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# **Monitoring of successive phosphorylations of thymidine using free and immobilized human nucleoside/nucleotide kinases by Flow Injection Analysis with High-Resolution Mass Spectrometry**

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## **Abstract**

Nucleosides and their analogues play a crucial role in the treatment of several diseases including cancers and viral infections. Their therapeutic efficiency depends on their capacity to be converted to the active nucleoside triphosphates form through successive phosphorylation steps catalyzed by nucleoside/nucleotide kinases. It is thus mandatory to develop an easy, rapid, reliable and sensitive enzyme activity tests. In this study, we monitored the three-step phosphorylation of thymidine to thymidine triphosphate respectively by (1) human thymidine kinase 1 (hTK1), (2) human thymidylate kinase (hTMPK) and (3) human nucleoside diphosphate kinase (hNDPK). Free and immobilized kinase activities were characterized by using the Michaelis-Menten kinetic model. Flow Injection Analysis (FIA) with High-Resolution Mass Spectrometry (HRMS) was used as well as capillary electrophoresis (CE) with UV detection. The three-step cascade phosphorylation of thymidine was also monitored. FIA-HRMS allows a sensitive and rapid evaluation of the phosphorylation process. This study proposes

simple, rapid, efficient and sensitive methods for enzyme kinetic studies and successive phosphorylation monitoring with immobilized enzymes.

**Keywords:** Capillary electrophoresis, Drug discovery, FIA-HRMS, Immobilized Kinases, Michaelis-Menten, Multi-step enzymatic cascade phosphorylation.

## 1. Introduction

The development of new drugs is a long time process. The accumulation of active or toxic substances could have an important impact on pharmacological and toxicological outcomes. Metabolic pathway of pharmaceutical drugs is an important aspect in drug discovery. Monitoring drug conversion (or non-conversion), mainly when successive enzymatic reactions are involved, brings better understanding of drug activity and toxicity. Therefore, good understanding of metabolic pathways helps to optimize drug development. In this work, the phosphorylation of some nucleosides and their analogues was studied in order to illustrate this statement. These compounds are the cornerstone of the treatment of several human diseases and are especially at the forefront of antiretroviral therapy. Once converted to their triphosphate counterparts they can exhibit their biological activity by incorporating viral DNA due to the action of the viral DNA polymerase. This can cause DNA chain termination, preventing virus proliferation [1-5]. The phosphorylation process of a nucleoside to its triphosphate counterpart involves three successive enzymatic phosphorylation reactions [6]. For instance, the conversion of thymidine (dT) to thymidine triphosphate (dTTP) can be catalyzed by human thymidine kinase (hTK1), human thymidylate kinase (hTMPK) and human nucleoside diphosphate kinase (hNDPK), respectively. These enzymes catalyze the phosphotransfer of the  $\gamma$ -phosphate of adenosine-5'-triphosphate (ATP) in the presence of a chelating agent, usually  $MgCl_2$  (Figure 1) [7]. The phosphorylation of nucleosides and nucleotides is highly compound dependent, and the first phosphorylation step is often rate-limiting [8]. Enzymatic cascade reactions are of great interest in drug discovery. They are sometimes easily conducted with the use of immobilized enzymes (stepwise immobilized enzymes, mixed immobilized enzymes and co-immobilized enzymes) [9-12], on various supports [13-16]. The use of immobilized enzymes for the study of enzymatic cascades has already been investigated [9, 11, 17].

To the best of our knowledge, the phosphorylation cascade process of a nucleoside into its triphosphate counterpart has not been investigated. Previous published studies mainly focused only on a single step of the phosphorylation process. The main techniques used for this are spectrophotometry [18, 19], radioisotopy [20, 21], high performance liquid chromatography [22] and capillary electrophoresis [23-25]. Assays conducted by spectrophotometry are based on the detection of ATP consumption. Even though widely used, this procedure suffers from a low sensitivity, a high substrate and enzyme consumptions, and a low robustness. Separative techniques may necessitate lengthy analyses and specific separation method for each kind of analysed molecules. Electrospray ionization mass spectrometry (ESI-MS), which is widely used for phosphorylated molecule analysis [26-28], is of general interest for application in drug discovery [29-33]. Mass spectrometer can be considered as a detector of choice for the study of enzymatic activity [34-38]. Its high sensitivity and detection specificity (exhaustive fingerprint) allows rapid and sensitive analysis after direct injection of enzymatic reaction mixtures without any prior separation.

A first part of this study was dedicated to develop a rapid, direct and simple kinase activity characterization by FIA-HRMS methodology. Validation of the developed method was performed by studying free hTMPK activity by FIA-HRMS. The Michaelis-Menten parameters (determination of  $K_M$  and  $V_{max}$ ) [39] obtained with UV-spectrophotometry and capillary electrophoresis (CE using UV detection) were compared with those obtained by FIA-HRMS. The FIA-HRMS methodology was then evaluated for monitoring the three successive phosphorylation steps of thymidine using free enzymes as well as enzymes immobilized on magnetic nanoparticles.

## **2. Material and methods**

### **2.1. Material**

Adenosine 5'-triphosphate magnesium salt (ATP), thymidine (dT), thymidine 5'-monophosphate disodium salt hydrate (dTMP), thymidine 5'-diphosphate sodium salt (dTDP), thymidine 5'-triphosphate sodium salt (dTTP), ammonium acetate and magnesium chloride were purchased from Sigma-Aldrich. Magnetic nanoparticles, which consisted of core-shell silica-coated iron oxide magnetic nanoparticles, were synthesized in our laboratory as previously described by our group [40]. Plasmids bearing hTMPK and recombinant hTK1 and hNDPK were a generous gift from University Pierre et Marie Curie

(UPMC). Recombinant hTMPK was produced and purified as described previously [41]. Briefly, cells transformed with the hTMPK-pET28 plasmid were grown at 37 °C in Luria broth (LB) containing 30  $\mu\text{g mL}^{-1}$  of kanamycin and 34  $\mu\text{g mL}^{-1}$  of chloramphenicol for 6 h 40 min until optical density  $E^{600} = 0.602$ . After induction with 400  $\mu\text{M}$  of isopropyl thio- $\beta$ -D-thiogalactopyranoside and growth for 3 h at 30 °C, cells were harvested, re-suspended in 20 mL of lysis buffer Tris/HCl 100 mM ionic strength, pH = 8.0 containing NaCl 40 mM and 2 mM DTT, and kept at -20 °C. Cells were suspended in a Tris/HCl 50 mM ionic strength, pH = 8 containing 1  $\mu\text{M}$  DTT, 1  $\mu\text{M}$  lysozyme, and 1 % PMSF (v/v). The solution was homogenized for 20 min at 4 °C. Cells were subjected to three thermal shocks (liquid nitrogen / water bath 37 °C) and 6 cycles of sonication (cycle 50 %, power 5, 30 s between each cycle). The supernatant was added onto a nickel column pre-equilibrated with 10 mL lysis buffer. The protein was manually eluted with 6 mL of lysis buffer containing 200 mM imidazole. The protein was pure as judged by 14 % SDS-PAGE. Protein concentration was determined using Bio-Rad Protein assay and absorbance at 595 nm. The protein was stocked in 50 % glycerol buffer at -80 °C at 2  $\text{mg mL}^{-1}$ .

