Electrochemical/Electrospray Mass Spectrometric Studies of Electrochemically Stimulated ATP Release from PP/ATP Films

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Polypyrrole (PP)/adenosine triphosphate (ATP) was chosen as a conducting polymer/anionic drug model to serve as a bioactive releasing material for ATP. The process of ATP release from PP/ATP films was investigated for the first time by electrochemical electrospray ionization mass spectrometry (EC/ESMS). This technique allowed the simultaneous and direct detection of ATP and its related species during electrochemical release. In the experiments, suitable solvent conditions were found for both the electrochemical release and the electrospray mechanisms. EC/ESMS results showed that continuous potential cycles allowed a higher ATP release rate than potential steps. It was also found that the film thickness is an important factor affecting the rate and the amount of electrochemical ATP release.   (J Am Soc Mass Spectrom 2007, 18, 919–926) © 2007 American Society for Mass Spectrometry

We chose to study the adenosine 5′-triphosphate (ATP) release system because ATP is a very important component of biological systems. It plays an important role in energy storage and conversion in all living cells. The controlled release of ATP from conducting polymers has been studied by several groups, for example, by both Reynolds and Genies [1–15]. These groups used optical spectroscopic, potentiometric, and conductiometric methods to examine ATP release. Because ATP is easily incorporated into the matrix of polymers during the electropolymerization process, and is easily exchanged with anions in solution, it is possible to make an ATP release device for a clinical system. In addition, ATP ions are thought to be inert during hydrolytic cleavage in both synthesis and potential driven release, and thus electrodes modified with PP/ATP polymer films may be useful as ATP supply devices [7].

In the work reported by Reynolds [7], the electrochemically stimulated and spontaneous release of ATP from PP/ATP-modified electrodes was investigated by ultraviolet–visible (UV–vis) spectroscopy. The results showed that over a much shorter time period, appreciable amounts of ATP were released during potential cycling with a peak at 260 nm. In contrast, the PP/ATP film was relatively stable to spontaneous ion-exchange processes using an open circuit. Genies reported that PP/ATP exhibits an ion dependent electrochemical response during cycling in various electrolyte solutions [8]. Two separate redox processes were observed from the cyclic voltammogram (CV) of PP/ATP film (of 450-nm thickness, on a Pt button) cycled in 0.1 M NaCl, NaPSS, and PVPy · HCl solutions, respectively. The results showed that the more anodic redox process is suppressed when the film is cycled in NaPSS, whereas the more cathodic redox peaks disappear when cycled in PVPy · HCl, indicating that the more anodic redox process was associated with anion-dominant transport, and the more cathodic redox process, with cation-dominant transport [11]. EQCM (electrochemical quartz crystal microbalance) studies showed that, in the initial cathodic redox process, the reduction was dominated by Na^+ penetration into the film. Subsequently, the diffusions of ATP sodium salt out of the film occurred with ease [7]. In the studies of Liljegren et al. [16], electrochemically controlled solid-phase extraction (EC-SPE), using conducting polymers (such as polypyrrole) as stationary phases, was demonstrated by coupling EC-SPE to electrospray ionization– or inductively coupled plasma–mass spectrometry (ESI-MS and ICP-MS, respectively) on line. The advantage of this relatively new technique was that both the extraction and desorption steps could be controlled merely by changing the applied potential of the conducting polymer-coated working electrode. In these experiments, the extraction of the negatively charged model analytes [$Fe(CN)_6^{3-}$ and Br^-] was performed by applying a potential of +800 mV to the working electrode modified with polypyrrole, using the flow cell. At this potential, the polypyrrole was oxidized, becoming positively charged, which resulted in an electrosorption (extraction) of anions [$Fe(CN)_6^{3-}$ and Br^-]. The polypyrrole was subsequently reduced to its neutral state by the application of a potential of −800 mV. During the latter step, anions

were again expelled (desorbed) from the stationary phase.

Previous studies on ATP release in PP/ATP system (electrochemically triggered release and spontaneous release) included analysis by CV, spectroscopy or EQCM; however, these techniques cannot give us detailed on-line information of electrochemical ATP release in the cell. To obtain real-time information in the electrochemical cell, electrospray to couple electrochemistry (EC) on-line with MS has been developed—that is, electrochemical electrospray mass spectrometry (EC/ESMS) [16–26]. In this technique, a high voltage (~4 kV) is used in ESI/MS to ionize and transfer the analyte mist into the mass spectrometer. The advantages of the high sensitivity and selectivity of mass spectrometry have long been recognized. Therefore, it is a very useful technique for detecting trace levels of analytes in the gas phase. In contrast to many ionization methods, such as EI and CI, electrospray (ES) is a very "soft" ionization method. It has the advantages of causing minimal decomposition and fragmentation of ions, gas-phase reactions of the analytes are minimal, and a wider variety of solvents can be used. Thus, the technique (EC/ESMS) has been applied to the study of organic molecules and to the identification of charged inorganic and organometallic species dissolved in organic solvents.

