Biosensors and Bioelectronics 62 (2014) 90–96

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

An efficient amperometric transketolase assay: Towards inhibitor screening

Nadia Touisni, Franck Charmantray, Virgil Hélaine, Laurence Hecquet *, Christine Mousty *

Clermont Université, Université Blaise Pascal, Institut de Chimie de Clermont-Ferrand, UMR-CNRS 6296, F-63000 Clermont-Ferrand, France

ARTICLE INFO

Article history:
Received 6 March 2014
Received in revised form
16 May 2014
Accepted 3 June 2014
Available online 14 June 2014

Keywords:
Transketolase
Galactose oxidase
Amperometric biosensor
Clay modified electrode
Inhibition
Layered double hydroxides.

ABSTRACT

This paper describes an innovative amperometric biosensor for the *in vitro* determination of activity of transketolase from *Escherichia coli* (TK*ec*) using commercially available TK substrates, namely D-fructose-6-phosphate a physiological donor and glycolaldehyde the best non-phosphorylated acceptor. A galactose oxidase (GAOx) biosensor, based on the immobilization of this enzyme within laponite clay, allows amperometric detection of L-erythrulose released upon TK-catalyzed reaction. A calibration curve has been established from 0.01 to 0.1 U ml^{-1} TK*ec* concentration in solution. These data are comparable to that obtained by a fluorometric method. In order to ensure a higher sensitivity and re-usability of the system, an original bienzymatic sensing system was further developed based on apoenzyme TK*ec* and GAOx separately immobilized on the electrode surface. The inner sensing layer contains GAOx@laponite and the outer layer TK*ec*@layered double hydroxide biohybrid. The biosensor response was validated by the determination of K_D^{app} for thiamine diphosphate, the TK cofactor and the inhibition action of two commercially available products, pyrophosphate, a TK cofactor analog and D-arabinose-5-phosphate, a substrate analog.

1. Introduction

Transketolase (TK; EC 2.2.1.1), a thiamine pyrophosphate (ThDP)-dependent enzyme, is a key enzyme in the non-oxidative branch of the pentose phosphate pathway. TK catalyzes the stereospecific formation of a C–C bond by a reversible transfer of the C1–C2 ketol unit from a ketose phosphate to an aldose phosphate. The new asymmetric center stereospecifically formed has an absolute (S) configuration (Scheme 1).

TK is a ubiquitous enzyme. The 3D protein crystal structures of the microbial TKs from *Escherichia coli* (Littlechild et al., 1995), *Saccharomyces cerevisiae* (Sundstrom et al., 1993), *Bacillus anthracis* (Maltseva et al., 2009) and human TK (Mitschke et al., 2010) have been resolved and show high structural homologies. All these TKs are homodimers with two active sites located at the interface between the contacting monomers. Both ThDP and divalent cations (Mg^{2+}) are strictly needed for their activity.

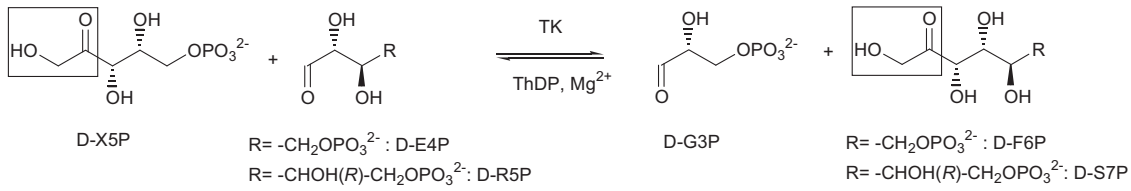
The catalytic properties and metabolic importance of TK support applications in biocatalysis for obtaining chiral polyols from non-physiological substrates and also in medicine. Indeed, some recent studies have shown that human TK is a target of neurodegenerative diseases (Zhao and Zhong, 2009), diabetes (Thornalley et al., 2001) and particularly cancer (Bentz et al., 2013; Du et al., 2004; Thomas et al., 2008a, 2008b) suggesting new therapeutic approaches. Therefore, the development of rapid, sensitive and efficient *in vitro* assays for TK activity profiling is highly sought to enable the identification of suitable inhibitors. In this context, new assays for TK activity detection have been recently developed based on cascade reactions involving one or more auxiliary enzymes and/or protein and/or non-protein agents.

The conventional method for measuring TK activity uses D-ribose-5-phosphate (D-R5P) as acceptor and D-xylulose-5-phosphate (D-X5P) as donor (Horecker and Smyrniotis, 1955). D-glyceraldehyde-3-phosphate (D-G3P) generated upon cleavage of the two-carbon unit from this donor substrate can subsequently be interconverted to dihydroxyacetone phosphate (DHAP) using triose phosphate isomerase. DHAP is then reduced into D-glycerol-3-phosphate using nicotinamide adenine dinucleotide NADH-dependent glycerol-3-phosphate dehydrogenase. Changes in NADH concentration can be monitored by either spectrophotometric absorbance at 340 nm or by fluorescent intensity with excitation at 330 nm and emission at 450 nm (Du et al., 2004). This method has been used for the identification of novel small-molecule inhibitors with high specificity and potency for human TK.

Because commercial supplies of D-X5P become limited, and because this compound is difficult to synthesize in a pure form (Zimmermann et al., 1999), similar multi-enzyme assays have been recently reported using systems able to generate this donor *in situ*

* Corresponding authors.
E-mail addresses: Laurence.Hecquet@univ-bpclermont.fr (L. Hecquet), Christine.Mousty@univ-bpclermont.fr (C. Mousty).

http://dx.doi.org/10.1016/j.bios.2014.06.019
0956-5663/



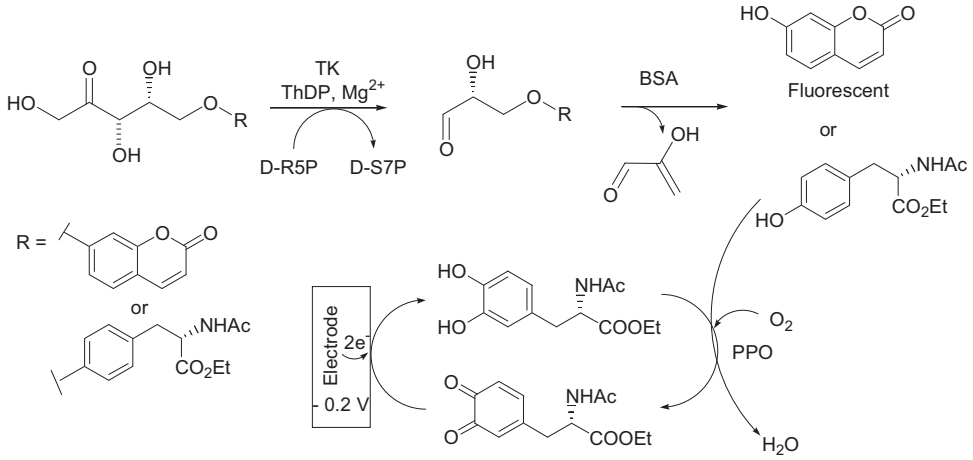
Scheme 1. *in vivo* TK reaction.