## 2.2. Instrumentation

Mass spectrometry experiments were performed on a Bruker maXis UHR-Q-TOF spectrometer (Bremen, Germany) in negative ion mode. The mass spectrometer was coupled on-line with a Dionex UltiMate 3000 RSLC (ultra-high-performance liquid chromatography, UHPLC) system (Germering, Germany). Before each experiment, calibration was performed using ES-TOF tuning mix (Agilent). MS parameters were optimized to enhance ionization and to minimize in-source fragmentation of dTDP and dTTP (Figure S1). The capillary voltage was set at -4000 V, the nebulizer pressure at 0.6 bar, the dry gas flow at 7.0  $\text{L min}^{-1}$  and heated at 200 °C. One  $\mu\text{L}$  of sample was injected in Flow Injection Analysis (FIA-HRMS) mode at 200  $\mu\text{L min}^{-1}$  using  $\text{H}_2\text{O}/\text{ACN}$  65/35 (v/v) as mobile phase. For all experiments, mass spectra in negative mode were recorded in the range of  $m/z$  50 to 1350. Extracted Ion Chromatograms (EIC) of dT ( $m/z$  241.0838,  $[\text{M}-\text{H}]^-$ ), dTMP ( $m/z$  321.0482,  $[\text{M}-\text{H}]^-$ ), dTDP ( $m/z$  401.0145,  $[\text{M}-\text{H}]^-$ ), and dTTP ( $m/z$  480.9808,  $[\text{M}-\text{H}]^-$ ) were plotted within  $\pm 0.005$   $m/z$ . All data were analyzed using DataAnalysis 4.4 software (Bruker).

The capillary electrophoresis system used was a PA800+ automated CE instrument from AB Sciex (Brea, CA, USA) equipped with a photodiode array detection system. The control of CE was performed

using 32 Karat software. Uncoated fused-silica capillaries (60 cm x 50  $\mu\text{m}$ ), purchased from Polymicro Technologies (Phoenix, AZ, USA) were used. New capillaries were conditioned by performing successive rinse cycles: 1 M NaOH (10 min) and Back Ground Electrolyte (BGE) ammonium acetate (80 mM ionic strength, pH = 9) for 30 min. At the end of each working day, the capillary was rinsed with water (10 min) to ensure good cleaning. Between analyses, the capillary was rinsed with BGE. All rinse cycles were carried out at 50 psi. Hydrodynamic injections were performed at the anodic side of the capillary (0.5 psi, 10 s). Normal polarity was used with a separation voltage of +15 kV. Electrophoretic separations were performed at 37 °C in the BGE. Detection was performed at 254 nm.

### **2.3. Validation of FIA-HRMS methodology for kinase activity characterization using free enzymes**

#### **2.3.1. Influence of $\text{MgCl}_2$ and ATP concentrations on kinase efficiencies**

Six solutions of 25  $\mu\text{M}$  of dT, dTMP and dTDP, were prepared with three concentrations of ATP (50, 150 and 350  $\mu\text{M}$ ) and  $\text{MgCl}_2$  (25, 200 and 400  $\mu\text{M}$ ), in ammonium acetate buffer (50 mM ionic strength, pH = 7). Solutions were respectively incubated with free hTK1 (0.48 g  $\text{L}^{-1}$ ), free hTMPK (0.04 g  $\text{L}^{-1}$ ) and free hNDPK (0.04 g  $\text{L}^{-1}$ ) in a heating block at 37 °C for 30 min. One  $\mu\text{L}$  of each solution was injected as previously described. Each solution was performed in triplicate.

#### **2.3.2. Linearity in dependence of time**

hTK1 (0.0875 g  $\text{L}^{-1}$ ), hTMPK (0.004 g  $\text{L}^{-1}$ ) and hNDPK (0.002 g  $\text{L}^{-1}$ ) kinetics were determined by monitoring the accumulation of dTMP, dTDP and dTTP over the time ( $\text{EIC}_{(\text{dTMP})}$ :  $m/z$  321.0482,  $\text{EIC}_{(\text{dTDP})}$ :  $m/z$  401.0145,  $\text{EIC}_{(\text{dTTP})}$ :  $m/z$  480.9808) by FIA-HRMS. Enzymatic reactions were conducted at 37 °C in an Eppendorf tube placed in an HPLC autosampler. dT (10  $\mu\text{M}$ ), dTMP (10  $\mu\text{M}$ ) or dTDP (2  $\mu\text{M}$ ) were incubated in ammonium acetate (50 mM ionic strength, pH = 7) containing ATP (350  $\mu\text{M}$ ) and  $\text{MgCl}_2$  (200  $\mu\text{M}$ ). 12 successive injections were programmed over 6 minutes.

#### **2.3.3. Enzymatic assays - Determination of kinetic parameters**

FIA-HRMS based assays: 9 dT solutions (from 0 to 15 000  $\mu\text{M}$ ), 10 dTMP solutions (from 0 to 500  $\mu\text{M}$ ) and 9 dTDP solutions (from 0 to 500  $\mu\text{M}$ ) were respectively incubated with hTK1 (0.0875 g  $\text{L}^{-1}$ ), hTMPK 0.004 g  $\text{L}^{-1}$  and hNDPK (0.002 g  $\text{L}^{-1}$ ) ([ATP] = 350  $\mu\text{M}$ , [ $\text{MgCl}_2$ ] = 200  $\mu\text{M}$ ). Samples were placed in a heating block at 37 °C. After 5 min, reactions were stopped by heating at 95 °C for 5 min.

Each Michaelis-Menten assay was conducted in triplicate. The feasibility of kinase activity characterization by FIA-HRMS was evaluated by hTMPK characterization (fresh enzyme) with two complementary techniques, spectrophotometry and CE-UV.

UV-spectrophotometry ( $\lambda = 340$  nm) assays: hTMPK reactions were performed by mixing Tris-HCl buffer (50 mM ionic strength, pH = 7.4), KCl 50 mM, MgCl<sub>2</sub> 10 mM (or MgCl<sub>2</sub> 200  $\mu$ M), NADH 0.2 mM, dithiothreitol 1 mM, phosphoenolpyruvate 1 mM, pyruvate kinase 4U, lactate dehydrogenase 4U, ATP 5 mM (or 350  $\mu$ M). Reactions were initiated at 37 °C with hTMPK addition (0.004 g L<sup>-1</sup>) and dTMP substrate (from 2 mM to 500 mM, 9 points). Curves were fitted with Michaelis-Menten equation with GraphPad Prism 5.0 software.

Capillary electrophoresis based assays: hTMPK reactions were conducted at 37 °C in an Eppendorf tube by placing dTMP solutions (from 0 to 800  $\mu$ M, ammonium acetate 50 mM ionic strength, pH = 7) with hTMPK (final concentration 0.004 g L<sup>-1</sup>), ATP (350  $\mu$ M) and MgCl<sub>2</sub> (200  $\mu$ M). Samples were placed at 37 °C in a heating block. After 5 min, reactions were stopped by heating at 95 °C for 5 min.

