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A NOVEL CONDUCTOMETRIC BIOSENSOR BASED ON HEXOKINASE FOR DETERMINATION OF ADENOSINE TRIPHOSPHATE

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The paper presents a simple and inexpensive reusable biosensor for determination of the concentration of adenosine-5'-triphosphate (ATP) in aqueous samples. The biosensor is based on a conductometric transducer which contains two pairs of gold interdigitated electrodes. An enzyme hexokinase was immobilized onto one pair of electrodes, and bovine serum albumin - onto another pair (thus, a differential mode of measurement was used). Conditions of hexokinase immobilization on the transducer by cross-linking via glutaraldehyde were optimized. Influence of experimental conditions (concentration of magnesium ions, ionic strength and concentration of the working buffer) on the biosensor work was studied. The reproducibility of biosensor responses and operational stability of the biosensor were checked during one week. Dry storage at -18 °C was shown to be the best conditions to store the biosensor. The biosensor was successfully applied for measurements of ATP concentration in pharmaceutical samples. The proposed biosensor may be used in future for determination of ATP and/or glucose in water samples.

Keywords: conductometric biosensor, immobilized enzymes, hexokinase, adenosine triphosphate, glucose.

Introduction

Adenosine-5'-triphosphate (ATP) is an organic molecule (nucleoside triphosphate) which consists of adenine, ribose, and three phosphoric acid residues. ATP serves as a

temporary carrier of energy in all living cells, so it is a common substance in any organism. In the cells, new ATP molecules are synthesized during decomposition of organic matter. Energy stored in ATP is utilized for numerous processes of biosynthesis. ATP is also a source of energy for the function of cell membrane proteins, an important precursor of the second messenger - cyclic adenosine monophosphate, an allosteric regulator of a number of cell processes, etc. [1, 2].

Determination of ATP concentration is promising for estimation of the energetic state of cells and tissues. Also, ATP determination may be useful in medicine for studying the biological processes, in which it is involved, namely, the regulation of muscle contraction and platelet aggregation, maintenance of vascular tone, neurotransmission and regulation of the nervous system [3, 4]. The determination of ATP concentration in human blood is promising for the diagnosis of various diseases [5]. The creation of kinase inhibitors may include an evaluation of the amount of ATP used by kinases in the presence of inhibitors and their absence.

Modern standard methods of precise determination of ATP concentration, such as spectrophotometry [6] and liquid chromatography [7], require qualified personnel and sophisticated expensive equipment, need complex pretreatment of samples for analysis [8, 9]. Fluorescent, bio- and chemiluminescent methods are free from the above drawbacks; however, often they do not correspond with the demands of ATP monitoring [10]. Radioisotope methods of ATP analysis are highly accurate, but potentially dangerous [11]. Therefore, at present the development of easy-to-use, accurate, fast, selective and low-cost method for determination of ATP concentration in biotechnology and research is an actual challenge.

Today there are several laboratory prototypes of biosensors for ATP determination. They are based on pH-sensitive field effect transistors [12], amperometric glassy carbon electrodes [13], amperometric platinum microelectrodes [14], which are usually coated with the enzymes. A common drawback of these biosensors is quite complex structure of electrodes, which increases their cost and reduces the possibility of mass production. Moreover, often two-enzyme systems are used as biorecognition elements of biosensors, what increases overall complexity of the biosensors. Recently, sensors based on photo detection of ATP binding with different receptor molecules were developed [15, 16, 17]. However, the measurement procedure in these cases is quite complicated.

An alternative is an application of conductometric biosensors based on planar transducers. These biosensors are advantageous because of simple structure of transducers, low-cost manufacture and fast measurement procedure [18]. Also they do not need a reference or other additional electrodes, and their response time is quite fast. On the other hand, the conductometric transducers are sensitive to all charged substances, including ATP, which presents significant difficulties in measurement of real biological samples. This is the reason why conductometric biosensors are inferior to amperometric and potentiometric biosensors. To the best of our knowledge, no conductometric biosensor for ATP determination has yet been described.

The study was aimed at the development of an original conductometric hexokinase-based biosensor that would be simpler in structure and usage comparing with the existing ATP biosensors. To prevent an influence of charged particles, a differential two-step procedure of measurement was supposed.

Materials and methods

Materials

Enzyme hexokinase (HEX, EC 2.7.1.1) from *Saccharomyces cerevisiae* with activity 30.6 U/mg (Sigma-Aldrich, Germany) was used for creation of biorecognition elements of biosensors. Bovine serum albumin (BSA, fraction V), glucose, ATP (disodium salt hydrate, grade 1, ≥99%), glycerol, HEPES, magnesium chloride, and 50% aqueous solution of glutaraldehyde (GA) have been purchased from Sigma-Aldrich Chemie (Germany). All other chemicals were of p.a. grade.

Design of conductometric transducers

Biosensors were based on planar conductometric transducers. They were manufactured in V. Lashkaryov Institute of Semiconductor Physics of National Academy of Science of Ukraine (Kyiv, Ukraine) in accordance with our recommendations. Each transducer was 5 x 30 mm in size and contained two pairs of gold interdigitated electrodes deposited onto a ceramic support. The sensitive area of each electrode pair was about 1.0 x 1.5 mm. The width of each digit as well as interdigital space was 20 μm. The deposition of gold onto ceramic surface was done by vacuum sputtering.

A photograph and microphotographs of these transducers can be found in [19].

Transducers were intended to operate in a differential mode of measurements: biorecognition element (enzyme) was placed on one pair of electrodes, and reference element

(BSA membrane) – on another pair. Details about the differential mode can be found in the section "Measurement procedure".