(Lee et al., 2008). Other donor substrates have been investigated such as D-fructose-6-phosphate (D-F6P) (Naula et al., 2008) or L-erythrulose (L-ERY) (Hecquet et al., 1993). In these cases the released aldehydes, D-erythrose-4-phosphate (D-E4P) or glycolaldehyde (GA) respectively, are reduced by an NADH-dependent dehydrogenase. More recently, Lithium β-hydroxypyruvate, a non-physiological donor substrate has been used with different acceptors in colorimetric assays using 2.3.5-triphenyltetrazolium chloride (tetrazolium red) (Smith et al., 2006) or phenol red (Yi et al., 2012). This last assay is rapid, easy, inexpensive and applicable to high throughput screening of a wide range of different aldehydes as TK acceptors, but not suitable for the detection of very low TK activity particularly in the case of TK inhibition studies.

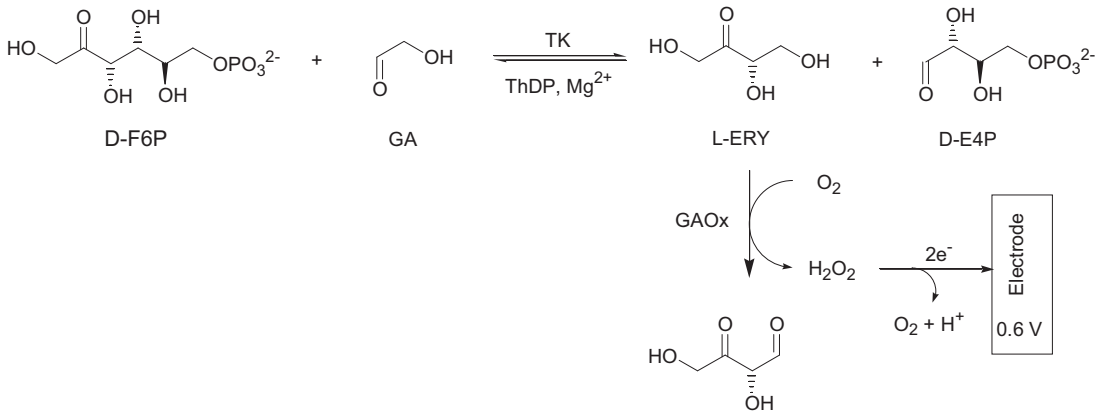
Alternative and more sensitive TK detection systems using fluorescence (Charmantray et al., 2010; Sevestre et al., 2003, 2006) or amperometry (Sanchez-Paniagua Lopez et al., 2010) technologies have been investigated in the last few years. In these cases, the donor substrates are not commercially available and thus are specially designed and synthesized. Such methods require a coupling reaction to produce the physical signal, catalyzed by either a weak base, usually the auxiliary protein Bovine Serum Albumin (BSA), or a mixture of BSA and another enzyme (Scheme 2). Although very sensitive, these multi-enzymatic cascade assays require the multi-step synthesis of specialized TK probes that prevents their use for the measurement of a large number of samples.

In this paper, we propose an amperometric biosensor for TK*ec* activity detection based on an auxiliary enzyme, galactose oxidase (GAOx), immobilized within laponite at the surface of the electrode (Charmantray et al., 2013) and working with commercially available TK substrates, such as D-F6P as physiological donor and GA, the best non-phosphrorylated acceptor. L-ERY formed by TK-catalyzed reaction was directly detected by galactose oxidase. The electro-oxidation of hydrogen peroxide released was monitored at the underlying platinum electrode (Scheme 3). This GAOx@laponite biosensor was first optimized for the detection of TK*ec* activity in solution. This system offers an original, rapid and more sensitive assay than that previously described.

The immobilization of TK*ec* on its apoenzyme form (ApoTK*ec*) onto a surface is of particular interest for the screening of ThDP or substrate analogs as TK inhibitors, for instance. Recently, we have optimized immobilization of ApoTK*ec* within layered double hydroxides (LDH), an inorganic lamellar material (Touisni et al., 2013). The resulting Tk*ec*@LDH biohybrid material, prepared by the coprecipitation method, depicts a stable, reproducible and high enzymatic activity for immobilized TK*ec*. Hence, an original bienzymatic sensing system was further developed based on ApoTK*ec* and GAOx separately immobilized on the electrode surface. The inner sensing layer contains GAOx@laponite and the outer layer TK*ec*@LDH biohybrid. The efficiency of this biosensor was demonstrated through the detection of few TK inhibitors. Indeed, several synthetic compounds and natural products have been reported to inhibit TK from different sources. A few donor and acceptor substrates analogs have been studied as TK inhibitors, *p*-hydroxyphenylpyruvate (Solovjeva and Kochetov, 1999) and D-arabinose-5-phosphate (D-A5P) (Sprenger et al., 1995). Most of the studies are focused on analogs of the TK cofactor, ThDP, for developing effective anticancer therapeutic agents. The importance of each part of the ThDP for its linking to the apoenzyme has been first considered (Kochetov et al., 1971). The most representative inhibitors that mimic the interactions with ThDP are oxythiamine (Raïs et al., 1999) and thiamine thiazolone diphosphate (Nilsson et al., 1993). More recently, in the aim of obtaining more selective TK inhibitors several thiamine antagonists (Le Huerou et al., 2008; Thomas et al., 2008a, 2008b) and also a novel family of compounds based on critical allosteric points of enzyme were designed (Obiol-Pardo et al., 2012). The bienzymatic biosensor based on TK-GAOx proposed in this paper may enable a sensitive and generic detection of TK inhibitors by the decrease of oxidation current of enzymatic generated hydrogen peroxide.



