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Efficient Immobilization of Yeast Transketolase on Layered Double Hydroxides and Application for Ketose Synthesis

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Abstract: Transketolase (TK) from *S. cerevisiae* was successfully immobilized on layered double hydroxides (LDH) using simple, affordable and efficient adsorption and coprecipitation based immobilization procedures. Optimization of the preparation was performed using zinc aluminium nitrate (Zn_2Al-NO_3) and magnesium aluminium nitrate (Mg_2Al-NO_3) LDH as immobilization supports, and the protein-to-LDH weight ratio (Q) was varied. The highest immobilization yields (98–99%) and highest relative specific activities (4.2–4.4 $U \cdot mg^{-1}$ for the immobilized enzyme compared to 4.5 $U \cdot mg^{-1}$ for the free enzyme) were both achieved when using a protein-to-LDH weight ratio (Q) of 0.38. Efficient lyophilization of the LDH-TK bionanocomposites thus synthesized was proven to allow easy use and storage of the supported TK with no significant loss of activity over a three-month period. The kinetic parameters of the LDH-TK enzyme were comparable to those of the free TK. The LDH-TK enzyme was finally tested for the synthesis of L-erythrulose starting from hydroxypyruvate lithium salt (Li-HPA) and glycolaldehyde (GA) as substrates. L-erythrulose was characterized and obtained with an isolated yield of 56% similar to that obtained with free TK. The reusability of the LDH-TK biohybrid material was then investigated, and we found no loss of enzymatic activity over six cycles.

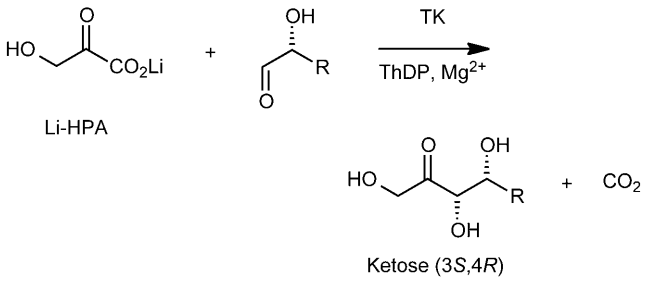
Keywords: biocatalysis; enzyme immobilization; L-erythrulose; ketoses; layered double hydroxides; transketolase

Introduction

Biocatalysis has become an essential tool for the synthesis of a wide variety of enantiomerically pure compounds on an industrial scale.[1] In the context of chemistry for sustainable development, the properties of enzymes (mild operating conditions, selectivity) make them an interesting alternative to chemical catalysis.[2] Enzymes share an inherent ability to create chiral centers that makes them particularly suitable for the synthesis of mono- and oligosaccharides widely used to produce important pharmaceuticals, cosmetics and food products.[2] Various studies have focused on enzyme-catalyzed stereospecific formation of C–C bonds such as lyases (including aldolases) and transferases (including transketolase) yielding enantiopure monosaccharides in one step from donor and acceptor substrates.[3] Transketolase (TK) is a thiamine diphosphate (ThDP)-dependent enzyme that is extremely efficient for synthesizing ketoses and analogues.[4a]

TK from yeast is a dimeric enzyme where the two-fold related active sites are located between the different domains of the two subunits. Residues from both subunits build up the active sites and are involved in binding the ThDP and divalent cation cofactors. Therefore, the dimer is the functional unit of TK.[4b]

In vivo, TK reversibly transfers a hydroxyacetyl unit from phosphorylated ketose as donor substrates onto phosphorylated aldoses as acceptor substrates in the presence of ThDP and Mg^{2+}. The newly-formed asymmetric center has the (*S*) configuration. TK is also highly enantioselective to α-hydroxyaldehyde acceptor substrates presenting the (*R*) configuration.



Scheme 1. Irreversible reaction catalyzed by TK in the presence of hydroxypyruvate lithium salt (Li-HPA) as donor substrate and α-hydroxy aldehyde as acceptor substrate.

Consequently, products are characterized by a ketose structure having a (3*S*,4*R*) configuration.

For synthetic applications, TK is used with hydroxypyruvate lithium salt (Li-HPA) as donor substrate, thereby rendering the ketol transfer irreversible, making TK an ideal tool for ketose synthesis (Scheme 1).[5]

Previous studies showed that this enzyme is also more flexible than the other ThDP-dependent enzymes towards acceptor-substrate aldehydes, preferentially when they are α-hydroxylated and differently substituted (R = alkyl, allyl, aromatic or heterocyclic moieties).[5b,6]

TK owes its synthetic potential to these special properties, which have been applied in the production of chemicals such as the pheromone α-*exo*-brevicomin[7], and also 6-deoxysugars[8], azasugars[9], thiosugars[10] phosphorylated sugars[11], and amino alcohols[12]. These reactions make TK a very attractive biocatalyst for industrial applications.[13] TKs from yeast[14] and *E. coli*[15] have previously been cloned and overexpressed, giving sufficient amounts of protein with good activity.

One of the main challenges for industrial applications of enzymes is their re-use over many reaction cycles. Immobilization of enzymes is seen as the only process able to solve commonly encountered problems such as high solubility, low stability and inhibition by high concentrations of substrates and products, inhibition by pH or temperature, and low activity and selectivity under non-conventional conditions. Indeed, immobilized biocatalysts are generally more stable and easier to use than their free counterparts. Enzyme immobilization may also lead to major improvements in their activity, selectivity and specificity, provided the bonding with the support is tightly controlled.[16] Various strategies have been developed through multipoint attachments or site-directed grafting *via* mutagenesis, and covalent spacers such as avidine-biotine complex as recently reported by Hernandez and Fernandez-Lafuente.[17]

There are many immobilization techniques using different supports with diverse activate groups (for covalent or physical adsorption) and even immobilization without using pre-exiting supports (CLEAs and CLEs).[18] The enzymes may be either covalently or electrostatically bonded to the carrier,[19] cross-linked with molecular linkers,[20] entrapped in polymers[21] or mesoporous inorganic frameworks.[22] Suspension of the enzyme,[23] lipid-coated enzymes,[24] enzyme microencapsulation in reverse-micelles,[25] enzyme immobilization on microgel matrices or within membranes,[26] and polymer-enzyme covalent attachment (pegylation)[27] have all been probed as techniques. In all cases, biocatalysis efficiency requires high enzyme loading and optimal diffusive properties. Simplicity and cost-effectiveness are two key points to be addressed when engineering new immobilization processes. However, these enzyme systems present varying catalytic activities and the modifications can result in a considerable loss of enzymatic activity.[24–28]

In order to improve synthetic processes based on TK-catalyzed reactions, a handful of studies has described TK immobilization methods including covalent immobilization of the enzyme by BrCN activation of Sepharose[29], on resin beads such as Eupergit-C[30], Amberlite XAD-7,[31] or nylon.[32] More recently, variants of the wild-type TK engineered with a his_6-tag were reversibly immobilized *via* Ni-NTA linkage to surface-derivatized units in a fused-silica capillary.[33]

Most of these publications have reported the use of either organic or semi-organic matrices only. In this study, we investigated the efficiency of an inorganic support, i.e., layered double hydroxides (LDH), as TK immobilizer in order to further perform an easy, affordable and biocompatible immobilization strategy.

The increasing interest in LDH as host structures for biomolecules[34] arises from their versatile properties in terms of chemical composition of both layers and interlayers, their high and tunable layer charge density and anion exchange capacities, and their two-dimensional structure which can accommodate large anionic and/or polar molecules. LDH display a layered structure built on a stacking of positive layers, $[M(II)_{1-x}M(III)_x(OH)_2]^{x+}$, separated by interlamellar domains consisting of anions and water molecules, $[X_{x/q}\cdot n\,H_2O]^{x-}$.[35] This flexible layered structure with unique anion exchange properties can adapt to accommodate large biomolecules such as proteins bearing an overall negative charge. Interestingly, LDH can be prepared in very soft conditions compatible with enzyme stability.[35a]

Immobilization of enzymes in LDH has recently been investigated, but mainly for electrochemical purposes.[36] LDH matrices have been proved to immobilize high amounts of enzymes while maintaining a fairly high fraction of their electrocatalytic activity. A few recent papers have reported the use of LDH as enzyme supports for biocatalytic applications.

