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A fluorescent probe for estimation of adenosine diphosphate and monitoring of glucose metabolism†

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An ADP selective fluorescent probe working in aqueous medium was identified and the change in fluorescence as a function of ADP concentration was standardized. Using this probe, all the steps of glycolysis coupled with ATP/ADP inter-conversion and oxidative breakdown of pyruvate in the mitochondria were monitored and the consumption/production of ATP/ADP at each step was quantified. The quantity of ADP present in the mitochondria, taken from different body parts of a pig, was also determined. It is hypothesized that an appropriate modification of the technique may provide a diagnostic tool for monitoring biochemical pathways as well as for quick estimation of ADP in the mitochondria and other cell organelles.

Introduction

Monitoring of a metabolic process carries immense biological and medicinal significance in exploring the working of natural systems and providing useful diagnostic information about the functioning of a biochemical pathway. Since most of the biochemical reactions are coupled to ATP–ADP inter-conversion,[1] approximately 0.005 ng of ATP is used in a typical cell every 1–2 min making a total of 50–75 kg of ATP to hydrolyze in the body everyday along with an equivalent amount of production of ATP as well.[2] Therefore, besides the use of tracer techniques[3] for monitoring biochemical processes, the quantification of formation/consumption of ATP/ADP during a biochemical reaction provides an alternative method to examine the working of a metabolic pathway. Although a number of ATP/ADP selective probes are reported only a few of them are employed for the monitoring of metabolic events.[4] Here, we report a highly fluorescent, ADP selective probe used for quantification of consumption/production of ATP/ADP during various steps of glycolysis and further breakdown of pyruvate to CO_2 in the mitochondrial system. The probe was also employed to quantify and compare the ADP present in the mitochondria taken from different body parts of a pig and a good correlation between the amount of ADP and the function of those particular tissues was observed.

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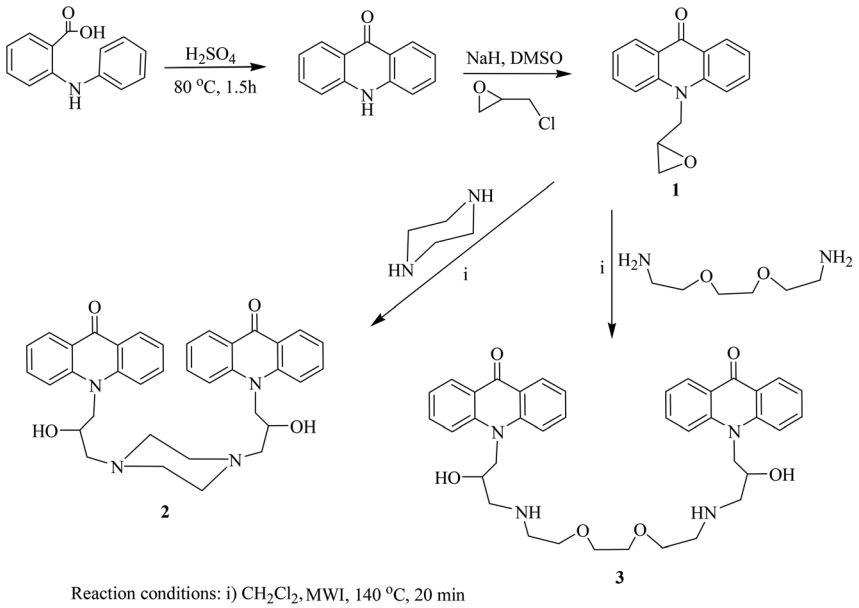
† Electronic supplementary information (ESI) available: NMR, Mass spectral data of compound **2** and **3** alone and in combination with ADP. See DOI: 10.1039/c3ob42505c

Results and discussion

The idea to increase the fluorescence intensity of the compound by having two acridone moieties in the molecule in comparison with one in our previous ATP selective probe[4m] led us to synthesize dimeric type compounds **2** and **3** by linking the two acridones through simple spacer groups. Synthesis of acridone from anthranilic acid and chlorobenzene was followed by treatment with epichlorohydrin to procure compound **1**. Further reaction of compound **1** with piperazine under microwave irradiation provided compound **2** (Scheme 1). In order to see the effect of the spacer group between two acridones on the nucleotide selectivity of the compound, another linker was chosen and compound **3** was prepared by the reaction of compound **1** with 2-[2-(2-aminoethoxy)ethoxy]ethylamine under microwave irradiation.

The fluorescence spectrum of compounds 2 and 3 and their non-selectivity/selectivity for ADP

The fluorescence spectrum of a solution of compound **2** (10 μM) in DMSO–HEPES buffer (1 : 4) exhibited strong emission at 427 nm (Φ = 0.63) when excited at 250 nm. Incremental (12 μL) addition of adenosine diphosphate (ADP) (0.5 ng in 12 μL HEPES buffer, 0.1 μM) to the solution of compound **2** (1.2 ml in DMSO–HEPES buffer, 1 : 4) decreased the fluorescence intensity of the solution (trace A to trace B, Fig. 1) after the addition of 360 μL ADP. No further change in the fluorescence intensity was observed when more ADP was added. However, addition of 360 μL ATP (the same concentration as for ADP) to the solution of compound **2** almost quenched the fluorescence (trace A to trace C, Fig. 1). It means



Scheme 1 Synthesis of compounds **2** and **3**.