Scheme 2. TK assays based on fluorometric and amperometric detection.



Scheme 3. TK assay based on GAOx@laponite biosensor.

2. Experimental

2.1. Reagents and materials

Galactose oxidase (GAOx, EC 1.1.3.9) from *Dactylium dendroides* (76 U mg^{-1}) was purchased from Worthington. Dihydroxyacetone (DHA), D-fructose-6-phosphate (D-F6P), glycolaldehyde (GA), L-erythrulose (L-ERY), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), glycyl-glycine (GlyGly), thiamine (Th), thiamine monophosphate (ThMP), thiamine pyrophosphate (ThDP), pyrophosphate, oxythiamine and D-arabinose-5-phosphate (D-A5P), D-ribose-5-phosphate (D-R5P), were purchased from Sigma. Laponite was obtained from Rockwood Specialities, Inc. (Princeton, NJ). Those reagents were of analytical grade and used without further purification.

TK*ec*@MgAl-GlyGly$_{cop}$ biohybrid was prepared by the coprecipitation route in the presence of TK*ec* (Touisni et al., 2013). A 0.1 M total molar concentration of metallic cations $Mg(NO_3)_2 \cdot 6H_2O$ and $Al(NO_3)_3 \cdot 9H_2O$ (Mg^{2+}:Al^{3+} molar ratio $r=2$) was added at a constant flow rate of 0.015 mL min^{-1} to 10 mL of a 0.025 M Gly-Gly buffer solution containing 10 mg of TK*ec*. The TK*ec*/LDH amount (w/w) was fixed at $Q_{th}=0.5$ for a theoretical amount (30 mg) of coprecipitated biohybrid. The pH of the solution was kept constant at 9.0 during the coprecipitation by the simultaneous addition of a 0.1 M NaOH solution. The reaction was left under stirring on ice bath for three hours and under N_2 pressure to avoid any possible contamination by atmospheric CO_2. The as-prepared material was recovered by centrifugation then suspended in 0.025 M Gly-Gly buffer solution (5 mg mL^{-1}, pH 7.5) before storage at 4 °C.

2.2. TK production and activity

N-terminal His_6-tagged *E. coli* transketolase A (TKtA gene) was expressed from *E. coli* BL21 (DE3) pET21 recombinant strain. Extraction of TK*ec* was conducted from cell pellets obtained after centrifugation. Cell paste was resuspended in lysis buffer (50 mM NaH_2PO_4 and 10 mM imidazole, pH 8) and cell lysis was performed by ultrasonication at 4 °C, under stirring. TK*ec* was purified from the cell-free extract using affinity chromatography on Ni-NTA resin. TK*ec* was eluted with 250 mM imidazole, and then ultrafiltrated for buffer exchange with 100 mM Gly-Gly buffer, pH 7.5, to obtain TK*ec* on its apoenzyme form. Purified apo-TK*ec* was then frozen and stored at −18 °C.

Enzyme activity of free TK*ec* was determined through a couple enzymatic continuous assay. In the first step, TK*ec* catalyzes the interconversion of L-ERY (85 mM, 120 mg mL^{-1}, 100 μL) as donor substrate and D-R5P (8.9 mM, 50 mg mL^{-1}, 50 μL) as acceptor substrate leading to D-sedoheptulose-7-phosphate (D-S7P) and GA as the products (Hecquet et al., 1993). The reaction was carried out in Gly-Gly buffer (100 mM, pH 7.5, 800 μL) with ThDP (106 μM, 10 mg mL^{-1}, 5 μL) and $MgCl_2$ (493 μM, 10 mg mL^{-1}, 10 μL) as cofactors. In a second step the GA formed was reduced by yeast alcohol dehydrogenase (25 units) to glycol in the presence of NADH (282 μM, 10 mg mL^{-1}, 10 μL, $\varepsilon_{340}=6220\ M^{-1}\ cm^{-1}$). The consumption of NADH was followed spectrophotometrically at 340 nm. TK activity was defined as the rate of formation GA and expressed in U mL^{-1} (μmol min^{-1} mL^{-1}). Specific activity at 25 °C was expressed as units of enzyme activity per mg of protein. Under our conditions, it was found 35 ± 2 U mg^{-1}.

The dissociation constant (K_D) for ThDP was determined using the previous assay. TK activities were measured for various ThDP concentrations ranging from 1 to 100 μM. K_D was determined from the linearization of the Michaelis-Menten curve by the Lineweaver Burk method.

2.3. Preparation of bioelectrodes

GAOx@laponite bioelectrodes were prepared following the same protocol described elsewhere (Charmantray et al., 2013). A colloid suspension of laponite (5.0 mg mL^{-1}) was dispersed overnight under stirring conditions in pure water. A drop (20 μL) of an aqueous mixture containing 50 μg GAOx and 25 μg laponite was spread on the surface of Pt electrode ($\Phi=0.5$ cm) previously polished with diamond paste (1 μm) (Charmantray et al., 2013). The coating was dried in the air at 4 °C overnight. The resulting electrode was then placed under saturated glutaraldehyde vapor for 15 min for the cross-linking of the membrane. The repeatability of the fabrication of the GAOx@laponite biosensor was tested with five different electrodes, the RSD is $<5\%$ for DHA determina]tion.

The bi-enzyme TK*ec*@LDH-GAOx@laponite biosensors were prepared by the sequential deposition of two inorganic biocoatings composed of GAOx@laponite and TK*ec*@MgAl-GlyGly$_{cop}$ biohybrid materials. The first layer containing the GAOx@laponite was prepared as described before but in this case the reticulation step with glutaraldehyde was omitted. Then, 50, 75 or 100 μg of the TK*ec*@MgAl-GlyGly$_{cop}$ suspension were deposited on the surface of the GAOx@laponite modified electrode and dried in the air at room temperature for 2.5 h. Finally, the bioelectrodes were rehydrated for 15 min by soaking in 0.05 M HEPES buffer solution (pH 7.0).

