Enzyme Immobilized Magnetic Nanoparticles for In-Line Capillary Electrophoresis and Drug Biotransformation Studies: Application to Paracetamol

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Abstract: Enzyme Immobilized Magnetic Nanoparticles (EMNPs) were injected and magnetically retained, as a microreactor, in the capillary of a capillary electrophoresis (CE) setup with UV detection. The enzyme horseradish peroxidase (HRP) was chemically immobilized onto commercially available magnetic 300 nm diameter nanoparticles. Paracetamol (acetaminophen: N-acetyl-*p*-aminophenol), a common analgesic drug, was used as model drug compound. The enzymatic reaction was studied in-line by CE in 12.5 mM phosphate buffer pH 7.4 containing 20 mg/ml sulfated-*β*-cyclodextrin and 0.1 mM hydrogen peroxide. By means of the developed setup, the apparent Michaelis Menten constant between HRP and acetaminophen (APAP) was determined as K_m^{app} = 53±5 μM. This approach was found to be of interest for enzyme kinetics studies with short time resolution condition. Based on our results and from the literature data, it was possible to infer that the in-line generated product was an APAP dimer. Higher enzyme immobilized beads loading in the CE setup generated the APAP dimer with two additional minor peaks likely attributing to APAP trimer and tetramer. N-acetyl-*p*-benzoquinone imine (NAPQI) was not generated during APAP short time migration through the in-line microreactor.

Keywords: Capillary electrophoresis, magnetic beads, biotransformation, paracetamol.

INTRODUCTION

Electrophoretically mediated microanalysis (EMMA) is a widely used in–capillary ultramicroreaction based analytical method which was firstly introduced by Bao and Regnier [1]. Most of the studies realized with EMMA methodology involve enzymatic reactions [2-4]. The use of EMMA methodology for studying enzymatic reactions provides high conversion efficiency due to increased enzyme turnover in a confined environment which compensates for the characteristically poor concentration sensitivity observed in capillary electrophoresis (CE). EMMA uses the capillary both as a reaction chamber and as a separation medium. The in capillary reaction can be (i) homogeneous with mixing of very small volumes of solutions of two or more compounds [5-8] and (ii) heterogeneous, where one of the reactants is immobilized onto the inner wall of the capillary [9-13].

In the homogeneous method, the plug–plug mode is the most commonly used, i.e. the reactant with the slower electrophoretic mobility is first injected into the capillary followed by the injection of the reactant with higher electrophoretic mobility. Upon the application of an electric potential, due to differences in their electrophoretic mobilities, the zones of the faster reactant catches up the slower zone of reactant, thereby the two zone mixes and the enzymatic reaction takes place. Sometimes for increasing the product yield, the potential can be turned off once the reactants zones have overlapped. After some reaction time, the potential is turned on, the unreacted substrate and the reaction product are separated and transported towards the detector, where as they are individually detected [7, 8]. In the heterogeneous assay, the enzyme can be immobilized onto the inner capillary wall *via* different ways, such as *via* a biotin-avidin-biotin link or ionic binding [9-13].

Magnetic particles (MPs) of micro-and nano-size coated with bioactive elements have been used in the preparation, separation and detection of biological molecules, such as DNA [14] cells [15] and proteins [16]. A very useful characteristic of MPs is that they can be easily manipulated by using external permanent magnets or electromagnets, independently of the microfluidic or biological process, which makes it easier, either to retain them in a desired place, or to readily trap them. MPs have been used in different microfluidic conditions, i.e. flow injection analysis [17-19], and liquid chromatography [20-23]. Trypsin immobilized nanobeads were used as on-chip microreactor for highly efficient proteolysis [24].

