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Chemical and Biological Analyses of the Role of the Poly- Para-Phenylenediamine in the Erythrocytes Resistance in Hypotonic Environment

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Abstract

The high chemical and biological reactivity of para-phenylenediamine (pPD) has been illustrated in several studies, including polymerization genotoxicity and oxidative stress at the level of the plasma membrane cells. However, its reactivity with red blood cells (RBC) was rarely explored.

The aim of this study is to help expound the molecular actions of para-phenylenediamine in hypotonic environment of human red blood cells by conducting biological tests for the globular resistance and physicochemical cyclic voltammetry and UV-visible spectroscopy.

Our results show that the para-phenylenediamine at low concentration gives red blood cells temporary resistance (30 min) against hemolysis in hypotonic environment with 4.5 g/L NaCl. Spectrometry results for UV-visible and cyclic voltammetry suggest that membrane stability is due to the presence of the polymer poly-para-phenylenediamine (poly-pPD).

Keywords: Para-phenylenediamine (pPD); Cyclic voltammetry; Red blood cells; The stability of the membrane; Temporary resistance Poly-pPD

Introduction

Red blood cells (erythrocytes) are one of the most used biological models in medicinal research, especially for the study of membrane transporters [1-5]. The erythrocyte membrane contains many transport systems which have mostly been characterized functionally and whose kinetics and pharmacology are well described [6,7].

The cell membrane, in order to function effectively, must combine fluidity and stability properties to enable signaling, transport and the exchange of molecules between the internal and external environments. The stability of the membrane represents the ability of the biological complex to maintain its structure under various conditions such as heat, hypotonia, extreme pH, presence of solutes and oxidative stress. This may be caused by a number of molecules including p- phenylenediamine (pPD) or p-aminobenzene [8-12]. This is an aromatic amine of mineral origins of the family of aniline, used since 1863 by women as black hair dye for coloring hair, or adjuvant henna for tattoos in several countries in Africa and the Middle East. It is also widely used in industry for dyeing furs and textiles in the manufacture of household products, cosmetic agents, wheels, caoutchoucs, in the synthesis of plastics or as a photographic developer [13-21].

Spontaneous oxidation pPD (Figure 1) leads to quinonediimine which pairs with a second molecule of pPD to form a diphenylamine, which, in turn, oxidizes then couples with a third molecule to provide a derivative pPD 3 benzene nucleus strongly colored in blue called of Bandrowski's Base (BB) [22].

In the study of the pPD polymerization mechanisms carried by Lakard et al. it was demonstrated that the electrochemical oxidation of the pPD leads to a poly-para-phenylenediamine (poly-pPD) [23]. The process of the electrochemical oxidation of the pPD is totally irreversible. The oxidation peak appears only for the first scan potential to + 1.6V / SER. In contrast, Yao et al. observed, in acetonitrile environment at a potential scan rate of 200 mVs-1, two voltametric waves on the platen; which corresponds to two successive and reversible processes to a single electron, leading to the formation of a diamine [24].

Moreover, the genotoxicity studies in vitro have shown that pPD, like several other aromatic amines which belong to the aniline family can react with DNA and cause breaks [25-47]. Picardo et al. have shown that pPD at high concentrations and for long exposure time induced cell cycle block and toxicity, but at low concentrations (10 µg/mL)and for shorter periods exposure (30 min) increased keratinocyte proliferation [39]. Habti et al. [6] have shown that pPD at low concentrations (5µg/mL) and for 2h exposure time gives RBC temporary resistance to hemolysis in hypotonic blend, the mechanism remains unexplained till now [6]. The aim of our study is to contribute to elucidate the molecular mechanism responsible for this temporary resistance.

Material and Methods

Collection of human blood sample

Blood samples from volunteer donors, and healthy aged 25-35 years were collected in tubes of 7 mL containing EDTA. Blood donors were nonsmokers and had no history of allergic reaction to pPD or

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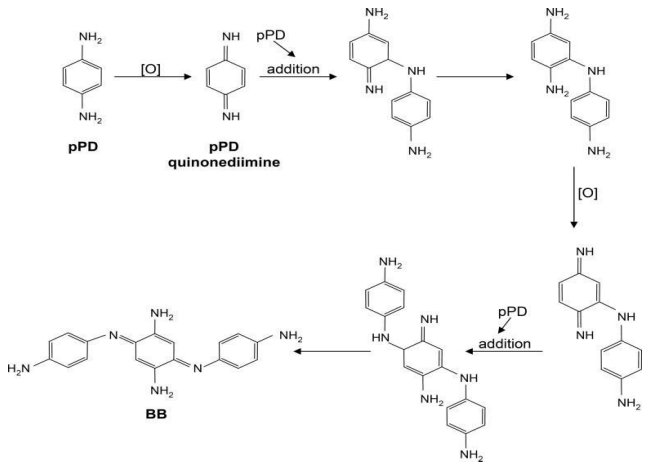


Figure 1: Propose doxidation of pPD and self-conjugation and the formation of the Bandrowski's Base (BB) [22].

exposure to hair dyes or tattoo. None had been exposed to drugs during the four weeks preceding the blood draw.