In this study, for the first time, we used EC/ESMS to investigate electrochemical ATP release processes from PP/ATP films. This technique allowed the simultaneous and direct detection of ATP and its related species during electrochemical release. By using EC/ESMS, we could precisely monitor the on-line ATP release profiles. In our research, electrochemical ATP release under three types of controlled potential was carried out. The electrochemical ATP release as a function of film thickness was also investigated in detail. The results show that the film thickness is an important factor affecting both the rate and the amount of electrochemical ATP release as previous studies suggested [11].

Experimental

Chemicals and Solutions

Adenosine 5'-triphosphate, disodium salt hydrate (99%), pyrrole, (98%), and ammonium acetate (99%) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). All salts were used as received. Electrolyte solutions of 2 mM NH_4Ac were prepared in freshly distilled CH_3CN, deionized water, and Et_4N (vol/vol/vol = 35/65/0.1).

Instrumental

A VG Trios 2000 (VG Biotech, Altrincham, UK) quadrupole mass spectrometer with VG electrospray source was used in these experiments. Here all the mass spectra were acquired in negative-ion mode coadding

30 scans at a scan rate of 0.25 scans/s. The ES tip voltage was −4.10 kV, the source temperature was kept at 110 °C, and the cone voltage was 20 V.

A custom-built electrochemical cell was made from a cylindrical Pyrex® tube (6 mm in diameter) with the solution flowing around the working gold disk electrode (3 mm in diameter) into a cupped Pyrex® collection tube. This cupped collection assembly led to the electrospray emitter through a fused silica capillary with 14 s of transfer time (2.3×10^{-3} mL of dead volume). A platinum mesh was used as counter electrode. An Ag/AgCl wire was used as the reference electrode, which was connected to the cell through a double salt bridge. The distance between working electrode and counter electrode was about 5 mm. Before use, the electrodes were cleaned by rinsing sequentially with distilled water, hydrofluoric acid 49%, piranha solution, distilled water, HPLC-grade methanol, and fresh acetonitrile. The electrochemical cell is shown in Figure 1.

A model CV-27 potentiostat [Bioanalytical Systems (BAS), West Lafayette, IN, USA] was used to apply potentials to the electrochemical cell and electrosynthesize the PP/ATP conducting polymer. All potentials are quoted versus aqueous Ag/AgCl. We held the potential at 0.8 V in the 0.1 M pyrrole and 20 mM ATP solution for a given time for PP/ATP film synthesis. The potential cycling ranged from 0.0 to −1.1 V at a scan rate of 10 mV/s; the potential step ranged from 0 to −1.1 V with the potential first at 0 V for 1 min, and then at −1.1 V for another 1 min. The entire electrochemical cell was maintained at room temperature. A BAS ε-EC potentiostat was used to obtain conventional CV for comparison purposes.

A Series 74,900 syringe pump (Cole-Palmer, Vernon Hills, IL, USA) with a flow rate of 0.6 mL/h was used to provide the constant mobile phase and produce the spray. To electrically isolate the electrochemical cell from the electrospray tip potential, the cell was connected to the electrospray probe tip through a 300- to 350-mm-long 0.1-mm ID untreated silica capillary, which caused 14 s of delay time to the electrospray emitter.

