Journal of Chromatography A, 1217 (2010) 600–604

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Short communication

Development of a microbioreactor with ecto-nucleoside triphosphate diphosphohydrolase 2 (NTPDase2) immobilized on a polyacrylamide-coated capillary at the outlet

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ARTICLE INFO

Article history:
Received 10 July 2009
Received in revised form
28 November 2009
Accepted 30 November 2009
Available online 11 December 2009

Keywords:
Capillary electrophoresis
Immobilized enzyme
Online enzymatic studies
Microbioreactor
NTPDase2
Kinetic studies

ABSTRACT

A simple and fast method of immobilization of cell membrane suspension containing human ecto-nucleoside triphosphate diphosphohydrolase 2 (NTPDase2) on a polyacrylamide-coated capillary was developed. The enzyme microbioreactor was prepared by hydrodynamic injection of a small plug of the polycationic electrolyte hexadimethrine bromide (HDB) followed by a suspension of an enzyme-containing membrane preparation. In order to shorten the enzyme assay time and to increase the throughput of the assay, the capillary was coated from the outlet end and all injections were performed from the outlet end of the capillary. For the monitoring of the enzymatic reaction, the substrate ATP dissolved in reaction buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM Hepes, pH 7.4, internal standard: 10 μM UMP) in the absence or presence of inhibitor was injected electrokinetically and incubated in the microbioreactor for 1 min with 1 kV of applied voltage. Then, the electrophoretic separation of the reaction products was initiated by applying a constant current of 60 μA. A 50 mM phosphate buffer (pH 6.5) was used for the separations and the products were detected by UV absorbance at 260 nm. The new method was compared with an at-capillary-inlet method without immobilization of the enzyme. The results (K_m values, K_i values for inhibitor) obtained with both methods were similar and comparable with literature data. The developed outlet immobilized enzyme microreactor using a coated capillary is very fast, simple and most economic allowing multiple use of the enzyme.

1. Introduction

Plasma membrane-bound nucleoside triphosphate diphosphohydrolases type 1, 2, 3 and 8 (NTPDase1, 2, 3 and 8) control nucleotide levels at the cell surface by hydrolyzing the γ- and β-phosphates of nucleotides [1,2]. NTPDase1, 3 and 8 hydrolyze both nucleoside tri- and diphosphates similarly well, yielding ADP and AMP as products, while NTPDase2 preferentially hydrolyzes nucleoside triphosphates yielding ADP as its main product [1,2]. The ratio of ADP to ATP hydrolysis appears to play an important role due to the differential effects of both nucleotides on purinergic receptors [3]. A selective inhibitor for NTPDase2 may exhibit cardioprotective and neuroprotective properties [3,4]. In the search for novel inhibitors of NTPDases, fast methods for analyzing compound libraries are required. Recent developments in the analysis of ultra-microquantities of biomolecules by capillary zone electrophoresis (CZE) include off- and on-capillary enzyme-catalyzed reactions for performing enzyme inhibiton assays and for characterizing alternative substrates [5–12]. In our laboratory, we previously developed CE-based enzyme assays for nucleoside and nucleotide metabolizing enzymes including off-capillary enzyme assays for thymidine kinase [13], as well as on-capillary CE enzyme assays for adenosine kinase [14], ecto-5′-nucleotidase [15] and NTPDases [16].

In recent years, enzyme-immobilized microreactors have been developed by several groups for studying enzyme activity and for inhibitor screening [17–20]. Enzymes can be covalently immobilized on a solid support by using either the in situ derivatization technique or by the batch-wise method, followed by packing into a column [21]. The sol–gel encapsulation method has been used for the preparation of a protein-doped matrix for biosensors: the studied enzymes can be covalently attached onto the surface of the monolithic bed by sol–gel entrapment [22,23]. The advantage of the covalent binding approach is that the leakage of the immobilized enzyme is minimized. However, the active site of the enzyme can be modified by the chemical reaction and the activity yield is therefore frequently low [24]. More recently, Kang et al. used cationic

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doi:10.1016/j.chroma.2009.11.100

electrolytes to immobilize enzymes via ionic interactions on the surface of fused-silica capillaries to obtain immobilized enzyme reactors for the screening of angiotensin-converting enzyme and acetylcholinesterase inhibitors by CE [19,20].

