Dyes and Pigments 95 (2012) 194–200

Contents lists available at SciVerse ScienceDirect

Dyes and Pigments

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

Spectral response of a methylimidazolium-functionalized polythiophene to phosphates

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ARTICLE INFO

Article history:
Received 31 January 2012
Received in revised form
20 April 2012
Accepted 23 April 2012
Available online 30 April 2012

Keywords:
ATP
Phosphate
Polythiophene
Fluorescence
UV/vis spectroscopy
Circular dichroism

ABSTRACT

We report on the colorimetric and fluorimetric responses of the cationic water-soluble polythiophene derivative poly[3-(2-(N-(N′-methylimidazole))ethoxy)-4-methylthiophene] in presence of different phosphates with a focus on ATP. UV/Vis absorbance and fluorescence measurements show that ATP concentrations can be determined in aqueous solutions with a sufficient selectivity to other phosphates. Measurements of circular dichroism reveal that the polymer forms a random coil structure that exhibits no signal in the CD spectrum. Upon addition of ATP the conformation changes to a helix, yielding in strong CD peaks. These experimental data suggest that this polymer is an attractive, simple and versatile colorimetric and fluorimetric probe for nucleoside triphosphates such as ATP.

1. Introduction

Conducting polymers have attracted a high attention in recent years due to their remarkable electrical and optical properties [1–3]. Extended π-systems are a common feature of such materials. Hence, aromatic heterocycles are often applied as monomer building blocks. Polythiophenes (PTs), for instance, have been implemented in organic LEDs, nonlinear optical devices, photoresists, antistatic coatings, batteries, artificial noses and smart or electrochromic windows [4–7], as well as in electrochemical or optical chemo- and biosensors [8–12].

Certain phosphates play an important role in biological systems, in the form of inorganic phosphates, pyrophosphate, nucleotides, as free species or assembled in the DNA backbone. Adenosine triphosphate (ATP) represents one of the most important members of this category of biomolecules, being referred to as the “currency of energy” of living organisms. This particular source of energy is featured in a variety of important processes. ATP is accumulated during metabolic processes such as glycolysis and is harnessed by enzymes such as different types of ATPases for transportation of certain ions (e.g. of alkaline and alkaline earth metals or of transition metals) and protons through the cell membrane against the gradient. Additionally, protein kinases are responsible for the phosphorylation of proteins using ATP as substrate in order to impact the biological function of the target molecule. On the other hand, adenylyl cyclases catalyze the production of the important cellular messenger molecule cyclic AMP (cAMP) from ATP. Thus, there is a lot of interest in the detection and monitoring of phosphates species in order to control the above mentioned processes. This enables also the screening of drugs for their impact on these pharmaceutically relevant enzymes.

An abundance of luminescent probes for the determination of ATP, pyrophosphate or related phosphate compounds have been designed in the past years [13,14]. Typical examples are zinc dipicolylamine [15–19] complexes, 3-hydroxy-4′-(dimethylamino) flavones [20], fluorophores bearing macrocyclic [21] or open chain polyamine receptors [22], and lanthanide complexes [23,24]. The advantages of using fluorescent molecular probes are that they can respond reversibly to ATP, no additional enzymes are required as in the case of the bioluminescent luciferase reaction, and also no antibodies as in case of the numerous immunoassays for the determination of phosphorylated biomolecules. However, all of the known probes show a limited applicability in complex biological matrices, because they are prone to interferences and often lose their selectivity for ATP recognition at physiological conditions. This is particularly the case for media with millimolar concentrations of Mg^{2+} which are required for many enzymatic reactions, e.g.

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doi:10.1016/j.dyepig.2012.04.012

for the hydrolases or phosphoryl transferases mentioned above. Indeed, a luminescent terbium norfloxacin complex was used to monitor the consumption of ATP by a bacterial adenylyl cyclase and to study the effect of enzyme regulators [25]. However, this probe cannot be adapted to other enzymatic reactions as it proved to be too prone to interferences.

Water-soluble poly(3-alkoxy-thiophene)s (PATs) can also interact with phosphates and enable their fluorescent determination in aqueous solution around pH 7. In particular, a PAT derivative with trimethylammonium side chains showed a significant bathochromic shift of its absorbance and undergoes fluorescence quenching in presence of ATP [26]. Furthermore, it was demonstrated that supramoleular complexes of this polythiophene derivative with ATP exhibit a circular dichroism (CD) effect [7]. Though, our own studies revealed that these macromolecular probes show only limited selectivity in buffer systems containing physiological concentrations of Mg^{2+}. The methylimidazolium derivative **PAT-1** was reported to undergo structural changes when being exposed to DNA [28,29]. Again, these are accompanied by a red shift of the absorbance of the PT backbone, a quenching of its fluorescence, and the appearance of CD signals. Imidazolium or naphthimidazolium receptors for selective recognition of phosphate anions were used in many different approaches [30–32].

