Determination of adenosine 5'-triphosphate by Flow Injection with Luminol Chemiluminescence Detection Using Immobilized Alkaline Phosphatase Enzyme Reactor

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Summary: A flow injection method is described for the determination of adenosine 5'-triphosphate using immobilized alkaline phosphatase based on luminol chemiluminescence detection. The molybdophosphoric heteropoly acid formed by phosphate and ammonium molybdate in acidic condition generated chemiluminescence emission by the oxidation of luminol. The detection limit (2s) was 1.0×10^{-7} M with a sample throughput of 45 h^{-1}. The calibration graph was linear over the range 2.0 – 10 x 10^{-6} M (r^2 = 0.9987), with relative standard deviation (RSD, n = 4) in the range 1.5 – 3.7%. Controlled porosity glass was used as a support for alkaline phosphatase immobilization and the immobilized enzyme showed good stability, and no deterioration in enzyme activity was recorded after use for three months.

Introduction

Adenosine 5'-triphosphate (ATP) is the biological energy currency and present in similar intracellular concentrations in all living cells. A large number of enzymes are involved in the production and consumption of ATP. The amount of ATP per cell is essentially proportional to the intracellular volume. Consequently most bacterial cells contain around 2×10^{-18} mol/ cell, while mammalian cells often contain 10^{-15} mol / cell. When the cell dies from natural causes, ATP is rapidly degraded by intracellular enzymes. These enzymes are eventually released and will degrade any ATP appearing outside cells. If cells are killed in a way that also inactivates enzymes, some ATP may appear even outside cells. ATP has also been used as a tool for detection of biological contamination [1-2].

Alkaline phosphatase (EC 3.1.3.1) is classically described as homodimeric nonspecific metalloenzymes, which catalyze phospho-monoesterase reactions [3]. The fact that they are widely found in nature from bacteria to mammals indicates that alkaline phosphatases are included in fundamen-tal biochemical processes [4]. Despite the fact that their physiological function is not clear, their induced production under inorganic phosphate starvation in many species (specially prokaryotic organism) indicates that they play a vital role in the phosphate metabolism. In mammals they are linked with transport mechanisms [5]. Alkaline phosphatase (ALP) is present in many tissues such as gastrointestinal mucosa, liver, spleen, vascular endothelium, renal tubule, thyroid, placenta, myeloid and osteoblasts [6-7]. This is a non-specific enzyme that splits phosphoric acid from phosphate esters, acting at alkaline pH, hence the name. It is dimeric allosteric enzyme [8] with four zinc ions, although only two of them are necessary for activity [9]. It is believed that ALP facilitates the transport of metabolites and lipids across the membrane. The enzyme is also associated with bone mineralization, but its function and natural substrates are still unknown [10]. ALP has been used for various purposes including, to check its inhibition by metal chelating agents, metal ions, and orthophosphate, related multi-charged anions, beryllium and some pesticides [11-15]. ALP is in common use in research laboratories for removal of the 5' phosphate groups from DNA and RNA. It will also remove phosphates from nucleotides and proteins.

Various methods have been reported for the determination of ATP in biomass based on ^{31}P nuclear magnetic resonance spectroscopy [16], electrochemical biosensors [17], spectrophotometric [18], chromatographic [19], and commonly bioluminometric [20 - 23] methods. Microbial quality can also be rapidly detected by microcolony fluorescence staining [24], enzyme linked immuno-assay [25], and polymerase chain reaction [26-27]. Chemiluminescence and bioluminescence (BL) detection systems have been found to provide rapid and sensitive methods for trace metals / anions and ATP due to its transient nature. BL methods with flow injection analysis have been reported for the

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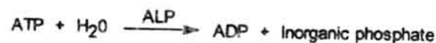
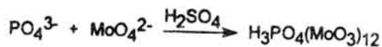
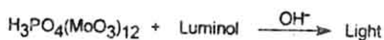
determination of ATP using soluble and immobilized luciferase enzyme [28-30]. A FI-CL system has been reported to determine ATP using a membrane-immobilized enzyme reactor with a flow injection luminol-CL detection. Alkaline phosphatase from *Escherichia coli* immobilized on nitrocellulose membrane by adsorption through which a sample of ATP was circulated for 20 min producing orthophosphate. This was coupled with molybdate resulting in heteropoly acid, which subsequently reacted with luminol in basic medium produced CL emission. The limit of detection for ATP was found 10 nM with sample throughput 3 h^{-1} [31].

This study reports a flow injection chemiluminescence method for the determination of ATP at submicro molar concentrations using a simple flow injection manifold. The method is based on the use of immobilized alkaline phosphatase enzyme covalently attached with control porosity glass produced phosphate from ATP which is coupled with ammonium molybdate resulted in heteropoly acid formation for the oxidation of luminol. The chemical and enzymatic reaction scheme can be shown as:







This provides a highly selective enzymatic procedure added by the sensitivity and selectivity of the CL detection. The stability of the immobilized enzyme column and its on-line application is the clear advantage of this method.