## **2.4. Enzymatic assays using immobilized enzymes**

### **2.4.1. Enzyme immobilization on magnetic nanoparticles (MPs)**

Functionalized MPs, consisting of core-shell silica-coated iron oxide, were synthesized and functionalized with glutaraldehyde as described in our previous study [40]. Separate immobilization by cross linking of the three enzymes was performed by placing the enzyme in contact with nanoparticles, with a final concentration of 4 mg mL<sup>-1</sup> of MPs diluted in phosphate buffer (50 mM, pH = 6.8) and 1.92 g L<sup>-1</sup> of hTK1, 0.6 g L<sup>-1</sup> of hTMPK and 0.6 g L<sup>-1</sup> of hNDPK. The three solutions were stirred for 7 h at 4 °C and then stored with 1 mM glycine at 4 °C overnight. Remaining free hTK1, hTMPK and hNDPK was removed by successive magnetic support washings (AcNH<sub>4</sub>, 50 mM ionic strength, pH = 7). Nanoparticle were finally suspended in ammonium acetate buffer to obtain three immobilized enzyme solutions at 0.96 g L<sup>-1</sup>, 0.3 g L<sup>-1</sup> and 0.3 g L<sup>-1</sup> for hTK1, hTMPK and hNDPK, respectively. Final washed solutions showed no activity which confirmed that all non-fixed free enzymes were removed.

### **2.4.2. Enzymatic assays - Determination of kinetic parameters of immobilized enzymes**

FIA-HRMS assays: 7 dT solutions (from 0 to 10 000  $\mu\text{M}$ ), 10 dTMP solutions (from 0 to 600  $\mu\text{M}$ ) and 9 dTDP solutions (from 0 to 500  $\mu\text{M}$ ) were incubated with hTK1, hTMPK and hNDPK at respective final concentrations 0.0875  $\text{g L}^{-1}$ , 0.004  $\text{g L}^{-1}$  and 0.002  $\text{g L}^{-1}$  as described in 2.3.2. After reaction and denaturation, immobilized enzymes were attracted by magnet and 1  $\mu\text{L}$  of each supernatant was analyzed by FIA-HRMS. Each Michaelis-Menten assay was conducted in triplicate.

## **2.5. Multi-step enzymatic reactions using FIA-HRMS**

### **2.5.1. Individual reaction conversion rate**

Using free enzymes, three solutions of 50  $\mu\text{M}$  of dT, dTMP and dTDP ( $[\text{ATP}] = 350 \mu\text{M}$ ,  $[\text{MgCl}_2] = 200 \mu\text{M}$ ) were incubated with respectively free hTK1 (0.48  $\text{g L}^{-1}$ ), free hTMPK (0.04  $\text{g L}^{-1}$ ) and free hNDPK (0.04  $\text{g L}^{-1}$ ) for 30 min at 37 °C. Similar conditions were used when working with the immobilized enzymes except that a 60 min incubation time was necessary for the immobilized hTK1. 1  $\mu\text{L}$  of each solution was analyzed by FIA-HRMS and each experiment was performed in triplicate.

### **2.5.2. Conversion rate study of multi-step enzymatic reactions**

Enzymatic cascades were performed with and without ATP addition before each phosphorylation step using free and immobilized enzymes.

Free enzymes: a solution of 50  $\mu\text{M}$  of dT was placed with free hTK1 0.48  $\text{g L}^{-1}$  at 37 °C for 30 min ( $[\text{ATP}] = 350 \mu\text{M}$ ,  $[\text{MgCl}_2] = 200 \mu\text{M}$ ). Then, the supernatant was placed with free hTMPK at a final concentration of 0.04  $\text{g L}^{-1}$  for 30 min at 37 °C. This process was repeated with 0.04  $\text{g L}^{-1}$  of free hNDPK. Before each enzyme addition, 1  $\mu\text{L}$  of supernatant was analyzed by FIA-HRMS. Same experiment was performed with the addition of 350  $\mu\text{M}$  of ATP before each phosphorylation step in order to evaluate the influence of ATP concentration in the reaction medium.

Immobilized enzymes: multi-step enzymatic reactions were performed with immobilized enzymes by placing 500  $\mu\text{M}$  of dT with immobilized hTK1 at 37 °C in a heating block for 1 h ( $[\text{ATP}] = 350 \mu\text{M}$ ,  $[\text{MgCl}_2] = 200 \mu\text{M}$ ). Experiments with and without ATP addition before each phosphorylation step were performed as previously described.

## **3. Results and discussion**

### **3.1. Enzymatic reaction conditions**



Buffers, phosphodonor (ATP) and chelating agent ( $\text{MgCl}_2$ ) concentrations (with free and immobilized enzymes) and mass spectrometry detection were optimized. Reaction conditions used in biological assays are often incompatible with mass spectrometry detection. Usually, enzymatic reactions are conducted with high phosphodonor (ATP) and chelating agent ( $\text{MgCl}_2$ ) concentrations (around mM). For mass spectrometry detection, these concentrations need to be decreased to  $\mu\text{M}$  level. Figure 2 shows the high influence of phosphodonor (ATP) and chelating agent ( $\text{MgCl}_2$ ) concentrations on the three enzymatic assays. Each enzymatic conversion was significantly increased with the ATP concentration. At 50  $\mu\text{M}$  ATP, hTK1 and hTMPK enzymes, known to be rate-limiting, show a conversion rate less than 30 %. Increasing ATP to 150  $\mu\text{M}$ , the hNDPK enzyme shows good conversion rate whereas the conversion rates of hTK1 and hTMPK do not exceed 50 %. Finally, the best conversion for the three enzymes was obtained with 350  $\mu\text{M}$  of ATP. The behaviour of the three kinases differed depending on the chelating agent concentration. The hTK1 enzyme seems to be more sensitive, with a conversion rate below 20 % of its maximum with 25  $\mu\text{M}$  of  $\text{Mg}^{2+}$ . As a compromise between reaction efficiency and molecule ionization in mass spectrometry, the chelating agent concentration was fixed at 200  $\mu\text{M}$ . At this concentration, ion intensities for ATP and dTMP were respectively reduced by 45 % and 23 %. In this work, reactions were performed with the volatile ammonium acetate buffer and a substrate concentration in the range of  $\mu\text{M}$ . Therefore, we first evaluated the influence of this concentration reduction on kinase activity. Moreover, due to ionization variations and the presence of in-source fragmentation of phosphate groups, the FIA-HRMS methodology can bring some uncertainties in the quantification of the obtained products. For this, the FIA-HRMS methodology validation was performed by the use of two complementary methods. A spectrophotometric method (indirect detection), performed with co-factor concentrations around mM and  $\mu\text{M}$ , was first used to validate the possible phosphorylation monitoring with low concentrations of phosphodonor and chelating agents. Capillary electrophoresis technique was also used since this separative technique with UV detection offers a direct detection and simultaneous quantification of the substrate and the different products. This study will validate the kinetic parameters  $K_M$  and  $V_{\max}$  obtained by the FIA-HRMS methodology since the influence of the ionization and the in-source fragmentations will be evaluated.