The goal of the present study was to develop an efficient on-capillary immobilized bioreactor for high-throughput NTPDase2 reaction monitoring, which should also be applicable to other ecto-NTPDases and related membrane-bound enzymes. We applied a new method based on immobilization of the enzyme on a polyacrylamide-coated capillary at the outlet end of the capillary allowing high reproducibility for a very long time combined with short analysis time.

2. Experimental

2.1. Reagents and chemicals

Suramin and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) were obtained from Sigma, Steinheim, Germany. ATP, ADP, AMP, UMP, $MgCl_2 \cdot 6H_2O$, and tris(hydroxymethyl) -aminomethane (Trizma Base), were from Sigma (Taufkirchen, Germany).

2.2. Preparation of membrane fractions containing human NTPDase2

Human embryonic kidney (HEK293) cells were transfected with human NTPDase2 cDNA as described and cell membranes were prepared [25,26].

2.3. CE instrumentation

All experiments were carried out using a P/ACE MDQ capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA) equipped with a UV detection system coupled with a diode-array detector (DAD). Data collection and peak area analysis were performed by the P/ACE MDQ software 32 KARAT obtained from Beckman Coulter. The capillary temperature was kept constant at 25 °C, and the temperature of the sample storing unit was also adjusted to 25 °C. The electrophoretic separations were carried out using an eCAP polyacrylamide-coated fused-silica capillary [(30 cm (20 cm effective length) × 50 μm internal diameter (I.D.) × 360 μm outside diameter (O.D.)), obtained from CS-Chromatographie (Langerwehe, Germany)]. The CE running buffer consisted of potassium phosphate 50 mM, pH 6.5. The analytes were detected using direct UV absorbance at 260 nm and a data acquisition rate of 8 Hz was used.

2.4. At-capillary-outlet immobilized enzyme microreactor using the short-end separation mode

A polyacrylamide-coated fused-silica capillary was rinsed with deionized water for 10 min by applying pressure (60 psi; 1 psi = 6894.76 Pa) and then the enzyme was immobilized at the inner surface of the capillary using a two-step protocol: (1) a 1.0 cm long plug of 0.1% (w/v) hexadimethrine bromide (HDB) solution prepared in running buffer (phosphate 50 mM, pH 6.5) was injected into the capillary with a pressure of 0.5 psi for 10 s to create a positively charged coating at the oulet end of the capillary; (2) the enzyme-containing suspension was injected from the outlet end of the capillary column by pressure of 0.5 psi for 10 s, giving almost the same plug length as that of the HDB solution. The injected volume of the human NTPDase2 suspension was ~20 nL (membrane preparation, 1.25 μg of protein/mL). The enzyme-containing membrane suspension was left in the outlet end of the capillary for 10 min to allow the enzyme to immobilize on the capillary wall via ionic binding, the positively charged HDB presumably binding to negatively charged phospholipids in the enzyme-containing cell membrane shreds. Finally, the capillary microreactor was rinsed with the running buffer for 5 min to remove the unimmobilized enzyme preparation from the outlet end of the capillary. The yield of the immobilization was estimated by quantifying the product of the enzymatic reaction. The renewal of the immobilized enzyme after about 50 runs was performed as follows: At first, the capillary was washed with 1 M aq. NaCl solution for 5 min and subsequently with 0.1 M HCl solution for 2 min to remove the residual enzyme and a new sample of enzyme suspension was immobilized again as before.

Hydrodynamic injection was achieved by placing the sample vial at the outlet and by applying pressure at the outlet end of the capillary. The separation was performed using an applied current of 60 μA with normal polarity to change the direction of migration. The capillary was conditioned by rinsing it with water for 2 min and subsequently with phosphate buffer (50 mM, pH 6.5) for 1 min. The enzymatic reaction was performed by electrokinetic injection (5 psi for 0.5 s) of a plug of 320 μM ATP as a substrate in reaction buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM Hepes, pH 7.4, containing 10 μM UMP as an internal standard) from the outlet end of the capillary. The mixture was incubated in the microbioreactor for 1 min with 1 kV of applied voltage. Then, the electrophoretic separation of the reaction products was initiated by applying a constant current of 60 μA. A 50 mM phosphate buffer (pH 6.5) was used for the separations and the products were detected by UV absorbance at 260 nm (see Fig. 1A Supporting Information). The NTPDase2 activity was determined by quantifying the corrected peak area of the ADP produced from the substrate ATP by the enzymatic reaction.