2.4. Electrochemical experiments by chronoamperometry

Electrochemical experiments were carried out with a potentiostat EA161 (EDAQ) connected to a thermostated cell (25 °C) with a

three-electrode system. A saturated Ag/AgCl electrode was used as reference electrode and a Pt wire was used as counter electrode. For GAOx@laponite biosensors, the steady-state chrono-amperograms (*I vs t*) were recorded at +0.6 V/Ag–AgCl under a rotating speed of 500 rpm in 5 mL of air saturated 0.05 M HEPES buffer solutions (pH 7.0). After baseline stabilization under applied potential, several compounds (or analytes) were assayed as possible GAOx substrates, namely dihydroxyacetone (DHA), L-ERY, GA or D-F6P. Successive additions of concentrated stock solutions of these analytes in 5 mL electrolyte solution were carried out. After every single addition of analyte, the current response was measured after its stabilization. The linear range of the calibration plots was fixed according to the best correlation coefficient (R^2) sensitivity was expressed as the slope of these plots. The reported values corresponded to the mean values of at least three independent measurements.

The amperometric signal at GAOx@laponite biosensor with TK in solution or at the bi-enzyme TK*ec*@LDH-GAOx@laponite biosensor was recorded under steady state condition (500 rpm) in 5 mL of 0.05 M HEPES buffer solution (pH 7.0) containing 0.2 mM ThDP, 1 mM $MgCl_2$, after the successive additions of (1) 1 or 2 mM GA as donor substrate, (2) D-F6P as acceptor substrate at 0.1, 1 or 5 mM and (3) 3×5 μM DHA as an internal reference.

TK inhibition experiments were carried out following two kinds of protocols depending on the chemical nature of the putative inhibitor assayed. While the inhibition action of the TK substrate analog can be directly analyzed after the addition of D-F6P within the reaction mixture, ThDP analogs instead were incubated for one hour in 0.05 M HEPES buffer solution containing 1 mM Mg^{2+}, 5 μM ThDP and different concentrations of inhibitors before assaying TK residual activity. After 1 h of incubation, the TK*ec*@LDH-GAOx@laponite biosensor was transferred into the electrolyte solution containing neither inhibitor norcofactor and the amperometric response was recorded as described before. To establish the calibration curves, all measurements were carried out in triplicate and data points represent the mean values of the determination.

3. Results and discussion

3.1. Amperometric responses of GAOx@laponite biosensor

An optimized single-enzyme GAOx@laponite modified electrode was reproduced as described elsewhere (Charmantray et al., 2013). This biosensor was aimed at detecting several analytes, namely DHA, its best substrate, and also L-ERY, never described as GAOx substrate and required in our TK/GAOx coupled system. Other substrates involved in the TK reaction mechanism (Scheme 3), namely GA and D-F6P, were also assayed in order to detect any interference on GAOx activity. The amperometric signal, measured under steady state condition at 0.6 V, corresponds to the electro-oxidation at the underlying Pt electrode of the enzymatically generated hydrogen peroxide. The buffer solution is 0.05 M HEPES (pH 7.0), since GlyGly buffer, generally used with TK*ec*, affects the stability of GAOx (Shleev et al., 2005). The biosensor characteristics, *i.e.* sensitivity and dynamic concentration range, are given in Table 1. L-ERY is effectively a good substrate of GAOx. Indeed, the sensitivity found for this molecule (55 mA M^{-1} cm^{-2}) is in the same order of magnitude than that obtained for galactose (85 mA M^{-1} cm^{-2}), its natural substrate (Charmantray et al., 2013). It should be noted that a small amperometric response to GA additions (9 mA M^{-1} cm^{-2}) was observed at GAOx@laponite electrode (Table 1), this anodic current may be due to GAOx catalyzed glycoladehyde oxidation. On the other hand, D-F6P is not a substrate of GAOx since no current response was observed in the presence of this compound. Finally, the presence of both cofactors of TK*ec,* namely 0.2 mM ThDP and 1 mM $MgCl_2$, in the electrolyte solution does not modify the response of the GAOx@laponite biosensor whatever the substrate assayed (L-ERY or DHA). Determination of L-ERY using GAOx biosensor is thus sensitive enough to meet our objective, the coupling with TK*ec*.

3.2. TK assay by amperometric method

As reported by Zhang et al. (2013) assays can be performed by monitoring changes in the electrochemical properties of enzyme solution for instance, as an alternative to optical enzyme assays. In a previous work, a proof of concept was described for determining *S. cerevisiae* TK activity by using a tyrosinase (PPO) biosensor for the amperometric detection of *N*-acetyl-L-tyrosine ethyl ester monohydrate (N-Ac-Tyr-OEt) at −0.2 V (Sanchez-Paniagua Lopez et al., 2010). This compound was released during an enzymatic reaction catalyzed by TK and BSA from N-acetyl-O-(2*R*, 3*S*, 5-trihydroxy-4-oxopentyl)-L-tyrosine ethyl ester used as a donor substrate (Scheme 2). This method was original since TK does not generate electroactive compounds and the assay could be achieved with the help of an adequate auxiliary enzyme. However this concept suffers from limitations such as (i) TK substrate must be synthesized which is time and money consuming, (ii) TK activity for this substrate is poor, and (iii) the release of N-Ac-Tyr-OEt from the donor substrate requires an additional step catalyzed by BSA which is limiting.

In the present work, we propose another bioassay for TK*ec* activity detection based on the coupling of TK*ec* with GAOx. Indeed, TK*ec* was shown to catalyze the transfers of C1–C2 ketol unit from a donor D-F6P to an acceptor GA to generate L-ERY which is directly oxidized by GAOx leading to the corresponding aldehyde along with hydrogen peroxide that was readily oxidized at Pt electrode at 0.6 V/Ag–AgCl (Scheme 3). Moreover, both D-F6P and GA are commercially available.

The amperometric assays were performed in the chrono-amperometric mode in a stirred 0.05 M HEPES solutions (pH = 7.0) containing different amount of TK*ec* (0.01–0.10 U mL^{-1}) and its cofactors (ThDP and Mg^{2+}). The concentrations in 1 mM

Table 1
Amperometric responses for GAOx@laponite biosensor towards several substrates (0.05 M HEPES pH 7.0, 25 °C, E_{app} = 0.6 V/Ag–AgCl at Pt 500 rpm).