Since the early description by Karger B. *et al* on using magnetic beads in isotachophoretic focusing to quantify femtomoles of an antigen by CE, relatively limited applications have appeared by using CE [25]. Okamoto Y. used affinity capillary electrophoresis to determine low-density lipoproteins (LDL) with high sensitivity [26]. The group of Noda N. used a similar approach for bacterial

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16sRNA quantification [27]. Girault H. *et al*. used magnetic beads on based immunoaffinity CE for total serum IgE assay [28]. Wang Y. *et al*. reported using *n*-octadecyltrichlorosilane bonded silica MPs packed inside a capillary for the separation of a mixture containing thiourea, toluene, naphthalene, fluorine, and anthracene by CE [29].

In this work, we prepared HRP immobilized magnetic nanoparticles (HRP-MNPs) and HRP immobilized microparticles (HRP-MMPs) for using in-line as a microreactor in CE for enzymatic transformation of paracetamol (APAP). HRP is a good model enzyme mimicking "*in vivo*" peroxidase systems (myeloperoxidase and cyclo-oxygenases) and to some extent the CYP450 system [30]. The HRP-MNPs were injected and magnetically retained in a short reaction zone in the capillary of the CE setup. Subsequently, the substrate APAP was injected in the microreactor based CE. The expected advantages of such a configuration are (i) a low biocomponent and analyte consumption, (ii) a high biocatalytic conversion efficiency, (iii) excellent time resolution between reaction and detection, (iv) on-line identification of bioreaction products (v) readily and reproducibly microreactor renewing. This set up could be a useful tool for *in vitro* drug biotransformation studies. As model analyte, paracetamol was selected because it is one of the world's most widely used pain relievers and because the oxidation of APAP by HRP in the presence of H_2O_2 was quite extensively studied in the literature [30-33]. A thorough report on paracetamol metabolism and tolerability was recently reported [30]. The HRP/H_2O_2 system generated the APAP free radical, N-acetyl-p-benzosemiquinone imine (NAPSQI), which rapidly polymerized giving rise to APAP dimers, trimers and tetramers [31-33]. It was reported that a minor reaction pathway occurred due to NAPSQI disproportionation to form N-acetyl-p-benzoquinone imine (NAPQI) and APAP [32]. In humans, APAP metabolization occurred mainly *via* the CYP450 system (CYP2E1) giving rise, by a two electrons, two protons transfer, to NAPQI which is highly reactive towards endogenous thiols. At high concentrations of APAP, glutathione reserves became depleted allowing NAPQI to react with liver cell proteins eventually causing tissue necrosis [30]. Human peroxidases such as myeloperoxidase and the peroxidase function of cyclo-oxygenase (COX isoenzymes) have also been reported to be involved in APAP metabolization [34] but their *in vivo* contribution *to* the production of NAPQI has not been determined. Dimer and further polymers are produced by peroxidases but their urinary output has not been determined yet [30].

EXPERIMENTAL SECTION

Materials and Reagents

Horseradish peroxidase (HRP) (EC 1.11.1.7 type 11, 240U/mg), was from Sigma (Bornem, Belgium). Superparamagnetic silica microparticles (MMPs) (5 µm) were duly provided by Fuji Silysia Chemical Ltd. (Kasugai, Japan). The superparamagnetic Amino-Adembeads (MNPs) (300 nm) were purchased from Ademtech (Pessac, France). It consisted of a MNP suspension at a concentration of 10 mg/mL. Acetaminophen (APAP) was purchased from SERVA (New York, USA). NAPQI was from Sigma (Bornem, Belgium), it was dissolved in pH 7.4 just prior use [35]. Hydrogen peroxide (30%) was from Vel (Leuven, Belgium). The background electrolyte (BGE) was made of phosphate buffer (PB) (pH 7.4) (final concentration of phosphate 12.5 mM) containing hydrogen peroxide (100 µM) and sulfated-β-cyclodextrin (sulfated-β-CD) at 20 mg/ml (Sigma, Belgium). Aminopropyltriethoxysilane (APTS) was from Chisso, Ltd. (Minimata, Japan). Glutaraldehyde 25% wt (GA) was from Aldrich (Belgium). All the solutions were prepared with reverse osmosis purified (ROP) water and filtrated through a 0.45 µm membrane filter before injection (Sartorius, Germany).