Preparation of bioselective elements

Hexokinase was immobilized by the following procedure. The initial solution for preparing the bioselective membrane of the biosensor contained 10% HEX (hereafter – w/w), 5% BSA and 10% glycerol in 20 mM phosphate buffer, pH 6.5; the solution for a reference membrane consisted of 15% BSA and 10% glycerol in the same buffer. Glycerol was added to the solutions to stabilize HEX and BSA during storage at -18°C and to prevent early drying of the solutions on the transducer surface. In case of solution for bioselective membrane, BSA was added to stabilize HEX in solution; also BSA and HEX formed intermolecular bindings during immobilization. These solutions were mixed with 0.5% aqueous solution of glutaraldehyde (cross-linking agent) in a ratio of 1:1 and immediately deposited onto the sensitive regions of transducer (approximately 100 nL of the mixture onto each region). Afterwards, the created biosensors were dried for 30 min in air at room temperature. During this time, GA formed covalent bonds between amino groups of enzymes and BSA. Then the biosensors were immersed in the working buffer for 10 min in order to stop immobilization and to wash out unbound components.

Measurement procedure

Conductometric transducers were connected to the portative device for conductometric measurements (9.5 cm * 2.5 cm * 13.5 cm) that was made in Institute of Electrodynamics of National Academy of Sciences of Ukraine (Kiev, Ukraine). This device applied sinusoidal potential with frequency of 36.5 kHz and amplitude of 14 mV allowed avoiding such effects as faradaic processes, double-layer charging and polarization of the microelectrodes. The nonspecific changes in the output signal induced by the fluctuations of ion concentrations, medium pH, etc. were decreased due to usage of differential mode of measurement: conductivity of solution measured by reference pair of electrodes was subtracted from the conductivity measured by pair of electrodes with biorecognition element. The measurements were carried out in a 2 ml plastic cell filled with 5 mM HEPES buffer, pH 7.4, under magnetic stirring.

All experiments were repeated three times. The data in the figures is either mean of three repeated results of the experiment or mean ± standard deviation (SD).

Results and discussion

Working principle of the biosensor

The operation of conductometric biosensor for ATP determination is based on the enzymatic reaction in bioselective membrane:

$$\text{D-glucose} + \text{ATP} \xrightarrow{\text{HEX}} \text{D-glucose-6-phosphate} + \text{ADP} \quad (1).$$

During the reaction (1), the local concentration of ions in the working membrane increases, thus, the conductivity of the solution in the near-electrode region changes, which is registered by a conductometric transducer. This change of conductivity is directly proportional to a concentration of glucose and ATP in the working cell. It is important that this reaction is catalyzed by a single enzyme, what is an advantage of the proposed biosensor over existing amperometric and potentiometric ones which require two enzymes to operate. This reaction itself neither generate electroactive products nor change solution pH, and can be registered directly only by conductometry.

A typical procedure for measuring the ATP concentration is as follows (Fig. 1, A). Initially, ATP or ATP-containing sample is added to the working cell. Since ATP is a charged substance, the biosensor generates a nonspecific signal (a peak at 50 s in Fig. 1, A). This signal is compensated by using the differential measuring mode if working and reference membranes are identical by their morphology and thickness (the peak disappears and the biosensor signal returns to baseline). After stabilization of the signal, a model glucose solution is added to the working cell, and the reaction (1) takes place in the enzyme membrane. This reaction results in the appearance of two new charged substances (ADP and phosphorylated glucose), thus the solution conductivity changes and the biosensor signal is generated. Since glucose is uncharged substance its addition does not cause any non-specific reaction of the biosensor, and the signal is generated exclusively due to the enzymatic reaction. Thus, the two-step procedure of addition of substances allows avoiding one of the main disadvantages of conductometric biosensors - sensitivity to any charged substances. The analysis lasts for about 5 min, which is quite acceptable. After measurements, the biosensor and the working cell should be washed from substrates during 5 min using the working buffer, and the biosensor can be used again.

As a control, the inverse experiment was carried out (Fig. 1, B), i.e. first glucose and then ATP were added to the working cell. As seen, there was no response to glucose, whereas the response after ATP addition coincided with the response, obtained in the previous experiment after the glucose addition.

Overall, experiments in Fig. 1 demonstrate that separate addition of glucose or ATP to the working cell does not produce a biosensor response. The response is generated only after both ATP and glucose appear in the cell. Furthermore, charged substances like ADP or ascorbic acid that can be present in a sample will not interfere measurements because the biosensor response depends only on concentrations of glucose and ATP, while possible non-specific response to ions (including ATP) is compensated by differential measurement mode (see section Measuring procedure).

Fig. 1.

Selection of conditions of hexokinase immobilization

Immobilization of biological material onto the transducer surface is an important step in the biosensor development. In our previous work with the amperometric ATP biosensor based on HEX and glucose oxidase we successfully used the co-immobilization of HEX with glucose oxidase, BSA, glycerol and GA [20]. GA covalently cross-linked the enzymes and BSA molecules whereas glycerol served as an auxiliary substance. This is why in the present work we used this method of HEX immobilization, but optimized it for conductometric transducers.

First, different HEX/BSA ratios in the composition of enzyme membrane were checked. The stock solutions with 3% – 15% HEX, and 0% – 5% BSA were used. These solutions were mixed with 0.5% or 0.8% GA (1:1) and then the biosensors were kept in air for 15-30 min. The highest biosensor responses were observed at initial concentrations of HEX - 10%, BSA - 5%, GA 0.5%, and duration of immobilization - 30 min.

Different concentrations of GA were also checked (from 0.3 to 0.9% before mixing with the enzyme solution). When using the GA concentration of 0.7% and 0.9%, almost no responses to glucose and ATP were observed because of a significant decrease in the HEX activity after immobilization. In the case of 0.3% and 0.5% GA, the biosensor responses were almost identical. Therefore, in further experiments 0.5% GA was used for immobilization.

An influence of composition of working buffer on biosensor operation

The composition of working buffer can to some extent influence the biosensor operation since the enzyme activity depends on the solution composition. Therefore, at the next stage of research such parameters as Mg^{2+} concentration, buffer capacity and ionic strength were investigated.