In this study we describe the effect of the single nucleotides ATP and GTP as well as a series of other phosphate species (ADP, cAMP, GDP, cGMP, phosphate and diphosphate) on the spectral properties of **PAT-1** in aqueous solution including UV/Vis absorption, fluorescence emission, and CD. The experiments reveal that ATP can easily be determined by fluorescence measurements within a broad dynamic range depending on the added polymer concentration.

2. Materials and methods

2.1. Reagents

Adenosine-5′-triphosphate disodium salt (ATP), adenosine-5′-diphosphate sodium salt (ADP), adenosine-3′,5′-cyclic monophosphate (cAMP), guanosine-5′-triphosphate sodium salt (GTP), guanosine-5′-diphosphate sodium salt (GDP), guanosine-3′,5′-cyclic monophosphate (cGMP), 3-methoxy-4-methylthiophene, 2-bromoethanol, sodium methoxide in methanol and 1-methylimidazole were from Sigma, Magnesium dichloride hexahydrate, tetrasodium pyrophosphate decahydrate (PP_i), trisodium phosphate dodecahydrate (P_i) and ready to use HEPES (2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethan sulfonic acid) buffer of pH 7.4 were purchased from Merck KgaA (Darmstadt, Germany).

2.2. Spectroscopy

All experiments were carried out in 1 cm quartz cuvettes. The UV/VIS absorption spectra were obtained by a Hitachi U-3000 spectrophotometer, the fluorescence spectra by an Aminco Bowman Series 2 luminescence spectrometer. The CD spectra were achieved with a Jasco J-710 spectropolarimeter. All measurements were performed in a 10 mM HEPES buffer at pH 7.4. The phosphate solutions were freshly prepared in the same media.

2.3. Microwell plate assay

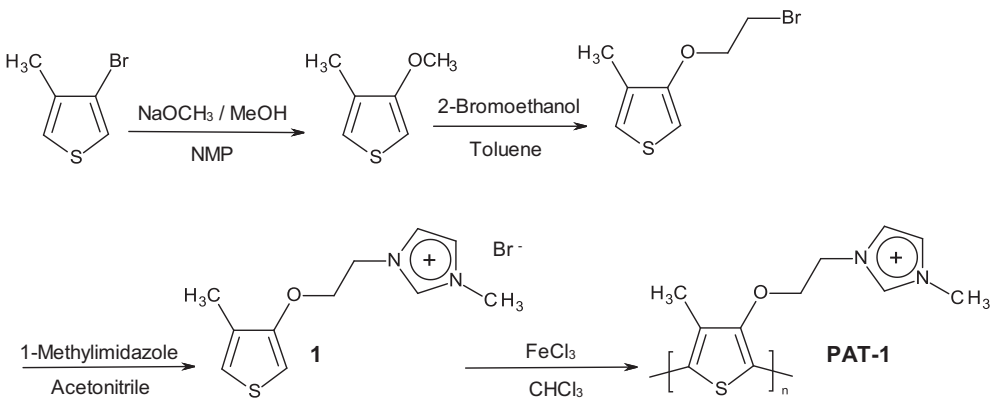
As an alternative to the measurements in cuvettes with fluorescence spectrometers commonly used microwell plate fluorescence readers can be used. In this case, the calibration of the fluorescence response of **PAT-1** in presence of ATP, ADP and PP_i was carried out with a commercially available reader from BMG Labtech (FLUOstar OPTIMA, equipped with a broadband excitation filter 400–440 nm and a t 520 nm emission filter for detection). The assays were performed in 96-microwell plates from Greiner Bio-One GmbH (Frickenhausen, Germany).