Rahman et al.[37] reported the immobilization of lipase from *Candida rugosa* on Mg, Zn and Ni-based LDH for ester synthesis. *Saccharomyces cerevisiae* lipase adsorbed on Mg_2Al LDH was investigated for biodiesel production.[38] The enzyme improved its activity about 7% compared to the free enzyme, and retained its high reactivity over ten reaction cycles with an ester conversion above 81%. Hybrids LDH have also been tested for enzyme adsorption. Borges et al.[39] recently succeeded in immobilizing an enzymatic extract from *Penicillium camemberti* onto a hybrid $Zn_2Al(CH_2)_7(COO^-)_2$ LDH. Lipoxygenase activity was maintained at 60%, and thermal stability was improved.

Herein, we describe a new and efficient immobilization process for yeast TK on LDH, and the application of the LDH-TK bionanocomposites for biocatalyzed synthesis of a ketose. Both adsorption and coprecipitation methods were studied. The immobilization process was optimized and the effects of varying protein-to-LDH weight ratios on the immobilization yields and TK relative activities were investigated. Kinetic parameters and stability over storage were evaluated. The LDH-TK biohybrids prepared under optimal conditions were then characterized using FTIR, XRD and TEM. The catalytic activity and reusability of the LDH-TK enzyme were tested using an established test reaction leading to the synthesis of L-erythrulose.[5a,40]

Results and Discussion

According to their M(II)/M(III) layer composition, LDH structures display tunable surface charge density and physico-chemical properties such as Brønsted basicity. Therefore, the choice of LDH matrices can be directed by the inherent properties of the enzyme to be immobilized on. We chose M(II)/M(III) = 2.0 as the ratio corresponding to the highest charge density of both matrices. Depending on the isoelectric point (IP) of the enzyme and its pH stability, M(II)M(III) LDH were selected in such a way that both their points of zero net charge (pznc) and surface pH ensure tight protein bonding and enzyme buffering effects. Accounting for this influence of LDH composition, the two LDH matrices Zn_2Al-NO_3 (Zn_2Al) and Mg_2Al-NO_3 (Mg_2Al), which have previously been described as highly compatible with the immobilization of enzymes (including proteases, lipases and peroxidases),[36b,37a,42] were investigated for TK immobilization. Zn_2Al and Mg_2Al LDH, with a pznc of about 10–11, display platelets with a net positive charge under working conditions (pH 8.0) while TK (IP = 6.9)[41] will bear an overall negative charge. Coulombic attractions might then lead to a stable LDH-enzyme assembly and a high yield of TK immobilization. Moreover, Zn_2Al and Mg_2Al LDH exhibit specific buffering properties due to their surface basicity allowing stabilization or even improvement of the bioactivity of the TK. The pH of the Zn_2Al and Mg_2Al suspensions (1 mg·mL^{-1}) are, respectively, 7.0–8.0 and 8.0–9.0. TK immobilization in both matrices made it possible to test the effect of LDH surface pH on the catalytic activity of the enzyme.

Preparation of LDH-TK Biohybrids

The immobilization of TK was carried out by two processes: adsorption (ad) and coprecipitation (co). Both methods present interests as far as enzyme immobilization is concerned.[36f] Adsorption of enzymes on LDH matrices, as in general on solids, is a mild surface reaction in favor of enzyme preservation, whereas the coprecipitation process, i.e., addition of the biomolecules directly to the LDH coprecipitation medium, has been developed to overcome the limitation of a low adsorbant surface area. Coprecipitation promotes a high immobilization yield and a good dispersion within the solid phase, thus avoiding subsequent leaching. Moreover, Zn_2Al LDH can be prepared by coprecipitation over a large range of pH values (7.0–11.0) that can be chosen to fit the pH of the optimal TK stability[14]. Yeast TK extract in glycylglycine buffer (50 mM) was obtained after disruption of overexpressed TK yeast cells[43] followed by centrifugation. Coprecipitation was performed at a pH of 8.5. Adsorption of TK on Zn_2Al was conducted in a non-conventional way. In order to overcome the constraint of a low specific surface area of LDH phases, TK was contacted with freshly coprecipitated LDH suspensions and washed. This modification of the standard adsorption process obviously favors TK diffusion at the surface of the suspended LDH platelets and should drastically increase adsorption yield. These experimental conditions were also used for the coprecipitation of Mg_2Al-TK_{co} with some modifications (coprecipitation pH 9.0) and for the adsorption of TK by Mg_2Al-NO_3.

In order to determine the immobilization capacities of LDH matrices, the amount of enzymes was adjusted by tuning the TK extract-to-LDH weight ratio in the synthesis solution, assuming a LDH precipitation yield of 100%, justified by the low LDH solubility. The initial protein-to-LDH weight ratio (Q_{in}) corresponds to the maximum amount of proteins that can be immobilized on a fixed amount of LDH (25 mg for adsorption or under a total coprecipitation of LDH matrix). Q_{in} was varied in the range 0.25, 0.38, 0.50, 0.75 and 1.0.

The experimental protein-to-LDH weight ratio (Q_{exp}) can, however, be defined as the real amount of proteins on the actual amount of LDH formed, which

was estimated by elemental analysis (using ICP-AES) of metal cations in supernatant solutions.

Characterization of the LDH-TK Biohybrids

The influence of the immobilization process (adsorption or coprecipitation) on the structural and textural properties of the biomaterials was investigated over a wide range of Q_{in} and Q_{exp} and in parallel with the measurement of TK activity. The results obtained with Zn_2Al are only discussed due to the higher Zn_2Al-TK catalytic activities over a larger range of Q_{in} compared to Mg_2Al (data shown later in Table 1).

The X-ray diffraction patterns of the Zn_2Al-TK samples prepared by either coprecipitation or adsorption are compared in Figure 1. The diffractogram of Zn_2Al-NO_3 LDH reference material is also plotted for comparison ($Q_{in}=0$). This latter pattern shows a structural feature of pure hydrotalcite-like compounds[35b] with the characteristic series of 00l and hk0 diffraction lines. The lattice parameters, refined from the hexagonal setting with a rhombohedral symmetry (space group: R-3m) are $a=0.3071$ nm and $c=2.669$ nm. The observed interlayer distance ($d=c/3=0.889$ nm) accounts for NO_3^- anion intercalation between the layers.

The X-ray patterns recorded for the LDH nanobiohybrids clearly evidenced the influence of the TK extract on the structural and textural properties of the inorganic materials. The powder X-ray diffractograms (PXRD) of both Zn_2Al-TK_{co} and Zn_2Al-TK_{ad} show a reduced number of strongly-enlarged diffraction lines. For all samples except Zn_2Al-TK_{co} ($Q_{in}=1.0$), the diffraction lines point at the same positions as those of Zn_2Al-NO_3, indicating that the biohybrid materials are pure LDH phases.

However, TK induces significant amorphization of both coprecipitated and adsorbed LDH materials due to a strong decrease of the coherent domain sizes. The 00l diffraction lines are very broad, indicating a very small number (~10) of randomly-oriented layers per crystallite. The layer stacking order along the c axis is almost lost, leading to turbostratic structures evidenced by dissymmetric (h0l) and (0kl) peaks. From the position of the (00l) diffraction lines, it can be concluded that TK does not intercalate in the structure, as a shift of the (003) to low-angle 2θ values (<2°) would have been observed. At a high rate of TK extract adsorption, a series of well-identified (00l) peaks corresponding to a basal distance of about 1.167 nm ($Q_{in}=1.25$) indicated some intercalation of anionic residues from the TK extract. Coprecipitated phases appeared more disordered than adsorbed phases. Under high protein loading ($Q_{in}\geq 1.0$), a secondary crystallized phase is formed whose FTIR spectra correspond to glycylglycine buffer.