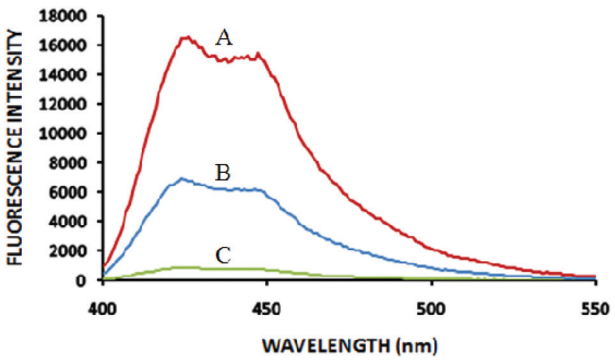


Fig. 1 Change in the fluorescence intensity of the solution of compound **2** (10^{-5} M) in DMSO–HEPES buffer (1 : 4), pH 7.2 (trace A) on addition of ADP (trace B), and addition of ATP (trace C). Excitation wavelength 250 nm.

compound **2** is responding to both ATP and ADP and exhibited non-selectivity between the two nucleotides.

The fluorescence spectrum of the solution of compound **3** (10 μM) in DMSO–HEPES buffer (1 : 4) also showed a strong emission band at 430 nm (Φ = 0.68) when excited at 250 nm. Stepwise (12 μL) addition of adenosine diphosphate (ADP) (0.5 ng in 12 μL HEPES buffer, 0.1 μM) to the solution of compound **3** (1.2 ml in DMSO–HEPES buffer, 1 : 4) (final concentration of ADP in 1.2 ml solution was 0.001 μM) resulted in a decrease in fluorescence intensity of the solution until complete quenching of fluorescence had taken place when 153 ng ADP (addition of 360 μL of ADP, 1 μM) was added to the solution (Fig. 2A). However, no change in the fluorescence of solution of compound **3** was observed in the presence of even 300 ng (in 12 μL HEPES buffer) ATP (45 μM), AMP (72 μM) and other nucleotides (GTP, GDP, CTP, CDP, UTP, UDP), indicating the selectivity of compound **3** for ADP (Fig. 2B). Addition of ADP to a solution of compound **3** in the presence of ATP/AMP decreased the fluorescence intensity to the same extent as with ADP alone pointing towards non-interference of ATP/AMP during the interaction of compound **3** with ADP (Fig. 2B). The ^{1}H NMR spectrum of compound **3** in the presence of ADP showed small changes in the chemical shifts of aromatic protons as well as the protons of the spacer group (Fig. S1†), while no such changes in chemical shifts were observed in the presence of ATP. On comparing the previously reported ATP selective monomeric type compounds[4m] with compounds **2** and **3**, it is apparent that the interaction of these compounds with ATP/ADP may be influenced by the orientation and nature of the substituent on acridone. Furthermore, to rationalize the selectivity of the compounds for ATP/ADP, some physicochemical parameters[5] of compounds **2** and **3** and the compounds reported previously[4m] were calculated (Table S1†). It was observed that compound **3** has Log *P* and total polar surface area (TPSA) quite different from the other compounds but relatively close to the Log *P* and TPSA of ADP. It may be due to this similarity in the topology of the two molecules that compound **3** is compatible to interact with ADP. The Benesi–Hildebrand plot indicated a 1 : 1 stoichiometry of the **3**·ADP complex (Fig. S2, ESI†) and their binding constant (*K*) was calculated as 2.14×10^5 M^{-1} (ESI†). Moreover, the solution of compound **3** did not exhibit any change in fluorescence on addition of Mg^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, Hg^{2+}, Pd^{2+}, Ag^{+}, Ni^{2+}, Na^{+}, and K^{+}, the common biological cations. The high resolution electrospray mass spectrum (ESI-HRMS) of the solution of compound **3** with ADP also showed formation of the **3**·ADP complex (Fig. 2C). Hence, a fluorescent probe, workable in aqueous medium and showing considerable selectivity for ADP, is identified which proved helpful in

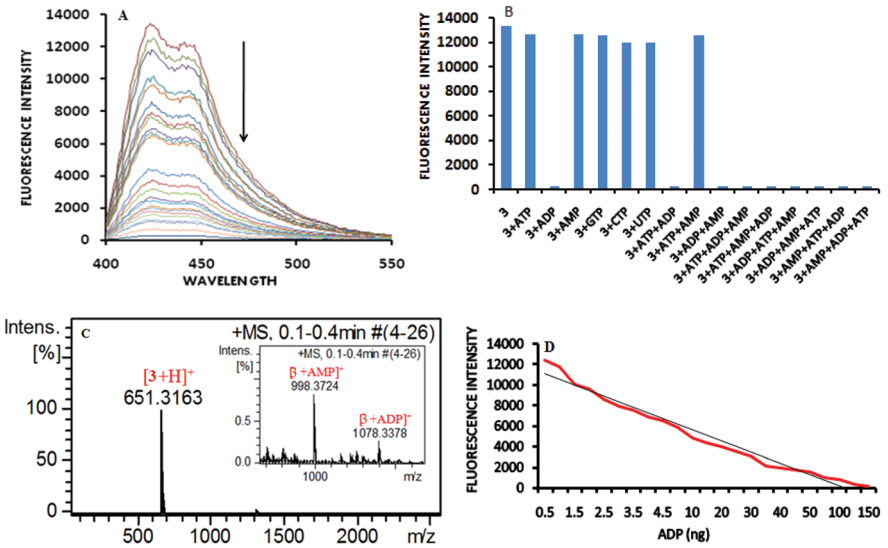
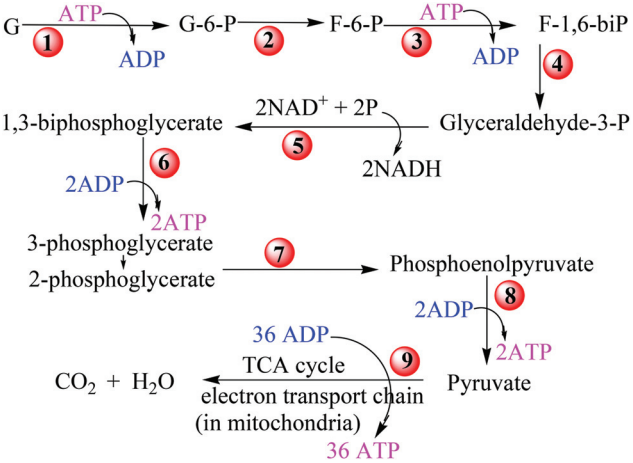