2.4. Polymer synthesis

The synthesis of PAT-1 was carried out according to Ho et al [28]. Briefly, the monomer unit 3-(2-(N-(N′-methylimidazole))ethoxy)-4-methylthiophene **1** was prepared in three steps. First, 3-bromo-4-methylthiophene was converted to 3-methoxy-4-methylthiophene with sodium methoxide. This was transformed to 3-(2-bromoethoxy)-4-methylthiophene with 2-bromoethanol. Finally, the monomer was obtained by conversion with 1-methylimidazole. The polymerization was accomplished by oxidation with $FeCl_3$ in anhydrous chloroform under argon atmosphere (Scheme 1). After stirring of 24 h at room temperature the solvent was evaporated and the crude product washed three times with methanol. The polymer was filtered and purified by Soxhlet extraction with acetone. Afterwards, the polymer was ingested in methanol and completely solubilized by addition of anhydrous hydrazine and strong stirring. The resulting product was precipitated by addition of a saturated solution of tetrabutylammonium chloride in acetone and washed again with acetone, and then dried under reduced pressure.

3. Results and discussion

The water-soluble cationic polymer **PAT-1** was synthesized via oxidative polymerization of 3-(2-(N-(N′-methylimidazole))



Scheme 1. Synthesis of monomer unit 3-(2-(N-(N′-methylimidazole))ethoxy)-4-methylthiophene and oxidative polymerization to **PAT-1**. NMP = N-Methyl-2-pyrrolidon [24].

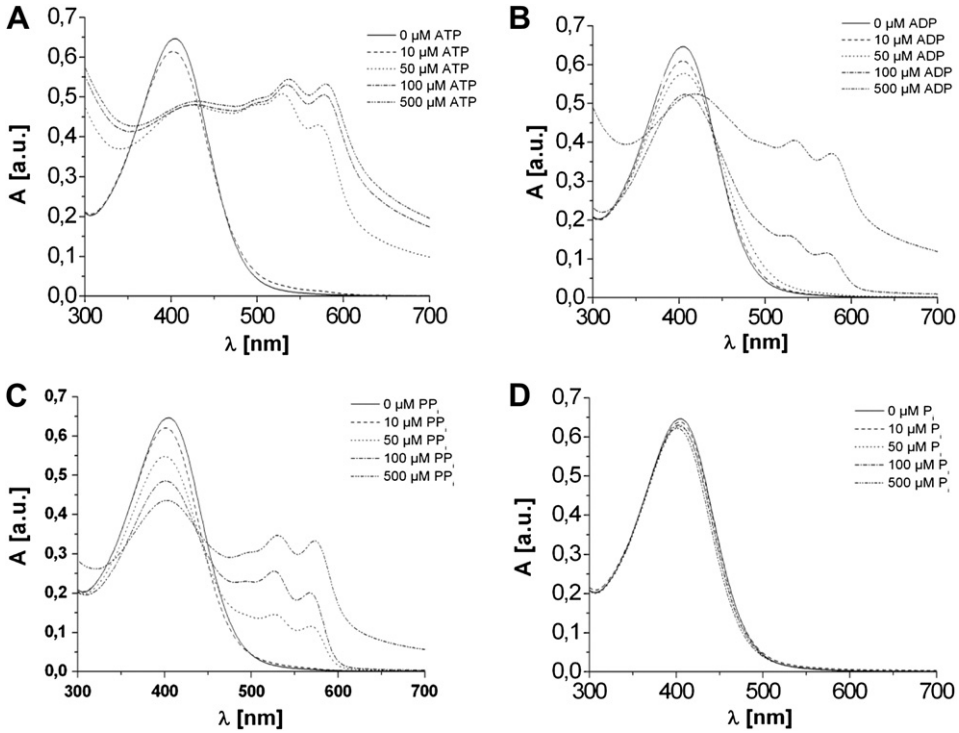


Fig. 1. Absorption (A) in arbitrary units of **PAT-1** at a concentration of $c = 100$ µmol L^{-1} based on monomer unit in absence and presence of different concentrations of ATP (A), ADP (B), PP_i (C), and P_i (D) in a 10 mM HEPES buffer at pH 7.4, $T = 25$ °C.

ethoxy)-4-methylthiophene with $FeCl_3$ in chloroform [28,29]. Usually, this method leads to polymers with an average molecular weight of 6–10 kDa [33,34].