Enzyme Immobilization

The magnetic particles had amino binding sites allowing proteins or nucleic acids to be conjugated to the particles by a bifunctional agent. The magnetic microparticles (MMP)s were functionalized with APTS, as described previously [18]. APTS-functionalized MMPs (10 mg) were then reacted with 1mL 2.5% GA solution (in 12.5 mM PB, pH 7.4) for 1 h and rinsed thoroughly with ROP water. Then, 1 mL HRP solution (3 mg/mL) was reacted with the GA-activated particles in 12.5 mM PB pH 7.4 for 1 h at room temperature. The suspension was rinsed with ROP water (5 × 1 mL) and the resulting HRP-MMPs were stored in 1 mL PB (2mM, pH 7.4).

The magnetic nanoparticles (MNPs, 1 mL) were washed firstly with phosphate buffer (5 × 1 mL), then reacted with 1 mL of a 2.5 wt.% GA solution (12.5 mM PB, pH 7.4) at room temperature for 30 min. After washing with PB (5 x 1 mL) the GA treated MNPs were reacted with 1 mL HRP (3mg/mL) solution during 1 h at room temperature to obtain the HRP-MNPs. The suspension was washed with PB (5 × 1 mL) and stored in 1 mL PB (12.5 mM, pH 7.4) at 4 °C (final concentration 10 mg/mL). All the washing steps were performed by trapping the magnetic particles to the vial wall with a magnet and releasing them after completion.

Visible spectrophotometry was applied for the determination of the amount of HRP immobilized onto the MNPs. After the immobilization of HRP, the suspension was diluted 20 times and the absorbance was measured. The amount of immobilized HRP was determined by measuring the initial and final concentration of HRP by referring to a calibration curve of HRP realized in PB of pH 7.4 (λ = 402 nm) and was found to be equal to 0.20 µmol/g. The same method was used to determine the quantity of HRP immobilized onto the MMPs (0.24 µmol/g i.e. ~ 10 µg/mg). An immobilization yield of 86 µg/mg was reported for trypsin by using GA as bifunctional reagent onto amino magnetized beads [24]. This high amount could be attributed to the distinct nature of the enzyme used (trypsin comparing to HRP) and to the higher specific surface area of small size beads (50 nm comparing to 300 nm).

Apparatus

The capillary electrophoresis instrument (Thermo Separation Products, Spectraphoresis 100) was equipped with a UV detector. The detector wavelength was set at 245nm. Fused silica capillaries (Composite Metal Services Ltd, UK) of 75 µm i.d. and 75 cm total length (50 cm from the inlet with 42 cm from the magnets to the detector) were

employed. When used for the first time, the capillary was conditioned with 1 M NaOH for 1 h. All the experiments were carried out in a positive mode (anode at the inlet and cathode at the outlet). The capillary was thermostated by air cooling at 25°C. The separation was performed at 90 μA (~18kV) for 10 min. At the beginning of each day, the capillary was conditioned by a wash cycle starting with a 5 min rinse with 0.1M NaOH, followed by a 5 min rinse with ROP water and a 10 min rinse with running buffer.

All injections of HRP-MNPs were realized by hydrodynamic (vacuum) mode.

Before the re-injection of new HRP-MNPs, the capillary was rinsed with 0.1M NaOH, ROP water and running buffer for 3, 3, and 6 min, respectively. At the end of each day, the capillary was rinsed with 0.1M NaOH for 5 min followed by ROP water for 5 min.

The capillary was sandwiched between two permanent magnets (i.d. 4 mm × 6 mm) Neody Magnet (Nd–Fe–B) purchased from As One, Ltd. (Osaka, Japan) situated 8 cm from the inlet.