Fig. 2 (A) Decrease in the fluorescence intensity of the solution of compound **3** (10^{-5} M) in DMSO–HEPES buffer (1 : 4), pH 7.2 on stepwise addition of ADP. Excitation wavelength 250 nm. (B) Fluorescence change of the solution of compound **3** in DMSO–HEPES buffer (1 : 4, pH 7.2) in the presence of other nucleotides (45 μM) showing selective and competitive binding of compound **3** with ADP. (C) HRMS of the solution of compound **3** and ADP showing formation of the **3** + ADP complex with *m*/*z* 1078.3378 (calcd *m*/*z* 1078.3471). (D) Normalized curve for the change in fluorescence intensity of solution of compound **3** as a function of ADP concentration (black line). The red line indicates the actual position of data points.

quantification of ADP during a biochemical reaction as well as in biological samples. Besides a recent report by Feng *et al.*[4b] about a mononuclear zinc complex appended with two anthracene groups as a highly selective ADP sensor, compound **3** is amongst the very few ADP selective probes reported so far. In order to utilize the results of these experiments for monitoring ATP–ADP coupled biochemical reactions, a curve showing changes in the fluorescence intensity of a solution of compound **3** as a function of ADP concentration was standardized (Fig. 2D).

Monitoring of glucose metabolism (Phase I)

A quick review of the process of glucose metabolism (Scheme 2) indicates formation of ADP in steps 1 and 3 and consumption of ADP in steps 6, 8 and 9. Starting with calculated amounts of glucose and other reagents required for reactions 1, 3, 6, 8 and 9, all these steps of glucose metabolism were monitored using probe **3**.



Scheme 2 Various steps of glucose metabolism showing formation/consumption of ADP/ATP.

Phosphorylation of glucose

A solution of D-glucose (50 μM), hexokinase (80 units), Mg^{2+} (50 μM) and compound **3** (10 μM in DMSO) in HEPES buffer (2 ml) showed the usual fluorescence intensity of compound **3** (trace A, Fig. 3A), indicating no effect of glucose and hexokinase on fluorescence behaviour of the compound. The moment 100 μL ATP (4 ng ATP in 100 μL HEPES buffer; 0.0036 μM in 2 ml final solution) was added to this solution, the fluorescence intensity started decreasing and became constant at 8000 A.U. (trace B, Fig. 3A). As calculated from the standard curve (Fig. 2C), the amount of ADP in the solution is ~3.1 ng (0.0036 μM) which indicates that all the ATP in the solution have been changed to ADP. With the same amount of reactants (as used above), kinetic analysis of this enzymatic reaction was studied by varying the concentration of hexokinase and it was found to be first order (Fig. 3B). The Lineweaver–Burk plot (Fig. 3C) gave the Michaelis–Menten constant (K_m) of 125 μM corresponding to the reported one 160 μM.[6] HRMS of the reaction mixture in this step of glucose metabolism (Fig. 3D) did not show any peak due to the mass of ATP while the peak at *m*/*z* 261.0355 corresponding to the mass of phosphorylated glucose (calcd *m*/*z* 261.0370 $[M + H]^+$) is quite intense. As a control experiment, the same reaction was performed in the absence of hexokinase. No change in the fluorescence intensity was observed in this experiment. Therefore,

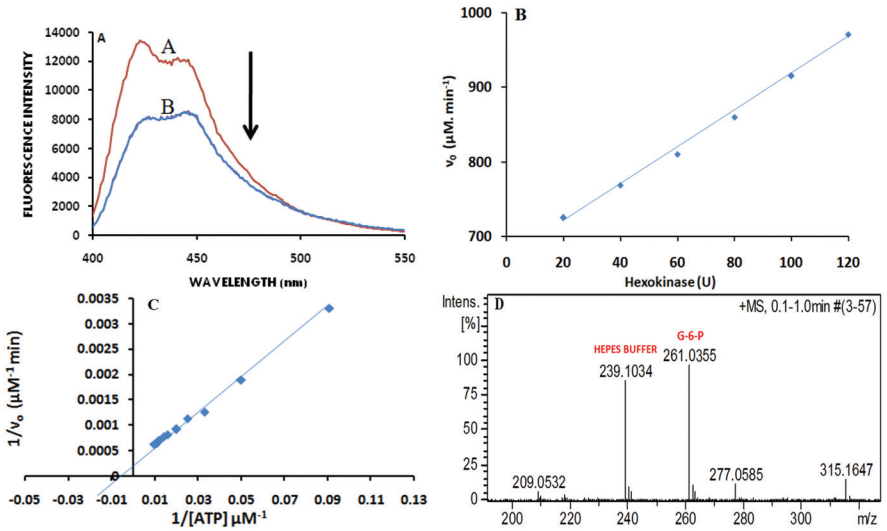


Fig. 3 (A) Change in fluorescence from A to B during the reaction in the first step of glucose metabolism. (B) Initial rate as a function of the amount of hexokinase. (C) Lineweaver–Burk plot. (D) A part of HRMS of the reaction mixture in the first step of glucose metabolism.

a simple protocol for quantification of consumption of ATP and production of ADP helped in monitoring the enzymatic phosphorylation of glucose.