The absorption spectra for **PAT-1** in aqueous solution are presented in Fig. 1A. The maximum at 400 nm represents the absorption of the random coil form of the polymer [26,28]. The torsion of its backbone leads to a reduced effective conjugation length. Upon addition of ATP this absorption band decreases while three new maxima at 495, 530 and 580 nm can be observed. Thus, a conformational change of the polymer backbone occurs due to the interaction with ATP. The bathochromic shift of the absorption indicates an elongation of the effective conjugation length. The phosphate anions shield the repulsion of the positively charged methylimidazolioum units which enables the formation of higher ordered structures. Solvatochromic studies of a polythiophene with oligo(ethylene oxide) side chains revealed the same effect [35]. A red shift of the absorbance was observed after addition of water to a solution of the polymer in THF. Alike, the new absorption band showed a vibronic fine structure. This can be attributed to an aggregation of the polymers which leads to an extended conformation and a planarization of adjacent monomer units due to π-stacking interactions [36]. This aggregation is particularly strong in presence of the triphosphate ATP and less pronounced after addition of ADP (Fig. 1B) or pyrophosphate (PP_i) anions (Fig. 1C), in which cases much higher concentrations are required to achieve a shift of the absorption band. Similar, but slightly higher responses can be observed for GTP and GDP, respectively (see Fig. 2). The conformational changes of cationic polythiophenes induced by interactions with nucleotides have been discussed by Ho et al [37].

This indicates that the number of negatively charged phosphate groups is the dominating factor for the formation of supramolecular aggregates. The nucleotide is not important for the shift of the absorption spectrum as can be deduced from the comparison between the responses for ADP and PP_i. This is in coincidence with

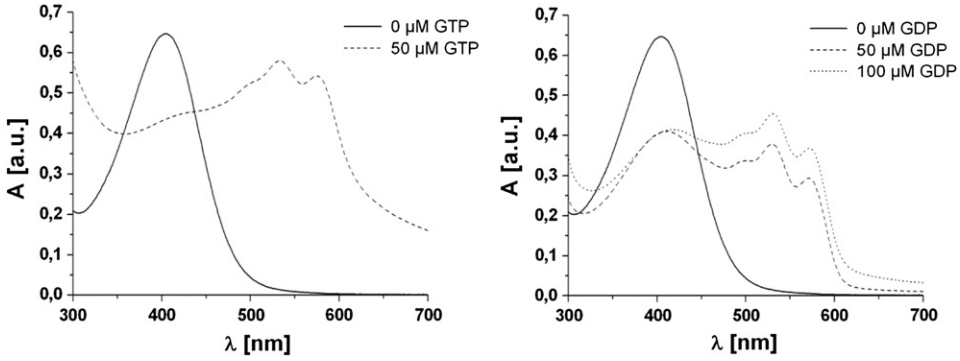


Fig. 2. Absorption (A) in arbitrary units of **PAT-1** ($c = 100$ µmol L^{-1} based on monomer unit) in absence and presence of GTP (left) and GDP (right), in a 10 mM HEPES buffer at pH 7.4, $T = 25$ °C.