The presence of Mg^{2+} ions in the working buffer is necessary for the HEX since magnesium stabilizes the ATP molecule and serves as a HEX activator. To determine the optimal Mg^{2+} concentration in the working buffer, the biosensor work was tested at Mg^{2+} concentration from 1 mM to 4 mM. In the experiment, the aliquots of concentrated magnesium chloride solution were initially added to the working buffer, then 100 μM of ATP was added; afterwards the baseline was obtained, and in 30 s 200 μM of glucose were injected.

The biosensor responses were evaluated after addition of glucose (the non-selective response to ATP in the absence of glucose did not depend on the magnesium concentration). The biosensor response, as expected, increased at higher concentrations of magnesium (Fig. S-1). At the magnesium concentration of 1 - 3 mM almost directly proportional increase in the biosensor response to ATP was observed whereas the magnesium concentration above 3 mM did not cause a significant increase in responses. Therefore, in future experiments 3 mM Mg^{2+} was added to the working buffer.

The working buffer capacity strongly affects the performance of conductometric biosensors. Therefore, at the next stage the biosensor work was tested at various concentrations of buffer solution (Fig. 2). As seen, the highest biosensor responses were observed at the minimal buffer concentration (1 mM), and the responses decreased exponentially with increase of the concentration. Such dependence is typical for conductometric biosensors. For further experiments, 5 mM HEPES buffer was used because it was necessary to keep pH of the working buffer stable after adding the samples with different pH. On the other hand, to analyze the samples with pH close to 7.4 much greater sensitivity to ATP can be achieved using less concentrated working buffer.

Fig. 2.

The ionic strength of the working solution can also affect the biosensor operation. Therefore, we investigated the dependence of biosensor responses on the concentration of potassium chloride (Fig. 3). As seen, the ionic strength did not significantly affect the value of

responses, but at higher KCl concentrations the signal noise, and thus the measurement error and limit of ATP detection increased.

Fig. 3.

HEX is the main part of biorecognition element of the proposed biosensor. Activity of HEX, as well as activity of all enzymes, depends on pH of the working buffer. Thus, sensitivity of the biosensors also depends on the solution pH. In our previous work HEX was a component of an amperometric ATP-sensitive biosensor, and there we studied the dependence of HEX work on solution pH [20]. The highest responses to ATP were obtained at pH 6.8-8.2 with a maximum at pH 7.5. Usage of pH 6.4 or 8.5 led to 30% decrease in the biosensor responses.

Analytical characteristics of the biosensor

The biosensor response depends on the concentration of both ATP and glucose. Therefore, the responses to glucose were compared at different ATP concentrations. In the experiment, ATP was added to the solution, then the glucose concentration was gradually increased (from 0 to 600 μM) and the responses were measured. The corresponding curves are shown in Fig. 4. As seen, for all ATP concentrations the close to maximal responses were observed at 200 μM glucose, and further increase in the glucose concentration did not lead to a significant increase of the response value. Thus, 200 μM glucose can be considered optimal for the ATP determination. Certainly, to obtain a maximal sensitivity to very high concentrations of ATP (over 300 μM), a higher concentration of glucose would be required. However, such ATP concentration is unnecessary because in real samples ATP concentration is not high and the measurement procedure requires the sample dilution.

Fig. 4.

When using 5 mM HEPES, pH 7.4, with 3 mM Mg^{2+} and 200 μM glucose, the limit of ATP detection was 15 μM (S/N = 3). The typical calibration curve of the biosensor for ATP determination is shown in Fig. 5. This curve is described by the equation $\sigma = 0.69 + 0.22 * C$ ($R^2 = 0.99$), where σ is the solution conductivity (steady-state response, μS), C is the ATP concentration (μM).

Notably, the biosensor determination of ATP to a great extent depended on the composition of working solution, particularly on the concentrations of a buffering agent, glucose and Mg^{2+}. Biosensor characteristics can be varied by changing these parameters depending on the task to be solved.

Fig. 5.

Reproducibility of biosensor responses

The proposed biosensor is reusable, and thus the next step of the work was an investigation of reproducibility of biosensor responses during several hours of continuous operation. One complete biosensor response to ATP and glucose lasted 5-7 min. During 10-min intervals between measurements the biosensor was washed from substrates, repeatedly changing working buffer.

The results obtained are presented in Fig. S-2. The response values did not reduce noticeably during 10 measurements; relative standard deviation was 10.3%.

Operational stability of biosensors

The possibility to use biosensors for a long time and perform plentiful measurements is very important characteristic. However, the partial leaching of components from the bioselective membrane on the transducer surface often occurs during biosensor operation. It can be due to the diffusion of weakly bound membrane components at vigorous stirring of buffer in the working cell. Additionally, some decrease in the enzyme activity can take place during the membrane operation and storage. This can result in some decrease of response of biosensors, and thus to the diminishing of their sensitivity.

The next phase of work was aimed at studying the operational stability of the biosensor developed. During one working day, five-six responses to glucose and ATP (0.2 mM and 0.1 mM, respectively) were measured; afterwards the biosensor was stored in the working buffer at 4 °C until the next use. On the next day, the responses to the same concentration of glucose and ATP were measured. The total period of the biosensor operation and storage was 6 days.

The results are presented in Fig. 6. As seen, a gradual decrease of responses was observed, which was accompanied by some deterioration of reproducibility. Nevertheless, during one week the biosensors remained suitable for daily measurements.

Fig. 6.

Long-term storage of biosensors

At the next stage, the biosensor stability was investigated at long-term storage. In the experiment, several responses of new biosensors to 0.1 mM ATP and 0.2 mM glucose were measured; afterwards the biosensors were stored under various conditions. After 11-day storage, several responses to the same substrate concentrations were measured again.

