support that may immobilize proteins through exfoliation-reassembly is an aminopropyl-functionalized magnesium (organophyllosilicate \([H_2N(CH_2)_3]_8Si_8Mg_6O_{16}(OH)_4\) which can be exfoliated in water by ultrasonication [32,33]. The layered protein-inorganic composites can be formed, when the exfoliated positively charged organo clay particles were directly used to entrap the negatively charged proteins, such as Mb and GOx. When the exfoliated organo clay particles were fractionated by gel chromatography, the small positively charged organo clay oligomers may be obtained, which can wrap the individual protein molecules to form protein-inorganic nanocomposite particles. These nanosheets or nanoclusters were also used as building blocks for the fabrication of DNA-based hybrid nanostructures, and thus enhanced the thermal, chemical and mechanical stability of DNA [52].

2.3. Coprecipitation method

Layered double hydroxides are very appropriate materials for biomolecular immobilization due to their anion exchange properties. Some negatively charged biomolecules such as ATP and DNA had been incorporated between the layers of LDH via simple ion exchange method [53-55]. But to large
molecules, such as proteins, the intercalation is often difficult. Recently, S. Vial et al successfully prepared LDH/urease composite nanomaterial via coprecipitation method [56,57]. At first an aqueous solution of ZnCl$_2$ and AlCl$_3$ was mixed with urease solution, the mixture was coprecipitated by the addition of NaOH solution and the pH was maintained at 8.0 to protect the urease from denaturizing. At last the as prepared LDH/urease composite material was centrifuged and dried. Structure and part of the activity of urease was preserved. There is a great affinity between the urease and LDH. The loading amount of urease could be tuned by adding different quantities of enzyme in the reaction system. Other LDH/enzymes composite materials could also be fabricated after careful optimization of the preparation procedure, which resulted in a wide range of new functional biomaterials for biochemistry and electrochemistry applications.

3. Application to biosensors

Inorganic layers (clay, LDH, etc.) may be applied to immobilize different kinds of proteins, such as tyrosinase, alkaline phosphatase, laccase, nitrite reductase, glucose oxidase and urease for electrochemical biosensors development [35-37,42]. Here, we introduce the biosensors based on the novel layered niobate-Hb composite materials, which were prepared by the exfoliation-reassembly method in mild pH media at room temperature [28,47]. The niobate-Hb composite gel was deposited on a pyrolytic graphite electrode and allowed to dry under ambient condition overnight. The process was shown in Scheme 4.

![Scheme 4](image)

**Scheme 4.** The preparation process of niobate-Hb modified electrode [47].

The niobate-Hb composite material has regularly layered structure with ideal layer-to-layer assembly, so the enzyme loading capacity is relatively larger than that of other kinds of matrices. As a semiconducting material, the niobate exhibits a certain electrical conductivity which may promote the direct electron transfer between the enzyme and the electrode. Thus, the niobate-Hb modified electrode has a fast transfer rate constant and a high detection sensitivity of about 172 µA mM$^{-1}$cm$^{-2}$ with relatively low detection limit of 1.7 µM to H$_2$O$_2$ (See in Figure 1). The inorganic layers have the protect effect on stabilizing the structure of Hb in the extreme media, which result in the detection of H$_2$O$_2$ under very acidic and basic conditions on the electrode. Similarly, enzymes immobilized by layered $\alpha$-zirconium phosphate and layered titanate were also could be applied to the construction of the biosensors [48,49]. These biosensors showed good stability to the extreme conditions because of the protection of the inorganic layer to the enzymes. The $\alpha$-zirconium phosphate-Hb modified electrode
displayed an excellent response to the reduction of H$_2$O$_2$ with good reproducibility. When applied the biosensor in high temperature solution, it retained high bioactivity even the temperature is as high as 85 °C. In addition, layered spongy Co$_3$O$_4$ was also reported, which showed a high efficiency for the entrapment of redox-active proteins. The Co$_3$O$_4$-Hb modified electrode had a fast direct electron transfer with a high sensitivity of 396 µA mM$^{-1}$cm$^{-2}$ to the detection of H$_2$O$_2$ [39].

![Figure 1](image)

**Figure 1.** Amperometric responses of (a) Hb–niobate and (b) reassembled HCa$_2$Nb$_3$O$_{10}$ modified electrodes at −400 mV upon successive addition of 50 µM H$_2$O$_2$ to 0.1 M pH 7.0 PBS. Inset: plot of catalytic peak current at Hb–niobate modified electrode vs. H$_2$O$_2$ concentration [47].

4. Conclusion

Inorganic layered materials are very useful to construct robust biosensors through entrapping enzymes. These materials not only have the advantages of high mechanical strength, thermal stability, resistance to organic solvents and microbial attacks, and easy regeneration, but also meet the requirements for a protein support: large surface area, permeability, insolubility, hydrophilic character, and high rigidity. The layered materials can overcome the shortages of those inorganic supports with three dimensional porous structures, where the minimal channel cross section of the rigid pores may restrict the application for immobilization of large molecules such as proteins. The inorganic layer immobilized enzyme composite material has regularly layered structure with ideal layer-to-layer assembly, and the enzyme loading capacity is relatively larger than that of other kinds of inorganic supports. As a semiconducting material, the inorganic layer, such as niobate, exhibits a certain electrical conductivity which may promote the direct electron transfer between the enzyme and the electrode. Thus, the niobate-Hb modified electrode has a fast transfer rate constant and high detection sensitivity with relatively low detection limit to H$_2$O$_2$. The inorganic layers have the protect effect on stabilizing the structure of enzyme in the extreme media, which result in the detection of H$_2$O$_2$ under very acidic and basic conditions on the electrode. The research on this field is very interesting and
more novel kinds of inorganic layer-enzyme composite materials are needed to prepare. Further exploration of the preparation method, application and the relations between them is necessary.

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References and Notes


49. Liu, Y.; Lu, C.; Hou, W.; Zhu, J. Direct Electron Transfer of Hemoglobin in Layered α-

pp 19-53.

51. Ding, Y.; Jones, D.J.; Maireles-Torres, P.; Roziere, J. Two-Dimensional Nanocomposites: 
562-571.

52. Patil, A.J.; Li, M.; Dujardin, E.; Mann, S. Novel Bioinorganic Nanostructures Based on 
Mesolamellar Intercalation or Single-Molecule Wrapping of DNA Using Organoclay Building 

53. Choy, J.H.; Kwak, S.Y.; Park, J.S. Intercalative Nanohybrids of Nucleoside Monophosphates and 


57. Vial, S; Prevot, V.; Leroux, F. Immobilization of Urease in ZnAl Layered Double Hydroxides by 