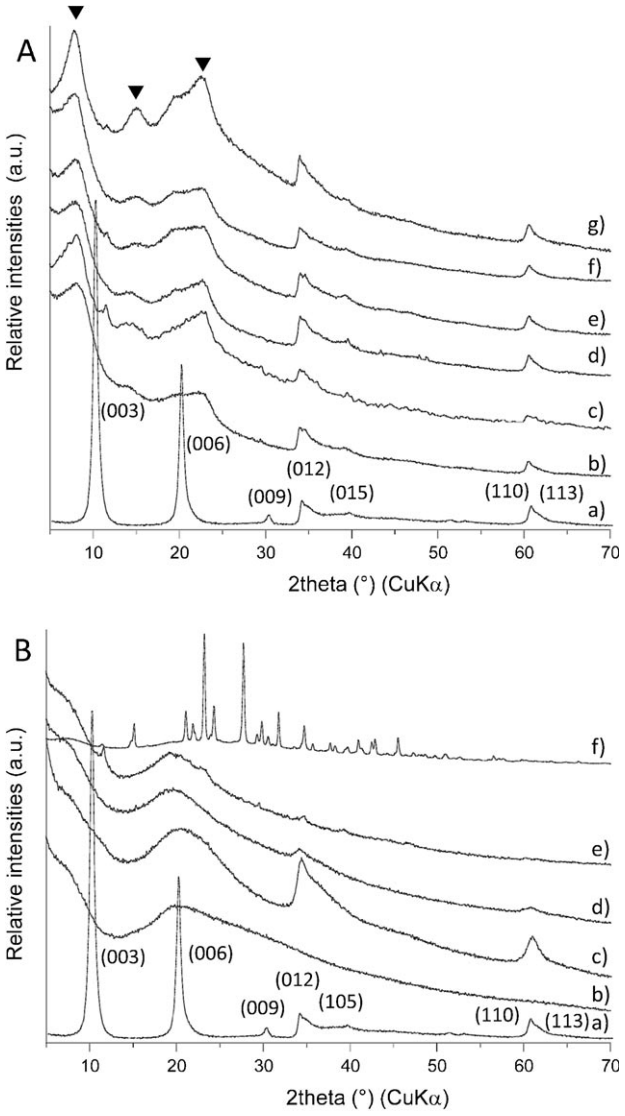


Figure 1. XRD patterns obtained for (**A**) Zn_2Al-TK_{ad} at $Q_{in}=0$ (**a**), 0.25 (**b**), 0.38 (**c**), 0.50 (**d**), 0.75 (**e**), 1.00 (**f**) and 1.25 (**g**) and (**B**) Zn_2Al-TK_{co} at $Q_{in}=0$ (**a**), 0.25 (**b**), 0.38 (**c**), 0.50 (**d**), 0.75 (**e**) and 1.00 (**f**).

FT-IR spectra were also recorded to further characterize the LDH-TK materials (Figure 2). For all Zn_2Al-TK biohybrids, vibration bands typical of the LDH structure are systematically observed, indicating that LDH is the major phase formed.

Large stretching ν_{OH} vibration bands (3000–3700 cm^{-1}, not shown) can be attributed to OH groups of hydroxylated layers and water molecules. Vibration bands in the range 400–800 cm^{-1} (621, 550 and 424 cm^{-1}) correspond to the ν_{MO} and δ_{OMO} lattice vibrations. For Zn_2Al-TK_{co}, these bands become broader as the TK loading increases due to the progressive amorphization of the structure. For the biohybrid materials, additional bands at 2700–2800 cm^{-1} (not shown) and 700–1700 cm^{-1}, characteristic of the

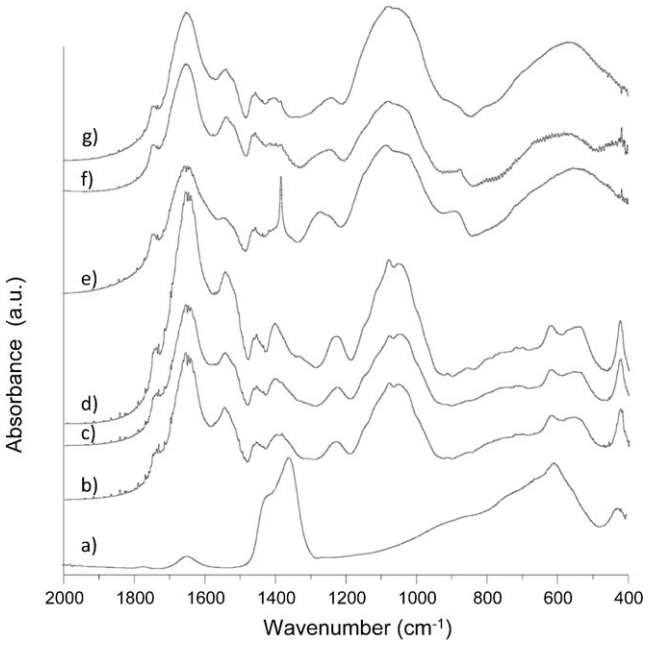


Figure 2. FTIR spectra of $Zn_2Al\text{-}NO_3$ (**a**), $Zn_2Al\text{-}TK_{ad}$ at $Q_{in}=0.25$ (**b**), 0.50 (**c**), 1.0 (**d**) and $Zn_2Al\text{-}TK_{co}$ at $Q_{in}=0.25$ (**e**), 0.50 (**f**), 1.0 (**g**).

ν_{CH} and amide vibration bands of the enzyme, are observed. Their high intensity confirms the efficiency of both enzyme immobilization processes. Interestingly, under the adsorption process, there was a clear decrease in $\nu_{3(NO_3)}$ intensity (1368 cm^{-1}) whatever the Q_{in}. Obviously, enzyme immobilization proceeds by an anionic exchange mechanism, with nitrate anions being replaced at the LDH surface by carboxylate groups of aspartate and glutamate TK surface residues.

Given that biomaterial morphology has a major impact on diffusion and accessibility properties, TEM experiments were also carried out on the bionanocomposites. TEM images of $Zn_2Al\text{-}TK$ prepared at $Q_{in}=0.38$ by adsorption and coprecipitation are presented in Figure 3. Two different morphologies can be distinguished according to the immobilization process. After TK adsorption by $Zn_2Al\text{-}NO_3$ LDH compound, large and dense aggregates formed by the intergrowth of platelet-like particles are observed. Since such a house of cards type morphology is usually obtained for Zn_2Al LDH precursors prepared by coprecipitation, it indicates that enzyme adsorption does not strongly modify particle architecture. In contrast, particle shape and aggregation appeared rather different when the coprecipitation was performed in the presence of enzyme. A similar effect has previously been underlined for LDH synthesis carried out in the presence of alkaline phosphatase.[36b] Since in this case the crystal growth occurs in interaction with proteins of the TK extract, small ill-defined LDH particles (30–50 nm) are formed which strongly aggregate together, leading to rather open three-dimensional networks of LDH particles. In conclusion, efficient immobilization of TK in LDH matrices by coprecipitation benefits from two positive converging factors: (i) a multipoint attachment due to anion exchange site reaction (NO_3^- being replaced in the structure by carboxylate groups of Asp and Glu) reinforced by a hydrogen bonding network favoured by highly hydrophilic LDH surface, and (ii) a porous and flexible 3D network of connected LDH nanoplatelets built around