## 3.2. Validation of FIA-HRMS approach for kinase activity characterization using free hTMPK

### 3.2.1. FIA-HRMS

Free hTMPK kinetic study was rapidly performed by successive automatic injections in FIA mode. 1  $\mu\text{L}$  of solution was automatically injected every 30 s without needle washing between each injection. No memory effect was found between injections, meaning that the absence of needle washing did not lead to any inter-analysis contamination. This injection programming, which increase the number of injections in a given timespan, produced clustered kinetic points and therefore a better depiction of the linear zone. As shown in Figure 3, hTMPK activity was found to be linear within 6 min. For Michaelis-Menten studies, an incubation time of 5 min was chosen to calculate the initial velocity. Values of catalytic efficiency obtained for hTMPK by FIA-HRMS methodology are summarized in Table 1. In Figure S2 is shown an example of mass spectra of dTMP conversion into dTDP. FIA-HRMS offers various advantages in enzymatic activity characterisation. Due to specific compound detection, analyses are rapidly performed without compound separation (1 min *per* analysis). This specific detection by EIC monitoring increases method versatility and precision.

### 3.2.2. UV-spectrophotometry

Kinase activity and kinetic studies are usually conducted by UV-spectrophotometry. As show in Figure 4, dTDP product is quantified according to a tri-enzymatic reaction, by coupling ATP consumption with NADH decrease. As shown in Table 1, catalytic efficiencies ( $k_{\text{cat}}/K_{\text{M}}$ ) for both co-factor concentrations ( $\mu\text{M}$  and  $\text{mM}$ ) were found to be in the same ranger;  $0.05 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  *versus*  $0.07 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ . This confirms that hTMPK is still active using a co-factor concentration of 200  $\mu\text{M}$ . These first results show the feasibility of kinase characterisation by FIA-HRMS with low co-factor concentrations.

### 3.2.3. Capillary Electrophoresis (CE-UV)

FIA-HRMS methodology feasibility was also validated by comparing to CE-UV [42]. As stated previously, FIA-HRMS methodology could bring imprecisions coming from no compound separation, ionisation fluctuation and in-source fragmentation. As shown in Table 1, the catalytic efficiency determined by CE-UV is in the same order of magnitude to the one obtained by FIA-HRMS ( $10^5 \text{ M}^{-1} \text{ s}^{-1}$ ). These values of catalytic efficiencies obtained with these two direct detection methods are close to those previously reported in the literature [6, 43]. Therefore, FIA-HRMS methodology does not bring

imprecision due to no compound separation and in-source fragmentation and turned out to be reliable methodology for kinase kinetic studies. Although the three values are in the same order of magnitude, deviation between the direct detection methods (FIA-HRMS, CE-UV) and the indirect detection method (UV-spectrophotometry) results is expected to be due to measure deviation coming from the tri-enzymatic reactions.

### 3.3. Free and immobilized kinase activity characterization by FIA-HRMS

Since FIA-HRMS methodology was shown suitable for hTMPK activity study it was applied to perform free and immobilized kinase characterization. Linearity study of free enzymes is shown in Figure 3. As immobilized enzymes showed lower reaction velocities, an incubation time of 5 min was chosen for both free or immobilized enzymes. Kinetic studies of immobilized hTK1, hTMPK and hNDPK were performed in triplicate. A calibration curve was realized for each Michaelis-Menten study to take into account any ionization influence (figure S3). Figure 5 shows the Michaelis-Menten plots for the three free and immobilized enzymes.  $K_M$  and  $V_{max}$  values are summarized in Table 2.

Similar  $K_M$  values for immobilized and free enzymes were obtained showing that the immobilization step does not influence enzyme affinities towards its substrate. As noted in graphs (b) and 4 (c), immobilized hTMPK and hNDPK showed a reaction speed decrease ( $V_{max}$  divided by 2). Immobilized hTK1 showed the highest loss of activity with a  $V_{max}$  value divided by 15. The repeatability of the hTK1 immobilization step was studied to confirm that this high loss of activity did not come from a problem occurring during the immobilization process. The same hTK1 batch was immobilized three times in the same conditions. The  $K_M$  and  $V_{max}$  values were found to be similar with  $K_{M(n=3)} = (7.9 \pm 1.1) \times 10^2 \mu\text{M}$  and  $V_{max(n=3)} = (7.1 \pm 0.3) \times 10^{-1} \mu\text{M min}^{-1}$ . This loss of activity may be caused by inappropriate binding geometry and possible inactivation of free enzymes during the immobilization process, and by the fact that the concentration of the enzyme fixed to the MPs is probably lower than the concentration used for the immobilization process.

Immobilized hTMPK and hNDPK activities were shown to be sufficient to conduct successive phosphorylation reactions. The first phosphorylation step from thymidine to thymidine monophosphate, catalyzed by hTK1 is shown in this work as a proof of concept.

### 3.4. Multi-step enzymatic reaction

### **3.4.1. Individual reaction conversion rate**

This work is dedicated to the proof of concept of dTMP conversion into dTTP by the use of free and immobilized enzymes. The feasibility of an enzymatic cascade was first assessed by determining the conversion rate of each step using free and immobilized enzymes. The challenge was to produce a sufficient amount of product, above the limit of detection, to perform the next reaction. Enzyme concentrations used for the enzymatic cascade study are higher than used for enzyme activity characterisation to facilitate reaction. Substrate concentration of 25  $\mu\text{M}$  was arbitrarily chosen for the enzymatic cascade.

A kinetic conversion study of the accumulation of three products (dT, dTMP and dTTP) was performed to estimate each reaction time. Figure S4 shows the accumulation of each product as a function of time. As noted in Figure S4, reaction time of 30 min for immobilized hTMPK and hNDPK should allow to obtain maximal conversion rate. As shown in Figure 6, free enzymes showed high conversion efficiency with 99.6 % for hTK1, 88.6 % for hTMPK and 76.3 % for hNDPK. Conversion efficiencies of 75 % were obtained within 30 min for immobilized hTMPK and of 85 % for immobilized. As previously mentioned, immobilized hTK1 showed lower activity with 10 % conversion efficiency. The challenge was therefore to accumulate enough dTMP to produce the final product (dTTP).

### **3.4.2. Conversion rate study of multi-step enzymatic reactions**

According to literature, hTK1 is inhibited by triphosphorylated products [44]. Therefore, the study of dT phosphorylation into dTTP was performed by off-line successive reaction of hTK1, hTMPK and hNDPK enzymes. Before studying the multi-step enzymatic reactions, the specificity of each reaction involving each enzyme one by one was evaluated. Each enzyme showed substrate specificity with no false positive conversion (< 10 %).

To evaluate the influence of an excess of ATP on the reaction efficiency, the multi-step reaction was performed with and without ATP addition before each reaction (Figure 7). The amount of ATP added was set at 350  $\mu\text{M}$  (excess of the phosphodonor).