2.5. At-inlet enzymatic microreaction procedure using the long-end separation mode

A new polyacrylamide-coated fused-silica capillary was washed with deionized water for 10 min and then equilibrated with the CE running buffer for 5 min. All injections were made at the cathodic side of the capillary. The in-capillary enzymatic reaction in the long-end separation mode was performed by pressure injection (5 psi for 0.5 s) of a plug of 320 μM ATP as a substrate in reaction buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM Hepes, pH 7.4, containing 10 μM UMP as an internal standard). Then injection of a plug of suitably diluted human NTPDase2 enzyme preparation (1.25 μg of protein/mL) at 5 psi for 0.5 s and finally another plug of 320 μM ATP in reaction buffer was injected (5 psi for 0.5 s). The plugs were then allowed to react for 1 min. Subsequently, a current of negative polarity (−60 μA) was applied and the reaction products moved towards the detector end of the capillary. Phosphate buffer (50 mM, pH 6.5) was used as the running buffer for separation. After each analysis the capillary was rinsed with CE running buffer for 2 min followed by deionized water for 1 min (see Fig. 1B Supporting Information).

2.6. Determination of Michaelis–Menten constant (K_m)

For the determination of the Michaelis–Menten constant (K_m) eight different substrate concentrations of ATP dissolved in reaction buffer were used, 10, 20, 30, 50, 100, 200, 250 and 1000 μM. The K_m value was determined using the enzyme-immobilized microreactor at capillary-outlet and at capillary-inlet enzymatic reaction as described in Sections 2.4 and 2.5. Data were analyzed using PRISM 3.0 (GraphPad, San Diego, USA). Three separate experiments were performed and each solution was analyzed in duplicate or triplicate.

2.7. Investigation of NTPDase2 inhibitor

For the determination of the K_i values of the standard NTPDase2 inhibitor suramin, 7–8 different concentrations of inhibitor spanning about three orders of magnitude were used in the presence of a fixed substrate concentration of 320 μM of ATP. Under the applied conditions less than 10% of substrate was converted by the enzyme. Suspensions of membrane preparations derived from transfected cells containing human NTPDase2 were used (1.25 μg protein/mL). Control experiments were performed using membrane preparations of cells transfected with the empty plasmid (pcDNA3). Substrate and inhibitor were dissolved in the reaction buffer. The Cheng-Prusoff equation was used to calculate K_i values from the IC_{50} values, determined by the non-linear curve fitting program PRISM 3.0.

3. Results and discussion

3.1. Preparation of an at-capillary-outlet immobilized enzyme microreactor

Several methods are available for enzyme immobilization on the inner surface of a fused-silica capillary: non-covalent binding with biotin–avidin [27], covalent immobilization [18,28] and immobilization by ionic binding [19,20]. No method has so far been reported for the immobilization of enzymes on a coated capillary. In most CE methods the long-end separation mode is used. A short-end injection procedure was developed by Altria et al. to decrease total analysis time [29], which was subsequently used for studying the enzyme kinetics of haloalkane dehalogenase, rhodanese and angiotensin-converting enzyme [30,31]. In this method the shorter outlet part of the capillary was used for enzymatic reactions. Hydrodynamic injection was achieved by placing the sample vial at the outlet. Electrophoresis was then performed with normal polarity to change the direction of migration. In the present study we investigated the possibility of combining the short-end separation mode with a polyacrylamide-coated fused-silica capillary containing immobilized enzyme at the outlet end in order to monitor enzymatic reactions of the membrane-bound ectonucleotidase NTPDase2. In order to retain enzymatic activity mild conditions for attachment to the capillary wall are required [32]. A polycationic electrolyte, hexadimethrine bromide (HDB), was used for modifying the capillary to create a positively charged coating. This was simply done by injecting a solution of HDB in running buffer to coat a small part of the capillary of 1.0 cm length (see Fig. 1A Supporting Information). By coating only the head of the capillary there was no effect on the electroosmotic flow (EOF). The resulting HDB coating was stable enough to withstand the flush of the running buffer between analyses. The HDB molecules possess ammonium functions which can interact with negatively charged groups in proteins and phospholipids in the cell membrane preparation and thus lead to immobilization [19]. The stability of the immobilized enzyme was investigated by assaying the enzyme activity after 24 h: no loss in the enzyme activity was observed (data not shown). The performance of the immobilized enzyme microreactor was high enough to rapidly catalyze the reaction. This was due to the high surface-to-volume ratio of the capillary [19]. On observing a drop in enzymatic activity, a fresh enzyme sample was immobilized. This was required after approximately 50 runs. In this enzyme-immobilized microreactor-CZE system, enzymatic reaction and separation can be simultaneously performed in a single capillary (see Fig. 1A Supporting Information). We did not see any interactions with the matrix because of the neutral nature of the capillary. If there were any matrix effects this would change the migration time of the compounds in the capillary and affect the enzymatic activity, which we did not observe.