Phosphorylation of fructose 6-phosphate

Similar to the monitoring of the above reaction, phosphorylation of fructose-6-phosphate (step 3, Scheme 2) was performed. The fluorescent solution of compound **3** (10 μM), fructose-6-phosphate (F-6-P) (50 μM) and ATP (4 ng ATP in 100 μL HEPES buffer; 0.0036 μM in 2 ml final solution) underwent a decrease in fluorescence (trace A to trace B, Fig. 4A) when phosphofructokinase (80 units) was added. Estimated from the standard curve, the quenching of fluorescence corresponds to the presence of 3.1 ng ADP (0.0036 μM) in the solution. Kinetic analysis of this reaction indicates it to be a first order reaction (Fig. 4B). The Michaelis–Menten constant K_m was calculated as 7 μM (lit.[7] 3 μM) (Fig. 4C). HRMS of the reaction mixture showed a peak at *m*/*z* 339.9961 which corresponds to the mass of fructose diphosphate (calcd *m*/*z* 339.9955 $[M]^+$) (Fig. 4D). Hence, showing the quantitative conversion of ATP to ADP, the second phosphorylation step of glucose metabolism was also successfully mimicked. In this experiment too, no change in fluorescence was observed in the absence of phosphofructokinase.

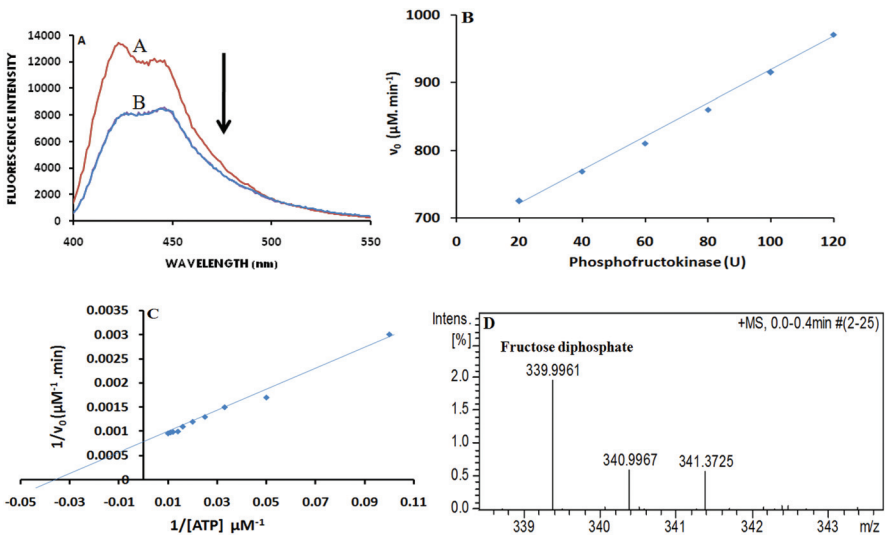


Fig. 4 (A) Change in fluorescence from A to B during the reaction in the third step of glucose metabolism. (B) Initial rate as a function of the amount of phosphofructokinase. (C) Lineweaver–Burk plot. (D) A part of HRMS of the reaction mixture in the third step of glucose metabolism.

Formation of 3-phosphoglycerate

During the dephosphorylation steps of glucose metabolism, the measurement of the increase in fluorescence intensity of the non-fluorescent solution of compound **3** (in the presence of ADP) also gave quantitative information about ADP consumed and the consequent formation of ATP during the reactions 6, 8 and 9 in Scheme 2. To demonstrate step 6 of glucose metabolism, a solution of 1,3-bisphosphoglycerate (50 μM), ADP (50 ng ADP in 100 μL HEPES buffer; 0.058 μM in 2 ml final solution) and compound **3** (10 μM) was taken in 2 ml HEPES buffer. The moment phosphoglycerokinase (80 units) was added to this solution; its fluorescence started increasing and attained the same intensity as that of the pure compound (trace A to trace B, Fig. 5A). This observation clearly indicated the consumption of almost all the ADP (formation of ~55 ng ATP, 0.05 μM in 2 ml final solution) during this reaction while formation of 3-phosphoglycerate was also detected in HRMS of the reaction mixture (Fig. 5D) (calcd *m*/*z* 208.9822 $[M + Na]^+$). Kinetic analysis indicated this reaction to be first order (Fig. 5B) and the experimental Michaelis–Menten constant (142.85 μM) corresponds to the reported one (180 μM)[8] (Fig. 5C).

Conversion of phosphoenolpyruvate to pyruvate

For monitoring the last step of the payoff phase of glycolysis *viz.* conversion of phosphoenolpyruvate to pyruvate (step 8, Scheme 2), a solution of phosphoenolpyruvate (50 μM), compound **3** (10 μM) and ADP (50 ng ADP in 100 μL HEPES buffer; 0.058 μM in 2 ml assay solution) in 2 ml HEPES buffer (pH 7.2) was prepared. After the addition of pyruvate kinase (80 units) to the reaction mixture, the solution slowly turned fluorescent (Fig. 6A) and within 2 min, it gained the same fluorescence as observed for the solution of compound **3** alone. This change in fluorescence intensity of the solution clearly indicated the consumption of all the ADP (formation of ~55 ng ATP, 0.05 μM). Here also the reaction was found to be first order and the Michaelis–Menten constant K_m 250 μM corresponds to the reported value (260 μM)[9] (Fig. 6C). Formation of pyruvate (as sodium pyruvate) was also ascertained from HRMS of the reaction mixture (Fig. 6D) (calcd *m*/*z* 132.9872 $[M + Na]^+$).