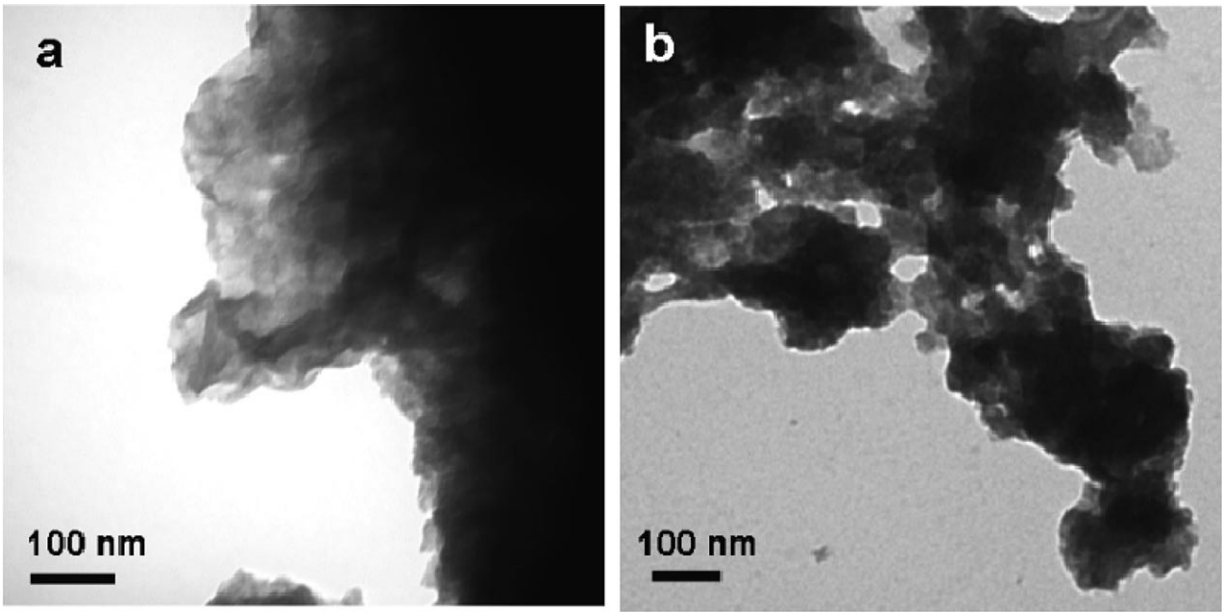


Figure 3. TEM images of the LDH/TK nanocomposites: (**a**) $Zn_2Al\text{-}TK_{ad}$ and (**b**) $Zn_2Al\text{-}TK_{co}$ at $Q_{in}=0.38$.

encapsulated TK enzyme leading to a gel-like biohybrid material. This behavior points out the interest of the enzyme-LDH immobilization process that does not need to set up single point attachment strategies which are cost- and material-consuming. Moreover, when immobilized TK retains its full bioactivity, the immobilization processes used keeps the multimeric form of the enzyme.

TK Immobilization Yield: Adsorption *vs.* Coprecipitation

The immobilization yields were measured for the bionanocomposites synthesized either by direct coprecipitation or adsorption at various Q_{in}. Figure 4 (upper panel) presents the results for Zn_2Al-TK_{ad} (◆) and Zn_2Al-TK_{co} (◇).

On testing the Zn_2Al-TK_{co} biohybrids, optimum immobilization yields (99%) were achieved when an initial protein-to-LDH Q_{in} weight ratio of about 0.38 was used. On increasing Q_{in} from 0.25 to 0.38, the immobilization yield increased from 50 to 99%, which can be explained by the increasing amounts of proteins initially used (from 6.25 to 9.38 mg) for the same amount of coprecipitated LDH (25 mg). On further increasing the Q_{in} ratio (from 0.38 to 1), immobilization yield dropped from 99% to 25%. Larger amounts of proteins used (from 9.38 to 25 mg) may thus be responsible for undesirable side reactions such as metal cation TK surface complexations that subsequently hinder LDH-TK formation.

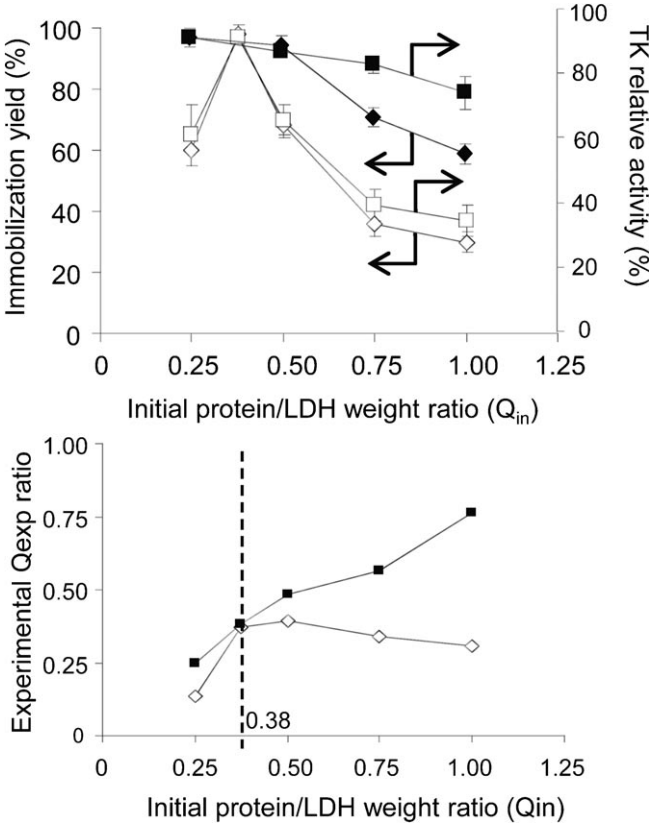


Figure 4. (*Upper panel*) Influence of the initial protein-to-LDH (Q_{in}) weight ratio on immobilization yield and TK catalytic activity in the presence of L-erythrulose and D-R5P as substrates of TK for Zn_2Al-TK_{ad} (◆ and ■, respectively) and Zn_2Al-TK_{co} (◇ and □, respectively). (*Lower panel*) Correlation between the initial (Q_{in}) ratio and the experimental (Q_{exp}) ratio for Zn_2Al-TK_{ad} (■) and Zn_2Al-TK_{co} (◇).

The adsorption method did however appear more efficient than the coprecipitation method, since higher immobilization yields (64–100%) were obtained for Zn_2Al-TK_{ad} compared to Zn_2Al-TK_{co}. However, there was a continuous tendency towards a simultaneous loss of TK loading and catalytic activity (data described later). This behavior must be related to the increase of Zn^{2+} and Al^{3+} quantities in the supernatant, as measured by ICP-AES analysis (data not shown). Obviously, under high amounts of TK extract ($Q_{in} > 0.5$), the Zn_2Al matrix undergoes a slight dissolution that affects TK immobilization yield. Similarly, the lower performance of the coprecipitation method may account for the reduction of the coprecipitation yield under higher amounts of TK extract ($Q_{in} > 0.5$). In the coprecipitation process, the inorganic salts and the base were simultaneously added to the TK extract suspension and stirred at the required pH (8.00) for three hours. Formation of Zn^{2+} or Al^{3+} complexes with available in-extract ligands, such as amino acids, polypeptides or even glycylglycine buffer, may occur. For instance, complexation of Zn^{2+} by amino acids has been extensively reported in the literature.[44]

For a better understanding of these results, we determined the experimental TK-to-LDH weight ratio (Q_{exp}). Q_{exp} values were correlated to Q_{in} values in order to compare the immobilization capacity of both as modified and *in situ*-coprecipitated Zn_2Al LDH (Figure 4, lower panel).

The Q_{exp} values obtained for LDH-TK_{ad} [Figure 4 (lower panel ■)] were found to correlate well with the corresponding Q_{in} values over the full Q_{in} range. In this concentration range, no saturation is obtained, which suggests that greater amounts of TK could be loaded if $Q_{in} > 1.0$ was used. Almost 100% of the protein amount is adsorbed up to $Q_{in} = 0.50$. Over $Q_{in} > 0.50$, there is a slight deviation to the possible maximum adsorption ($Q_{exp} < Q_{in}$). This was explained by a slight dissolution of Zn_2Al in the presence of higher protein extract residues, as confirmed by ICP-AES chemical analysis of Zn^{2+} in the equilibrium solution.

The Q_{exp} values (0.13–0.39) for LDH-TK_{co} [Figure 4 (lower panel, ◇)] did however appear to be significantly lower than the corresponding Q_{in} values (0.25–1.0). In particular, the amount of LDH actually produced during coprecipitation decreased (from 25 to 19.9 mg) significantly at Q_{in} values higher than 0.38,

as demonstrated by ICP-AES analysis. The best correlation between Q_{exp} and Q_{in} was achieved for a Q_{in} of 0.38 for the LDH-TK_{co} bionanocomposites. This supported the hypothesis that the coprecipitation of LDH was not favored at higher amounts of protein (>9.2 mg).