Biological tests: osmotic stability tests of RBC

The test of resistance of the RBC in hypotonic hemolysant environments was performed under the conditions described below; either in the presence of pPD solubilized with or without antioxidant, or in the presence of poly-pPD fixed on an electrode. The hemolysis of RBC was assessed in the supernatants of the incubation liquids by UV-Vis spectrometry at 412 nm. The percentage of the hemolysis was calculated as follows [40,41]:

% Hemolysis = (Absorbance of sample / total absorbance) × 100

Meanwhile, pH and conductivity were measured using a pH meter (Eutech Instruments, Salt 6, and France) and a conductivity meter (Hand-held Ecoscan series TDS6, France).

pPD effect of osmotic stability RBC

To assess the impact of PPD on RBC at different NaCl concentrations, we prepared two sets of 16 liquids recipients with a volume of 1 ml and containing 0 to 9 g/L NaCl in distilled water. In the first set of recipients we added, 5 µg/mL of pPD wile added nothing in the other.

In the both sets we added 5% red cells and made it incubated with at room temperature for 30 min, 1 h 30 min and 2h. Supernatants were then harvested after centrifuging the tubes at 500 g for 5 min.

pPD solubilized effect in the absence and presence of antioxidant

The effect of the pPD on the globular resistance, in the existence of an antioxidant, glutathione (GSH) or beta- mecraptoethenol (BME), was investigated in the supernatants of the RBC liquids 5%, NaCl 4,5 g/L and 5 g/L, pPD 5 µg/mL and antioxidant 1 mM, after 30 min, 1 h 30 min and 2 h incubation at ambient temperature. The experience was done 6 times.

Fixed poly-pPD on an electrode effect

This effect has been investigated in the supernatants after 30 min incubation and 1 h 30 min at room temperature RBC blends of 5%, NaCl 4.5 g/L and 5 g/L in the presence of a platinum electrode covered with a pPD film (polymer poly-pPD). The experiment was performed 4 times.

Statistical Analysis

The results are validated by the statistical test one-way ANOVA.

Physicochemical tests

Fixing of Poly-pPD on a platinum electrode using cyclic voltammetry: All experiments were performed at room temperature (≈ 21°C). The H_2SO_4 solution (0.5 M) was prepared from bi-distilled water and a concentrated solution of H_2SO_4 (96%). The pPD solution was prepared from bi-distilled water and pPD powder purity > 98% (Sigma Aldrich, France). The solutions were carefully deoxygenated by bubbling purified nitrogen.

The measurement cell is made of Pyrex glass, with a capacity of 100 mL and provided with five apertures for a platinum electrode, said working electrode, the opposite-electrode in a platinum wire of high surface area, reference saturated calomel electrode (ECS), thermometer, and input-output gas bubbled. The potential and the current are controlled using a potentiostat (VoltaLab, PGZ100 Radiometer Analytical, France) controlled by a computer (software Volta Master 4).

Antioxidants effect on pPD polymerization evaluated by UV-visible spectrophotometry: The pPD solution at 50 µM was incubated at room temperature in the absence and presence of 1 mM of GSH or 1 mM of BME during periods of time ranging from 30 min to 5 days. UV spectra were recorded using a UV-Visible spectrophotometer (UviLine 9400 - SECOMAM, France).

Results

Biological test

pPD effect of osmotic stability RBC: Figure 2 shows the resistance of erythrocytes to hemolytic effects of hypotonic liquids of decreasing ionic strength. An increase in membrane resistance was observed in the presence of pPD at a concentration of 5 mg/mL: the hemolytic curve is shifted to low levels of NaCl. After 30 min, 50% hemolysis was observed in the hypotonic solution at 3.25 g/L NaCl in the presence of pPD instead of 4.32 g/L in his absence. In contrast, after 30 min and this hemolysis of 61% and 65% of RBC in the hypotonic blend to 1.5 g/L in the presence and absence of pPD respectively. After 2h, 50% hemolysis was observed in the hypotonic solution at 4.25 g/L of NaCl instead of 5 g/L. however, after 2h the hemolysis is 96% and 98% of RBC in the presence or absence of pPD, respectively.

