Spongy gel-like layered double hydroxide–alkaline phosphatase nanohybrid as a biosensing material†

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Formation of new bio-nanohybrid material was obtained by immobilization of alkaline phosphatase within a Mg_2Al LDH by "soft chemistry" coprecipitation synthesis, resulting in an original spongy gel-like morphology allowing the preservation of the enzyme structure and activity even at low pH values thanks to the buffering property of the basic host structure.

One key step in the development of biosensors is the immobilization of a biological element (enzyme, antibody, microorganism) to the sensor surface. A number of innovative immobilization techniques have been reported, including the design of new bio-nanohybrid materials that present advantages in addition to molecular recognition, such as thermal stability, physical ruggedness and pH buffering.[1,2] The fact that the immobilized enzymes are capable of operating under aggressive environment, for instance under extreme pH conditions, may be required for biosensor applications in real sample analysis. Indeed, the performance of the biohybrid system is governed by the properties of both enzyme and host structure. The inorganic partner is not only seen as a protective matrix for the preservation or exaltation of the bioactivity or the lengthening of the lifetime of the biological material but it may contribute to novel multifunctionalities by a synergic effect at the nanoscale level of its own specific properties. For instance, alkaline phosphatase (AlP) is an enzyme of great agronomic value because it hydrolyses phosphate monoesters into alcohols. Alkaline phosphatase activity has been also used for the development of electrochemical immunoassays and biosensors. As the name of the enzyme implies, its catalytic activity is optimal at basic pH values (9.5). It has been shown recently that the entrapment of AlP into sol–gel matrix can protect the enzyme against harsh pH condition.[3]

Among the various inorganic supports available, layered double hydroxides (LDH) with their bidimensional structure and unique anion exchange properties, appear as very favourable host structures for biomolecule immobilization, particularly for enzymes with isoelectric point varying in a large pH domain.[4] They are synthetic materials with positively charged brucite-like layers of mixed metal hydroxides separated by interlayered hydrated anions, defined by the general formula $[M_{1-x}^{2+}M_x^{3+}(OH)_2]^{x+}[(A^{n-})_{x/n}, yH_2O]$ (abbreviated as $M^{2+}_{(1-x/x)}M^{3+}$–A).

The "Soft Chemistry" coprecipitation route used for the preparation of LDH phases is a very tuneable process allowing to choose specific conditions (pH, temperature, buffer, solvent, reagents concentration) avoiding structural change of the enzyme and denaturation of enzyme activity. Since AlP has an isoelectric point around 5 and an optimum working pH of 9.5, it is possible to fit the LDH chemical composition to the desired charge density and basicity properties. Among the wide variety of M^{II}–M^{III} LDH combination, MgAl LDH appears as the best candidate. Indeed, the nature of metal cations in the LDH layers has been reported to have a buffer effect.[5] The base strength of MgAl LDH is significantly higher than ZnAl and ZnCr LDH.[5,6] The basic nature of the LDH material could stabilize the enzyme at low pH, thus enhancing its acid resistance. Moreover, MgAl LDH can act not only as an encapsulation matrix for AlP preservation and a pH buffering medium but also as substrate adsorbent which could facilitate diffusion of organophosphate anion substrate into the MgAl–AlP nanocomposite membrane. Immobilization of AlP in MgAl LDH matrix was successfully realized by coprecipitation under tightly controlled conditions and the effect of the AlP : LDH mass ratio (Q) on the structure, morphology and activity of the hybrid biomaterials was investigated.