Electrode configuration	Substrate	Sensitivity (mA M^{-1} cm^{-2})	Linear range (M)	R^2 (*n*)
GAOx@laponite	DHA	237	5.0×10^{-7}–2.9×10^{-4}	0.999 (52)
TK*ec*@LDH/GAOx@laponite	DHA	208	6.3×10^{-7}–4.0×10^{-4}	0.999 (56)
GAOx@laponite	L-ERY	55	1.0×10^{-6}–1.5×10^{-3}	0.999 (26)
TK*ec*@LDH/GAOx@laponite	L-ERY	48	9.7×10^{-5}–1.9×10^{-3}	0.999 (18)
GAOx@laponite	GA	9	2.3×10^{-4}–1.5×10^{-2}	0.998 (20)
TK*ec*@LDH/GAOx@laponite	GA	6.5	2.1×10^{-4}–1.8×10^{-2}	0.998 (27)
TK*ec*@LDH/GAOx@laponite[a]	ThDP	77	5.0×10^{-7}–10×10^{-6}	0.986 (7)

[a] 1 mM $MgCl_2$, 2 mM GA, 1 mM d-F6P.

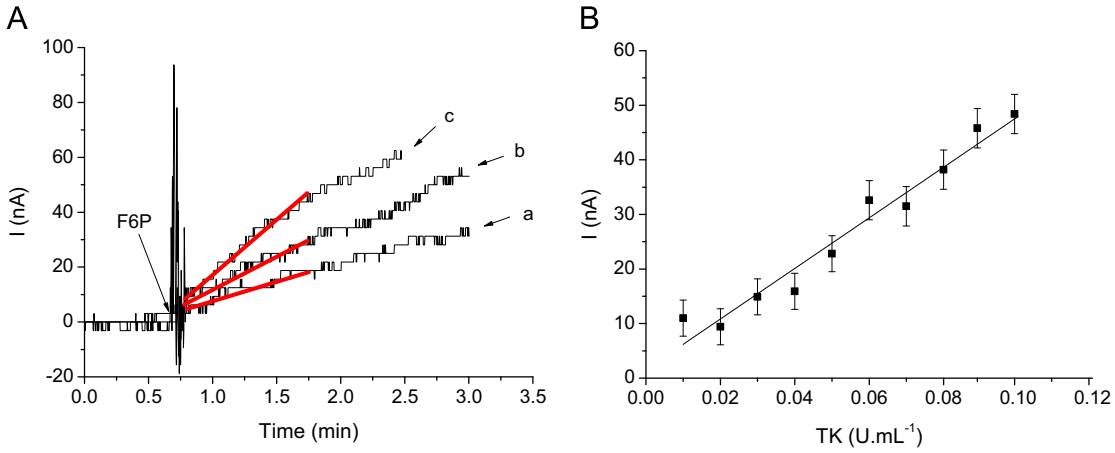


Fig. 1. (A) TK assay based on GAOx@laponite biosensor for TK*ec* concentrations (a) 0.01 U mL^{-1}, (b) 0.04 U mL^{-1}, and (c) 0.1 U mL^{-1}. (B) TK*ec* calibration curve.Experimental conditions: 0.05 M HEPES pH 7.0, 25 °C, 0.2 mM ThDP, 1 mM MgCl2, 1 mM GA, 1 mM d-F6P, and *E*app = 0.6 V/Ag-AgCl, 500 rpm.

Table 2
Comparison of TK assay linearities.

TK assay	Dynamic range (U mL^{-1})	Reference
Spectrophotometric (DH-NADH)	1–10	Hecquet et al. (1993)
Fluorometric (umbelliferone)	0.01–0.1	Charmantray et al. (2010)
Amperometric (PPO biosensor)	0.25–1	Sanchez-Paniagua Lopez et al. (2010)
Amperometric (GAOx biosensor)	0.01–0.1	This work

GA as donor substrate and in 1 mM D-F6P as acceptor substrate were fixed to obtain a steady and reproducible amperometric signal corresponding to the concentration of L-ERY released within the linear dynamic range of the GAOx biosensor. To avoid any artifacts due to the possible saturation of GAOx biosensor by a high concentration of formed L-ERY, an internal calibration was performed with three successive additions of 5 μM DHA. These current steps are used to calibrate the biosensor.

After baseline stabilization following the addition of GA, D-F6P was added to the electrolyte solution and the increase of the anodic current was then recorded as a function time (Fig. 1A). The enzyme assay was therefore performed by measuring the I *vs* t slope (initial reaction rate for 1 min). The observed signal is linked with the amount of TK*ec* present in the solution. For a given amount of TK*ec* in solution (0.1 U mL^{-1}), the amperometric response depends on the concentration of ThDP, as TK cofactor. The dissociation constant (K_D) determined by the Lineweaver Burk method is 1.0 μM. This value is very close to the K_D value (1.8 μM) determined in UV–Vis for the free enzyme in GlyGly solution using L-ERY and D-R5P as substrates (see Section 2). This new amperometric assay for TK activity detection is thus validated. The reproducibility of enzyme assays for each TK*ec* or each ThDP concentrations was verified with three independent electrodes giving a relative standard deviation (RSD) of 3%. Moreover, the same biosensor can be used 10 times to determine a TK*ec* activity.

A calibration plot has been then established for a TK*ec* concentration range between 0.01 and 0.1 U mL^{-1} (Fig. 1B). This dynamic concentration range can be compared to those found by other methods (Table 2). By comparison with the tyrosinase amperometric biosensor formerly developed (Sanchez-Paniagua Lopez et al., 2010), the detection limit in TK revealed a 25-fold increase and thus becomes comparable to that obtained by the fluorometric method (Charmantray et al., 2010). This clearly shows the interest of the amperometric assay mode by comparison with the other methods described in the literature, since the electrochemical apparatus are known to be quite cheap and the electrochemical cell can be easily miniaturized.

3.3. Optimization of the bi-enzyme electrode

In a second step, a bienzymatic electrode consisting in the co-immobilization of TK*ec* and GAOx at the electrode surface was optimized. Following our strategy, we have conceived an innovative bienzyme sensing system based on TK*ec* and GAOx immobilized separately on the electrode surface. TK*ec* on its apoenzyme form was immobilized in LDH by the coprecipitation method as recently described in Touisni et al. (2013). The resulting TK*ec*@LDH biohybrid material can be coated as a thin film on the electrode surface.