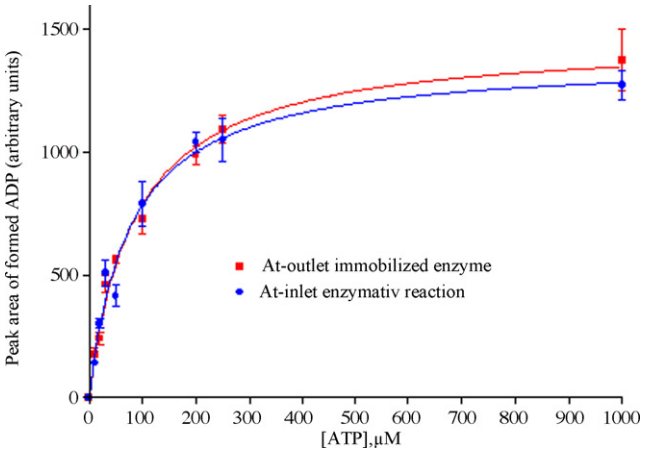

Fig. 1. Michaelis–Menten plots of the initial ATP concentration with respect to the reaction velocity (peak area of ADP formed) for the determination of K_m values of human NTPDase2 by capillary electrophoresis using on-column immobilized enzymatic microreaction (■) and in-capillary reaction at-capillary-inlet (●). For enzyme activity assay see Sections 2.4 and 2.5 and for CE conditions see Figs. 2 and 3. Data points represent means ± SD from three separate experiments each run in duplicate. The K_m values determined by the immobilized enzyme microreactor was 86 ± 8 μM, and the K_m value obtained with at-inlet capillary enzyme reaction was 76 ± 5 μM.

3.2. Linearity of enzymatic activity

In order to confirm that the enzymatic activity was linear with the amount of immobilized enzyme, different quantities of enzyme-containing membrane preparation were used: 0.625, 1.25, 2.5, 5, 10 and 20 ng/mL of protein. The substrate concentration was kept constant at 320 μM and a waiting period of 1 min was applied. A straight line could be drawn by linear regression analysis. In the regression equation y is the corrected peak area of ADP generated by the enzymatic reaction and x represents the enzyme concentration in ng/ml. The following equation was obtained: $y = 4{,}753{,}000x + 71{,}500$; $S_{y,x} = 995$ and the correlation coefficient, r, was 0.9942. Thus, the enzymatic reaction proceeded linearly with increasing amounts of enzyme preparation.

3.3. Michaelis–Menten analysis

For the determination of the Michaelis–Menten constant (K_m) of human NTPDase2, eight different ATP concentrations ranging from 10 to 1000 μM were used (Fig. 1). The enzyme kinetic studies were conducted by measuring the changes in absorbance signals for ADP produced from different substrate concentrations. The reaction conditions were within the linear range of product formation in the substrate concentration curves. The K_m value for human NTPDase2 determined with the immobilized enzyme microreactor at capillary outlet was 86 ± 8 μM (Fig. 1).