For the off-line experiments, 20μl HRP-MNPs suspension (10 mg/mL) were mixed with 1 mL PB (pH 7.4) containing APAP (1×10^{-4}M) and H_2O_2 (1×10^{-4}M). After incubation at room temperature for certain time (1, 10, 20, 30, 50 min), a magnet was placed onto the reaction tube to trap the HRP-MNPs, then the supernatant was electrokinetically injected into the capillary by applying a constant current of 90μA for 10s.

Spectrophotometric experiments were performed with a Pye Unicam PU 8650-Philips.

RESULTS

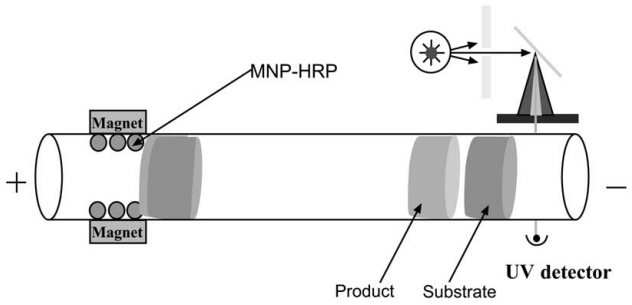
A schematic drawing of the analysis concept is depicted in Fig. (**1**). The enzyme immobilized beads were introduced into the capillary by hydrodynamic injection and spontaneously trapped by the magnets. The key component of the system was the microreactor sandwiched between two permanent magnets. The retention of the magnetic beads by the magnets was clearly observed by taking a macroscopic photo of the transparent capillary as illustrated in Fig. (**1**).

Preliminary Experiments

The injection of HRP-MMPs into the CE was first investigated, but a base line with many parasite peaks was obtained. This could be related to the relative broad particle size domain and low magnetite content in the MMPs [18] making them not sufficiently retained and readily flushed away by the electroosmotic flow leading to progressive leaking of the particles out of the capillary. Subsequent experiments were performed with the HRP-MNPs which possessed a higher magnetite content (> 70%) and were coated by a polymer core shell structure with a uniform diameter of 300 nm (RSD: 20%) and a specific area of 10 m^2/g [36].

Sulfated-β-CD was used as micellar additive to the electrolyte [7]. At the studied pH, both APAP and NAPQI

(**A**)



(**B**)

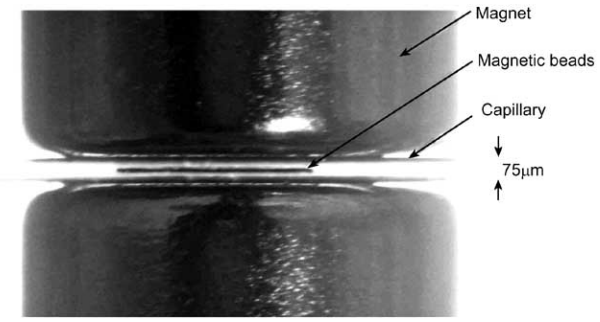


Fig. (1). (**A**) Schematic illustration of the EMMA device used for biotransformation studies. (**B**) Optical image of a microreactor after packing with magnetic nanoparticles (MNPs).

were neutral species making their separation difficult but possible in the presence of sulfated-β-CD. This was likely due to a stronger interaction of APAP than NAPQI to the negatively-charged sulfated-β-CD, similarly as reported in the literature for the CE separation of hydroquinone and 1,4-benzoquinone [7].