Results and Discussion

Synthesis of PP/ATP Film

In our experiments, the film thicknesses were calculated by the charge passed during electropolymerization [8]. For an example, a gold electrode with a PP/ATP film was prepared potentiostatically from 0.1 M pyrrole in 20 mM ATP aqueous solutions at +0.8V (versus Ag/AgCl) for 10 min. From chronoamperometry of film synthesis process, the charge passed was recorded as 44.6 mC. Because the electrode surface area is 0.07 cm^2, the charge density can be calculated as 637.5 mC/cm^2. The film was prepared on the electrode surface with estimated thickness of 2.5 μm based on the assumption
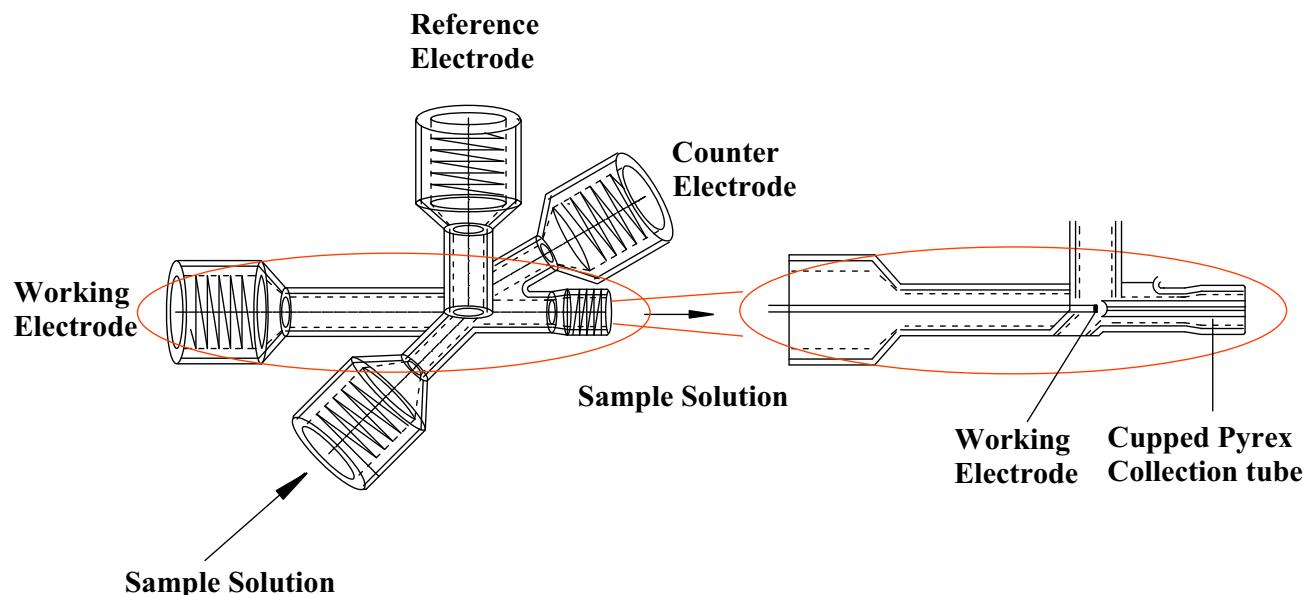
Figure 1.   Diagram of electrochemical cell.

that 255 mC/cm^2 gives 1-μm film thickness [8]. Because the doping level of PP/ATP was 0.27 in the above synthesis solution [7], 44.6 mC electropolymerization charge corresponded to 5.3 mC doping charge from ATP tri-anions; as a result, 1.83×10^{-8} moles of ATP tri-anions were doped in the matrix of PP/ATP film.

Selection of Suitable Conditions for the Detection of ATP by ESMS

One difficulty in implementing ESMS is the selection of suitable conditions for the detection of ATP. In previous works on ATP release, all experiments were carried out in aqueous solution. However, a pure aqueous solution is not suitable for electrospray because the electric field required for the onset of electrospray increases with the surface tension of the solvent. Water has very high surface tension, which requires high onset fields, and these fields can lead to electrical discharges that partially suppress the ES process. Therefore, 100% H$_2$O is not a good solvent choice for ESMS [27]. In addition, in our experiments, we found that pure water does not form a good spray from the capillary tip. To improve the signal of possible released ATP, we mixed water with an organic solvent, such as acetonitrile. The most efficient system for good ionization of the compounds of interest (ATP, ADP, AMP, etc.) was the mixture of acetonitrile/water/triethylamine (35/65/0.1 vol/vol/vol). Before mixing with acetonitrile, the pH of the aqueous solution was 7.6. In this system, the addition of the Et$_3$N (neutral) helped to increase the pH without the addition of sodium ions (addition of NaOH). These might decrease the signal of analyte arising from the increased electrolyte concentration, leading to the suppression of analyte signal, because an electrospray ion source is analogous to a controlled-current electrolytic flow cell [28]. The addition of Et$_3$N not only increased the ionization of the nucleotide phosphates but also decreased the formation of sodium ion adducts from Na$_4$ATP. This explains why mostly H$_3$ATP$^-$ was observed.