The X-ray diffraction patterns of the coprecipitated MgAl–AlP samples are compared to the diffractogram of the reference MgAl–CO_3 LDH material (Fig. 1). The AlP containing LDH coprecipitated phases shows a strong decrease of crystallinity. When increasing the Q = AlP/LDH mass ratio

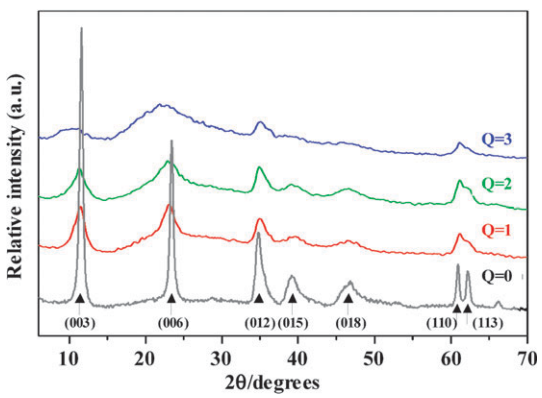

Fig. 1 Powder XRD patterns of MgAl–AlP LDH with various theoretical ratios for AlP : LDH.

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from 1 to 3, one observes a strong decrease of the (00l) diffraction lines intensities and an enlargement of the diffraction lines that confirms both a reduction of the particle size according the Laue–Scherrer law, and a greater disorder of the structure with a net turbostratic effect. The preservation of the (012) and (110) diffraction lines for all the biohybrid materials evidences the formation of the layer structure.

These structural observations show the successfully presence of the LDH layers although the presence of AlP influences the precipitation of the inorganic compounds. Higher the concentration of the enzyme in the reaction medium higher the dispersion of the layers in the biohybrid nanocomposite. The absence of diffraction lines shifted at low Bragg angles of 2θ indicates that the immobilization process does not lead to the intercalation of AlP within the LDH layers. Even at low concentration of enzyme when surface saturation has not yet been reached, formation of ordered stacked MgAl–AlP LDH is not obtained. AlP from porcine kidney is a voluminous tetrameric metalloenzyme with a total molecular weight of 156 kDa[7] whose cross section area is much larger than exchange site surface of the LDH (2.5 nm^2 per e^-) and cannot be intercalated in LDH host structure. Electrical charge balance is then insured by co-immobilization of inorganic anions.

The presence of AlP was also confirmed by FTIR. The amide I ($\nu_{C=O}$ stretching coupled with in-plane N–H bending and ν_{C-N} stretching) bands of the confined enzyme (centered at 1638 cm^{-1}) are well resolved and superimposable with that of the native AlP (centered at 1635 cm^{-1}) indicating that the α-helix segment is not disturbed under immobilization. The three gaussian components of the amide II (δ_{N-H} bending and ν_{C-N} and ν_{C-C} stretching) bands (1501 cm^{-1}, 1518 cm^{-1} and 1540 cm^{-1}) are shifted by about 15 cm^{-1} at a higher energy (1514 cm^{-1}, 1541 cm^{-1} and 1556 cm^{-1}). Such a shift arises from the formation of hydrogen bonds between the LDH matrix and the protein.

The scanning and transmission electron microscopy images clearly evidence the influence of the biomolecules on the LDH particles and their aggregation. Whereas MgAl–LDH coprecipitated in absence of AlP displays a sand rose like aggregation (Fig. 2A), the bio-nanocomposite MgAl–AlP shows a novel spongy gel-like structure with a wide range of macropores formed by interconnected LDH platelets network in which the individual inorganic particles or aggregates cannot anymore be distinguished (Fig. 2B).

Such a spongy morphology has never been reported for LDH nanocomposite and is highly in favour of molecular diffusion. Note that on the TEM image the darker part traduces the presence of the inorganic particles dispersed around the organic matter.

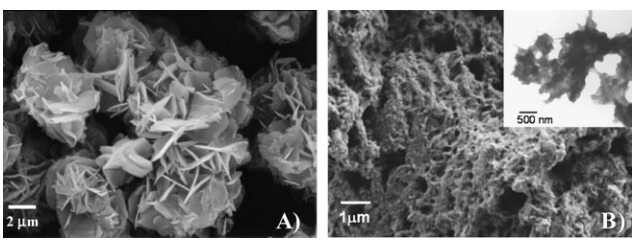

Fig. 2 SEM images of MgAl–CO_3 (A) and MgAl–AlP nano-biohybrid (Q = 1) (B) with corresponding TEM image in inset.