Three storage modes were under investigation: dry storage at the temperatures +4 °C and -18 °C, and storage in the working buffer at +4 °C (only sensitive transducer parts coated with the enzyme and BSA were immersed to a buffer). Dry storage at +4 °C occurred to be the worst, after storage no responses were observed. After storage in the buffer at +4 °C the responses decreased by 10-15%. The best results were obtained at storage in the freezer, the responses decreased by 7-12%. During longer periods of time, activity of HEX will continue to decrease almost linearly, and thus sensitivity of the biosensor will also decrease. In case of the best storage mode, after one month storage the responses decreased by 30%. Often enzyme biosensors demonstrate better storage stability, but in our case HEX is not so stable enzyme as glucose oxidase or urease, and this is the reason of quite quick decrease of biosensor responses.

Measurement of pharmaceutical samples

To test the developed biosensor we purchased pharmaceutical vials with ATP (nominal concentration 10 mg/ml or 18.15 mM) produced by "Darnitsa" (Kiev, Ukraine) for intravenous and intramuscular injection. The measurement procedure was following. We obtained a calibration curve for ATP determination. Then the aliquot of the sample was added to the working cell, baseline stabilized, and glucose was added. The value of biosensor response was compared with the calibration curve. Volume of the sample that was added to the working cell was much smaller than the volume of working buffer. Thus, the sample was diluted directly in the working cell (73-fold, 91-fold and 114-fold dilutions were used, what is indicated in Table S-1).

Furthermore, 5 mM of ATP was added to one sample (thus total concentration of ATP in the sample was 23.15 mM). This sample was also successfully measured by the biosensor. The results and coincidence between real and obtained concentration of ATP are shown in the

Table S-1. As can be seen, accuracy of biosensor measurements of the real sample is quite acceptable. Difference between the biosensor results and real concentration was usually less than 10%.

Reproducibility of measurements of ATP concentration in one sample was also determined. For this purpose, aliquots of the sample were added 6 times to the working cell (with washing between additions), and responses of the biosensor were recorded (Fig. S-3). Relative standard deviation of biosensor responses was 13.7%.

Comparison with other biosensors

At the end, it would be useful to compare the proposed conductometric biosensor with previously developed ATP-sensitive biosensors (Table 1). As seen, the proposed conductometric biosensor has longer response time than other biosensors; very small response time have microbiosensors due to small size of their sensitive regions. Linear working range and stability of the proposed biosensor are comparable with most of other biosensors. Biosensors based on ATPases are an exception, since they have very wide working range, but also very bad stability. Advantage of the proposed biosensor is a simple transducer; its price is about 1 USD or even less in case of mass production. Another advantage is a price of measuring setup, since conductometric device usually costs much less than potentiostat for amperometric or impedance measurements.

Table 1. Comparison of analytical characteristics of the proposed biosensor with other electrochemical biosensors for ATP determination.

Biorecognition element	Type of transducer	Time of response	Linear or working range	Stability (residual enzyme activity)	Ref.
H^+-ATPase	ISFET	1-1.5 min	200-1000 μM	10% after 18 day	[12]
Na^+, K^+-ATPase	Teflon film	10 min	6.3-1000 μM	15 hours	[21]
Glucose oxidase + HEX	Pt disk electrode	5 min	15-100 μM	80% after 34 days	[22]
Glucose oxidase + HEX	Glassy carbon electrode	15 s	0.5-20 μM	65% after 22 days	[23]
Glycerol kinase + glycerol-3-phosphate oxidase	Pt/Ir microelectrode	10 s	0.2-50 μM	6 month	[24]
Glycerol kinase + glycerol-3-phosphate oxidase	Graphene-modified Pt microelectrodes	20 s	1.3–12 μM	No data	[25]
salicylate hydroxylase, glucose phosphate dehydrogenase, and HEX	Clark-type oxygen electrode	2 min	0.1 mM to 8 mM	90% after 7 days	[26]
dual-labeled aptamers complex	Poly(phenylenediamine) modified glassy carbon electrodes	No data, incubation time 45 min	10 nM–2 mM	94% after 3 weeks	[13]
HEX	Interdigitated gold electrodes	5 min	15-300 μM	90% after 11 days	This work

Conclusions

A reusable conductometric biosensor for determination of ATP and glucose has been developed. The best immobilization of HEX was obtained as a result of HEX and BSA cross-linking by glutaraldehyde during 30 min. The biosensor characteristics depended on the composition of working buffer. 5 mM HEPES, pH 7.4, with 3 mM magnesium ions was found to be the optimal buffer solution. The investigation of relationship between the biosensor sensitivity to ATP and concentration of glucose showed that the optimum glucose concentration was 0.2 mM. Limit of ATP detection was 15 μM. The relative standard

deviation of 10 consecutive measurements of biosensor responses to glucose and ATP was 10.3%. The biosensor developed remained suitable for daily measurements during at least one week. It can be applied for determination of ATP concentration in pharmaceutical vials or other water samples.

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Figure captions

Fig. 1. A: typical biosensor responses during measurements of the ATP concentration (first ATP or sample is added, and then glucose). B: control experiment, first glucose is added, and then ATP. Concentration of ATP was 100 μM, glucose – 200 μM. Working buffer - 5 mM HEPES, pH 7.4, with 3 mM of Mg^{2+}.

Fig. 2. Dependence of biosensor responses on concentration of working buffer, pH 7.4. Concentration of ATP in buffer - 100 μM, glucose - 200 μM, magnesium ions - 3 mM.

Fig. 3. Dependence of biosensor responses on KCl concentration. Working buffer - 5 mM HEPES, pH 7.4. Concentration of ATP - 100 μM, glucose - 200 μM, magnesium ions - 3 mM.

Fig. 4. Dependence of biosensor response on glucose concentration at different ATP concentrations in the measurement cell. ATP concentrations are depicted near corresponding curves. Working buffer - 5 mM HEPES, pH 7.4, with 3 mM of Mg^{2+}.

Fig. 5. Typical calibration curve of HEX-based biosensor for determination of ATP concentration. Glucose concentration - 200 μM. Working buffer - 5 mM HEPES, pH 7.4, with 3 mM of Mg^{2+}.