Therefore, using fluorescent probe **3**, all those steps of glucose metabolism, which involve either consumption of ADP or generation of ADP (steps 1, 3, 6 and 8; Scheme 2) were monitored. It is hypothesized that starting from the calculated amount of glucose and by performing all the steps in a sequence (transferring the solution from the first vial to the next one), phase I of glucose metabolism, as shown in the animated figure, could be demonstrated in one go (animated Fig. S1, see ESI†). It is worth mentioning that the formation of ADP during steps 1 and 3 and ATP during the reactions of 6th and 8th steps was also quantified with the help of LC-MS using a QuantAnalysis2.0. The results of mass spectral experiments and fluorescence experiments were very close (Table S2†) with a difference of <4%.

Quantification of ADP in the mitochondria

A solution of 20 μL of compound **3** (DMSO) and 10 mg mitochondrial mass in 100 μL resuspended buffer (extracted from the pig liver and refined as per the reported procedure[10]) in 1 ml final solution in HEPES buffer (compound **3** is of 10 μM) showed fluorescence as per trace B of Fig. 7. This decrease in fluorescence intensity of the otherwise fluorescent solution of compound **3** (trace A, Fig. 7) was assumed to be due to the

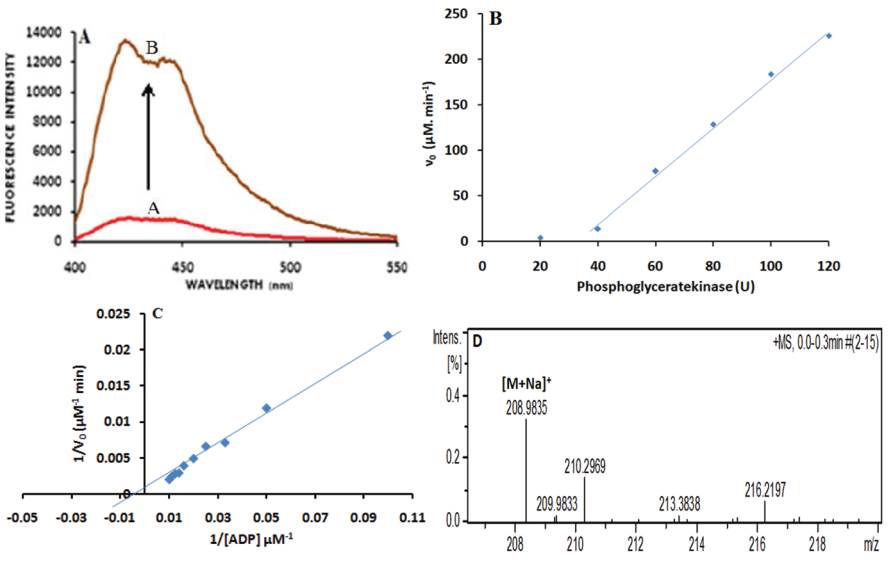


Fig. 5 (A) Change in fluorescence from A to B during the reaction in the sixth step of glucose metabolism. (B) Initial rate as a function of the amount of phosphoglyceratekinase. (C) Lineweaver–Burk plot. (D) A part of HRMS of the reaction mixture showing the formation of phosphoglycerate with *m*/*z* 208.9835 (calcd *m*/*z* 208.9822 $[M + Na]^+$).

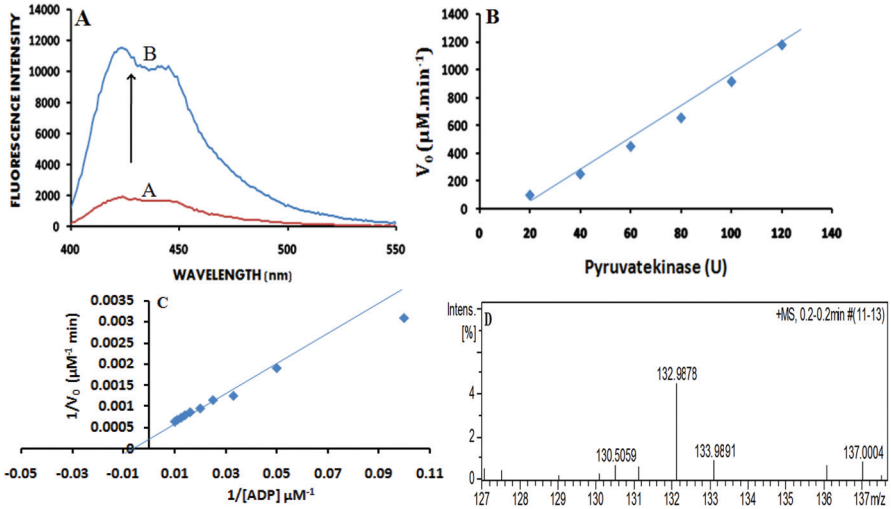


Fig. 6 (A) Change in fluorescence from A to B during the reaction corresponding to conversion of phosphoenolpyruvate to pyruvate. (B) Initial rate as a function of the amount of pyruvatekinase. (C) Lineweaver–Burk plot. (D) A part of HRMS of the reaction mixture showing the formation of sodium pyruvate with *m/z* 132.9878 (calcd *m/z* 132.9872 $[M + Na]^+$).