Results and Discussion

Yield of the cross-linking method

In the cross-linking procedure more than 80 % of the enzyme incubated with alkylaminated beads was covalently bound to the glass. About 8 -20 % of the protein incubated with glutaraldehyde derivatized glass beads was detected in the supernatant, after the reaction. The immobilized enzyme packed in the glass column was used for three months period (stored at 4 °C) without any appreciable change in their activity.

Optimization of the FI manifold

In order to establish optimal conditions for the lowest possible detection limit of ATP using immobilized ALP enzyme column, the effect of various parameters was investigated, i.e. the pH of the borate buffer, luminol, ammonium molybdate and sulfuric acid concentrations, sample volume, flow rate, incubation coil length and immobilized column temperature. All of these studies were performed with a 1×10^{-5} M ATP standard solution and a detector (PMT) voltage of 1000 ± 5.0 V.

Luminol chemiluminescence (CL) is particularly dependent on the reaction pH. In the proposed FI-CL system with borate/NaOH buffer (pH 12.5), the chemiluminescence intensity was higher than with carbonate buffer and therefore the pH optimum for the luminol reaction with borate buffer was further investigated in the range 10.0 - 12.5. Maximum CL emission was observed at pH 12.5 as shown in Fig. 2(a). The use of sodium hydroxide (0.25 M) solution was also investigated but the CL response, although of similar intensity, resulted in irreproducible peaks and a non-steady baseline. Therefore borate buffer (pH 12.5, 0.1 M) was used for all subsequent studies.

The effect of luminol concentration on the determination of ATP was studied over the range 1.0 - 100 x 10^{-6} M. As shown in Fig. 2(b), the CL intensity increased from 1.0 - 50 x 10^{-6} M but no appreciable increase in intensity was observed above this concentration due to saturation. A luminol concentration of 50 x 10^{-6} M was therefore used for all subsequent experiments. The difference in reagent sensitivity was observed as the luminol solution aged for some time as found by other workers [32].

The effect of ammonium molybdate was studied over the range 1.0 - 30 x 10^{-4} M. As shown in Fig. 2(c), the CL response increased up to 1.0 - 15 x 10^{-4} M ammonium molybdate, above which the response decreased due to an unfavorable acid / molybdate ratio [33]. Therefore, 15 x 10^{-4} M ammonium molybdate was used for all subsequent studies. The effect of sulfuric acid was also studied from 0.01 - 0.07 M as shown in Fig. 2(d) and 0.04 M and was used for all further studies.

The effect of pH on the activity of immobilized alkaline phosphatase was investigated using

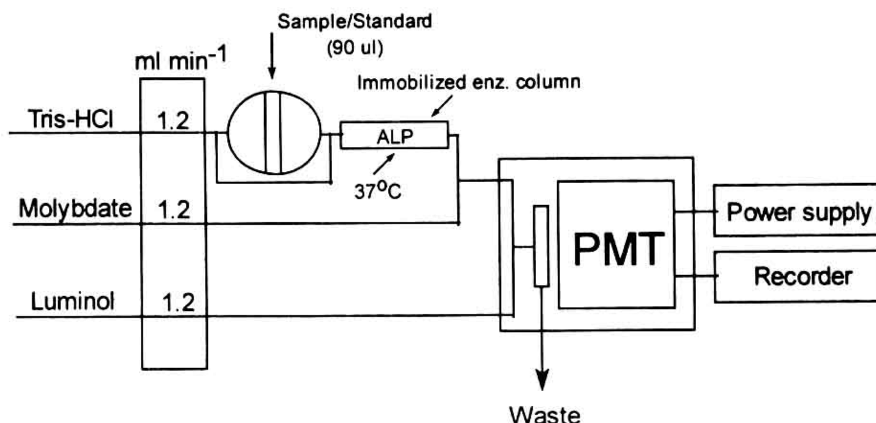


Fig. 1. Flow injection chemiluminescence (FI-CL) manifold for the determination of ATP.

Table-1:Effect of key physical parameters on the determination of ATP using FI-CL manifold.

Tris-HCl buffer (0.1 M) pH	8.5	8.75	9.0	9.25	9.5
CL signal (mV)*	4.6	6.1	8.5	7.6	6.2
RSD (%)	1.9	2.0	2.4	1.7	1.5
Flow rate (ml min^{-1})	0.3	0.6	0.9	1.2	1.5
CL signal (mV)*	3.8	5.7	8.0	8.4	6.7
RSD (%)	1.7	2.3	1.9	2.5	1.8
Sample volume (μL)	30	60	90	120	150
CL signal (mV)*	3.2	6.0	7.8	8.0	8.2
RSD (%)	1.8	1.5	2.0	2.3	1.6
Mixing coil (cm)	Without	30	60	90	120
CL signal (mV)*	4.0	5.8	8.0	8.2	8.3
RSD (%)					
Temperature (°C)	20	30	40	50	60
CL signal (mV)*	4.5	6.7	8.5	9.2	7.6
RSD (%)	2.2	2.0	1.8	2.1	1.9

*Mean of four injections.