Fig. 6. Operational stability of biosensor during six days. Working buffer - 5 mM HEPES, pH 7.4. Concentration of ATP - 100 μM, glucose - 200 μM, magnesium ions - 3 mM.

- A conductometric biosensor for determination of adenosine triphosphate was created.
- It is based on enzyme hexokinase immobilized on interdigitated planar electrodes.
- Quick response, simplicity, and low cost are advantages of the biosensor.
- The biosensor can be used in for determination of ATP in water samples.

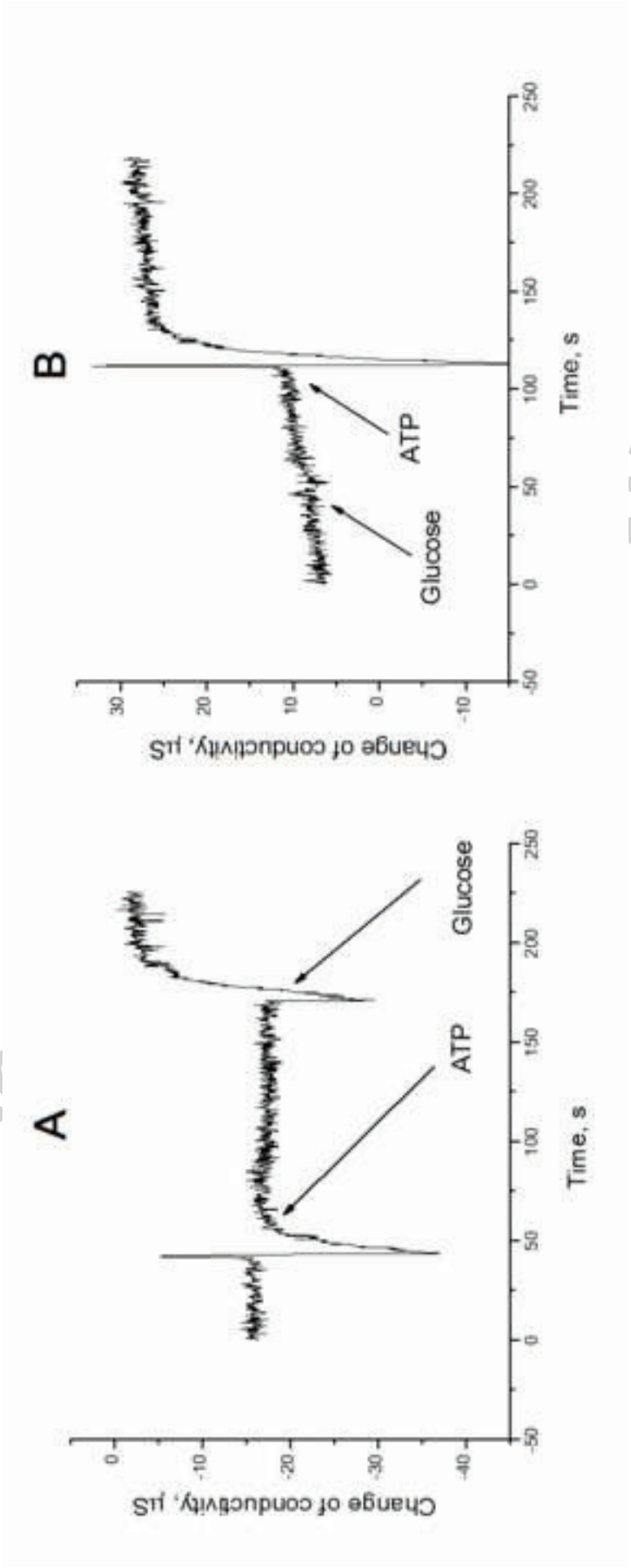

Figure 1

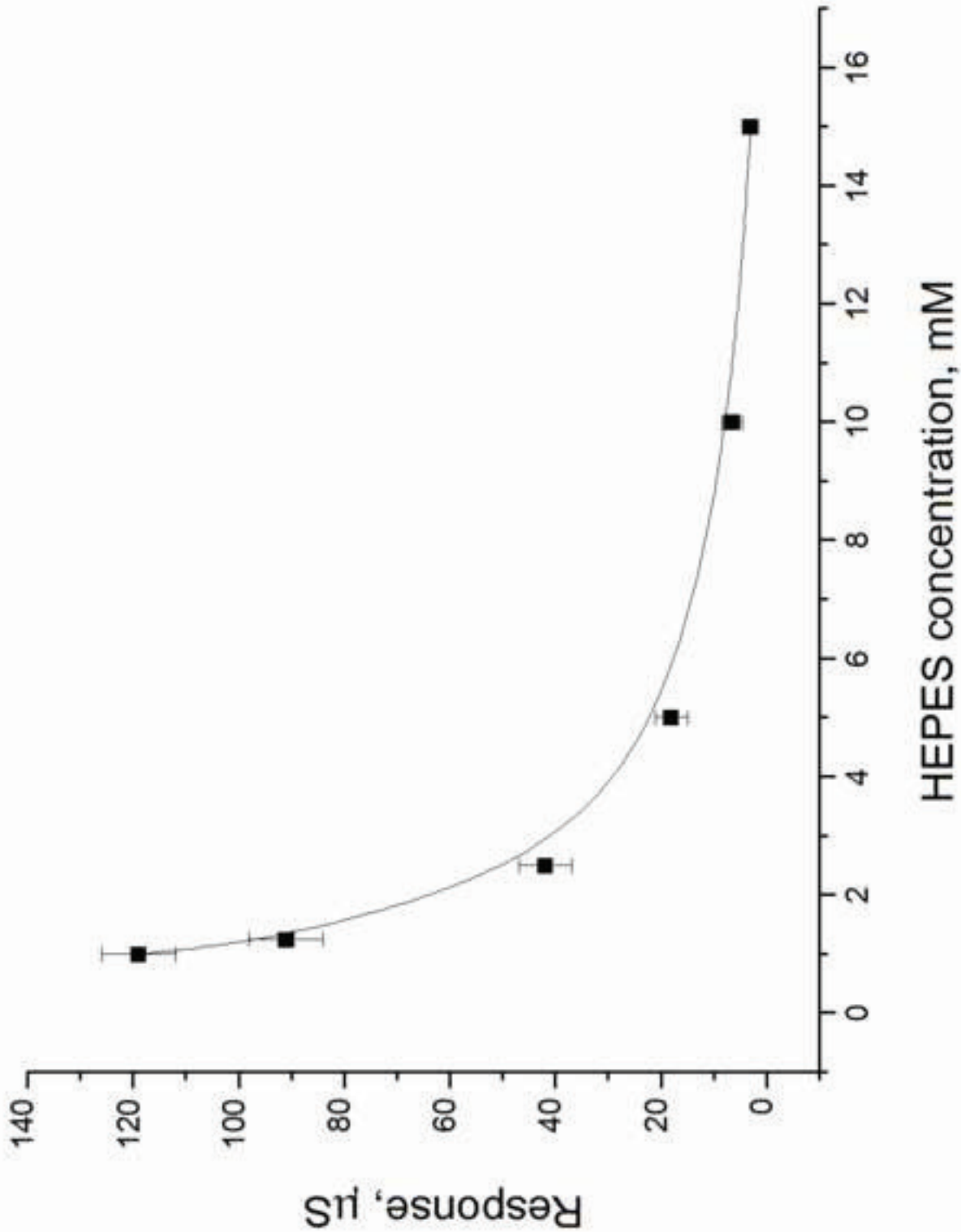
Response, μS
0
20
40
60
80
100
120
140
0
2
4
6
8
10
12
14
16
HEPES concentration, mM