Enzyme Kinetics Studies

The concentration of H_2O_2 in the CE running buffer was 100μM. The reaction rate was determined by measuring the peak area of the product at 245nm. The HRP-MNPs (10 mg/mL) were first introduced into the capillary by hydrodynamic (vacuum) injection for 3 seconds. Under an applied current of 90 μA and during 3 min they moved to and were trapped by the magnets. Different concentrations of APAP (10, 30, 50, 70 100 μM) were subsequently introduced by electrokinetic injection at 90μA for 10 s. As illustrated in Fig. (**2**), only one product was obtained besides the APAP peak. The repeatability (n =3) of the assay was checked at 50μM APAP with (i) the same HRP-MNPs microreactor; product peak area RSD= 6.7%, product migration time RSD = 2.5% and (ii) with different HRP-MNPs reactors; RSD = 4.7% (peak area), RSD = 0.3% (migration time). It was observed that by using the same microreactor and beyond five APAP injections, there was a progressive decrease of signal which could be attributed to the inhibition of the enzyme activity by APAP oxidation polymer products or to gradual release of HRP linked to the MNPs [24].

The conversion efficiency in the microreactor was calculated according to the ratio of the APAP (100μM) peak area with and without the microreactor in the CE set up. For a 3s injection of HRP-MNPs, the conversion efficiency of the microreactor was approximately 6%. This low yield can be explained by the short reaction time within the in-capillary microreactor, which can be estimated to be 1-2s based on the electroosmotic flow magnitude and microreactor length. As developed below, this peak likely corresponded to an APAP dimer (A2).

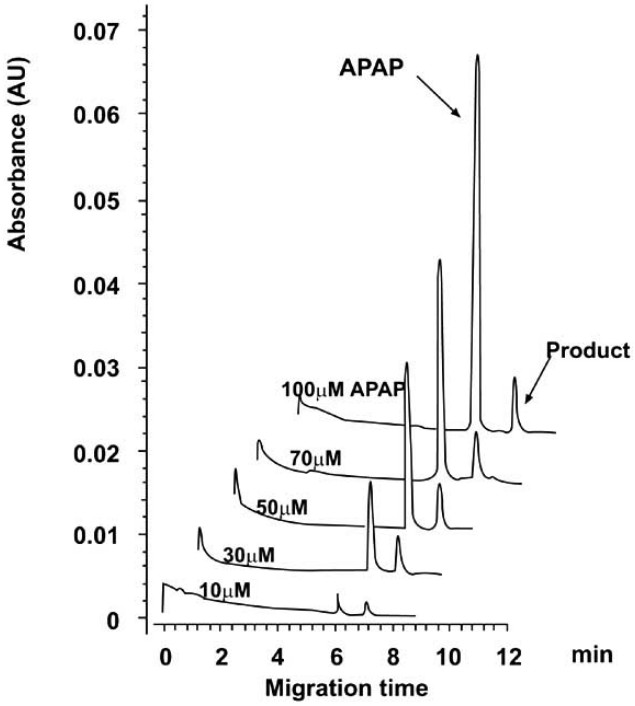


Fig. (2). Electropherograms of in-capillary reactions at concentrations of APAP between 10-100μM. Separation conditions: background electrolyte 12.5mM PB (pH 7.4), separation current 90μA (18kV), detection at 245 nm, temperature of capillary 25°C. Injection: 1mg/ml HRP-MNPs in PB; hydrodynamic mode for 3s, APAP in PB (12.5mM) and sulfated-β-CD(20mg/ml); electrokinetic mode 18kV for 10s.

Determination of K_m

The initial reaction velocity was calculated by considering the peak area of the reaction product with respect to APAP concentration. A typical Michaelis–Menten plot was obtained (Fig. **3A**). The K_m for the HRP reaction was estimated by linear regression from a Lineweaver–Burk plot. Five APAP concentrations were studied ranging from 10 to 100 μM. Each concentration was analyzed in triplicate. The double reciprocal plot of these data (Fig. **3B**) gave an apparent K_m value of $K_m^{app} = 53 \pm 5\mu M$.