The incubation time for the first phosphorylation step (from dT to dTMP) was optimized to obtain the same amount of dTMP as that obtained with free enzymes in order to compare the enzymatic cascade using free and immobilized enzymes. As the immobilized hTK1 enzyme was weakly active, the initial

reaction was performed with a high amount of substrate and longer reaction time in order to obtain a sufficient amount of product for the next reaction. Of the 500  $\mu\text{M}$  of dT placed with hTK1, 50  $\mu\text{M}$  were converted into dTMP (conversion efficiency of 10 %). An excess of ATP increased conversion efficiencies for both free and immobilized hTMPK and hNDPK. Reaction catalyzed with free enzymes showed low conversion efficiencies with 3.1  $\mu\text{M}$  of dTTP accumulated at the end of the multi-step enzymatic reaction (6.2 % of dTMP conversion efficiency). Although conversion was more efficient with ATP addition, conversion rates remained low and the amount of dTTP can be considered insignificant. As shown in Figure 7, conversion efficiencies are higher with immobilized enzymes. An amount of 26.0  $\mu\text{M}$  of dTTP is obtained at the end of the multi-step reaction corresponding to 52 % of dTMP conversion efficiency. The low free enzyme efficiencies could come from high enzyme concentrations in solution which could lead to ionisation competition. Immobilized enzyme, removed from solution before analysis by magnet, could avoid these interferences. Therefore, multi-step enzymatic reaction study was only possible with the use of immobilized enzymes.

This experiment proved that the enzyme immobilization methodology is suitable for multi-step enzymatic phosphorylation study by FIA-HRMS. It has several advantages going from removal of storage buffer, easy removal of enzymes from solution and increasing of enzyme stability. Although the first phosphorylation step was found to be rate-limiting, dTTP accumulation was proved significant in the case of immobilized enzymes.

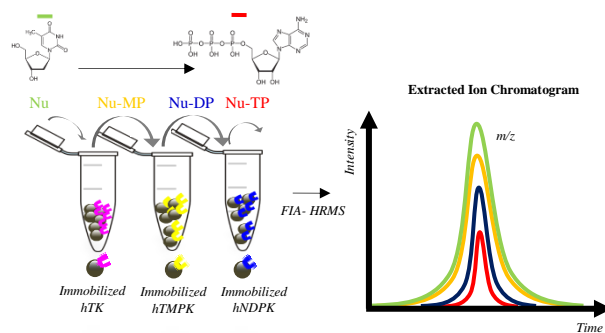
## **Conclusion**

This work shows for the first time the feasibility and the potential of FIA-MS methodology for the monitoring of nucleoside multi-step enzymatic phosphorylation. As a proof of concept, the three-phosphorylation steps from thymidine to thymidine triphosphate were monitored using an FIA-HRMS methodology for a rapid and direct enzyme activity characterization as well as CE-UV. Validation was conducted by the Michaelis-Menten study of hTMPK using three complementary methodologies: FIA-HRMS, UV-spectrophotometry and CE-UV. Once FIA-HRMS validated, this methodology was also used for immobilized hTK1, hTMPK and hNDPK activity characterisations. Both free and immobilized enzymes were used for the three-step phosphorylation monitoring using FIA-HRMS. However,

significant results were only obtained using immobilized enzymes. This study shows the interest of the FIA-HRMS approach for kinase activity characterization thanks to a speed of analysis (1 min *per point*), sensitive analysis (around  $\mu\text{M}$ ) and specificity of detection (direct visualization of products). It also shows the potential of immobilized methodology for the monitoring of multi-enzymatic cascade notably in terms of the possibility to remove enzyme before the analysis of supernatant by FIA-HRMS and to decrease ionic suppression. This development can be applied to the study of synthetic (pro)drugs in order to bring better comprehension of a potential drug metabolic pathway; phosphorylation steps, specific activity regarding human or viral enzymatic strains...

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## GRAPHICAL ABSTRACT



## References

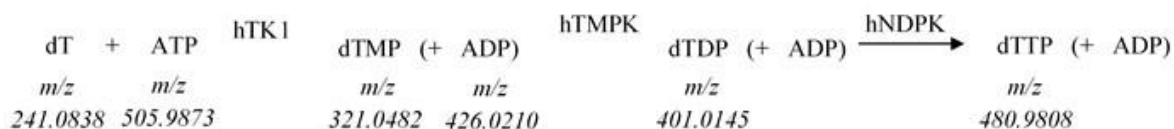
- [1] J. Bourdais, R. Biondi, S. Sarfati, C. Guerreiro, L. Lascau, J. Janin, M. Véron, Cellular Phosphorylation of anti-HIV nucleosides, *J. Biol. Chem*, 271 (1996) 7887-7890.
- [2] A. Varga, E. Graczer, L. Chaloin, K. Liliom, P. Zavodszky, C. Lionne, M. Vas, Selectivity of kinases on the activation of tenofovir, an anti-HIV agent, *Eur J Pharm Sci.*, 48 (2013) 307-315.
- [3] T.S. Topalis, R.; Andrei, G., Tenofovir activating kinases may impact the outcome of HIV treatment and prevention, *Ebiomedicine*, 2 (2015) 1018-1019.
- [4] T. Cihlar, A.S. Ray, Nucleoside and nucleotide HIV reverse transcriptase inhibitors: 25 years after zidovudine, *Antiviral Res.*, 85 (2010) 39-58.
- [5] A.R. Van Rompay, M. Johansson, A. Karlsson, Substrate specificity and phosphorylation of antiviral and anticancer nucleoside analogues by human deoxyribonucleoside kinases and ribonucleoside kinases, *Pharmacol. Ther*, 100 (2003) 119-139.
- [6] D. Deville-Bonne, C. El Amri, P. Meyer, Y. Chen, L.A. Agrofoglio, J. Janin, Human and viral nucleoside/nucleotide kinases involved in antiviral drug activation: structural and catalytic properties, *Antiviral Res*, 86 (2010) 101-120.
- [7] A. Biswas, A. Shukla, R.S. Vijayan, J. Jeyakanthan, K. Sekar, Crystal structures of an archaeal thymidylate kinase from *Sulfolobus tokodaii* provide insights into the role of a conserved active site Arginine residue, *J. Struct. Biol*, 197 (2016) 236-249.
- [8] A. Lavie, M. Konrad, Structural requirements for efficient phosphorylation of nucleotide analogs by human thymidylate kinase, *J. Med. Chem.*, 4 (2004) 351-359.
- [9] J. Luo, A.S. Meyer, R.V. Mateiu, M. Pinelo, Cascade catalysis in membranes with enzyme immobilization for multi-enzymatic conversion of CO<sub>2</sub> to methanol, *N. Biotechnol.*, 32 (2015) 319-327.
- [10] S. Xia, X. Zhao, B. Frigo-Vaz, W. Zheng, J. Kim, P. Wang, Cascade enzymatic reactions for efficient carbon sequestration, *Bioresource Technol.*, 182 (2015) 368-372.
- [11] Q. Ji, B. Wang, J. Tan, L. Zhu, L. Li, Immobilized multienzymatic systems for catalysis of cascade reactions, *Process Biochem.*, 51 (2016) 1193-1203.
- [12] S.R. Tabaei, F. Guo, F.U. Rutaganira, S. Vafaei, I. Choong, K.M. Shokat, J.S. Glenn, N.J. Cho, Multistep Compositional Remodeling of Supported Lipid Membranes by Interfacially Active Phosphatidylinositol Kinases, *Anal. Chem.*, 88 (2016) 5042-5045.
- [13] B. Krajewska, Application of chitin- and chitosan-based materials for enzyme immobilizations: a review, *Enzyme Microb. Technol.*, 35 (2004) 126-139.
- [14] Z.G. Wang, L.S. Wan, Z.M. Liu, X.J. Huang, Z.K. Xu, Enzyme immobilization on electrospun polymer nanofibers: An overview, *J. Mol. Catal. B: Enzymatic*, 56 (2009) 189-195.
- [15] F.C. Yajun Wang, Mesoporous silica spheres as supports for enzyme immobilization and encapsulation, *Chem. Mater*, 17 (2005) 953-961.
- [16] C. Mateo, O. Abian, R. Fernandez-Lafuente, J. M. Guisan, Reversible enzyme immobilization via a very strong and nondistorting ionic adsorption on support-polyethylenimine composites, *Biotechnol. Bioeng.*, 68 (1999) 98-105.
- [17] R. Xue, J.M. Woodley, Process technology for multi-enzymatic reaction systems, *Bioresour. Technol.*, 115 (2012) 183-195.
- [18] P. Schelling, G. Folkers, L. Scapozza, A spectrophotometric assay for quantitative determination of k<sub>cat</sub> of herpes simplex virus type 1 thymidine kinase substrates, *Anal. Biochem.*, 295 (2001) 82-87.
- [19] T. Spector, Studies with GMP synthetase from ehrlich ascites cells, *J. Biol. Chem.*, 250 (1975) 7372-7376.
- [20] B.N. Stretcher, A.J. Pesce, P.T. Frame, D.S. Stein, Pharmacokinetics of Zidovudine Phosphorylation in Peripheral Blood Mononuclear Cells from Patients Infected with Human Immunodeficiency Virus, *Antimicrob. Agent. Chemother.*, 38 (1994) 1541-1547.
- [21] R.R. Drake, T.N. Wilbert, T.A. Hinds, K.M. Gilbert, Differential ganciclovir-mediated cell killing by glutamine 125 mutants of herpes simplex virus type 1 thymidine kinase, *J. Biol. Chem.*, 274 (1999) 37186-37192.