Figure 2

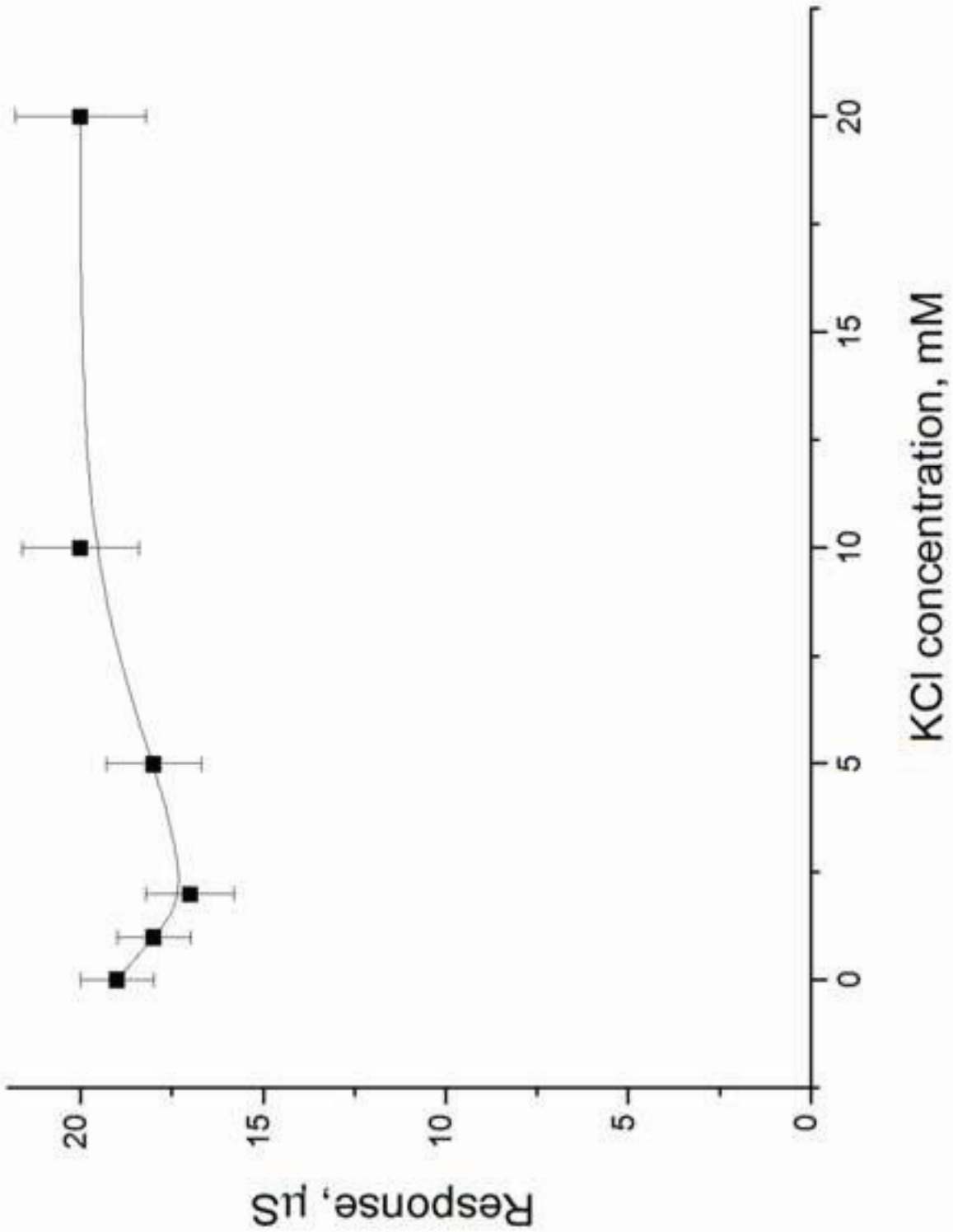

Figure 3

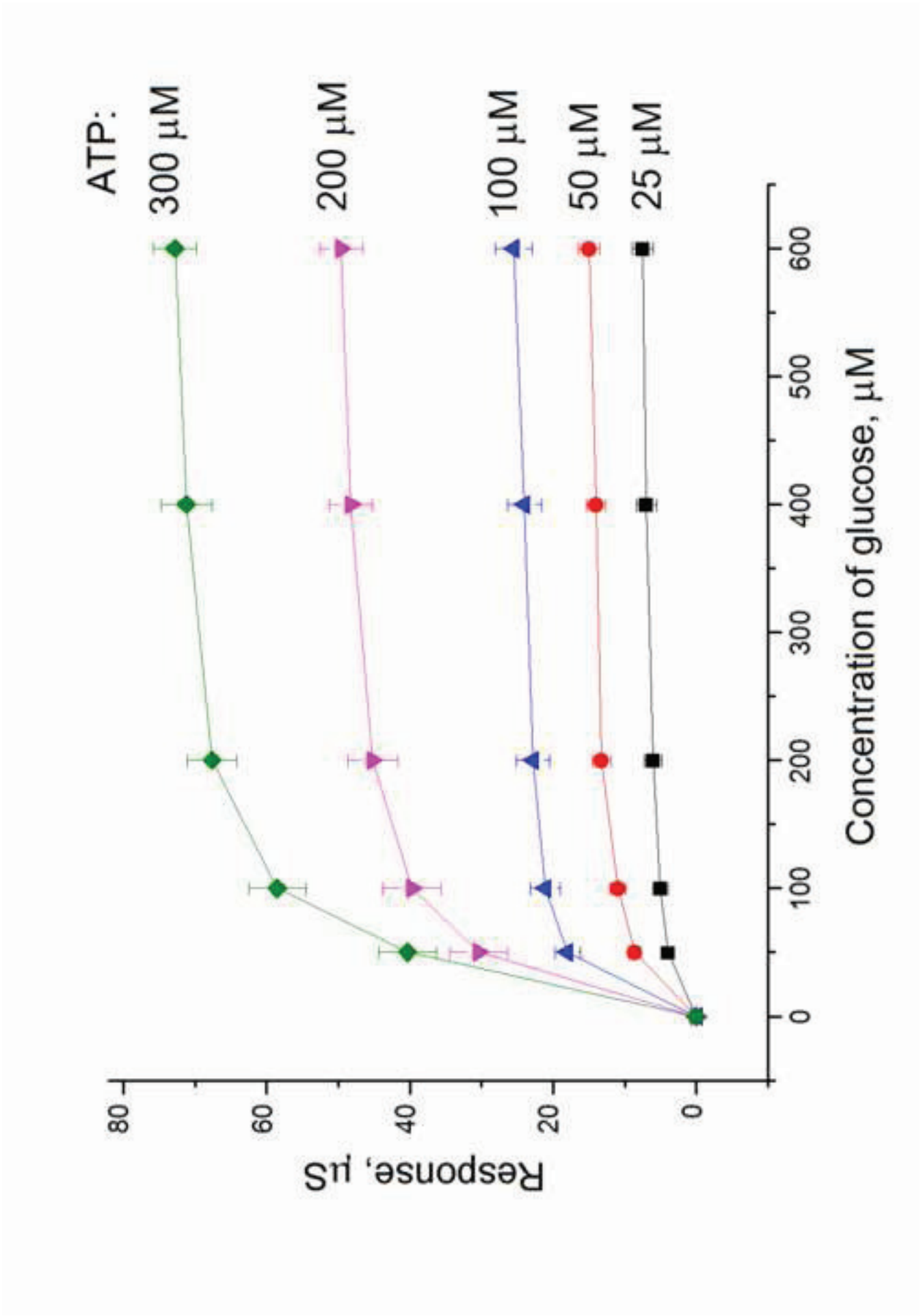
ATP:
300 µM
200 µM
100 µM
50 µM
25 µM
Response, µS
0
20
40
60
80
0
100
200