In order to determine the best coating configuration for this bi-enzyme electrode, three different amounts of TK*ec*@LDH biohybrid (50, 75 or 100 μg) were used to prepare the outer layer. The inner sensing layer GAOx@laponite was fixed at the same composition than previously described (*vide supra*). However in this case, the reticulation step with glutaraldhyde was avoided. Indeed, there is no enzyme leaching when GAOx@laponite film was covered of another LDH layer.

To verify that the diffusion of analytes through 100 μg TK*ec*@LDH outer layer is not a limiting step, the amperometric responses of the bi-enzyme electrode for the three analytes, L-ERY, GA and DHA, were also recorded and compared to that obtained at the GAOx@laponite electrode (Table 1). These substrates can be detected by the GAOx inner layer with a small decrease of 12% for the sensitivities and within roughly the same dynamic concentration ranges. It should be noted that under these experimental conditions, TK*ec* was not active.

Activation of ApoTK*ec* requires the presence of its cofactors, 0.2 mM ThDP and 1 mM $MgCl_2$ in the electrolyte solution or its previous activation by incubation of the biosensor in 0.2 mM ThDP and 1 mM $MgCl_2$ solution. In both cases, the same amperometric response is observed upon addition of 1 mM D-F6P as shown in Fig. 2. Moreover, these current steps (*I vs t*) are very fast with a t_{90} of 36 s. Indeed the electroenzymatic reaction occurs directly at the vicinity of the electrode surface within the LDH layers. The reusability of the bienymatic electrode was studied. A current response is systematically higher for the first use of the biosensor, afterwards the current values become reproducible for five independent uses of the biosensor in the same day (RSD = 1.2%). We will consider only these reproducible responses.

The current step measured after D-F6P addition depends on the concentration of GA and D-F6P (Table 3). The highest response is obtained with 2 mM GA and 1 mM D-F6P (350 nA) or 5 mM D-F6P (414 nA), however in the last case the current response is less stable. In further experiments, the GA and D-F6P concentrations will be fixed at 2 mM GA and 1 mM D-F6P, respectively. Anodic currents depend on the amount of TK*ec* immobilized on the electrode surface with 185 ± 5, 217 ± 1 and 350 ± 2 nA for 50, 75 and 100 μg TK*ec*@LDH, respectively. A 100 μg amount of TK*ec*@LDH$_{cop}$ biohybrid was thus chosen as the best biomembrane composition. The reproducibility of the fabrication of this bi-enzyme biosensor was tested with five different electrodes, leading to a relative standard deviation value (RDS) of 3.8% for the amperometric current measured for 1 mM D-F6P addition.

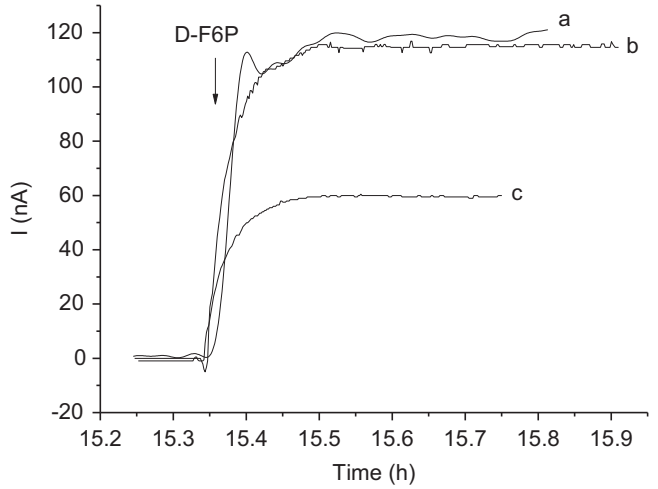


Fig. 2. Amperometric responses for TK*ec*@LDH/GAOx@laponite biosensor adding 1 mM D-F6P in 0.05 M HEPES pH 7.0, 2 mM GA and (a) 5 μM ThDP, 1 mM $MgCl_2$; (b) without ThDP and $MgCl_2$ in solution (after 1 h incubation in 5 μM ThDP and 1 mM $MgCl_2$); and (c) after 1 h incubation in 0.05 mM pyrophosphate, 5 μM ThDP and 1 mM $MgCl_2$ (25 °C, E_{app}=0.6 V/Ag-AgCl, 500 rpm).

Table 3
Amperometric response for TK*ec*@LDH/GAOx@laponite biosensor as a function of GA and D-F6P concentrations (0.05 M HEPES pH 7.0, 25 °C, 1 mM $MgCl_2$, 0.2 mM ThDP, E_{app}=0.6 V/Ag–AgCl, 500 rpm).

[GA] (mM)	[D-F6P] (mM)	*I* (nA)
1	1	202 ± 5
2	0.1	72 ± 3
2	1	350 ± 4
2	5	414 ± 5

The response for TK*ec*@LDH/GAOx@laponite biosensor according to the cofactor ThDP concentration was also analyzed. A linear relationship is obtained between 0.5 and 10 μM with a sensitivity of 77 mA M^{-1} cm^{-2} (Table 1). A maximum of current (I_{max}) of 350 nA is obtained for ThDP concentrations equal or higher than 50 μM. An apparent dissociation constant (K_D^{app}) of 9 μM is evaluated for the immobilized TK*ec* by the Lineweaver–Burk method. This value is slightly higher to the value determined in solution (K_D=1.0 μM). This effect is generally reported for immobilized enzymes due to partition coefficient and diffusional constraints of the analytes (Rodrigues et al., 2013).

3.4. Detection of TK inhibitors

As mentioned in the introduction, there is a growing interest in the search for specific TK inhibitors, particularly in anticancer field (Bentz et al., 2013; Du et al., 2004; Kochetov et al., 1971; Le Huerou et al., 2008; Nilsson et al., 1993; Obiol-Pardo et al., 2012; Raïs et al., 1999; Solovjeva and Kochetov 1999; Sprenger et al., 1995; Thomas et al., 2008a, 2008b). In this context, the emergence of rapid and inexpensive bioassays dedicated to TK activity detection in the presence of cofactor or substrate analogs is of importance. The bi-enzyme electrode was therefore applied for TK-inhibitor detection as an analytical model to evaluate the potential of this new biosensor.