- [22] M. Faria, M.S. Halquist, E. Kindt, W. Li, H.T. Karnes, P.J. O'Brien, Liquid chromatography-tandem mass spectrometry method for quantification of thymidine kinase activity in human serum by monitoring the conversion of 3'-deoxy-3'-fluorothymidine to 3'-deoxy-3'-fluorothymidine monophosphate, *J. Chromatogr. B*, 907 (2012) 13-20.
- [23] J. Iqbal, L. Scapozza, G. Folkers, C.E. Muller, Development and validation of a capillary electrophoresis method for the characterization of herpes simplex virus type 1 (HSV-1) thymidine kinase substrates and inhibitors, *J. Chrom. B*, 846 (2007) 281-290.
- [24] Y. Qi, Y. Li, J.J. Bao, Development of a capillary electrophoresis method for analyzing adenosine deaminase and purine nucleoside phosphorylase and its application in inhibitor screening, *Anal. Biochem.*, 506 (2016) 31-44.
- [25] C.C. Liu, J.S. Huang, D.L. Tyrrell, N.J. Dovichi, Capillary electrophoresis-electrospray-mass spectrometry of nucleosides and nucleotides: application to phosphorylation studies of anti-human immunodeficiency virus nucleosides in a human hepatoma cell line, *Electrophoresis*, 26 (2005) 1424-1431.
- [26] S. Neubauer, A. Rugova, D.B. Chu, H. Drexler, A. Ganner, M. Sauer, D. Mattanovich, S. Hann, G. Koellensperger, Mass spectrometry based analysis of nucleotides, nucleosides, and nucleobases--application to feed supplements, *Anal. Bioanal. Chem.*, 404 (2012) 799-808.
- [27] M. Mateos-Vivas, E. Rodriguez-Gonzalo, J. Dominguez-Alvarez, D. Garcia-Gomez, R. Ramirez-Bernabe, R. Carabias-Martinez, Analysis of free nucleotide monophosphates in human milk and effect of pasteurisation or high-pressure processing on their contents by capillary electrophoresis coupled to mass spectrometry, *Food Chem.*, 174 (2015) 348-355.
- [28] D. Moravcova, M. Haapala, J. Planeta, T. Hyotylainen, R. Kostianen, S.K. Wiedmer, Separation of nucleobases, nucleosides, and nucleotides using two zwitterionic silica-based monolithic capillary columns coupled with tandem mass spectrometry, *J. Chromatogr. A*, 1373 (2014) 90-96.
- [29] M.C. Jecklin, D. Touboul, R. Jain, E.N. Toole, J. Tallarico, P. Drucekes, P. Ramage, R. Zenobi, Affinity classification of kinase inhibitors by mass spectrometric methods and validation using standard IC50 measurements, *Anal. Chem.*, 81 (2009) 408-419.
- [30] S.A. Hofstadler, K.A. Sannes-Lowery, Applications of ESI-MS in drug discovery: interrogation of noncovalent complexes, *Nat. Rev. Drug Discov.*, 5 (2006) 585-595.
- [31] A. Salwinski, R. Délépée, B. Maunit, Continuous-flow step gradient mass spectrometry based method for the determination of kinetic parameters of immobilized mushroom tyrosinase in equilibrating conditions: comparison with free enzyme, *Rapid Commun. Mass Spectrom.*, 25 (2011) 3549-3554.
- [32] M.C. Jecklin, D. Touboul, C. Bovet, A. Wortmann, R. Zenobi, Which electrospray-based ionization method best reflects protein-ligand interactions found in solution? A comparison of ESI, nanoESI, and ESSI for the determination of dissociation constants with mass spectrometry, *J. Am. Soc. Mass Spectrom.*, 19 (2008) 332-343.
- [33] J.M. Wiseman, Z. Takats, B. Gologan, V.J. Davisson, R.G. Cooks, Direct characterization of enzyme-substrate complexes by using electrosonic spray ionization mass spectrometry, *Angew. Chem. Int. Ed. Engl.*, 44 (2005) 913-916.
- [34] N. Pi, J.A. Leary, Determination of enzyme/substrate specificity constants using a multiple substrate ESI-MS assay, *J. Am. Soc. Mass. Spectrom.*, 15 (2004) 233-243.
- [35] X. Ge, T.L. Sirich, M.K. Beyer, H. Desaire, J.A. Leary, A strategy for the determination of enzyme kinetics using electrospray ionization with an ion trap mass spectrometer, *Anal. Chem.*, 73 (2001) 5078-5082.
- [36] H. Gao, J.A. Leary, Multiplex inhibitor screening and kinetic constant determinations for yeast hexokinase using mass spectrometry based assays, *J. Am. Soc. Mass. Spectrom.*, 14 (2003) 173-181.
- [37] L.M. Danan, Z. Yu, A.J. Hoffhines, K.L. Moore, J.A. Leary, Mass spectrometric kinetic analysis of human tyrosylprotein sulfotransferase-1 and -2, *J. Am. Soc. Mass. Spectrom.*, 19 (2008) 1459-1466.
- [38] G. Grasso, M. Fragai, E. Rizzarelli, G. Spoto, K.J. Yeo, A new methodology for monitoring the activity of cdMMP-12 anchored and freeze-dried on Au (111), *J. Am. Soc. Mass Spectrom.*, 18 (2007) 961-969.