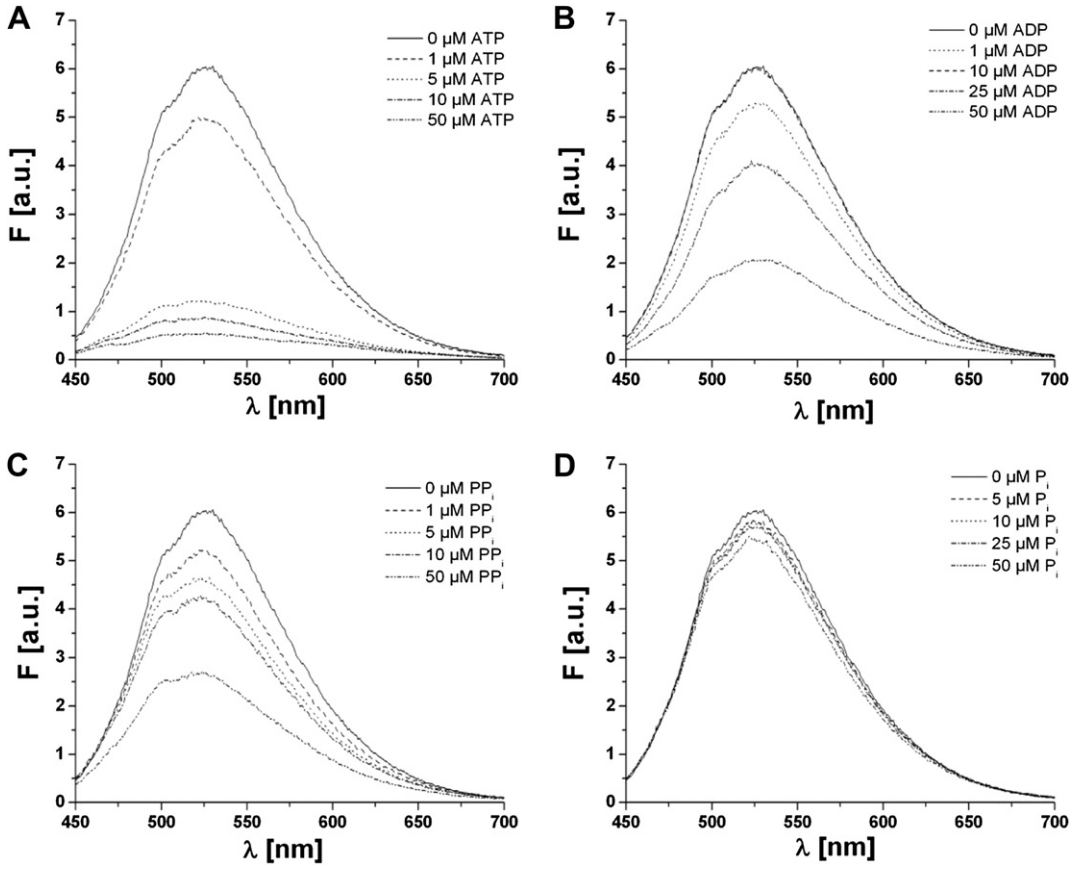


Fig. 3. Fluorescence emission (F) in arbitrary units of **PAT-1** ($c = 10$ µmol L^{-1} based on monomer unit) in absence and presence of different concentrations of ATP (A), ADP (B), P_i (C) and PP_i (D) in 10 mM HEPES buffer at pH 7.4, $\lambda_{ex} = 405$ nm, $T = 25$ °C.

the behaviour of the trimethylammonium-derived polythiophene studied by Li et al [26]. The addition of phosphate (P_i, Fig. 1D) as well as of AMP, cAMP or cGMP (results not shown) did not induce any spectroscopic changes. ATP concentrations ranging from 10 µmol L^{-1} to 500 µmol L^{-1} can be determined with **PAT-1** concentrations of 100 µmol L^{-1} in relation to the monomer unit with high selectivity to other phosphate species by measuring the increase of the longwave absorption.

In biological assays and in optical sensing fluorimetric techniques are preferred compared to colorimetric methods because of

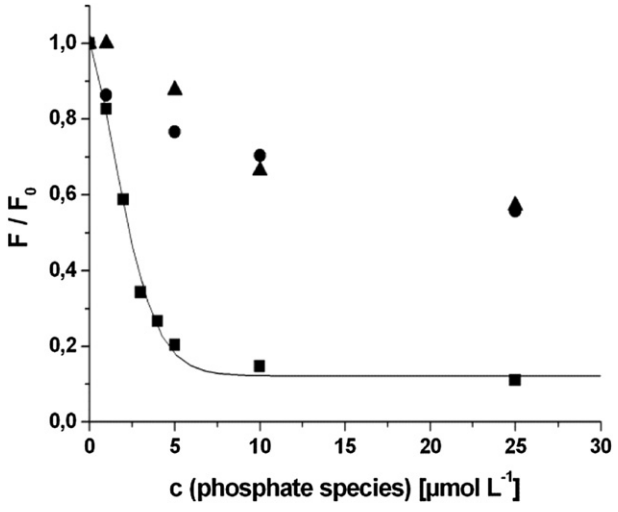


Fig. 4. Quenching of the fluorescence emission (F) of **PAT-1** ($c = 10$ µmol L^{-1} based on monomer unit) in presence of ATP (■), ADP (▲), and PP_i (●), in 10 mM HEPES buffer at pH 7.4, $\lambda_{ex}/\lambda_{em} = 405/520$ nm, respectively, $T = 25$ °C. The values are referenced against the fluorescence intensity of **PAT-1** (F_0). The single plots can be fitted sigmoidally, which is shown exemplarily for ATP.