Tris-HCl buffer (0.1 M) in the range 8.5 - 9.5 as a sample carrier stream (Table-1). Maximum CL emission was observed at pH 9.0 and used in further investigation of conditions.

The effect of flow rates, sample volume, coil length and temperature is shown in Table-1. Flow rates for each of the three channels was simultaneously investigated over the range 0.3 -1.5 mL min^{-1} in terms of sensitivity, sample throughput and reagent consumption. Maximum CL intensity was observed at a flow rate of 1.2 mL min^{-1} with a steady baseline and reproducible peak height (rsd < 1.4 %, n = 4). The effect of reaction coil length on the formation of the heteropoly acid was investigated in the range 10 - 120 cm and the optimum (60 cm) was used for all further studies. The effect of sample volume on the

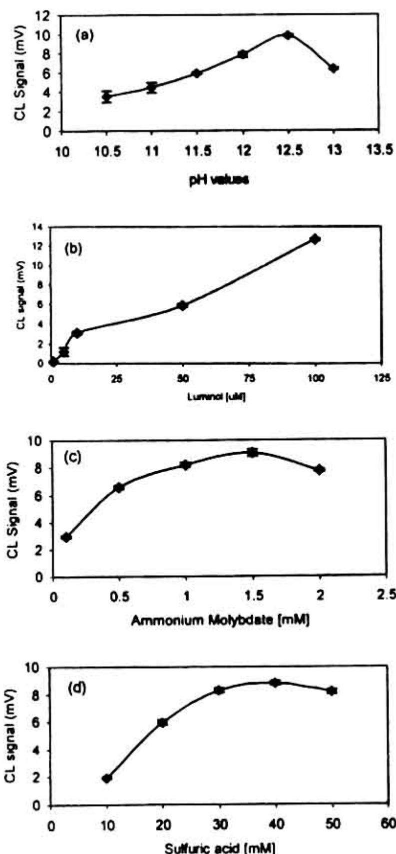


Fig. 2. Variation of CL intensity with: (a) pH of borate buffer (0.1 M); and concentrations of (b) luminol; (c) ammonium molybdate; and (d) sulfuric acid.

sensitivity of the flow system was studied in the range 30 - 120 μL, with a maximum CL signal for volumes of 90 μL and above. The effect of temperature on the activity of immobilized enzyme was studied over the range 20 – 60 °C. There is an increase in intensity with increase in temperature up to 50 °C. However, the column was maintained at 37 °C to protect the enzyme from denaturation and to increase the lifetime of the enzyme column. The immobilized ALP enzyme showed good operational stability over a storage period of 60 days and more than 430 injections were made during 20 days of use, displayed 90 % conversion when stored at 4 °C. After 60 days the percent substrate conversion was slightly decreased.

Analytical figures of merit

Under the optimized conditions established, a calibration data was obtained for the determination of ATP in the range 2 – 10 $\times 10^{-6}$ M given in Table-2. The correlation coefficient was 0.9987 (n = 5) and regression equation y = 0.9973x - 0.5075 [y = CL signal, x = concentration (M)]. The rsd of the method was 1.5 – 3.7 % (n = 4) over the range studied. The limit of detection (2s) was 2.0 $\times 10^{-7}$ M ATP with a sample throughput 45 h^{-1}, which is sufficiently low and rapid to monitor ATP concentrations in contaminated samples of milk and sludge filtrate.

Interferences

The effect of some metal ions on the luminol-CL system (in the absence of immobilized column) and on the activity of immobilized alkaline phosphatase was studied. Calcium(II); 100 mg l^{-1}, Ni(II) and Zn(II); 1 mg l^{-1}, and Fe(III); 0.1 mg l^{-1}, had no significant effect. Cobalt(II) and Fe(II); 0.01 mg l^{-1} enhanced the CL signal due to their action as catalysts for luminol oxidation in the presence of molecular oxygen [34-35]. Magnesium(II); 100 mg l^{-1} had an enhancing effect on both the CL signal blank and on the activity of immobilized enzyme and reported as an activator for alkaline phosphatase [36]. Zinc(II), Ni(II), Co(II) and Fe(III) were found to be inhibitors except calcium.