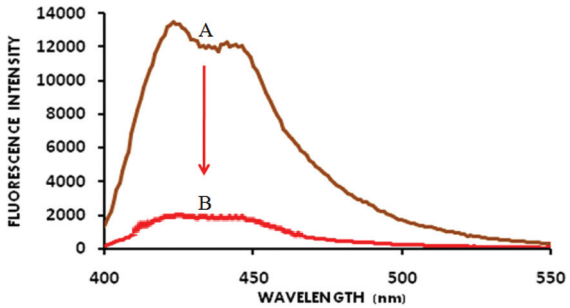


Fig. 7 Change in fluorescence of the solution of compound **3** from trace 2 to trace 1 when mitochondrial solution was added.

presence of ADP in the mitochondrial system because almost all the other components associated with the mitochondria were removed.[10] As per the standard curve (Fig. 2D), ~50 ng of ADP seems to be there in the tested sample of mitochondria. A high resolution mass spectrum of mitochondrial solution also showed a mass peak at *m/z* 428.0377 corresponding to the mass of ADP (calcd *m/z* 428.0367, $[M + H]^+$) (Fig. S4†) and 48 ng ADP in 10 mg mitochondrial mass was quantified by LC-MS using QuantAnalysis2.0. For comparison, mitochondria from heart muscles and leg muscles of a pig were also isolated. Performing the same experiment as for the liver mitochondria, here, respectively 100 ng and 40 ng of ADP were estimated in 10 mg mitochondrial mass extracted from heart muscles and leg muscles. The amount of ADP estimated in three types of tissues taken from different body parts seems to be almost consistent with the functions of these tissues. Hence the experiments demonstrated here provide a practical approach for estimation of ADP in the biological samples.

Breakdown of pyruvate in the mitochondria (phases II and III of glucose metabolism)

Interesting observations were recorded while performing experiments corresponding to phases II and III of glucose metabolism *viz.* breakdown of pyruvate to CO_2 (step 9, Scheme 2). In order to perform the experiment for breakdown of pyruvate in the mitochondria, 30 mg mitochondria (carrying ~150 ng ADP), extracted from the pig liver, was taken in 100 ml HEPES buffer (pH 7.2) in a two necked round bottom flask (rbf) (Fig. S5†). Through a rubber tube, one end of the rbf was connected to a test tube containing lime water. On addition of pyruvate solution (1.5 mg in 5 ml HEPES buffer) through a dropping funnel fixed in the second neck of rbf, the solution in the rbf started turning fluorescent and bubbling was observed in lime water. Within 2–3 min, the solution in the rbf became fluorescent (Fig. 8) and a solid appeared in lime water. Since CO_2 is generated during the breakdown of

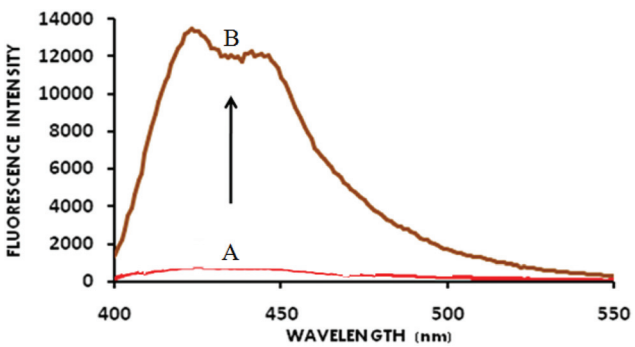
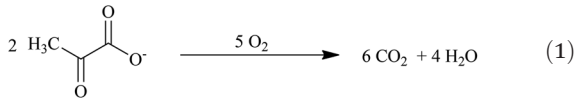


Fig. 8 Change in fluorescence of the solution of compound **3** and mitochondrial mass from trace A (non-fluorescent in the presence of mitochondrial mass) to trace B on addition of pyruvate.

pyruvate in the mitochondria, 4 mg $CaCO_3$ was isolated from the lime water which corresponds to the formation of 0.052 mmol of CO_2. Consistent with oxidative transformation of pyruvate (eqn (1)), in this experiment, the total available pyruvate (1.5 mg) had undergone breakdown to generate CO_2. Therefore, the results of this experiment demonstrated the part of glucose metabolism which takes place in the mitochondria.



(1)

Combination of the process of glycolysis with oxidative breakdown of pyruvate

As per the results of above experiments, production/consumption of ADP can be quantified during various reactions of phase I (glycolysis) of glucose metabolism. Moreover, the amount of pyruvate metabolized in the mitochondria can be calculated, if the step corresponding to conversion of fructose diphosphate to phosphoenolpyruvate is also achieved; starting from step 1, sequentially performing all the eight reactions of phase I, the reaction mixture in the last step can be passed onto mitochondria (phase II/III) and hence breakdown of glucose to CO_2 can be demonstrated (animated Fig. S2, see ESI†) by looking at the fluorescence change at each step. In order to check the workability of this connection between phase I and phase II/III reactions, the reaction mixture in the last step of phase I of glucose metabolism (containing pyruvate, ATP) was subjected to mitochondrial solution. The fluorescent solution of step 8 (Scheme 2) turned non-fluorescent when it was added to 30 mg mitochondria taken in 50 ml HEPES buffer (trace A, Fig. 9) indicating the presence of ~150 ng ADP in the mitochondrial solution. Within 2–3 min, the solution started turning fluorescent and attained 5000 AU fluorescence (trace B, Fig. 9). This has probably happened due to the metabolism of pyruvate in mitochondrial solution and conversion of ADP to ATP. Using the standard curve (Fig. 2D), it was calculated that 15 ng ADP was present in the solution and approximately 105 ng was converted to ATP. Hence, it is possible to link phase I reactions of glucose metabolism to phase II/III and breakdown of glucose to CO_2 could be studied with the help of probe 3. Interestingly, the amount of ATP produced in this experiment was in excellent agreement with that calculated from the LC-MS experiment using QuantAnalysis.