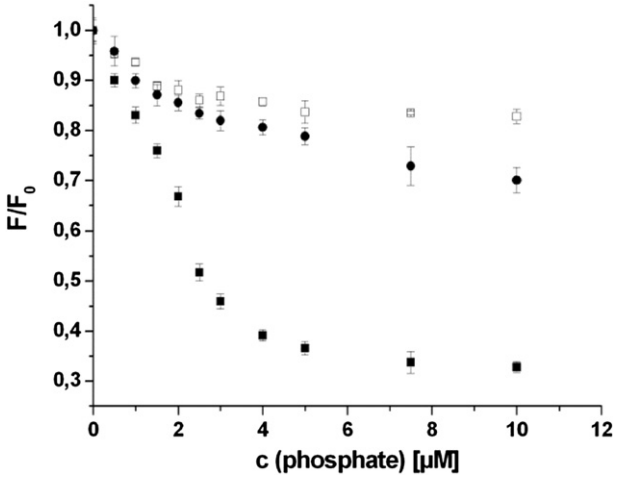


Fig. 5. Quenching of the fluorescence emission (F) of **PAT-1** ($c = 10$ µmol L^{-1} based on monomer unit) in presence of ATP (■), ADP (□), and PP_i (●), in 10 mM HEPES buffer at pH 7.4, $T = 25$ °C obtained by a microwell plate reader. The values are referenced against the fluorescence intensity of PAT-1 (F_0). $\lambda_{ex}/\lambda_{em} = 400–440/520$ nm.

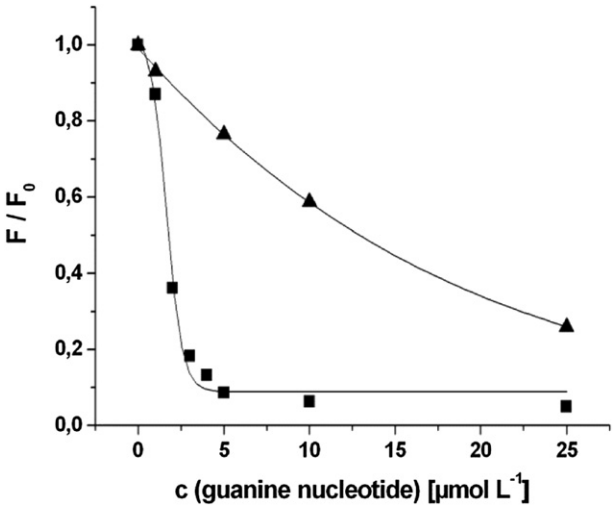


Fig. 6. Quenching of the fluorescence emission (F) of **PAT-1** ($c = 10$ µmol L^{-1} based on monomer unit) in presence of GTP (■) and GDP (▲), with sigmoidal fits, in 10 mM HEPES buffer at pH 7.4, $\lambda_{ex}/\lambda_{em} = 405/525$ nm, respectively, $T = 25$ °C. The values are referenced against the fluorescence intensity of **PAT-1** (F_0).

Table 1
Quenching efficiency F/F_0 for **PAT-1** of different nucleotides at a molar ratio of 1:2 of nucleotide to monomer unit.

Nucleotide	ATP	GTP	ADP	GDP
F/F_0[a]	0.20	0.09	0.88	0.77

[a] Fluorescence intensity of PAT-1 in absence of quencher.

their higher sensitivities. Therefore, we studied the fluorescence response of **PAT-1** in presence of different phosphate species. The resulting emission spectra are presented in Fig. 3. Excitation at 405 nm leads to an intense fluorescence emission with a maximum at 525 nm. Upon addition of ATP the emission intensity decreases depending on the concentration of ATP (Fig. 3A). Hence, the formation of planar conformations and polymer aggregates causes a strong quenching of the polythiophene fluorescence due to π-π interactions. Again, the quenching in presence of ADP (Fig. 3B) and PP_i (Fig. 3C) is much weaker. No fluorescence quenching can be observed in presence of P_i (Fig. 3D), and cAMP (data not shown). The resulting response curves are displayed in Fig. 4. The referenced fluorescence intensity F/F_0 at $\lambda = 525$ nm decreases approximately linearly up to concentrations of 5 µmol L^{-1} of ATP, where quenching reaches saturation. This point represents a ratio of 1:2 of ATP to monomer unit. The assays can also be performed in a conventional microwell plate fluorescence reader which shows similar response curves (Fig. 5).

The guanosine phosphates GTP and GDP quench the fluorescence similarly to ATP and GDP, respectively. However, the quenching efficiencies are significantly higher compared to the corresponding adenosine phosphates (Fig. 6, Table 1). In contrast to cAMP, cGMP quenches the fluorescence slightly, whereas no red-shifted absorption bands can be observed in presence of cGMP. The corresponding fluorescence spectra are shown in Fig. 7. These findings indicate that the guanosine unit exerts an additional quenching effect which is independent from structural changes. It exhibits a lower oxidation potential than the other nucleosides and, therefore, can induce a nonradiative relaxation of the excited state of the fluorophor by photoinduced electron transfer processes