300
400
500
600
Concentration of glucose, µM

Figure 4

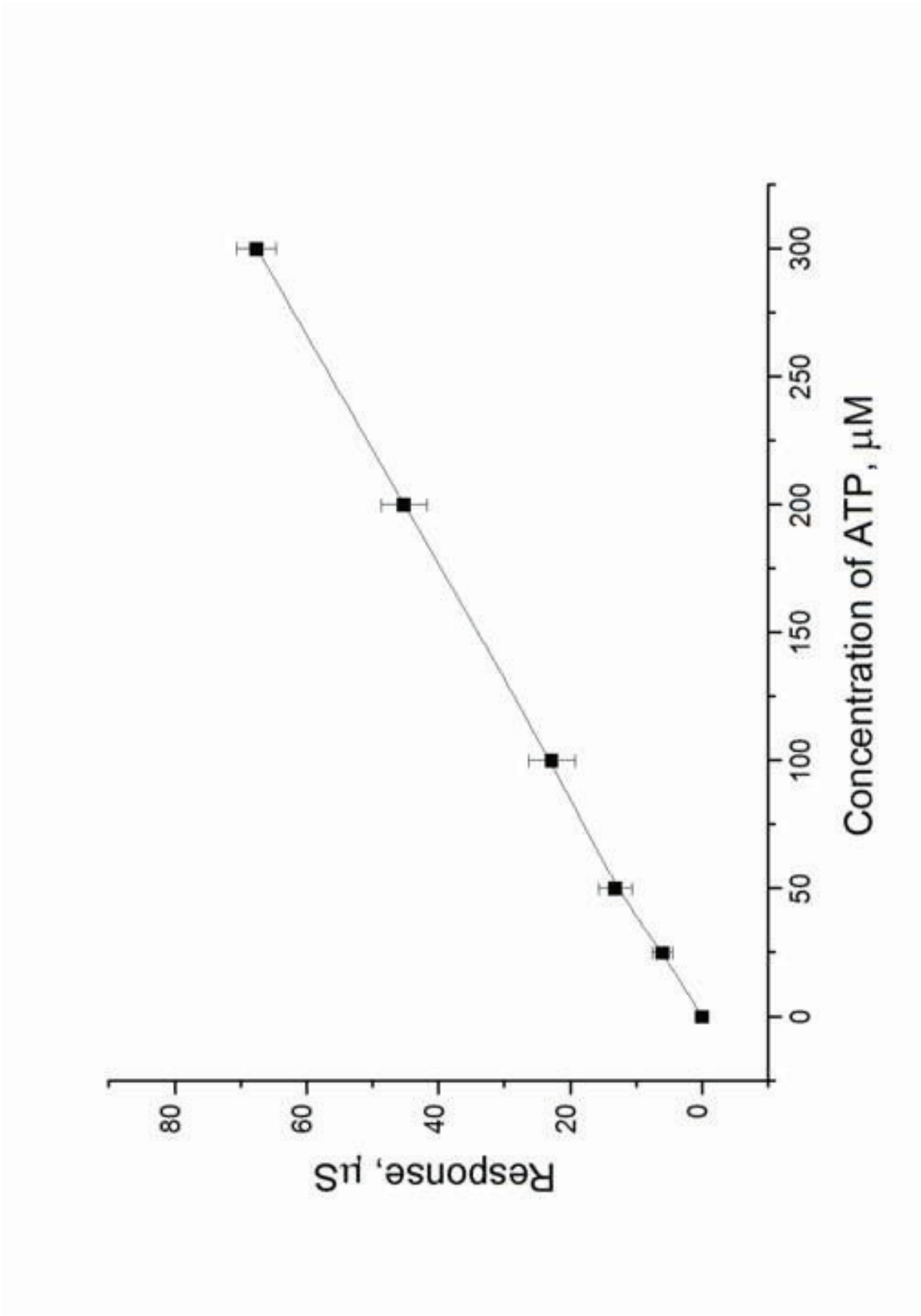
Response, μS
0
20
40
60
80
0
50
100
150
200
250
300
Concentration of ATP, μM