For comparison an at-inlet enzymatic reaction method was applied. In this method, which has previously been described for rat NTPDase1–3 [16] the plugs of enzyme and substrate were introduced into the capillary by consecutive pressure injections and were allowed to react during a predetermined waiting period. During this period the plugs were gradually mixed, which might cause a dilution of the substrate solution over time [31]. In contrast, when the enzyme is immobilized the substrate is in direct contact with the enzyme without any dilution processes, which may be advantageous. Using the at-inlet enzymatic method without immobilization of the enzyme a K_m value of 76 ± 5 μM was obtained

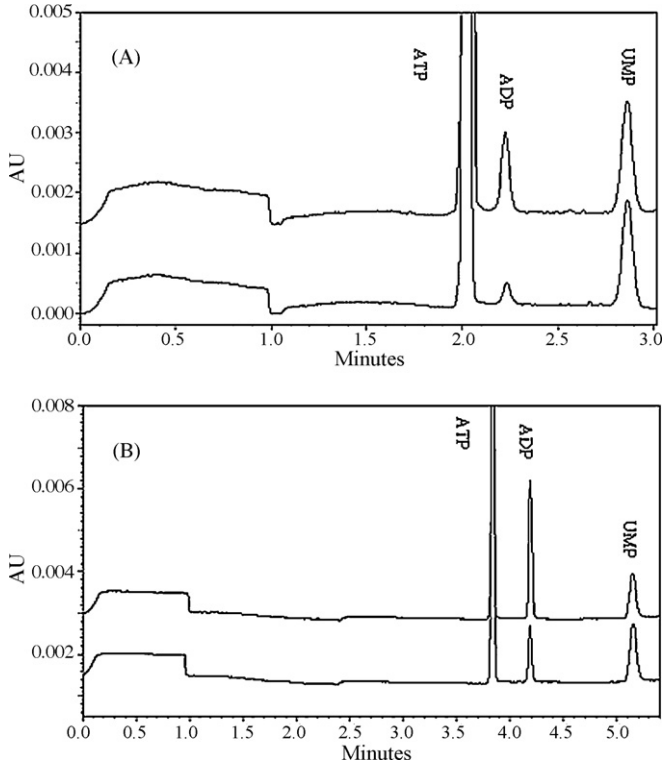

Fig. 2. Typical electropherograms of human NTPDase2 control and inhibition assays. A concentration of 320 μM of ATP was used without inhibitor in control assays, and in inhibition assays the concentration of the inhibitor suramin was 3 μM, which was below the detection limit. UMP (10 μM) was added as internal standard. The quantitation of ADP was used to determine the inhibitory potency of the human NTPDase2 inhibitor. The reaction buffer consisted of 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM Hepes, pH 7.4. (A) At-capillary-outlet immobilized enzyme microbioreactor; separation conditions: 50 mM phosphate, pH 6.5, neutral capillary, 30 cm length (20 cm to the detector), 50 μM I.D, 60 μA, detection at 260 nm, pressure injection from the outlet side of the capillary, normal polarity. (B) At-capillary-inlet enzymatic reaction, long-end injection mode, reverse polarity; the separation buffer was the same as in (A).

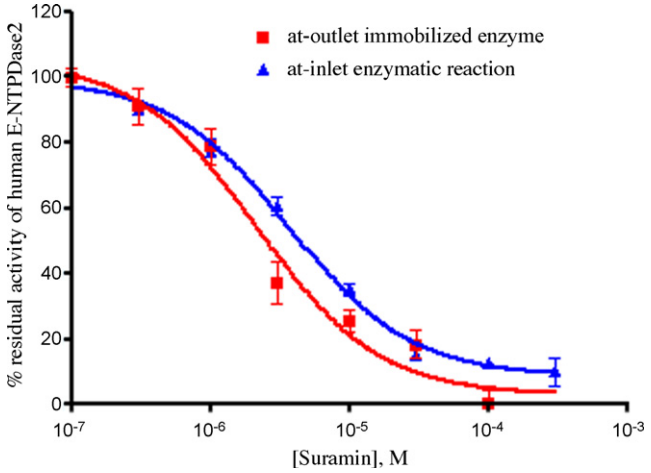

Fig. 3. Concentration-dependent inhibition of human NTPDase2 by suramin determined by capillary electrophoresis using an at-outlet immobilized enzyme microbioreactor (■) and at-inlet enzymatic reaction (▲). A substrate concentration of 320 μM of ATP was used. The reaction buffer consisted of 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM Hepes, pH 7.4, and various concentrations of inhibitor. The separation conditions were 50 mM phosphate buffer, pH 6.5, neutral capillary, 30 cm length (20 cm to the detector), 50 μM I.D.; detection at 260 nm. Data points represent means ± SD from three separate experiments, each run in duplicate. A K_i value of 0.45 ± 0.01 μM was determined with the at-outlet immobilized enzyme microbioreactor. Using the at-inlet enzymatic reaction a K_i value of 0.72 ± 0.02 μM was obtained.