Using the adsorption method gave higher immobilization yields (64–82%) than those achieved by coprecipitation (25–36%). An evaluation of adsorption *vs.* coprecipitation for Zn_RAl-urease[36f] revealed that adsorption was clearly less efficient than protein embedding by the direct coprecipitation route. However, using a fresh suspension of non-aggregated LDH platelets, in this study, clearly increased the amount of adsorbed proteins. TEM images showed that the Zn_2Al-TK_{ad} preparation and adsorption conditions used prevented crystal growth and aggregation by Ostwald ripening of individual LDH particles. As-prepared LDH platelets offer a higher surface for adsorption than rehydrated platelets. On the other hand, coprecipitation cannot necessarily ensure a high enzyme immobilization yield when metal cations may be complexed by ligands preventing them precipitating.

Catalytic Activity of Zn_2Al-TK Obtained by Adsorption and Coprecipitation

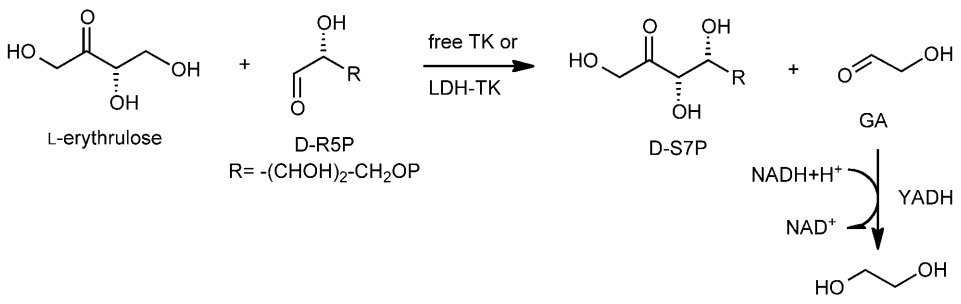
TK activity was determined in the presence of L-erythrulose as donor and D-ribose 5-phosphate (D-R5P) as acceptor. L-erythrulose, a non-natural substrate, was used in order to replace D-xylulose 5-phosphate, a natural donor substrate not commercially available (Scheme 2).

This assay was chosen because it allows a direct measurement of TK initial velocity by following the formation of glycolaldehyde (GA) over time using alcohol dehydrogenase (YADH) and NADH by spectrophotometry at 340 nm[45]. The specific catalytic activity of free TK extract was 4.5 $U \cdot mg^{-1}$. The results of the catalytic activity determination of TK highlighted the major interest of LDH as embedding matrices. For both series of biohybrid catalysts Zn_2Al-TK_{ad} and Zn_2Al-TK_{co}, catalytic activity follows the same tendency as immobilization yield as Q_{in} varied (Figure 4, upper panel). Moreover, TK activity was always as high as could be expected from the amount and even higher for $Q_{in} > 0.5$.

It can be concluded that TK keeps its full activity once in LDH, whatever the immobilization process. On testing the Zn_2Al-TK_{co} and Zn_2Al-TK_{ad} biohybrids, optimal relative TK activities (98–99%) were achieved when an initial protein-to-LDH Q_{in} weight ratio of about 0.38 was used. For other TK loadings ($Q_{in} \neq 0.38$), percent catalytic activity was nearly equal to immobilization percentage or in some cases even greater, as mentioned above. Greater TK activity than expected for full immobilization can be explained by a strong selective affinity of Zn_2Al LDH matrices toward TK proteins.

Influence of Intralayer LDH Cations (Zn^{2+} *vs.* Mg^{2+}) on Immobilization Yield and TK Activity

The influence of the chemical composition of the LDH layer (Mg_2Al *vs.* Zn_2Al) obtained for both processes on the immobilization yields and on TK activity was examined for different TK loadings. Results are compiled in Table 1 for $Q_{in} = 0.38$, which was considered the best condition for optimal Zn_2Al-TK immobilization and activity. As shown in Table 1, the immobilization of TK in Mg_2Al was comparable to that of Zn_2Al in terms of both immobilization yield and activity, whatever the method of preparation. Although Zn_2Al-TK and Mg_2Al-TK display different basic properties, Mg_2Al-TK being more basic, this difference does not affect the catalytic activity of the embedded TK. Even though the coprecipitation pH was higher for Mg_2Al-TK (pH 9.0), Mg_2Al-TK_{co} displayed a similar activity to the other three samples for $Q_{in} = 0.38$. In order to assess whether the pH used had a negative effect on free TK activity, TK was left stirring at pH 9.0 for three hours in similar conditions to Mg_2Al-TK coprecipitation. Measuring back the cat-



Scheme 2. Assay used for free or immobilized TK activity determination in the presence of L-erythrulose and D-ribose 5-phosphate (D-R5P) as substrates leading to D-sedoheptulose 7-phosphate (D-S7P) and glycolaldehyde (GA).

Table 1. Immobilization yields and TK enzymatic activities obtained for Mg_2Al-TK and Zn_2Al-TK LDH prepared by either coprecipitation or adsorption for a protein-to-LDH (Q_{in}) weight ratio of 0.38 and 1.

LDH matrix	$Q_{in}=0.38$		$Q_{in}=1$	
	Immobilization yield [%]	Specific activity [$U\cdot mg^{-1}$][a]	Immobilization yield [%]	Specific activity [$U\cdot mg^{-1}$][a]
Mg_2Al-TK_{co}	96	97	36	24
Mg_2Al-TK_{ad}	95	100	82	54
Zn_2Al-TK_{co}	97	100	25	37
Zn_2Al-TK_{ad}	98	100	64	83

[a] TK extract activity was measured in the presence of L-erythrulose as donor and D-R5P as acceptor: one unit (U) of TK leads to 1 μmol of glycolaldehyde per minute and per mg of proteins at pH 7.5 (glycylglycine 50 mM buffer) and at 25 °C.

alytic activity in standard conditions (pH 8.0), the enzyme was found to recover only 80% of its initial activity (results not shown). Clearly, precipitation of TK with Mg_2Al matrix has a protective effect against partial basic inhibition due to an embedding of the enzyme, starting from the beginning of the coprecipitation reaction.

However, measurements of immobilization yields and catalytic activities for higher Q_{in} demonstrated a better behavior for Zn_2Al-TK than for Mg_2Al-TK, particularly at $Q_{in}=1.0$. Indeed, even if immobilization yields proved better for Mg_2Al-TK (82% and 36% for Mg_2Al-TK_{ad} and Mg_2Al-TK_{co}, respectively, *vs.* 64% and 25% for Zn_2Al-TK_{ad} and Zn_2Al-TK_{co}), the relative activities were far greater for Zn_2Al-TK (54% and 24% for Mg_2Al-TK_{ad} and Mg_2Al-TK_{co}, respectively, *vs.* 83% and 37% for Zn_2Al-TK_{ad} and Zn_2Al-TK_{co}). However, at these Q_{in} values, it is important to take into account the formation of secondary phases (as shown on X-ray data) which may not ensure TK protection. For the Mg_2Al matrix, the drop in TK activities, particularly for Mg_2Al-TK_{co}, could be partially explained by a lower Mg_2Al-mediated protective effect against partial basic inhibition than at for $Q_{in}=0.38$.

For all these reasons, Zn_2Al-TK appeared to be more suitable for TK catalytic activity than Mg_2Al-TK over a larger Q_{in} range. This preliminary study led to encouraging results using Mg_2Al and Zn_2Al for both immobilization yield and activity, provided that Q_{in} value was lower than 0.50. In both adsorption and coprecipitation scenarios, a Q_{in} of 0.38 was identified as the optimum, since it gave excellent immobilization yields ($\geq 98\%$) and relative activities ($\geq 99\%$). Therefore, this Q_{in} value (0.38) was used from then on for all LDH-TK biohybrid synthesis.