The most difficult problem coupling electrochemistry on-line with ESMS is that most traditional supporting electrolytes for common electrochemistry are not suitable for electrospray mass spectrometry. For example, tetraalkylammonium perchlorates, sodium perchlorate, and sodium chloride would give rise to very strong background peaks, thus resulting in the suppression of the analyte signal. To optimize the supporting electrolyte used in EC/ESMS for the ATP release system, ESMS measurements of solutions (CH$_3$CN/H$_2$O/Et$_3$N 35/65/0.1 vol/vol/vol) with different supporting electrolytes were carried out. The results showed that ammonium acetate is the best electrolyte in this system. Figure 2 shows an ESMS mass spectrum of 1 mM Na$_2$H$_2$ATP with 1 mM NH$_4$Ac as supporting electrolyte. It gave us a very strong molecular ion species signal at m/z 506. The m/z 506 and 528 were attributed to (H$_3$ATP)$^-$ and ATP plus
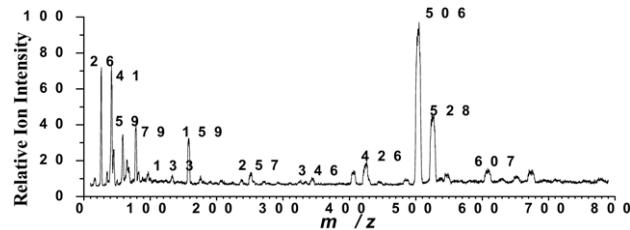
**Figure 2.**   Negative ion ESMS of 1 mM Na$_2$H$_2$ATP in acetonitrile/water/triethylamine (vol/vol/vol = 35/65/0.1) solution, with electrolyte 1 mM NH$_4$Ac. Flow rate 0.6 mL/h; source temperature 110 °C.
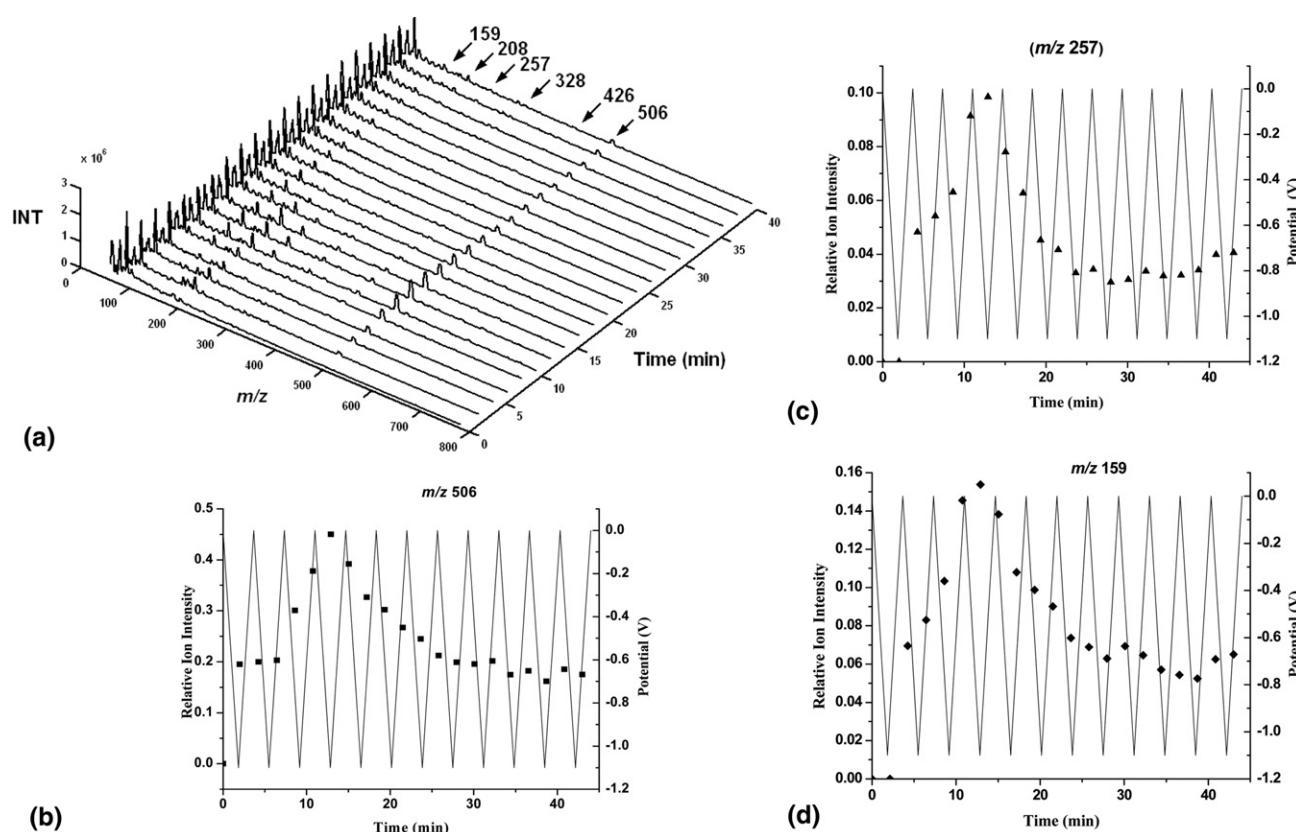
Figure 3. (**a**) Negative ion on-line EC/ESMS as a function of time. m/z 506, $(H_3ATP)^-$; m/z 426 $(H_2ADP)^-$; m/z 328, $(HAMP-H_2O)^-$; m/z 257, $H_3P_3O_{10}^-$; m/z 208, $[H_3ATP \cdot (CH_3COO)_2]^{3-}$; m/z 159, $HP_2O_6^-$. Flow rate 0.6 mL/h; source temperature 110 °C. Potential cycling from 0 to −1.1 V, $\nu = 10$ mV/s (~5-μm-thick PP/ATP film on the gold electrode). (**b**) Time-resolved relative ion intensity responses for m/z 506, (**c**) m/z 257, (**d**) m/z 159 obtained from EC/ESMS experiments (on-line electrolysis cycling from 0 to −1.1 V, scan rate 10 mV/s in which PP/ATP film was reduced in the optimal solution (~5-μm-thick PP/ATP film on the gold electrode).