Experimental

Reagents and solutions

All glass ware used during the experiments and for storage of reagents and standards was precleaned with 20% HCl for 48 h, thoroughly rinsed with ultra high purity (UHP) deionised water (18.2 MΩ cm^{-1}, Elgastat, Maxima, England), stored in plastic bags to prevent contamination and used as required. All reagents were of analytical grade, supplied by Merck BDH, UK, unless stated otherwise, and solutions were prepared in UHP water.

Table 2: Calibration data for ATP.

ATP ($\times 10^{-6}$ M)	CL signal (mV)*	RSD (%)
Blank (Tris-HCl Buffer)	0.04 ± 0.005	1.8
2.0	1.4 ± 0.10	3.1
4.0	3.4 ± 0.11	3.7
6.0	5.6 ± 0.08	2.0
8.0	7.4 ± 0.13	1.8
10.0	9.3 ± 0.1	1.6

*Mean of four injections.

Adenosine 5'-triphosphate stock solution (0.001 M) was prepared by dissolving 28 mg of adenosine 5'-triphosphate (sodium salt, BDH, England) in 50 mL of UHP water. Working standards were prepared by suitable dilution as required. Standard solutions (1000 mg L^{-1}) of Ca(II), Mg(II), Co(II), Ni(II), Zn(II) and Fe(III) were prepared from their respective salts (BDH, England) in UHP water and various working solutions were prepared from these stock solutions in Tris-HCl buffer (0.1 M, pH 9.0) for interference studies.

Ammonium molybdate stock solution (0.01 M) was prepared by dissolving 1.24 g of ammonium molybdate (VI) tetra hydrate ($(NH_4)_6MoO_{24}.4H_2O$) in 100 mL of UHP water. A working solution (0.0015 M) was prepared by diluting 15.0 mL of the stock in 100 mL of water containing sulfuric acid (0.04 M).

Luminol stock solution (0.01 M) was prepared by dissolving 0.178 g of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Aldrich) in 20 mL of carbonate buffer (0.1 M, pH 10.5) followed by sonication for 30 min, made up to 100 mL with water and stored at 4 °C. A working luminol solution (5×10^{-5} M) was prepared by diluting 0.5 mL of the stock solution to 100 mL with borate buffer (0.1 M) and adjusting to pH 12.5 with sodium hydroxide solution (2.0 M).

Enzyme immobilization

Alkaline phosphatase (25 units) was immobilized on 0.5 g of aminopropyl derivatized CPG by

cross-linking with glutaraldehyde, following the procedures reported previously [37-38]. The immobilization was carried out by incubating the derivatized glass beads overnight at 4 °C with the enzyme dissolved in 0.5 ml of phosphate buffer (0.1 M, pH 6.0). After immobilization, the aqueous phase was measured for protein content according to the reported method [39] to evaluate the yield of the immobilization procedure. Approximately 80% of the enzyme incubated with the glutaraldehyde treated beads was covalently bound and only 8 - 20 % of the protein was detected in the aqueous phase. The immobilized enzyme was packed in a glass column (2.5 x 50 mm) and stored in Tris-HCl buffer (0.1 M, pH 9.0) at 4 °C until use. The immobilized column was utilized for about 100 h without any appreciable decrease in their activity and the enzymatic activity was completely preserved after two months storage at 4 °C.

Instrumentation and procedures

The flow-injection chemiluminescence manifold used for this study is shown in Fig. 1. A peristaltic pump (Ismatec Reglo 100, 4 channel, Switzerland) was used to deliver the sample carrier and reagent solutions at a flow rate of 1.2 mL min^{-1}. A rotary injection valve (Rheodyne 5020, Anachem, Luton, UK) was used to inject ATP standards (90 µL) into Tris-HCl buffer (0.1 M, pH 9.0) stream which was passed through a immobilized ALP enzyme column (50 mm x 2.0 mm i.d.) and merged with a stream of ammonium molybdate (15×10^{-4} M in 0.04 M sulfuric acid) before passing through a reaction coil (60 cm x 0.75 mm i.d). This stream was then merged at a T-piece with the chemiluminescence reagent stream. The merged stream traveled 3.0 cm before passing through a quartz glass spiral flow cell (1.1 mm i.d, 130 µL internal volume) placed directly in front of an end window photomultiplier tube (PMT, 9798QA, Electron Tubes, Ruislip, UK). The PMT, glass coil and T-piece were enclosed in a light tight housing [40-41]. The detector output was recorded using a chart recorder (Kipp & Zonen Ltd., Lincoln, UK). The immobilized enzyme packed glass column was thermostated at 30 °C by flowing water through a water jacket [42] around the enzyme column when in operation.

Conclusions

A simple flow injection method was established for ATP determination based on chemiluminescent detection. The advantage of the present method on the other methods derives from the use of immobilized enzyme on a solid support. The use of immobilized enzyme in FI-CL system makes the procedure of ATP quantitation very easy, selective and economical. The effect of various ions showed no effect on the enzymatic release of hydrogen peroxide and CL reaction.

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