Kinetic Parameters of the Free TK and the Zn_2Al-TK

The effect of donor substrate (L-erythrulose) and acceptor substrate (D-R5P) concentrations was determined for the reactions catalyzed by free TK and by Zn_2Al-TK obtained by adsorption and coprecipitation. The kinetic parameters were determined using the standard Michaelis–Menten model and compared to those obtained for the free TK. The results obtained by the Lineweaver–Burk plot ($n=3$) are summarized in Table 2. V_{max} is the maximum rate of reaction ($U\cdot mg^{-1}$), K_m is the Michaelis constant (μM).

For L-erythrulose and D-R5P, comparable K_m values ($3.0–5.9\cdot10^3$ μM and 79–152 μM, respectively) were obtained for the free[14,45] and Zn_2Al-TK-immobilized enzymes, showing that the affinity of these substrates for TK remained unchanged after immobilization on LDH.

Similar V_{max} values in the presence of the free TK[14,45] and Zn_2Al-TK-immobilized enzymes were obtained for L-erythrulose (10.9–12.1 $U\cdot mg^{-1}$) and D-R5P (12.1–15.1 $U\cdot mg^{-1}$). This suggested that immobilization did not alter the velocity of the reaction towards L-erythrulose and D-R5P.

These results showed that K_m and V_{max} values of Zn_2Al-TK and free TK were fairly similar. The type

Table 2. Kinetic parameters obtained for free TK and the Zn_2Al-TK-immobilized enzyme (at $Q=0.38$).[a]

Biocatalyst	L-erythrulose		D-R5P	
	K_m [μM]	V_{max} [$U\cdot mg^{-1}$]	K_m [μM]	V_{max} [$U\cdot mg^{-1}$]
Free TK	$5.9\cdot10^3\pm200$	11.3 ± 0.8	100 ± 10	15.1 ± 0.9
Zn_2Al-TK_{co}	$3.0\cdot10^3\pm600$	12.1 ± 0.7	152 ± 8	15.1 ± 1.0
Zn_2Al-TK_{ad}	$6.5\cdot10^3\pm500$	10.9 ± 0.9	79 ± 9	12.1 ± 0.8

[a] Initial velocities of TK (free extract or Zn_2Al-TK) were measured by varying L-erythrulose and D-R-5-P concentrations in the presence of NADH and YADH by spectrophotometry at 340 nm. One unit (U) of TK leads to 1 μmol of glycolaldehyde per minute at pH 7.5 (glycylglycine 50 mM buffer) and at 25 °C.

Table 3. Influence of storage process (buffered solution or lyophilized powder at −18 °C) on relative activity (%) of free TK and Zn_2Al-TK.

Time [days]	Buffered solution relative activity [%][a]			Lyophilized powder relative activity [%][a]		
	Free TK	Zn_2Al-TK_{co}	Zn_2Al-TK_{ad}	Free TK	Zn_2Al-TK_{co}	Zn_2Al-TK_{ad}
0	100	100	100	100	100	100
30	80	96	92	99	99	98
60	74	94	88	88	97	96
90	70	88	76	83	92	85

[a] TK activities were measured using L-erythrulose and D-R-5-P in glycylglycine buffer (50 mM, pH 7.5) at 25 °C.

of support and immobilization process did not modify the kinetic parameters of TK. Moreover, substrates of the enzymatic reaction are free to diffuse through the LDH-TK biohybrid materials.

Influence of Storage Process on the Activity of the TK in Zn_2Al by Adsorption and Coprecipitation

We studied the influence of lyophilization on the free and Zn_2Al-TK activity at −18 °C and we compared the results with those obtained for free and Zn_2Al-TK stored in buffered solution at −18 °C. The TK activities were measured in the presence of L-erythrulose and D-R5P as substrates. Zn_2Al-TK nanocomposites were synthesized by either coprecipitation or adsorption at $Q_{in} = 0.38$.

First, we showed that after lyophilization of free TK and Zn_2Al-TK the initial TK activity was fully recovered. Thus, lyophilisation did not alter TK activity and provides a useful and efficient storage process for the TK. Second, we followed the TK activity over a three-month storage period. Table 3 indicates that free TK and Zn_2Al-TK lyophilized powders retained between 83–92% of their initial activity after three months of storage at −18 °C. These relative activities were better than those (70–88%) obtained for free and Zn_2Al-TK stored in buffered solution at −18 °C.

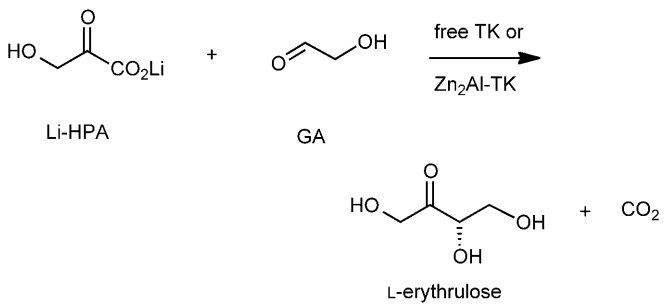
Whatever the storage process used, substantial activities (89–92%) were conserved for the Zn_2Al-TK_{co} over three months in storage, whereas lower activities (76–85%) were observed for the Zn_2Al-$TK_{ad.}$ material. This may be explained by a possible desorption of the proteins from the support when the adsorption method was used. This indicated that coprecipitation produced more robust LDH-TK biohybrid materials.

Synthesis of L-erythrulose Catalyzed by the Zn_2Al-TK Nanocomposites

We chose an established test reaction[40] from hydroxypyruvate lithium salt (Li-HPA) as donor substrate rendering the reaction irreversible by carbon dioxide release and glycolaldehyde (GA) as acceptor substrate in order to study the efficiency of the Zn_2Al-TK biohybrid material over time. The synthesis of L-erythrulose was performed on a preparative scale (Scheme 3).

The catalytic efficiency of the Zn_2Al-TK was compared to that of the free TK, and control reactions were set up without catalyst and with only Zn_2Al LDH. The conversion of Li-HPA over time was quantified by spectrophotometry at 340 nm from an aliquot of the reaction mixture in the presence of L-lactate dehydrogenase as auxiliary enzyme and NADH. The results are illustrated in Figure 5. The inertia of Zn_2Al toward TK substrates was confirmed by control reactions realized without TK (Figure 5). In the presence (◆) or not (×) of Zn_2Al, the decrease in Li-HPA was similar in both cases and was due to the slight decomposition of Li-HPA without enzyme.[5b]

When using the Zn_2Al-TK_{co} nanocomposites, total conversion of Li-HPA was observed after only a 30-minutes reaction time at 25 °C, which was comparable to the results obtained when the free TK was used. However, the catalytic activity of the LDH-TK_{ad} biohybrids was significantly lower than that of the LDH-TK_{co}. Under comparable conditions, the conversion of Li-HPA catalyzed by LDH-TK_{ad} was completed after a three-day reaction (data not shown). As shown by TEM images (Figure 3), the method used for the preparation of Zn_2Al-TK influenced the nanocompo-



Scheme 3. Synthesis of L-erythrulose catalyzed by free and Zn_2Al-TK in the presence of hydroxypyruvate lithium salt (Li-HPA) and glycolaldehyde (GA).