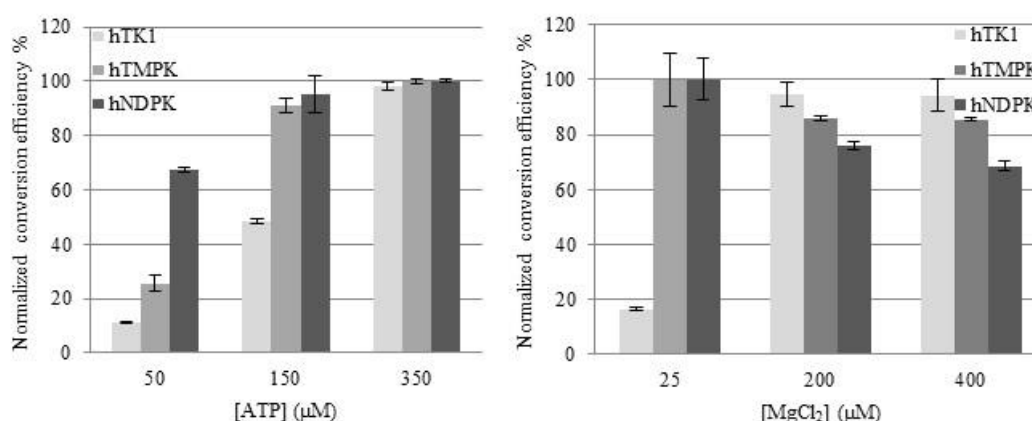


- [39] L. Michaelis, M.L. Menten: Die Kinetik der invertinwirkung, *Biochemistry*, 49 (1913) 333-336.
- [40] A. Salwinski, D. Da Silva, R. Delepee, B. Maunit, Enzyme-coupled nanoparticles-assisted laser desorption ionization mass spectrometry for searching for low-mass inhibitors of enzymes in complex mixtures, *J. Am. Soc. Mass Spectrom.*, 25 (2014) 538-547.
- [41] S. Pochet, L. Dugue, G. Labesse, M. Delepierre, H. Munier-Lehmann, Comparative study of purine and pyrimidine nucleoside analogues acting on the thymidylate kinases of *Mycobacterium tuberculosis* and of humans, *Chembiochem*, 4 (2003) 742-747.
- [42] R. Nehme, P. Morin, Advances in capillary electrophoresis for miniaturizing assays on kinase enzymes for drug discovery, *Electrophoresis*, 36 (2015) 2768-2797.
- [43] J.A.C. Alexandre, B. Roy, D. Topalis, S. Pochet, C. Périgaud, D. Deville-Bonne, Enantioselectivity of human AMP, dTMP and UMP-CMP kinases, *Nucleic Acids Res.*, (2007) 1-10.
- [44] M. Welin, U. Kosinska, N.E. Mikkelsen, C. Carnrot, C. Zhu, L. Wang, S. Eriksson, B. Munch-Petersen, H. Eklund, Structures of thymidine kinase 1 of human and mycoplasmic origin, *PNAS*, 101 (2004) 17970-17975.

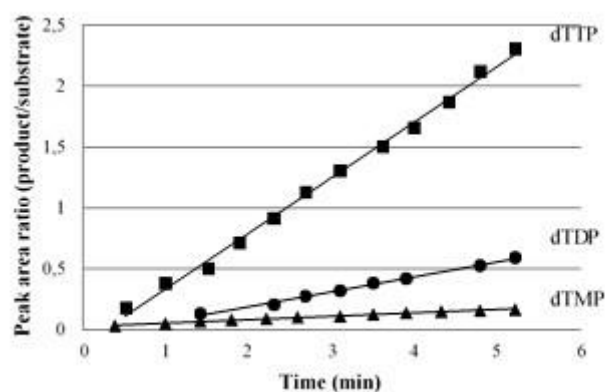
## Figures



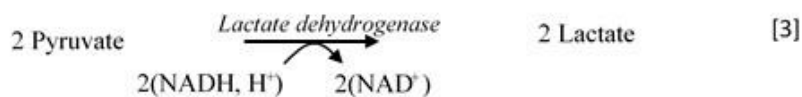
**Figure 1:** Conversion of dT, dTMP and dTDP catalyzed by hTK1, hTMPK and hNDPK respectively *via* the transfer of the  $\gamma$ -phosphate from phosphodonor ATP.



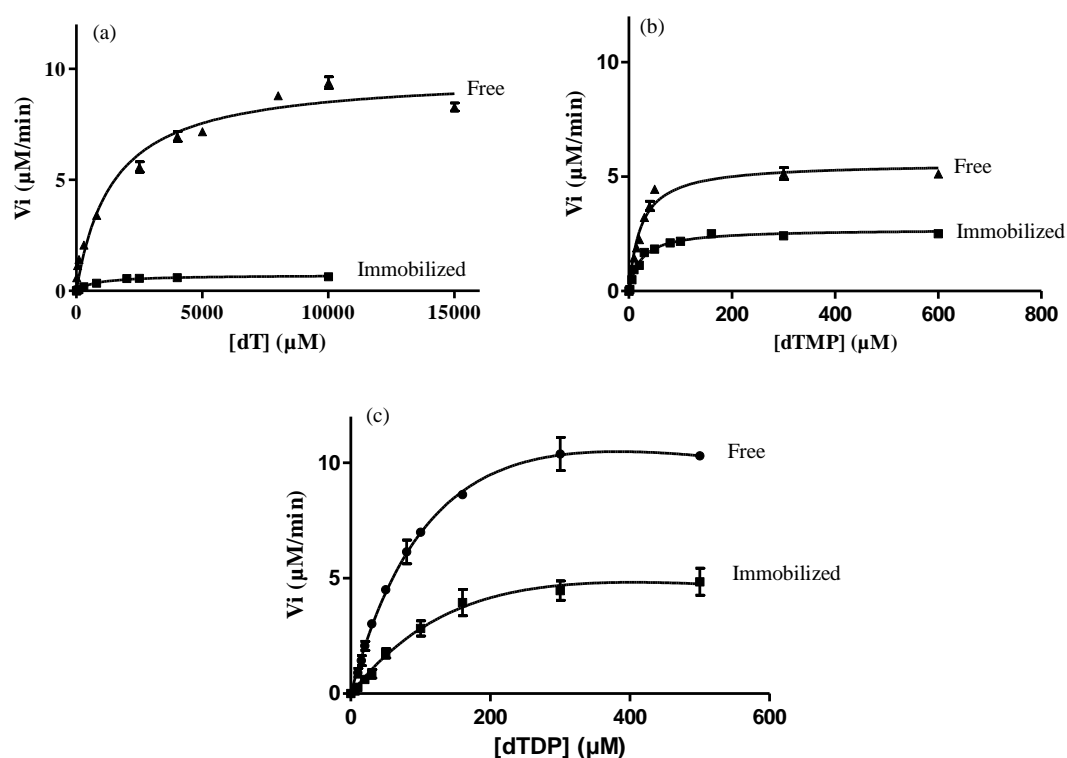
**Figure 2:** Influence of [ATP] (50 μM, 150 μM and 350 μM) and [MgCl<sub>2</sub>] (25 μM, 200 μM, 400 μM) on conversion efficiency of hTK1, hTMPK and hNDPK. Each value is represented as mean and standard deviation over 3 independent experiments.