(Fig. 1). This means that both methods yielded nearly identical values.

Reported K_m values for human NTPDase2 obtained with spectrophotometric methods were 70, and 210 μM [25,33], respectively, and therefore in very good agreement with the values determined in the present study by two different methods.

3.4. Enzyme inhibition studies

Inhibition of NTPDase2 was determined by a range of concentrations of inhibitor, in the presence of a fixed amount of ATP (320 μM). The results were obtained by injecting mixtures of the standard inhibitor suramin along with substrate dissolved in assay buffer into a capillary containing immobilized human NTPDase2. Typical electropherograms are shown in Fig. 2A. In the assays in which the inhibitor suramin (3 μM) was present the peak size for ADP used for quantification was significantly smaller compared to the control assay without inhibitor. Suramin itself – despite its negative charge – did not interfere with the measurements. The obtained concentration-inhibition curve for the standard inhibitor suramin is shown in Fig. 3. For comparison, a concentration-inhibition curve for suramin was also determined using the at-capillary-inlet enzymatic reaction without immobilization of the enzyme. Electropherograms from inhibition assays using the at-capillary-inlet reaction method are shown in Fig. 2B and a corresponding concentration inhibiton curve for suramin is depicted in Fig. 3. A K_i value of 0.72 μM was obtained, which was very similar to that determined with the new immobilization method (K_i 0.45 μM, see Fig. 3). It is interesting to note that suramin was >90-fold more potent at the human NTPDase2 than at rat NTPDase2 (K_i 65.4 μM [16]) indicating considerable species differences.

3.5. Comparison with previously developed NTPDase assays

Classical methods used for testing NTPDase inhibitors include radioactive, spectrophotometric and HPLC methods [34–36]. Radiometric assays [34] are sensitive, but require radioactive substrates. The frequently used spectrophotometric malachite green phosphate assay [35] requires large amounts of materials and suffers of a high background noise due to phosphate being frequently present as an impurity. HPLC methods [36] suffer from the requirement of large quantities of solvents, relatively high prices for columns, and the requirement of sample pretreatment. Online-HPLC assays for NTPDases cannot be performed. In our previously developed capillary electrophoresis-based method for NTPDase assays [16] a plug of enzyme was sandwiched between two plugs of substrates. The enzymatic reaction took place by mixing of the plugs of substrate and enzyme. The inlet side of the capillary was used for the enzymatic reaction and several injection steps were performed for substrate and enzyme. In contrast, the current manuscript describes the immobilization of the enzyme NTPDase2 at the outlet side of the capillary using HDB as an immobilizing agent. The current work performed with a human NTPDase2-immobilized microbioreactor is much easier in terms of operation and regeneration, with much shorter overall analysis time than all previously published procedures.

4. Conclusions

By coupling the enzyme-immobilized microreactor with the short-end separation mode the resulting analysis time was reduced by almost half as compared with the previously applied long-end separation mode. Along with reduction in assay time the method is very simple as there is no need to change the main CE separation

parameters of the long-end separation method, like background electrolyte, separation voltage, injection, or separation buffer. The only change is that the injection is done from the opposite end of the same capillary with normal polarity of separation voltage. In addition, the separation voltage and current stays the same as in the case of the classic long-end separation mode and consequently buffer depletion is minimized.

In the present study, an immobilization procedure was used to successfully bind a cell membrane preparation containing human NTPDase2 to a polyacrylamide-coated fused-silica capillary and combined with a short-end separation mode. Enzyme activity was preserved for more than 24 h. By using the short-end separation mode the assay time was dramatically reduced to less than 3 min. Further studies could be continued towards the transformation of this system onto a microchip-based platform, which is required for an ultra-high-throughput assay system.

Acknowledgement

Jamshed Iqbal is grateful for a Deutscher Akademischer Austauschdienst (DAAD) scholarship (STIBET programme).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2009.11.100.

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