A series of commercially available inhibitors of TK*ec* were selected from the literature. Most of them are analogs of ThDP its cofactor such as thiamine (Kochetov et al., 1971), thiamine monophosphate (Kochetov et al., 1971), pyrophosphate (Kochetov et al., 1971), oxythiamine (Obiol-Pardo et al., 2012; Wood and Fletcher, 1978). Another molecule was selected as acceptor substrate analog, D-A5P (Sprenger et al., 1995). Pyrophosphate shows the lowest inhibition constant (K_i=0.28 mM), however most of those commercial molecules are not good inhibitors for TK, with a K_i value ranging from 1 to 34 mM.

GAOx is a T2 copper enzyme and it is reported that compounds containing amino groups, such as GlyGly buffer, may affect the stability of the enzyme, probably due to complexation of the copper site (Petersen and Steckhan, 1999). As some of the selected molecules contain an amine moiety such as Th, ThMP or ThDP its cofactor as well, they could inhibit GAOx. Their putative inhibition activities were then first tested using 20 μM L-ERY as substrate. The inhibition was quantified as an inhibition percentage (In%) corresponding to the ratio of the current decrease (I-$I°$) *versus* the

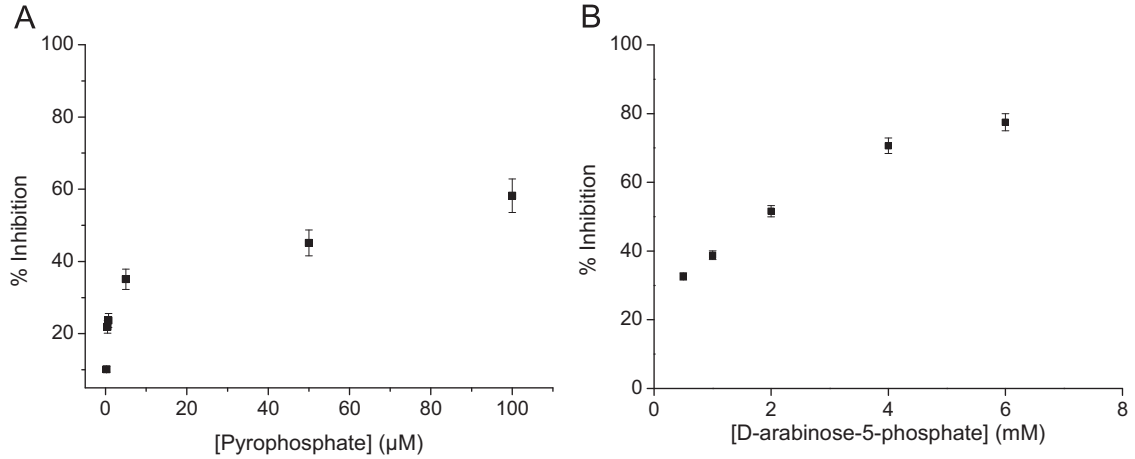


Fig. 3. Inhibition calibrations curves determined for TK*ec*@LDH/GAOx@laponite biosensor with (A) pyrophosphate and (B) D-arabinose-5-phosphate.

original current I° (without inhibitor) in the steady state. The presence of ThDP, ThMP and Th into the electrolyte at a concentration of 10 mM ($3 \times K_i$ for the TK) causes 47, 33 and 30% inhibition, respectively. It should be noted that within the concentration range used in this study [ThDP] ≤ 0.2 mM, ThDP does not inhibit GAOx. The most important inhibition is obtained with oxythiamine (62%), whereas no inhibition was observed with pyrophosphate and D-A5P. Therefore, those latter molecules were selected to validate the inhibition concept for TK.

Curve c in Fig. 2 shows the typical biosensor response to 1 mM de D-F6P addition recorded after 1 h incubation in 0.05 mM pyrophosphate. The presence of pyrophosphate in the incubation medium caused a significant decrease of the steady state current. The normalized inhibition curves were obtained by plotting $(I-I^\circ)/I^\circ \times 100\%$ *vs* various inhibitor concentrations (Fig. 3). The concentration of inhibitors corresponding to 50% of the biosensor response and hence to 50% of the inhibition process (C_{50}) are determined from these data. They are respectively 0.05 and 2 mM for pyrophosphate and D-A5P, respectively. Of course, these C_{50} values obtained with the bi-enzyme electrode are apparent values due to the combination of mass transport, enzyme kinetics and immobilization effects (Streffer et al., 1998). However it confirms the best inhibitor action of pyrophosphate, a TK cofactor analog and the inhibition by a substrate analog, D-A5P.

4. Conclusion

An innovative amperometric biosensor for TK*ec* activity determination based on commercially available TK substrates, such as D-F6P a physiological donor and GA, the best non-phosphrorylated acceptor, was developed by coupling with commercially available auxiliary enzyme, GAOx. We showed that L-ERY released upon TK-catalyzed reaction was effectively oxidized by GAOx biosensor. This device allows the *in vitro* detection of TK*ec* activity as low as 0.01 U mL^{-1}. By comparison to the tyrosinase amperometric biosensor developed formerly, the detection limit in TK was improved by 25-fold increase and is comparable to that obtained by the fluorometric method. Encouraging preliminary results have been recently obtained with the human TK which open the perspective to adapt this biosensor for the detection of human TK activity in cell cultures and biological fluids.

Secondly in order to ensure highest sensitivity and re-usability of the system an original bienzyme sensing system was performed based on apoenzyme TK*ec* and GAOx separately immobilized on the electrode surface. The biosensor response was validated with the determination of the K_D^{app} of ThDP and of C_{50} of TK inhibitors already described in the literature.

This system offers an original, rapid and more sensitive response than that obtained with multi-enzymatic cascade assays known in the literature. The miniaturization of this bienzymatic biosensor could offer promising prospects for the screening of various human TK inhibitors.

Acknowledgments

Nadia Touisni would like to thank Region Auvergne and CNRS for her Ph.D. fellowship (Program Research and Innovative Technology n°1130).