one sodium atom $(NaH_2ATP)^-$; m/z 426 and 448 were attributed to $(H_2ADP)^-$ ion and ADP plus one sodium atom $(NaHADP)^-$; m/z 346 and 328 were attributed to $(HAMP)^-$ and $(HAMP-H_2O)^-$, respectively. The observation of ADP and AMP species in the mass spectra arose from the fragmentation of ATP ions that took place during the electrospray process. In addition, other fragment ion species at m/z 257, 159, 97, and 79 were shown in the negative-ion mass spectrum. In Figure 2, m/z 181 was probably attributable to m/z 159 plus one sodium atom minus one proton, $(NaP_2O_6)^-$. There was a small peak at m/z 133, which was attributed to adenine $C_5N_5H_3^-$, a fragment ion of ATP. A peak at m/z 607 was attributed to the clusters of $(ATP \cdot Et_3N)^-$. We did not expect to see ATP^{-2} or ATP^{-3} anions because the pK_a of the phosphates increases significantly from −1 to −2 to −3 and because of the instability resulting from a large amount of charge present in such a localized area of the molecule.

Moreover, the UV results under this condition verified that ATP is still electrochemically released very well. Therefore, the most efficient system we found for ATP detection by ESMS is $CH_3CN/H_2O/Et_3N$ 35/65/0.1 vol/vol/vol with 2 mM NH_4Ac.

ESMS Results of Electrochemical ATP Release

It has been suggested that the amount and rate of ATP release can be tuned by carefully controlling the potential [1]. In our experiments, the electrochemical ATP release under three types of controlled potentials was investigated.

On-line EC/ESMS Results of Potential Cycling

In the experiment, ATP was electrochemically released from PP/ATP film in the above solvent system. Before the experiment, the polymer was oxidized in the synthesis process; the oxidation reaction is shown as follows:

$$nPyrrole \xrightarrow{-ne^-} \text{oxidized polypyrrole } (PP)^+ \qquad (1)$$

$$3PP^+ + HATP^{3-} \longrightarrow (PP^+)_3 \cdot HATP^{3-} \qquad (2)$$
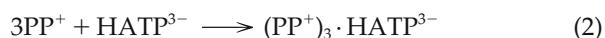
The electrochemical release of ATP from PP/ATP film was generated by applying potential cycling with a range of 0.0 to −1.1 V at a scan rate of 10 mV/s. ATP was released out of the film as a result of the reduction
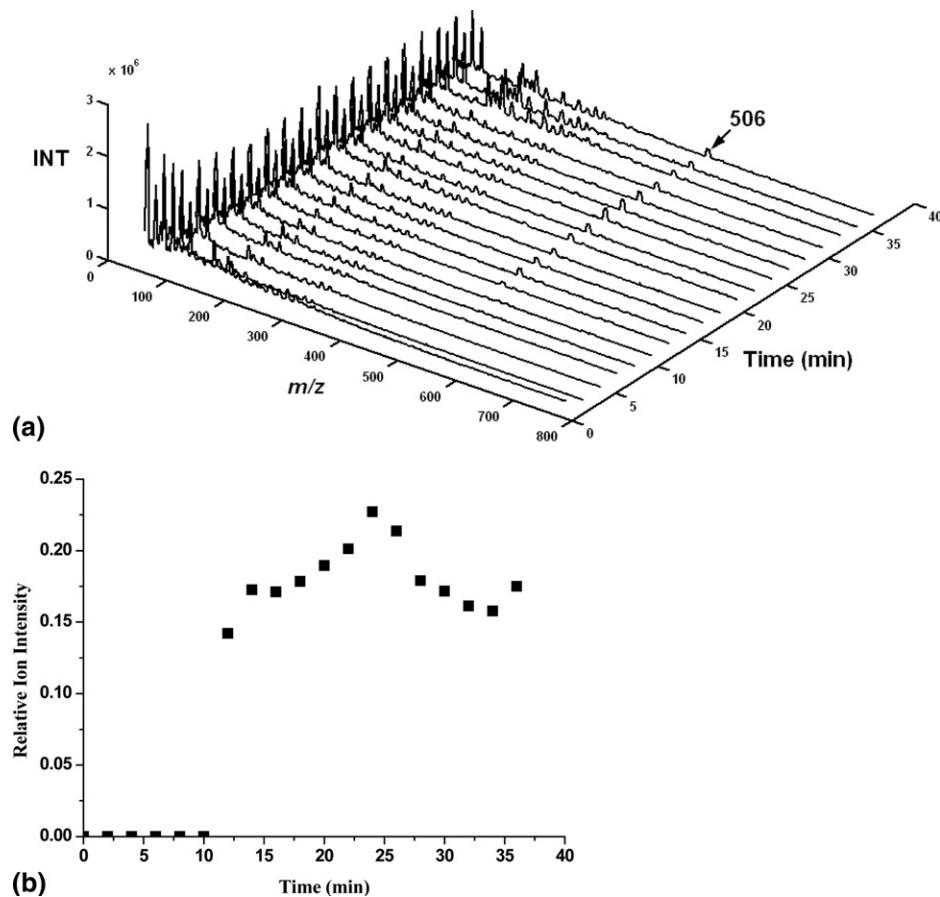
Figure 4. (**a**) EC/ESMS negative mass spectra as a function of time. Flow rate 0.6 mL/h; source temperature 110 °C. Holding the potential at −1.1 V (~5-μm-thick PP/ATP film on the gold electrode). (**b**) Time-resolved relative ion intensity responses for *m/z* 506 obtained from EC/ESMS experiments (on-line electrolysis holding potential at −1.1 V) in which PP/ATP film was reduced in the optimal solution (~5-μm-thick PP/ATP film on the gold electrode).