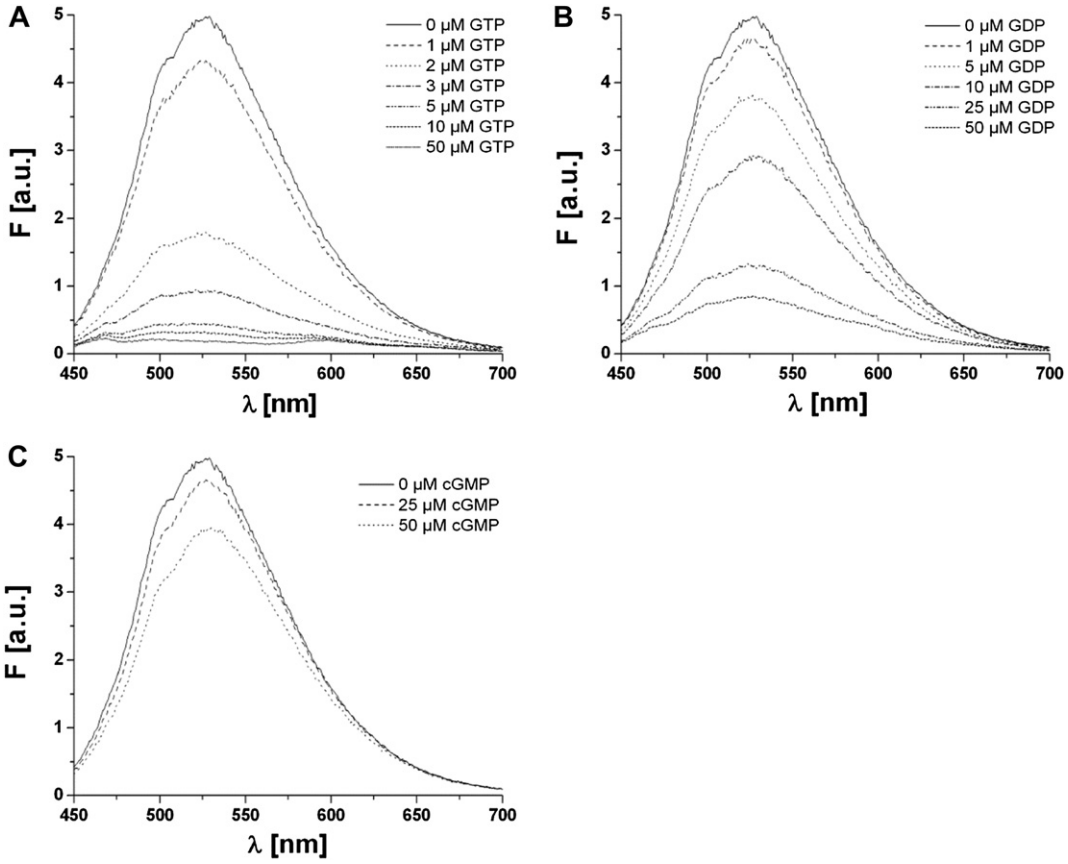


Fig. 7. Fluorescence emission (F) in arbitrary units of **PAT-1** ($c = 10$ µmol L^{-1} based on monomer unit) in absence and presence of different concentrations of GTP (A), GDP (B), and cGMP (C) in 10 mM HEPES buffer at pH 7.4, $\lambda_{ex} = 405$ nm, $T = 25$ °C.

[38,39]. However, as the response of the red-shifted absorption is also more distinct in case of GTP and GDP (Fig. 2) compared to ATP and ADP (Fig. 1A and B), respectively, also other factors must be involved and the occurrence of electron transfer processes remains as a hypothesis.

These data show that this macromolecular probe is applicable to the fluorescent determination of ATP with high sensitivity and sufficient selectivity compared to other phosphate species. The dynamic range of the response can be adjusted by changing the polymer concentration. Furthermore, the magnitude of fluorescence quenching at a certain concentration of ATP exhibits a good reproducibility with low standard deviations. The changes in absorbance and fluorescence emission occur immediately.