References

Bentz, S., Cee, A., Endlicher, E., Wojta, I.K.A., Naami, A., Pesch, T., Lang, S., Schubert, P., Fried, M., Weber, A., Coy, J.F., Goelder, S., Knüchel, R., Hausmann, M., Rogler, G., 2013. Digestion 88, 182–192.
Charmantray, F., Legeret, B., Hélaine, V., Hecquet, L., 2010. J. Biotechnol. 145, 359–366.
Charmantray, F., Touisni, N., Hecquet, L., Mousty, C., 2013. Electroanalysis 25, 630–635.
Du, M.X., Sim, J., Fang, L., Zheng, Y., Koh, S., Stratton, J., Pons, J., Wang, J.J.-X., Carte, B., 2004. J. Biomol. Screen. 9, 427–433.
Hecquet, L., Bolte, J., Demuynck, C., 1993. Biosci. Biotechnol. Biochem. 57, 2174–2176.
Horecker, B.L., Smyrniotis, P.Z., 1955. Methods Enzymol. 1, 371–375.
Kochetov, G.A., Izotova, A.E., Meshalkina, L.E., 1971. Biochem. Biophys. Res. Commun. 43, 1198–1203.
Le Huerou, Y., Gunawardana, I., Thomas, A.A., Boyd, S.A., de Meese, J., deWolf, W., Gonzales, S.S., Han, M., Hayter, L., Kaplan, T., Lemieux, C., Lee, P., Pheneger, J., Poch, G., Romoff, T., Sullivan, F., Weiler, S., Wright, S., Lin, J., 2008. Bioorg. Med. Chem. Lett. 18, 505–508.
Lee, J.Y., Cheong, D.E., Kim, G.J., 2008. Biotechnol. Lett. 30, 899–904.
Littlechild, J., Turner, N., Hobbs, G., Lilly, M., Rawas, A., Watson, H., 1995. Acta Crystallogr., Sect. D: Biol. Crystallogr. 51, 1074–1076.
Maltseva, N., Kim, Y., Kwon, K., Joachimiak, A., AndersonW.F., 2009. RCSB protein data bank (PDB entries 3HYL and 3M49, submitted 06/2009 and 04/2010).
Mitschke, L., Parthier, C., Schröder-Tittmann, K., Coy, J., Lüdtke, S., Tittmann, K., 2010. J. Biol. Chem. 285, 31559–315570.
Naula, C., Alibu, V.P., Brock, J.M., Veitch, N.J., Burchmore, R.J., Barrett, M.P., 2008. J. Biochem. Biophys. Methods 70, 1185–1187.
Nilsson, U.Y.L., Kluger, R., Schneider, G., 1993. FEBS Lett. 326, 145–148.
Obiol-Pardo, C., Alcarraz-Vizán, G., Cascante, M., Rubio-Martinez, J., 2012. PLoS One 7, e32276.
Petersen, A., Steckhan, E., 1999. Bioorg. Med. Chem. 7, 2203–2208.
Raïs, B., Comin, B., Puigjaner, J., Brandes, J.L., Creppy, E., Saboureau, D., Ennamany, R., Paul Lee, W.-N., Boros, L.G., Cascante, M., 1999. FEBS Lett. 456, 113–118.
Rodrigues, R.C., Ortiz, C., Berenguer-Murcia, A., Torres, R., Fernandez-Lafuente, R., 2013. Chem. Soc. Rev. 42, 6290–6307.
Sanchez-Paniagua Lopez, M., Charmantray, F., Helaine, V., Hecquet, L., Mousty, C., 2010. Biosens. Bioelectron. 26, 139–143.
Sevestre, A., Charmantray, F., Hélaine, V., Lasikova, A., Hecquet, L., 2006. Tetrahedron 62, 3969–3976.
Sevestre, A., Hélaine, V., Guyot, G., Martin, C., Hecquet, L., 2003. Tetrahedron Lett. 44, 827–830.
Shleev, S., Tkac, J., Christenson, A., Ruzgas, T., Yaropolov, A.I., Whittaker, J.W., Gorton, L., 2005. Biosens. Bioelectron. 20, 2517–2554.
Smith, M.E.B., Kaulmann, U., Ward, J.M., Hailes, H.C., 2006. Bioorg. Med. Chem. Lett. 14, 7062–7065.
Solovjeva, O.N., Kochetov, G.A., 1999. FEBS Lett. 462, 246–248.
Sprenger, G.A., Schörken, U., Sprenger, G., Sahm, H., 1995. Eur. J. Biochem. 230, 525–532.
Streffer, K., Kaatz, H., Bauer, C.G., Makower, A., Schulmeister, T., Scheller, F.W., Peter, M.G., Wollenberger, U., 1998. Anal. Chim. Acta 362, 81–90.
Sundstrom, M., Lindqvist, Y., Schneider, G., Hellman, U., Ronne, H., 1993. J. Biol. Chem. 268, 24346–24352.
Thomas, A.A., De Meese, J., Le Huerou, Y., Boyd, S.A., Romoff, T.T., Gonzales, S.S., Gunawardana, I., Kaplan, T., Sullivan, F., Condroski, K., 2008a. Bioorg. Med. Chem. Lett. 18, 509–511.
Thomas, A.A., Le Huerou, Y., De Meese, J., Gunawardana, I., Kaplan, T., Romoff, T.T., Gonzales, S.S., Condroski, K., Boyd, S.A., Ballard, J., 2008b. Bioorg. Med. Chem. Lett. 18, 2206–2210.
Thornalley, P.J., Jahan, I., Ng, R., 2001. J. Biochem. 129, 543–549.
Touisni, N., Charmantray, F., Helaine, V., Forano, C., Hecquet, L., Mousty, C., 2013. Colloid Surf. B 112, 452–459.
Wood, T., Fletcher, S., 1978. Biochim. Biophys. Acta (BBA)—Enzyme 527, 249–255.
Yi, D., Devamani, T., Abdoul-Zabar, J., Charmantray, F., Hélaine, V., Hecquet, L., Fessner, W.-D., 2012. ChemBioChem 13, 2290–2300.
Zhang, M., Karra, S., Gorski, W., 2013. Anal. Chem. 85, 6026–6032.
Zhao, J., Zhong Neurosci., C.-J., 2009. Neurosci. Bull. 25, 94–99.
Zimmermann, F.T., Schneider, A., Schörken, U., Sprenger, G.A., Fessner, W.-D., 1999. Tetrahedron: Asymmetry 10, 1643–1646.