of polypyrrole during the negative potential scan (0 to −1.1 V). The reduction reaction is shown as follows:

$$3PP^+ \cdot HATP^{3-} \xrightarrow{+3e^-} (PP)_3 + HATP^{3-} \qquad (3)$$
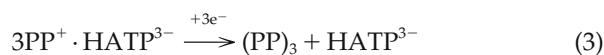
From the cyclic voltammogram in our experiments, polypyrrole was still released during the reverse scan (−1.1 to −0.6 V). From −0.6 V to even more positive potential (0 V), a little oxidation current occurred, indicating polypyrrole started to be oxidized. The oxidation reaction is shown as

$$(PP)_3 + HATP^{3-} \xrightarrow{-3e^-} 3PP^+ \cdot HATP^{3-} \qquad (4)$$
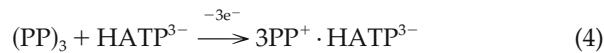
To obtain the time profile of released ATP, we performed electrochemical reactions on-line, where the species generated in the cell flowed into the electrospray probe and were monitored through mass spectrometry. Mass spectra as a function of time are shown in Figure 3a. We observed a small indicative peak of ATP *m/z* 506 occurring in the second spectrum taken after application of potential cycling for 2 min, indicat-

ing that the delay time was only 2 min long. Here we ignored the transfer time between the electrochemical cell and electrospray tip (14 s). The peak grew quickly, up to about 13 min, where it reached its maximum value and then gradually decreased almost to zero. From this 3-D plot, it was observed that the intensity of several other peaks also varied with time.

Figure 3b, c, d show the plots of the relative ion intensity for the molecular ion and fragment species of ATP at *m/z* 506, 257, and 159 obtained as a function of time in a potential cycling experiment. The potential as a function of time was also plotted. In general, the type of relationship between ion abundance and time for release of all the ATP species is similar. In the middle of the fourth cycle, at a potential of −1.1 V, the polymer was fully reduced and the ion intensity had a strongest value. The ion intensity started to increase sharply at 6 min, until 13 min, then decreased slowly thereafter, which indicates that for all the ATP species, their associated mass intensity reached its maximum value at about 13 min. By further experiments, we found that no significant release occurred above a given gate potential (about −0.6 V).
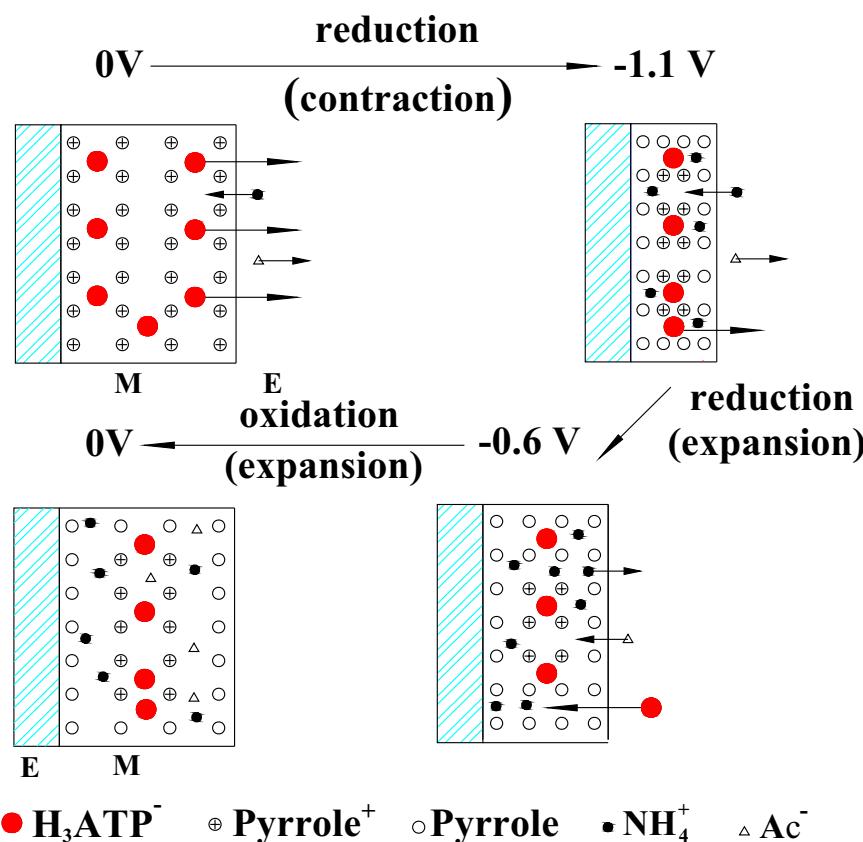
Figure 5. Schematic diagram showing the control of ion flux and electrochemically stimulated ATP release from PP/ATP films. E, electrode; M, membrane.