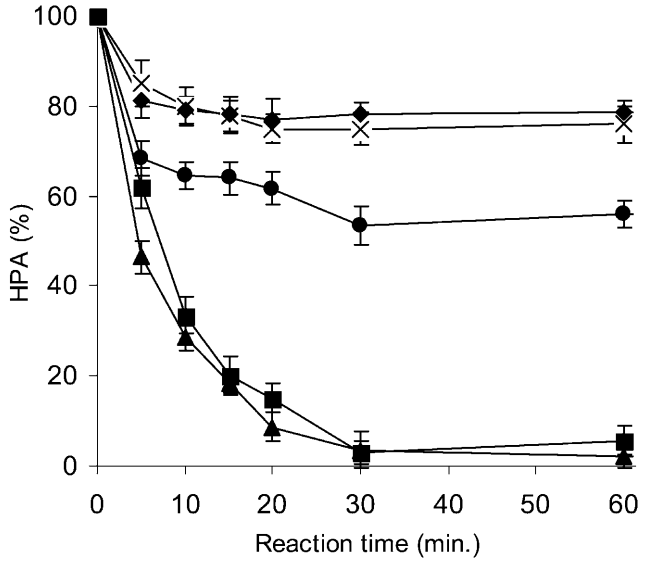


Figure 5. Conversion of Li-HPA (50 mM) in the presence of GA (50 mM), ThDP (2.4 mM), $MgCl_2$ (3.0 mM) in glycylglycine buffer (50 mM; pH 7.5) catalyzed by TK (3.5 $U{\cdot}mL^{-1}$), either on Zn_2Al-TK_{ad} (●) or Zn_2Al-TK_{co} (■) (Q = 0.38), or as a free suspension (▲). Control reactions in the presence of Li-HPA, GA, ThDP and $MgCl_2$ without Zn_2Al LDH and without TK (×) with Zn_2Al LDH only (◆).

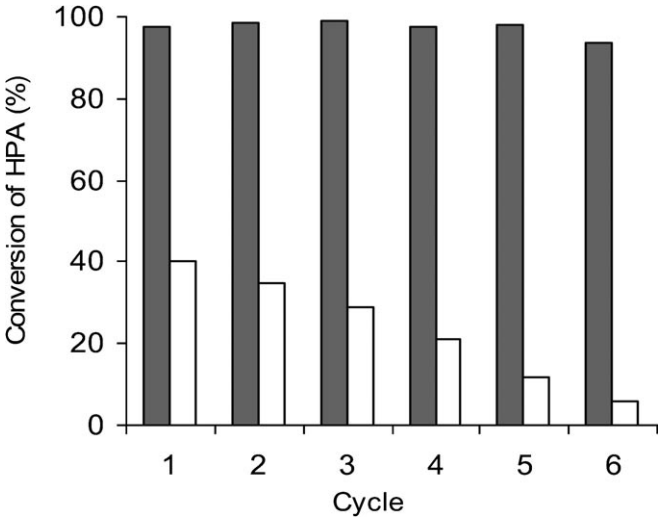


Figure 6. Reuse of Zn_2Al-TK (Q = 0.38). Zn_2Al-TK_{co} (gray) or Zn_2Al-TK_{ad} (white) were used to catalyze the synthesis of L-erythrulose. For each cycle, Zn_2Al-TK was added to Li-HPA (50 mM), GA (50 mM), ThDP (2.4 mM) and $MgCl_2$ (3 mM) in glycylglycine buffer (50 mM; pH 7.5), stirred at 25 °C for 30 min, and then centrifuged. Li-HPA was measured in the presence of L-lactate dehydrogenase and NADH by spectrophotometry at 340 nm.

site structure. LDH-TK_{ad} are highly aggregated materials, and substrate diffusion over time may be more difficult in LDH-TK_{ad} materials than in LDH-TK_{co} materials, leading to a slower conversion of Li-HPA.

The conversion rate of Li-HPA in the presence of TK and GA was calculated as 80%, taking into account the 20% degradation of Li-HPA without enzyme. L-erythrulose was produced with an isolated yield of 56%, and the optical rotation of the isolated product $[\alpha]_D^{25}$: +6.3 (*c* 1.1, H_2O) was comparable to that obtained for the free TK and to published values.[40] This indicated that the stereospecificity of TK was not altered through immobilization.

In summary, Zn_2Al-TK biohybrids were found to be highly compatible with the enzymatic synthesis of L-erythrulose starting from Li-HPA and GA.

Reusability of the Zn_2Al-TK Nanocomposites

The reusability of the Zn_2Al-TK biohybrids for catalyzing the synthesis of L-erythrulose was tested, and the results are shown in Figure 6. The LDH-TK_{co} enzyme was successfully used to catalyze the reaction between Li-HPA and GA with a constant Li-HPA conversion rate ($\geq$95%) observed over six cycles.

LDH-TK_{ad} did however lead to an 80% decrease of Li-HPA conversion after six cycles, probably due to leaching of the protein from the support. Furthermore, proteins were detected in the supernatant after centrifugation of the reaction mixture. This indicated a higher stability of the Zn_2Al-TK_{co} enzyme compared to Zn_2Al-TK_{ad} highlighting the interest of the *in situ* coprecipitation process.

Conclusions

Zn_2Al and Mg_2Al layered double hydroxides were successfully used as supports for the immobilization of TK according to a coprecipitation or an adsorption procedure. Best results were obtained under the optimized conditions of TK/LDH = 0.38 w/w. The protocol achieved high immobilization yields of the LDH-TK bionanocomposites with no significant catalytic activity loss. The stability of these biohybrids over storage as lyophilized powder was tested, and excellent activity was observed. For synthetic purposes, the coprecipitation process offered the same conversion rate as free TK, without any significant loss of activity over six cycles.

This cheap and efficient procedure revealed the promising potential of LDH supports for practical applications of TK, which could be extended to a wide range of TK acceptor substrates. The protective effect of LDH on TK should now be investigated against pH, temperature and organic solvents. The interesting properties of these bionanohybrids could find future applications for the development of microfluidic bioreactors.

Experimental Section

General

Chemicals and solvents were purchased from Aldrich and Acros and were reagent grade. Bradford reagent was obtained from Bio-Rad. Proteins and enzymes were obtained from Sigma.

Yeast cells were disrupted using a Constant Systems Limited One Shot cell disruptor. Centrifugation was carried out using a Beckman Coulter Avanti-J26 XP centrifuge. Lyophilization was carried out with a Serail RP2V Freeze dryer. Absorbance was measured using a Hitachi U-2010 UV-visible spectrophotometer. Merck 60 F254 silica gel TLC plates and Merck 60/40–63 mesh silica gel for Liquid Flash Chromatography (Armen Instruments Spot) were used. NMR spectra were recorded in D_2O on a Bruker Avance 400 spectrometer. The optical rotation was determined with a polarimeter Perkin–Elmer 241 at the given temperature and wavelength (Na D line: D = 589 nm) in a cell 10 cm long. $[\alpha]_D$ values are given in $10^{-1}\cdot deg\cdot cm^2\cdot g^{-1}$ (concentration given as g/100 mL).

Powder X-ray diffraction (PXRD) patterns of solids were recorded using a Philips X-Pert Pro diffractometer equipped with a graphite monochromator using Cu Kα radiation (λ = 0.15415 nm). Patterns were recorded over the 2–70° (2θ) range in steps of 0.0668 degree with a counting time per step of 400 s. Transform infrared (ATR-FT-IR) spectra were measured over the range 400–4000 cm^{-1} on an FT-IR Nicolet 5700 (ThermoElectron Corporation) spectrometer employing the KBr dilution technique. Transmission electron microscopy (TEM) images were taken using a Hitachi 7650 microscope at an acceleration voltage of 80 kV. Samples were dispersed in ethanol and then one droplet of the suspension was applied to a 400 mesh holey carbon-coated copper grid and left to dry in air. Chemical analyses of metals were performed by inductively coupled plasma atom emission spectroscopy (ICP-AES) using a Jobin Yvon/HORIBA ULTIMA 2C Series spectrometer.

Synthesis of the LDH-TK Nanocomposites

TK was immobilized on LDH using either coprecipitation or adsorption methods. All experiments were carried out under an N_2 atmosphere to prevent any competitive carbonization and the resulting precipitates were extracted as described in coprecipitation section.