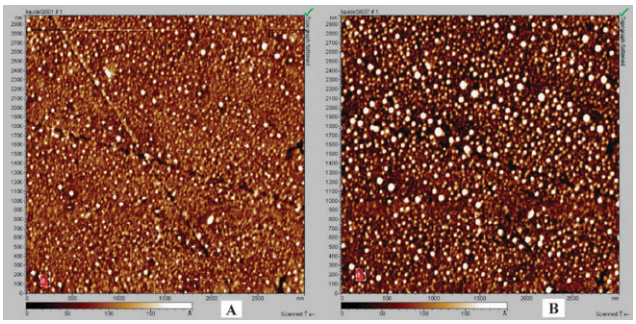

Fig. 3 AFM image of the MgAl–AlP coating in a Tris buffer solution t = 3 (A) and 35 min (B).

AFM analysis was performed on MgAl–AlP film coated on silicon wafer dried and immersed in electrolyte solution in order to sort out the aspect and behaviour of the deposit. The dried MgAl–AlP deposit shows a very smooth coating with the presence of some dispersed crystal particles of the biohybrid material (not shown). AFM images of the same sample were further recorded in Tris buffer solution at different times to study the rehydration of the film. Fig. 3A and 3B show images recorded at t = 3 and t = 30 min. We clearly observe between these two different states a deep increase of the contrast related to the water swelling of the macrostructure simultaneous to the macropore sizes increase. The porous morphology is evidenced by the dark and brown network of LDH interconnected walls whereas some LDH particles appear as white hexagonal platelets on the top of the surface. Higher is the swelling time, better the porous background network and the surface particles are observed. This phenomenon is associated to a progressive z displacement of the piezoelectric ceramic (about 3 µm). These results are in good agreement with SEM images. They suggest the progressive swelling of the nanostructured film in the electrolyte solution and the spongy gel-like behaviour of the nanohybrid MgAl–AlP material.

Permeability, which is also a characteristic of porosity of the biohybrid film, was determined by linear sweep voltammetry at a rotating disk electrode, using hydroquinone as the electroactive permeant. The values of permeability for the MgAl–AlP films are 2.3–2.6 × 10^{-2} cm s^{-1}. These values are slightly higher than the permeability values obtained with the Mg_2Al–CO_3 films in the presence or not of absorbed AlP (0.9 and 1.1 × 10^{-2} cm s^{-1}, respectively).

Enzyme assays show that the residual activity of confined enzyme depends strongly on the storing conditions of the samples. Two different samples of MgAl–AlP (Q = 1) were prepared showing, respectively 44 and 36% of residual activity. A slow drying in air of the MgAl–AlP biohybrids caused a drastic decrease of the enzyme activity (0.1–0.4%) due to the shrinkage of LDH aggregate framework and the loss of the native AlP structure. However when the samples were stored as frozen aqueous suspensions at −20 °C, their residual activities remained equal to 42 and 36% after 80 days of storing. Moreover, the half of their initial activity was still observed after 8 months. These results show the good

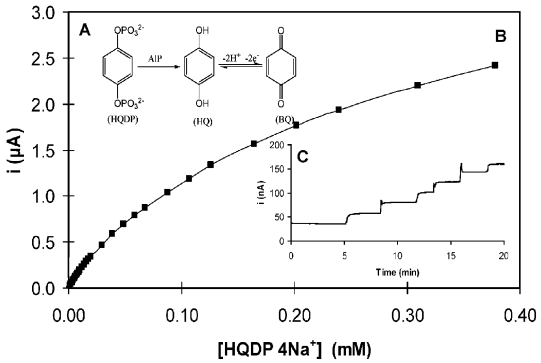

Fig. 4 (A) Electro-enzymatic reaction; (B) calibration curve of MgAl–AlP biosensor; (C) dynamic response after subsequent additions of 1 µM HQDP (E_{app} = 0.4 V/Ag–AgCl, pH = 8.5).

reproducibility and storage stability of MgAl–AlP biohybrids prepared by the coprecipitation method.