On-line EC/ESMS Results of Potential Step

Another EC/ESMS on-line experiment was done as follows: the experiment was performed with exactly the same solution and flow rate, except the potential was held at E = −1.1 V instead of potential cycling. The mass spectra as a function of time are shown in Figure 4a. Among the first six mass spectra, no ATP molecular ion peak at m/z 506 was observed. At 10 min, a peak appeared at m/z 506, reached its maximum value at about 24 min, and then decreased slowly, indicating that a fixed amount was released. The release profile of ATP relative ion intensities versus time is shown in Figure 4b. In addition, it is worth noting that ATP is still released slowly even after 40 min. This is likely attributable to structural changes that occur in the polymer during redox switching and the multi-ionic nature of the dopant. It has been suggested that the physics associated with this unusual behavior arises from the inhibited diffusion process that occurs as a consequence of initial pore restriction in the first step of the release [29]. Compared with Figure 3, we saw that ATP diffused out of the membrane later than in the potential cycling experiment. The intensity was smaller as well, which is in accordance with the results from the literature [7, 28]. Here again, the transfer time of 14 s was also ignored as negligible, when compared with the large delay time (10 min).

On-line EC/ESMS Results of Repeated Potential Steps

We repeatedly stepped the electrode potential down from 0 to −1.1 V for 1 min, and then up to 0 V for 1 min, while monitoring the ion signal. After 45 min, we did not observe any ATP (m/z 506) release. This indicated that potential cycling was the best way to release ATP out of the film. We suggest that potential steps may have resulted in polymer films lacking porosity, so that anions could not escape. In this case, cations compensate more easily for charge balance.

From our experiments, it was observed that repeated slow potential cycling gave a higher ATP release rate than did a sustained single potential step of equal range, a result in accordance with that from the study reported by Pyo [7]. This phenomenon can be explained as follows. Cathodic reduction of the polymer film tends to expel the ATP anions out of the film. During the negative potential scan from 0 to −1.1 V, the film composed mainly of PP$^+$ is supposed to undergo slight contraction because of a pore-closing process occurring as increasingly more ATP anions are pushed out of the PP backbone into the solution [29]. In the meantime, some NH$_4^+$ ions in the solution phase are getting into the film, whereas Ac$^-$ ions are moving in the opposite direction. During the reverse scan of the potential, the
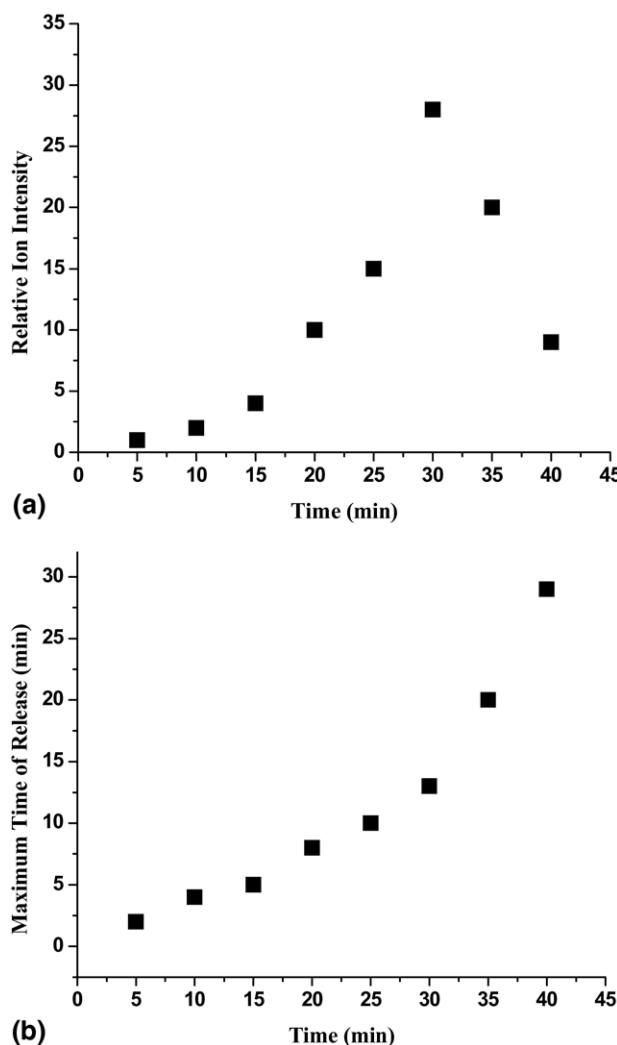
Figure 6. (**a**) Profile of relative ion intensity of H_3ATP^- (m/z 506) at the largest detected amount from EC/ESMS versus synthesis time when we made the film. (**b**) Profile of time at which the intensity of H_3ATP^- reaches its maximum value versus synthesis time.