Coprecipitation: LDH biohybrids were prepared following the procedure described by Vial et al.[35f] Typically, a mixed aqueous solution of $M(NO_3)_2\cdot 6H_2O$ (M = Mg, Zn) and $Al(NO_3)_2\cdot 9H_2O$ (M^{2+}/Al^{3+} molar ratio R = 2 and total molar concentration of metallic cations of 0.1 M) was added at a constant flow rate of 0.015 $mL\cdot min^{-1}$ to the TK extract (7 $U\cdot mg^{-1}$) suspended in glycylglycine buffer (8 mL, 50 mM, pH 7.5) in a four-neck glass vessel. TK per LDH amount (w/w) was fixed at Qin = 0.25, 0.38, 0.50, 0.75, 1.0 assuming total coprecipitation of the mineral matrix. The pH of the solution was maintained constant at either 8.0 (Zn_2Al LDH) or 9.0 (Mg_2Al LDH) during the coprecipitation by the simultaneous addition of a 0.1 M NaOH solution. The reaction was left stirring in an ice bath for three hours under N_2 pressure to avoid any possible contamination by atmospheric CO_2. The suspension thus obtained was centrifuged at 10,000 rpm for 20 min and washed three times with decarbonated water. The precipitate was recovered and stored at −18 °C either as a suspension (10 $mg\cdot mL^{-1}$) or as a powder. The supernatant was also collected and stored at −18 °C to allow quantifications of residual protein concentrations (hence immobilization yields). Samples were labelled Zn_2Al-TK and Mg_2Al-TK.

Adsorption: Adsorptions of TK by LDH were realized by contacting freshly prepared suspensions of coprecipitated LDH with a solution of enzyme. After washing, the fresh LDH suspensions (25 mg) were added to the TK yeast extract in glycylglycine buffer (50 mM, pH 7.5) and the reaction was left stirring under N_2 pressure in an ice bath for three hours. The precipitates were recovered as described for the coprecipitation.

Enzyme free LDH: $M_2Al(OH)(NO_3)_2\cdot n H_2O$ LDH (M = Mg and Zn) were synthesized and stored as described above but without any enzyme in the reaction medium.

Protein Assay

Protein concentration was determined using the Bradford assay. Aliquots (100 μL) of the TK crude extracts were added to the Bradford reagent (900 μL) and incubated in the dark for 10 min at 25 °C. Absorbance of the samples was measured at 595 nm. The concentration of protein in the samples was determined using a standard curve plotted against bovine serum albumin (BSA) at concentrations ranging from 0.2 to 1.2 $mg\cdot mL^{-1}$. Aliquots of the samples were diluted to the standard concentration range when needed. Immobilization yields, Q_{in} and Q_{exp} values were then calculated using Equation 1, Equation 2 and 3 respectively.

Equation 1: Immobilization yield (%) = amount of proteins (mg)/amount of total proteins (mg) × 100. Amounts of proteins were estimated by subtracting the amounts of proteins present in the supernatant to the total amounts of proteins.

Equation 2: Theoretical protein/LDH (Q_{in}): Q_{in} = total amount of proteins used (mg)/amount of maximum theoretical LDH formed (mg).

Equation 3: Experimental protein/LDH (Q_{exp}): Q_{exp} = amount of proteins (mg)/actual amount of LDH formed (mg). Actual amounts of LDH formed were estimated using ICP-AES.

Transketolase Enzymatic Activity

TK enzymatic assay was performed in the presence of L-erythrulose and D-ribose 5-phosphate (D-R5P) leading to D-sedoheptulose 7-phosphate (D-S7P) and glycolaldehyde (GA).[45] The GA formed is reduced by yeast alcohol dehydrogenase (YADH) to glycol in the presence of nicotine adenine dinucleotide, reduced form (NADH). L-erythrulose (88 mM, 10 $mg\cdot mL^{-1}$), D-R5P (3.0 mM, 0.8 $mg\cdot mL^{-1}$), ThDP (2.4 mM, 1.1 $mg\cdot mL^{-1}$), $MgCl_2$ (3.0 mM, 0.6 $mg\cdot mL^{-1}$), NADH (140 μM, 0.1 $mg\cdot mL^{-1}$), YADH (50 $U\cdot ml^{-1}$, 0.15 $mg\cdot mL^{-1}$) and either the TK suspension (10 μL) or the TK powder (5 mg) were added into disposable plastic cuvettes and completed to 1 mL with glycylglycine buffer (50 mM, pH 7.5). The disappearance of NADH was followed at 340 nm by spectrophotometry (value ε_{340} = 6220 $M^{-1}\cdot cm^{-1}$).

Transketolase Production

TK from yeast was produced from *S. cerevisiae* strain H402×pTKL1 (which was kindly donated by Prof. G. Schneider, Sweden). Yeast culture was prepared according to Wikner et al. (1994).[14] TK yeast extraction was conducted from cell pellets obtained after centrifugation. Typically, 10 g of cells were suspended in 100 mL of glycylglycine buffer (0.1 M, pH 7.5) and lysis was carried out using a cell disruptor at a pressure of 2.0 kbar. The crude lysate was then centrifuged at 15,000×*g* for 20 min at 4 °C. The precipitate was removed. The supernatant was recovered and stored at −18 °C in solution or as a lyophilized powder. Protein content in the supernatant was 1.25 ± 0.25 mg·mL^{-1}. The TK crude extract had an activity of 5.2 ± 1.8 U·mL^{-1} and a specific activity of 4.5 ± 1.1 U·mg^{-1}.

Synthesis of L-erythrulose Catalyzed by the Zn_2Al-TK Nanocomposites

Synthesis of L-erythrulose:[40] The synthesis wase performed from glycolaldehyde (GA) and hydroxypyruvate lithium salt (Li-HPA) prepared according to the procedure described in the literature.[46] The substrates Li-HPA (50 mM), GA (50 mM) and the cofactors ThDP (2.4 mM), $MgCl_2$ (3.0 mM) were dissolved in 5 mL of glycylglycine buffer (50 mM). Once the pH of the solution was adjusted to 7.5 with 0.5 N NaOH, TK (35 U·mL^{-1}) either on Zn_2Al LDH (Q = 0.38) by adsorption or coprecipitation, or free TK suspension was added. The reactions were stirred at 25 °C. The conversion of Li-HPA was monitored at 340 nm from an aliquot of the reaction mixture in the presence of L-lactate dehydrogenase (3 U·mL^{-1}, 0.03 mg·mL^{-1}) and NADH (140 μM, 0.1 mg·mL^{-1}). After total conversion of Li-HPA, the reaction mixture was discarding by centrifugation 10,000×*g* for 20 min. For the reaction catalyzed by free TK three volumes of CH_3OH were added to the reaction mixture before centrifugation. The supernatant was collected and concentrated under reduced pressure. The crude product was purified by liquid flash chromatography using dichloromethane/methanol v/v 80:20 as eluent to give a colorless oil; yield:16.8 mg (56%). ^{1}H NMR (400 MHz, D_2O): δ = 4.58 [1H, d, $J_{H,H}$ = 19.4 Hz, C(1)H], 4.51 [1H, d, $J_{H,H}$ = 19.4 Hz, C(1)H], 4.44 [1H, t, $J_{H,H}$ = 4.1 Hz, C(3)H], 3.85 [1H, dd, $J_{H,H}$ = 4.1 Hz, $J_{H,H}$ = 12.1 Hz, C(4)H], and 3.83 [1H, dd, $J_{H,H}$ = 4.1 Hz, $J_{H,H}$ = 12.1 Hz, C(4)H]; ^{13}C NMR (100 MHz,D_2O): δ = 65.8 (C1), 212.3 (C2), 75.8 (C3), 62.9 (C4); MS (HR-ESI): *m*/*z* = 143,0335 (M + Na), calcd. for $C_4H_8NaO_4$: 143,0320; $[\alpha]_D^{25}$: +6.3 (*c* 1.1,H_2O), lit. $[\alpha]_D^{25}$: +6.7 (*c* 1.48, H_2O).[40]

Reusability of Zn_2Al-TK: For the reuse of Zn_2Al-TK obtained by adsorption and coprecipitation, the reaction mixture was centrifugated at 10,000 rpm for 20 min and the precipitate was washed three times with Tris buffer (50 mM, pH 7.5). The precipitate (containing the LDHs-TK bionanocomposites) was recovered, a fresh batch of substrates was then added and the experiment was repeated 5 times as described above. The conversion rate of Li-HPA after 30 min was estimated by measurement of Li-HPA concentration in the presence of L-lactate dehydrogenase and NADH as described above.

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