The activity of MgAl–AlP (Q = 1) biofilm was also tested by electrochemistry using hydroquinone diphosphate (HQDP) as a model of substrate of AlP.[8] Hydrolysis HDQP by AlP produces hydroquinone (HQ). The reversible oxidation of HQ proceeds *via* a two-electron transfer and deprotonation to produce benzoquinone (BQ) (Fig. 4A). The amount of HQ, enzymatically formed, was then determined amperometrically at low applied potential (0.4 V/Ag–AgCl) without electrode fouling.[8] MgAl–AlP modified electrodes have be obtained through only one step deposition on the electrode surface with a response reproducibility of 98% for three different electrodes. A typical calibration HQDP curve based on steady state measurement is depicted in Fig. 4B, with some dynamic response curves shown in the inset (Fig. 4C). The bioelectrode presents a fast, stable and reproducible response with a relative standard deviation of 5% for 5 successive additions of 1 µM HQDP. It should be noted that the response time (t_{80}) decreases with the swelling time of the bio-film in the electrolyte solution, from 36 s for 1 h to 14 s after 4 h. This phenomenon is associated with an increase in the amperometric current from 14 nA for 1 h swelling to 21 nA after 4 h, confirming the spongy gel-like behavior of the nanohybrid MgAl–AlP material observed in AFM. When the biosensor was stored in buffer solution at 4 °C, 93% of its maximum response was still observed after two months. For comparison, the activity of free AlP or in liposome system constantly decreased during the storage at 4 °C with a $t_{1/2}$ of ~21 days or 46 days, respectively.[9,10] The storage stability was slightly improved when AlP was immobilized onto Fe_3O_4 nanoparticles with a constant activity of 60% between the 1st and 16th week.[10]

The calibration curve follows Michaelis–Menten kinetics with a constant K_M^{app} = 74 µM. This value is comparable to those generally reported for AlP substrates with the free enzyme in solution, ranging between 40 and 130 µM, and it is lower than the K_M^{app} value (300 µM) reported for HQDP using AlP-labeled antibodies anchored on IrOx electrode.[8] The activity of free AlP varies in a narrow pH range with a maximum value at 9.5 (Fig. 5A). Immobilization of AlP into LDH nanohybrid leads to a broader pH profile over 2 pH units with a shift of the optimum pH value between 8 and 8.5 (Fig. 5B).

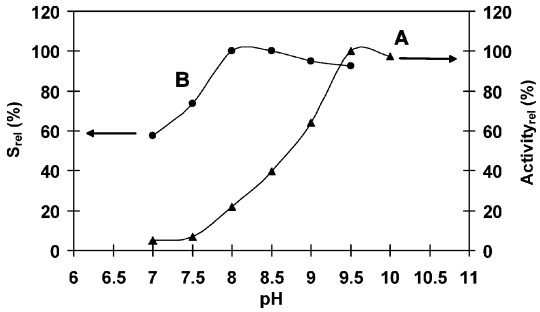

Fig. 5 pH effects on free AlP activity (A) and on MgAl–AlP biosensor response (B).

Even at pH 7, the biosensor response remains equal to 60% of its maximum value whereas it is only 28% when AlP is immobilized into a polypyrrole based biosensor, for example.[11] This fact confirms the expected buffer effect of MgAl LDH host material, enhancing the enzyme stability against acidic attack. This property is very useful for environmental applications in real aqueous media where pH usually ranges from 6.5 to 9.0.

In conclusion, efficient immobilization of alkaline phosphatase in Mg_2Al layered double hydroxide by the soft chemistry coprecipitation method is demonstrated for the first time. Thanks to its novel spongy morphology, the resulting bio-nanohybrid material forms a gel in aqueous solution that preserves the enzyme structure and activity even at low pH value. A biosensor can be prepared using this bio-hybrid material with a simple and cheap method that overcomes an additional cross-linking step which often induces the denaturation of the enzymes.[12]

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