PP^+ matrix is likely to undergo expansion instead of contraction. However, for the first part of the reverse scan from -1.1 to -0.6 V, the current remains cathodic as observed in the CV. This means the reduction of polypyrrole is still in continuation, although to a lesser and decreasing extent and, consequently, some more ATP anions are still expelled from the film. Only later in the more positive potential range from -0.6 to 0 V, there appears a very small anodic current, probably attributable to the reoxidation of the partly reduced polypyrrole. Yet, as a result of the relatively low concentration of the ATP anion in the vicinity of the polymer electrode, a very small amount of ATP anions might be reincorporated into the film. As a result, for the whole cycle of the potential scan, many more ATP anions are released than are reincorporated. Thus we might explain why repeated slow potential cycling is more effective than single potential step in releasing

ATP anions from the polymer film. The polypyrrole film doped with ATP is somewhat analogous to a sponge soaked with water. If we repeatedly squeeze it and release it for many times, more water will be squeezed out than just squeezing it once and holding the squeeze. Figure 5 is a schematic diagram of a PP/ATP film and the flow of ions under electrochemically stimulated ATP release. In Figure 5 we assume an ATP doping level of roughly 25%, which corresponds to one ATP anion per four pyrrole repeat units [7].

Electrochemical ATP Release as a Function of Film Thickness

We observed that the amount of released ATP from PP/ATP film was dependent on film thickness. The longer we electropolymerized a PP/ATP film, the thicker the film we obtained. In our experiments, we polymerized films over different times, from 5 and 10 min, up to 40 min, which corresponded to film thicknesses of 1.25, 2.5, and 10 μm, respectively. We then electrochemically released the ATP and recorded the strongest peak intensity in the mass spectrum for each film thickness. Figure 6a shows the profile of relative ion intensity of H_3ATP^- (m/z 506) versus film synthesis time. The points are normalized to a film polymerized for 5 min. Figure 6b shows the profile of time at which the intensity of H_3ATP^- reached its maximum value in the on-line EC/ESMS experiments for each film thickness versus film synthesis time. From Figure 6a, it is observed that the amount of ATP released is proportional to the film synthesis time up to 30 min. Before 30 min, the total amount of ATP released could also be adjusted by controlling the membrane thickness, providing the polymer remained electroactive [29]. The longer the time of electropolymerizing the film, the more ATP that was entrapped into the film, and thus more could be released during the reduction. From a synthesis time of 30 min onward, the relative ion intensity of the film at m/z 506 decreased. There is evidence from the literature that thicker films become less electroactive [9], which leads to less ATP entrapped in the polymer film, and thus less ATP would be released from the film during the reduction. We also observed that the release time at which the peak intensity at m/z 506 reached its maximum value for each film thickness increased with film synthesis time. For a synthesis time of 30 min, the film was much less electroactive, so the maximum release time increased sharply. We suggest that thicker film has greater resistance after it has lost electroactivity. Thus, a smaller current passes through the film and it has slower ion compensation, which produces a slower release rate.

Conclusion

For the convenience of ATP detection in the EC/ESMS experiment, the best conditions were found at $CH_3CN/$

H_2O/Et_3N (vol/vol/vol = 35/65/0.1) with 2 mM NH_4Ac as the supporting electrolyte. Under these conditions, ATP could be released during the potential cycling process, and the results show a significant electrochemical ATP release in a relatively short time. The parent ions (ATP molecular ion m/z = 506) and these offspring ions (m/z = 426, 328, 257, 159) have very similar release profiles, indicating that ATP fragmentation occurs in the electrospray process. In addition, it is noticed that slow potential cycling allowed a higher ATP release rate than did a rapid potential step. Therefore, by carefully controlling potential, the ATP release rate is adjustable. In addition, the film thickness is an important factor affecting both the rate and the amount of electrochemical ATP release.

Acknowledgments

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