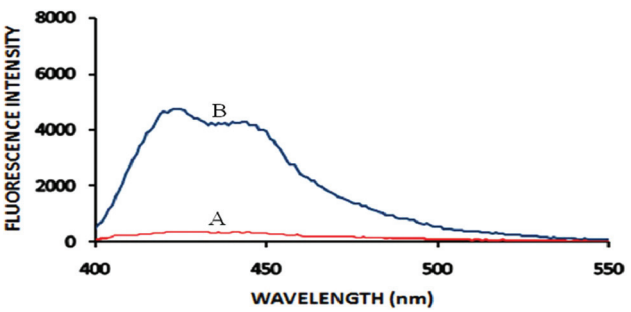


Fig. 9 Fluorescence change (from trace A to trace B) when the solution in step 8 of phase I was subjected to mitochondria.

Conclusions

A highly versatile approach for monitoring biochemical reactions is demonstrated. Using a fluorescent and ADP selective probe, the consumption/production of ATP/ADP during the four reactions of phase I of glucose metabolism was quantified. The same probe was also used for estimation of ADP in the mitochondria of the pig liver, heart and leg muscles and breakdown of pyruvate to CO_2 in the mitochondria was monitored. The experimental protocol could be highly useful to show breakdown of glucose to CO_2, monitor ATP/ADP coupled biochemical reactions, compare the healthy/unhealthy state of mitochondria from different body parts and with certain modifications, it may prove as medicinally significant in diagnosis.

Experimental

General note

^1H and ^{13}C NMR spectra were recorded on JEOL 300 MHz NMR spectrometer using DMSO-d_6 as a solvent. Chemical shifts are given in ppm with TMS as an internal reference. *J* values are given in hertz. Mass spectra were recorded on a Bruker micrOTOF Q II Mass spectrometer. The low intensity of desired peaks was due to more intense HEPES peaks in the mass spectra because all these reactions were performed in HEPES buffer. The reactions corresponding to epoxy ring opening with amines were performed in a microwave synthesizer (BIOTAGE INITIATOR EXP – EU) at 75 watt and 140 °C. Reactions were monitored by thin layer chromatography (TLC) on glass plates coated with silica gel GF-254. Column chromatography was performed with 60–120 mesh silica. IR and UV spectral data were recorded on FTIR (VARIAN 660 IR) and BIOTEK Synergy H1 Hybrid Reader instruments, respectively. The fluorescence spectra were recorded on a BIOTEK Synergy H1 Hybrid Reader spectrofluorophotometer (excitation at 250 nm). Glucose, hexokinase, fructose-6-P, phosphofructokinase, phosphoglycerokinase, pyruvate kinase, phosphoenolpyruvate, pyruvate, ATP, ADP, AMP, GTP, CTP, UTP and other nucleotides were purchased from Sigma-Aldrich. 1,3-Bisphosphate glycerate was prepared in step 5 (Scheme 2). Pig tissues from different body parts were taken from a local butcher's shop.

Experimental procedure

N-Phenyl anthranilic acid (5 g) was treated with 10 ml conc. H_2SO_4 at 90–100 °C for 1.5 h and 10*H*-acridin-9-one (5 g) was procured. NaH (200 mg) was washed with dry hexane and added to 20 ml DMSO followed by addition of 5 g acridone and 2 ml epichlorohydrin. The reaction mixture was heated at

70 °C for 18 h (TLC monitoring). After the addition of water (20 ml) to the reaction mixture, it was extracted with ethyl acetate (5 × 50 ml). The organic phase was dried over anhydrous Na_2SO_4. The solvent was distilled off and the residue was column chromatographed using ethyl acetate and hexane as eluents to isolate the pure compound **1**. A solution of compound **1** (500 mg) and piperazine/(2-[2-(2-aminoethoxy)ethoxy]-ethylamine (150 μL) was taken in a reaction vial containing 2 ml dichloromethane and was irradiated in a microwave synthesizer for 20 min. After the completion of reaction (TLC monitoring), the reaction mixture was column chromatographed using chloroform and methanol as eluents to isolate compound **2/3**.

Compound 2. Recrystallization from ethanol yielded 58% light green solid: 159 °C; ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6): δ = 3.30 (m, 8H, piperazine 4 × CH_2), 3.81 (d, 4H, J = 5.4 Hz, 2 × NCH_2), 4.39–4.42 (m, 2H, 2 × CHOH), 4.64–4.71 (m, 4H, 2 × NCH_2), 5.68 (d, 2H, J = 5.1 Hz, 2 × OH), 7.29 (m, 4H, ArH), 7.75 (m, 4H, ArH), 7.89 (d, 4H, J = 12 Hz, ArH), 8.44 (d, 4H, J = 7.8 Hz, ArH) ppm; ^{13}C NMR (75 MHz, DMSO-d_6): δ = 45.92, 48.03, 67.54, 115.15, 119.89, 121.08, 126.22, 132.68, 141.39, 194.66 ppm; IR (KBr): 1593, 3261 cm^{-1}; HR-ESI-MS for $C_{36}H_{36}N_4O_4$ required 589.2809 $[M + H]^+$. Found 589.2810.

Bis-10-[2-hydroxy-3-(2-methoxy-ethylamino)-propyl]-10*H*-acridin-9-one (3). Using the above mentioned procedure, after recrystallization from ethanol yielded 61% green solid: 164–170 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.58–2.76 (m, 8H, NCH_2), 3.46–3.52 (m, 8H, OCH_2), 3.90–4.05 (m, 2H, CHOH), 4.42–4.57 (m, 4H, 2 × NCH_2), 7.24–7.28 (m, 4H, ArH), 7.71–7.75 (m, 4H, ArH), 7.88–7.99 (m, 4H, ArH), 8.28–8.45 (m, 4H, ArH) ppm; ^{13}C NMR (75 MHz, DMSO-d_6): δ = 46.2, 50.2, 65.3, 66.2, 114.5, 118.7, 119.1, 124.0, 131.3, 140.0, 174.1, 191.3 ppm; IR (KBr): 1559, 3400, 3447 cm^{-1}; HR-ESI-MS for $C_{38}H_{42}N_4O_6$ required 651.3183 $[M + H]^+$. Found 651.3123.