Biotransformation Studies

In order to obtain mechanistic information on the reaction product(s) generated in the microreactor, studies were realized with the developed setup by performing the APAP enzymatic conversion both in-line and off-line. As reported in the literature, the oxidation of APAP by HRP is a time dependent process giving rise first to NAPSQI radical which rapidly dimerized, with eventually subsequent formation of trimers and tetramers of APAP [31, 32]. In-line CE studies as a function of HRP-MNPs hydrodynamic injection time (5 and 8 s) showed (i) the APAP peak (ii) the main product (ii) and (iii) two minor additional peaks (data not shown). Such longer injection times likely gave a higher loading of HRP-MNPs in the microreactor which may explain the additional peaks observed. The three peaks detected in-line, post APAP migration, did not correspond to NAPQI as inferred by injecting a NAPQI standard solution. Injection of the latter gave a peak appearing before the APAP peak. This pre-APAP peak was detected when reacting off-line the HRP-MNP/H_2O_2 with APAP for longer reaction times (10 min) i.e. during a time period allowing for a higher amount of metabolites to be generated (Fig. **4**). The nature of the pre-APAP peak; however, was not certified due to NAPQI, as it could also correspond to hydroxylated APAP. The three post-APAP peaks were not directly identified in line. It was inferred, based on the literature data, that these peaks may be APAP dimer (A2), trimer (A3) and tetramer (A4) as reported in Schemes **1** and **2**.

CONCLUSIONS

This work aimed at showing the utility of enzyme immobilized magnetic beads for drug biotransformation

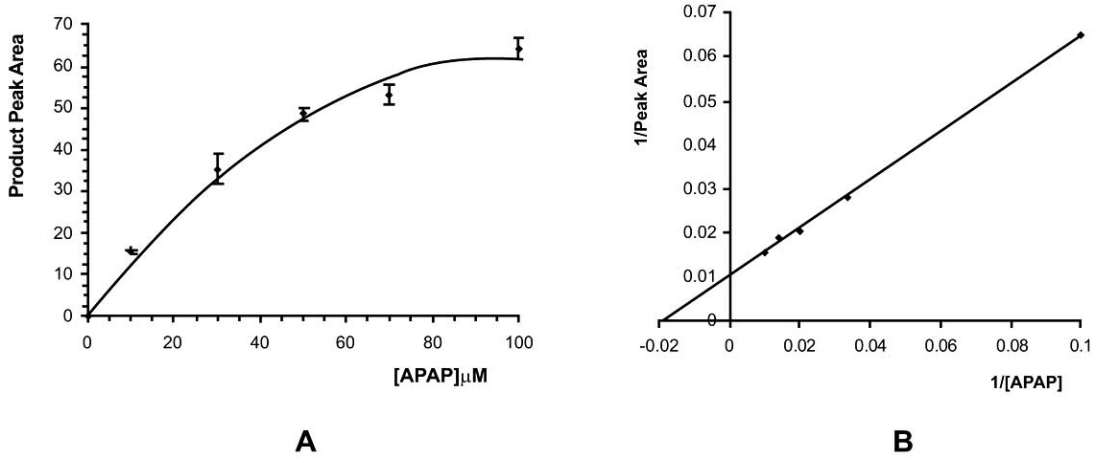
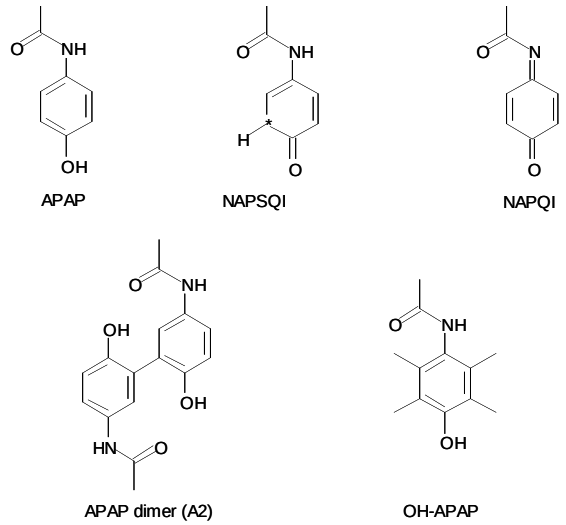
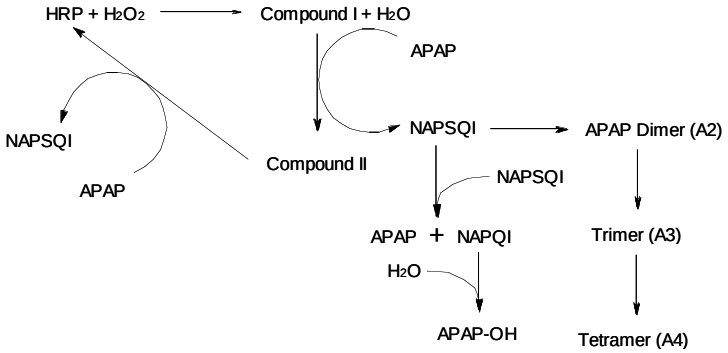