The appearance of circular dichroism (CD) in presence of ATP is another noticeable effect to study conformational changes of the polymer backbone. The CD spectrum of **PAT-1** is presented in Fig. 8.The random coil structure of the polymer shows no CD signal, as expected. But as the 3D structure is changed upon addition of ATP, a strong split-type CD of the $\pi-\pi^*$ transition band can be observed. The CD spectrum of the supramolecular complex between ATP and **PAT-1** displays two maxima at 540 and 575 nm with a negative CD, and a third maximum at 460–470 nm, depending on the ATP concentration, with a positive CD. Thus, the vibronic fine structure of the absorption spectrum is retained. The zero crossing points are in the range between 500 and 515 nm. The reverse CD bands are a hint for the formation of a right-handed helical form and exciton coupling between aggregated polymer chains [36,40]. The CD signal in presence of ATP is specific because no signals can be obtained with ADP, cAMP and PP_i up to concentrations of 500 μmol L^{-1}. Thus, the interaction with the nucleoside triphosphate is essential for the formation of the chiral supramolecular complexes. It can be assumed that π-stacking interactions between the nucleobases and the methylimidazolium units induce the helical superstructure of the polymer chains [27,40].

It has to be emphasized that the response to ATP (and also GTP) is significantly decreased and the selectivity compared to ADP and PP_i disappears if the concentration of Mg^{2+} exceeds 0.5 mmol L^{-1}. This applies likewise to the colorimetric, fluorescent, and CD based assay. Thus, the probe is not suitable to determine ATP selectively in more complex biological samples or to monitor certain enzymatic ATP conversions, as the corresponding enzymes (e.g. kinases, adenylyl cyclases or ATPases) require mmolar concentrations of Mg^{2+}. The concentration of Mg^{2+} in plasma is usually between 0.75 and 1.1 mmol L^{-1}. This loss of response was also observed for the trimethylammonium analogue of the polymer. Nevertheless, this probe is applicable to the fluorescent determination of ATP if the concentration of Mg^{2+} can be kept below 0.5 mmol L^{-1} in form of cheap and straightforward assays. It is also appropriate for high-throughput analysis using conventional fluorescence microwell plate readers.

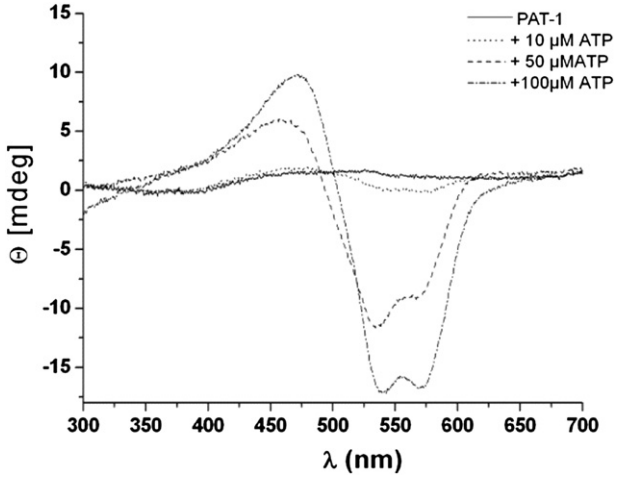


Fig. 8. CD spectra of **PAT-1** (c = 100 μmol L^{-1} based on monomer unit) in absence and presence of different concentrations of ATP in 10 mM HEPES buffer at pH 7.4, T = 25 °C.

4. Conclusions

We have examined the potential of the water-soluble polythiophene derivative **PAT-1** as a probe for colorimetric and fluorescent determination of ATP. The selectivity of the spectral response in comparison with other phosphate species such as ADP, cAMP, P_i, and PP_i has been studied. The findings demonstrate that this probe has a sufficient sensitivity and selectivity for the determination of ATP in buffered aqueous solution if the concentration of Mg^{2+} is lower than 0.5 mmol L^{-1}, particularly, if the fluorescence quenching effect is used as detection method. The response occurs very fast and can be utilized for rapid and easy to perform assays also in a high-throughput screening mode. Maximum quenching is achieved at a molar ratio of 1:2 of ATP to monomer unit. The dynamic range and lower detection limit of this assay can be easily adjusted by the choice of the polymer concentration. The quenching is induced by planarization and aggregation of polymer segments in presence of ATP. The characterization by CD spectroscopy reveals another interesting property of the supramolecular complexes between **PAT-1** and ATP, namely the formation of helical structures.

The guanosine phosphates GTP, GDP and cGMP generate similar spectral responses as the corresponding adenosine phosphates, but the guanine unit causes an additional quenching effect, presumably by a photoinduced charge transfer process. This reveals that two contributions are in charge of the fluorescence response: structural changes of the polymer backbone induced by interactions with the triphosphate units and fluorescence quenching induced by the single nucleobase units.

Acknowledgements

Financial support by the DFG (Deutsche Forschungsgemeinschaft, SCHA 1009/4-3) is gratefully acknowledged. We also thank Prof. Mario Leclerc for providing the protocol for monomer and polymer synthesis.

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