Figure 5

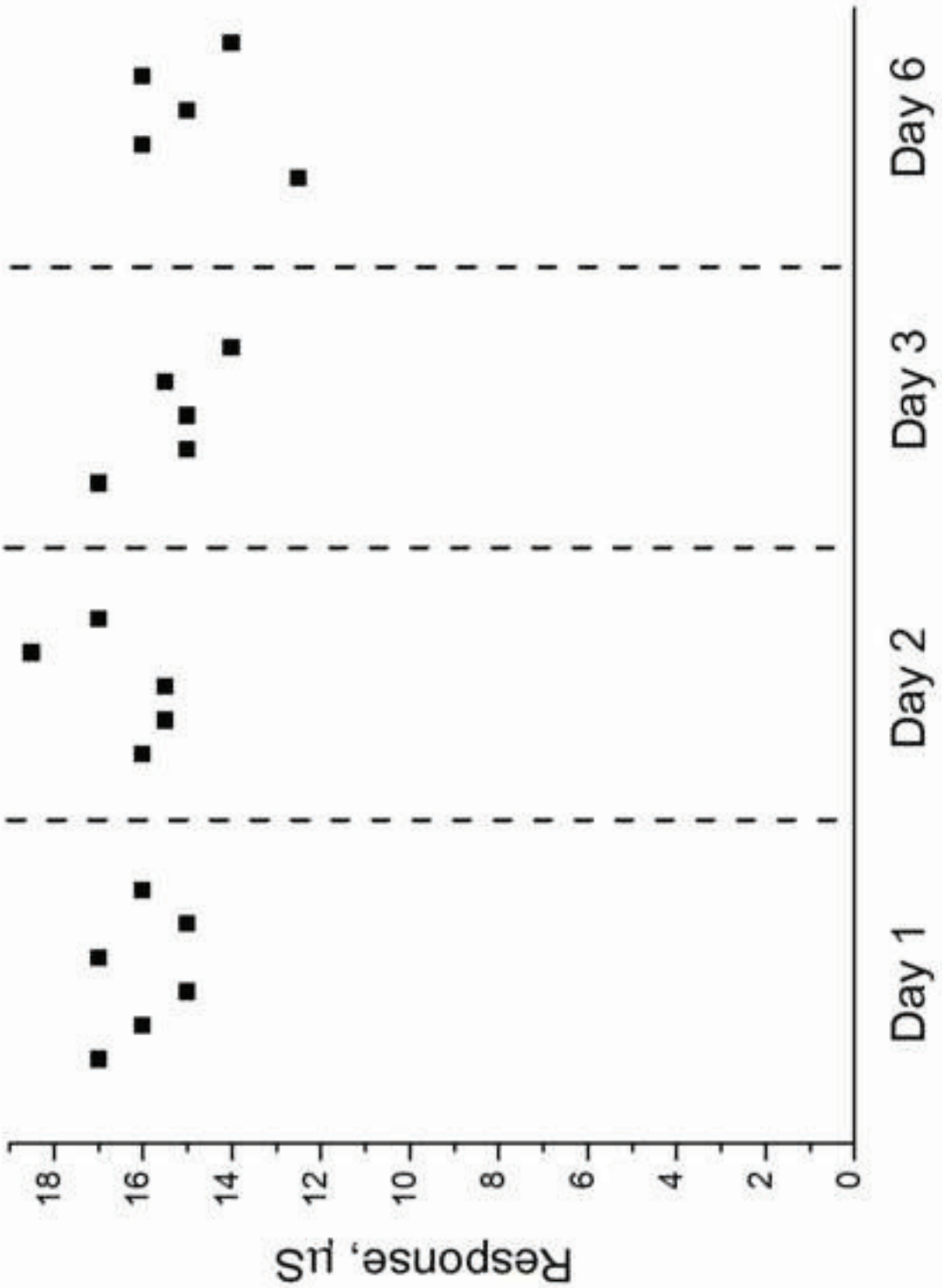

Figure 6

*Graphical Abstract (for review)