Fig. (3). (**A**) Michaelis–Menten plot for the in-line HRP reactor reaction with acetaminophen. (**B**) Corresponding linear Lineweaver–Burk plot where 1/Peak Area is plotted versus 1/[APAP].



Scheme 1. Chemical structure of paracetamol (APAP) and some derivatives : N-acetyl-p-benzosemiquinone imine (NAPSQI), N-acetyl parabenzoquinone imine (NAPQI), paracetamol dimer (APAP dimer A2), hydroxylated paracetamol (OH-APAP).



Scheme 2. Biotransformation pathway of paracetamol (APAP) in the HRP-MNPs /H_2O_2 suspension at pH 7.4.

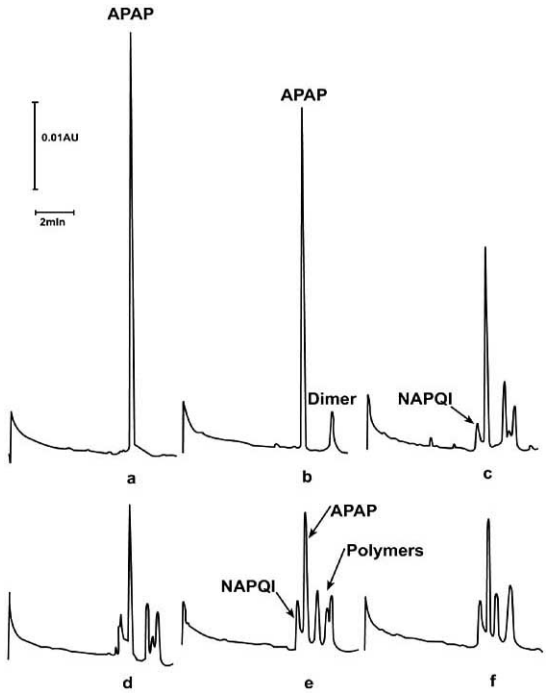


Fig. (4). Off-line biotransformation studies. [APAP]=10^{-4}M, 20µl of HRP-NPMs [H_2O_2] = 10^{-4}M. Reaction time a: 0 min b: 1 min, c: 10 min, d: 20 min, e:30 min, f:50 min.

studies in a capillary electrophoresis configuration. The magnetically trapped enzyme immobilized beads do not interfere with the detection system in line. The enzyme loading is low and the relatively short residence time of the substrate in the microreactor during in-line experiments can be of interest in order to discriminate between reaction kinetics. Affinity data (K_m^{app}) relative to paracetamol for the HRP-MNPs/H_2O_2 system have been obtained by the developed in-line CE set up. Further analytical development can be expected e.g. by using a longer microreactor, smaller magnetic beads with a higher enzymatic loading and by using a CE system coupled to a MS detector.

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