UV-vis and fluorescence studies with ADP

10^{-3} M stock solutions of compounds **2** and **3** were prepared in DMSO. A 10^{-3} M stock solution of ADP was prepared by dissolving NaADP in HEPES buffer. All the solutions were prepared in Eppendorf/glass vials and UV-vis and fluorescence spectra were recorded by taking 1 ml of the solution in a Biocell of 1 ml capacity. Taking the concentration of compounds **2** and **3** as constant (10 μM), incremental addition of ADP (0–50 μM) resulted in an increase in absorbance in the UV spectrum. For recording the fluorescence spectra, in 12 μL increments, ADP (0.001–1 μM) was added to 10 μM solutions of compound **3** which resulted in the decrease in fluorescence up to complete quenching.

Selectivity for ADP

To check the selectivity of compound **3** for ADP amongst its homologues ATP, AMP, *etc*., the stock solutions (10^{-3} M) of the nucleotides were prepared in HEPES buffer. Upon addition of even 45–60 μM of ATP/AMP/GTP/CTP/UTP to 10 μM solution of compound **3** to a final volume of 1 ml, no change in the fluorescence intensity was observed.

Phosphorylation of glucose

Taking the calculated volume of all the components, an assay solution of ATP (0.0036 μM), glucose (50 μM) and compound **3** (10 μM) was prepared in 2 ml HEPES buffer (pH 7.2). After addition of hexokinase (80 units) and Mg^{2+} (50 μM), the fluorescence intensity (excitation at 250 nm) of the solution was recorded. A 42% decrease in fluorescence intensity was observed. The concentration of ADP was calculated from the change in fluorescence intensity which on dividing by the time taken for the change gave the initial velocity (v_0, μM min^{-1}). The Michaelis–Menten constant (K_m, μM) was obtained from the Lineweaver–Burk plot.

Phosphorylation of fructose 6-phosphate

An assay solution having a final concentration of 0.0036 μM for ATP, 50 μM for fructose-6-phosphate and 10 μM for compound **3** was prepared in 2 ml HEPES buffer (pH 7.2). After addition of phosphofructokinase (80 units) and Mg^{2+} (50 μM), the fluorescence intensity (excitation at 250 nm) was recorded. About 43% decrease in the fluorescence intensity was observed. The concentration of ADP was calculated from the change in fluorescence intensity which on dividing by the time taken for the change gave the initial velocity (v_0, μM min^{-1}). The Michaelis–Menten constant (K_m, μM) was obtained from the Lineweaver–Burk plot.

Conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate

100 μL of glyceraldehyde-3-phosphate (10^{-3} M), glyceraldehyde phosphate dehydrogenase (80 units), 100 μL NAD^+ (10^{-3} M), 100 μL H_2PO_4 (10^{-3} M) and 100 μL $MgCl_2$ (10^{-3} M) were taken in 2 ml HEPES buffer. Reaction completion was confirmed from the NADH absorbance at 340 nm in the UV-vis spectrum of the reaction mixture.

Formation of 3-phosphoglycerate

Taking the calculated volume of ADP, 1,3-bisphosphoglycerate and compound **3**, their final concentration in 2 ml HEPES buffer (pH 7.2) was adjusted to 0.058 μM, 50 μM and 10 μM, respectively. After addition of PGK (80 units) and Mg^{2+} (50 μM), the fluorescence intensity (excitation at 250 nm) was recorded. An 87% increase in fluorescence intensity was observed. The concentration of ADP was calculated from the change in fluorescence intensity which on dividing by the time taken for the change gave the initial velocity (v_0, μM min^{-1}). The Michaelis–Menten constant (K_m, μM) was obtained from the Lineweaver–Burk plot.

Conversion of phosphoenolpyruvate to pyruvate

An assay solution of ADP (0.058 μM), phosphoenol pyruvate (50 μM) and compound **3** (10 μM) was prepared in 2 ml HEPES buffer (pH 7.2). After addition of PK (80 units) and Mg^{2+} (50 μM), the fluorescence intensity (excitation at 250 nm) was recorded. An 85% increase in fluorescence intensity was observed. The concentration of ADP was calculated from the

change in fluorescence intensity which on dividing by the time taken for the change gave the initial velocity (v_0, μM min^{-1}). The Michaelis–Menten constant (K_m, μM) was obtained from the Lineweaver–Burk plot.

Breakdown of pyruvate in the mitochondria

An assay solution containing mitochondrial pellets, 10 mg in 50 μL HEPES buffer, and compound **2** (10 μM) was prepared in 2 ml HEPES buffer (pH 7.2). After addition of pyruvate (50 μM), the solution started turning fluorescent and an 87% increase in fluorescence intensity was observed.

LC-MS and QuantiAnalysis

For quantification of ADP/ATP, the standard curve was obtained by recording the LC-MS of commercial samples of ADP and ATP. For LC-MS, the Dionex Ultimate 3000 system was linked to a mass spectrometer. A C-18 column (Acclaim® 120 C18) 5 μm 120 Å (4.6 × 250 mm) was used for HPLC. Acetonitrile–water (1 : 1) was used as the eluent. 2 μL of sample (injection volume) was loaded onto the column, the flow rate was kept at 0.2 ml and the absorbance was set at 200, 220 and 254 nm. Sodium formate was used as an internal calibrant. As per the protocol of QuantAnalysis2.0, the ADP and ATP in the reaction mixtures were quantified from the standard curves.

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