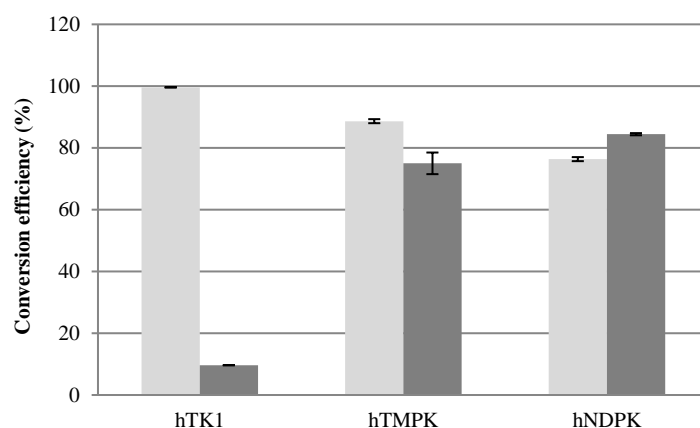
**Figure 3:** Time course production of dTMP (triangles,  $y = 0.0281x + 0.0252$ ,  $R^2 = 0.991$ ), dTDP (circles,  $y = 0.4566x - 0.01185$ ,  $R^2 = 0.996$ ) and dTTP (squares,  $y = 0.1228x - 0.0578$ ,  $R^2 = 0.995$ ) detected by FIA-HRMS upon dT, dTMP and dTDP phosphorylation catalyzed by hTK1, hTMPK and hNDPK.



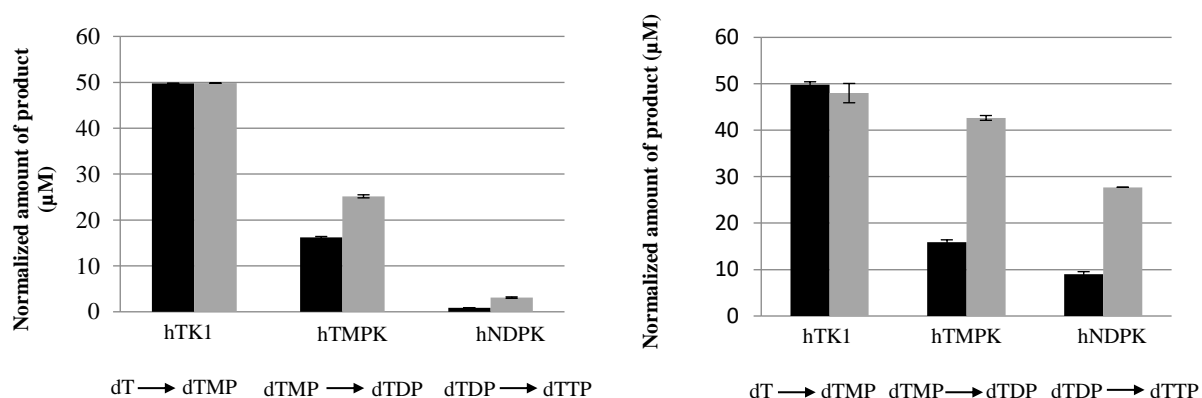
**Figure 4:** Spectrophotometric tri-enzymatic test for kinase activity assays



**Figure 5:** Michaelis-Menten of free (triangles) and immobilized (squares) for (a) hTK1, (b) hTMPK and (c) hNDPK enzymes. Each value is reported as mean and standard deviation over 3 independent experiments. Curves obtained by GraphPad Prism 5.0 and fitted by Michaelis-Menten equation.



**Figure 6:** Conversion efficiency for individual reaction catalyzed with free (light grey) and immobilized (dark grey). Reaction time of 30 min except for immobilized hTK1 (60 min). Analyses by FIA-HRMS (n = 3).



**Figure 7:** Normalized amount of product accumulated ( $\mu\text{M}$ ) for the three successive reaction catalyzed by (a) free and (b) immobilized hTK1, hTMPK and hNDPK. Black lines:  $[\text{ATP}]_{\text{initial}} = 350 \mu\text{M}$ , without ATP addition between each reaction step, grey lines:  $[\text{ATP}]_{\text{initial}} = 350 \mu\text{M}$ , with ATP addition ( $350 \mu\text{M}$ ) between each reaction step. Each value is the mean of 3 independent experiments. Analyses conducted by FIA-HRMS.

## Tables

**Table 1:** Catalytic efficiency ( $k_{cat}/K_M$ ) of hTMPK determined by FIA-HRMS, UV-spectrophotometry and CE-UV.

	$k_{cat}/K_M$ ( $M^{-1} s^{-1}$ )			
	FIA-HRMS	UV-Spectrophotometry ( $\lambda = 340$ nm)		CE-UV
	[ATP] = 350 $\mu$ M [MgCl <sub>2</sub> ] = 200 $\mu$ M	[ATP] = 350 $\mu$ M [MgCl <sub>2</sub> ] = 200 $\mu$ M	[ATP] = 5 mM [MgCl <sub>2</sub> ] = 10 mM	[ATP] = 350 $\mu$ M [MgCl <sub>2</sub> ] = 200 $\mu$ M
<b>hTMPK</b>	$0.21 \times 10^5$	$0.05 \times 10^5$	$0.07 \times 10^5$	$0.48 \times 10^5$

**Table 2:** Kinetic parameters of thymidine (dT), deoxythymidine monophosphate (dTMP), deoxythymidine diphosphate (dTDP) phosphorylation. Each value is the mean of 3 independent experiments. Analyses conducted by FIA-HRMS.

	Free enzymes		Immobilized enzymes	
	$K_M$ ( $\mu$ M)	$V_{max}$ ( $\mu$ M min <sup>-1</sup> )	$K_M$ ( $\mu$ M)	$V_{max}$ ( $\mu$ M min <sup>-1</sup> )
hTK1	$(14.5 \pm 2.1) \times 10^2$	$9.7 \pm 0.3$	$(7.9 \pm 1.2) \times 10^2$	$(7.2 \pm 0.3) \times 10^{-1}$
hTMPK	$(3.4 \pm 0.5) \times 10^1$	$6.9 \pm 0.5$	$(2.8 \pm 0.4) \times 10^1$	$2.9 \pm 0.2$
hNDPK	$(9.3 \pm 1.3) \times 10^1$	$(1.3 \pm 0.1) \times 10^1$	$(1.3 \pm 0.2) \times 10^2$	$6.4 \pm 0.4$