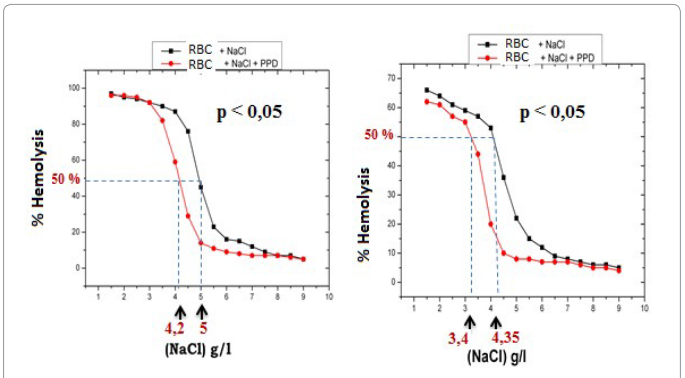


Figure 2: Effect of pP Don the osmotic fragility after 30 min (a) and after 2h (b).

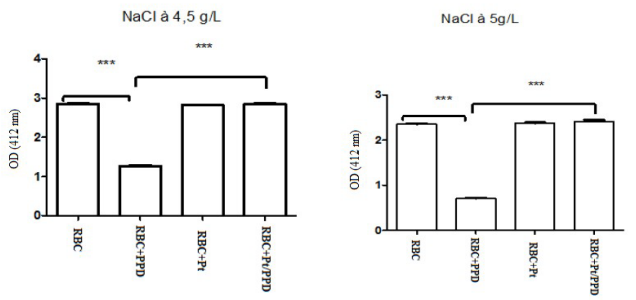


Figure 3: Absorbance of haemoglobin released after 1 h 30 min RBC incubation in hypotonic solutions to 4.5 g/L and 5 g/L NaCl in the presence of pPD attached to a platinum wire.

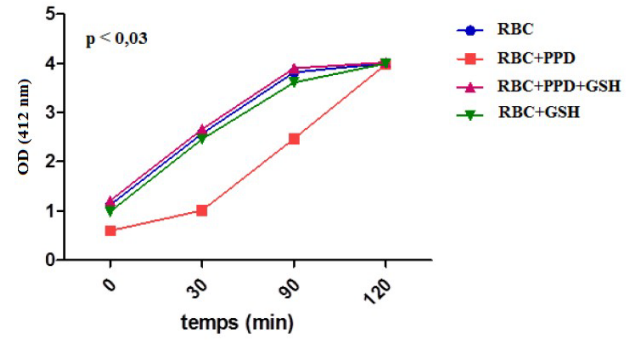


Figure 4: Absorbance of hemoglobin released as a function of time in the presence of solubilised pPD and in the presence of solubilised pPD with antioxidant in a hypotonic solution at 4.5 g/L NaCl.

Fixed poly-pPD on an electrode effect: Figure 3 shows the released hemoglobin absorbance obtained in the presence of pPD in the free form or fixed (cured) on a Pt electrode. Hemolysis in the presence of the fixed pPD (polymerized) on the electrode (OD = 2.85) is higher than that obtained in the presence of the solubilized pPD (OD =1.26). The stabilizing effect of RBC by the pPD is canceled when it is fixed (polymerized) on the platinum electrode.

Values represent averages obtained with 4 experiments for each concentration of NaCl. OD = optical density RBC = NaCl blend with 5% red blood cells; RBC + Pt / PPD = NaCl blend with 5% red cells + pPD attached to a platinum wire; Pt = RBC + NaCl solution with 5% red cells + a bare platinum wire; PPD = RBC

+ NaCl fluid with 5% red cells + 5 µg/mL of solubilised pPD; ***: P<0.0001.

pPD solubilized effect in the absence and presence of antioxidant: Figure 4 shows that the protective effect of pPD on hemolysis of RBC hypotonic liquids (4.5 g/L NaCl) is annulled in the presence of GSH or BME.

Physicochemical Tests

Fixing of Poly-pPD on a platinum electrode using cyclic voltammetry

Polymerization of pPD on the platinum electrode: The results of the study of the anodic oxidation of pPD on the platinum electrode were published in 2013 [42]. The decrease of the oxidation peaks with repeated potential cycling between 0.8 and 1.35 V/ECS show a second oxidation of the pPD accompanied by fixation of pPD on the electrode (or polymerization) until saturation Pt pPD sites (Figure 5). This oxidation is quasi-reversible.

Antioxidants effect on pPD polymerization evaluated by UV-Visible spectrophotometry

UV spectra obtained with an aqueous fluid of pPD: In this section we report the results of the described experiments. In fact we report the optical absorption results (UV-visible) of pPD in an aqueous environment. The two figures we show in below are related respectively to optical absorption at time 0 (Figure 6a). The second spectrum (Figure 6b). Demonstrates the existence of an absorption band appears with a maximum at 500 nm at 43 h. This result is supported by the appearance of violet-blue color instead of the original transparent of the solution (Figure 6c). The absorption intensity becomes greater after 5 days. This band corresponds to the formation of a poly-pPD by a self-polymerization process.

Spectra obtained with an aqueous solution of pPD in the presence of antioxidants: In this section we report the absorption spectrums at different times for solutions of pPD with antioxidants in solutions (Figure 8).

In the presence of 1 mM of GSH, the solution remains transparent

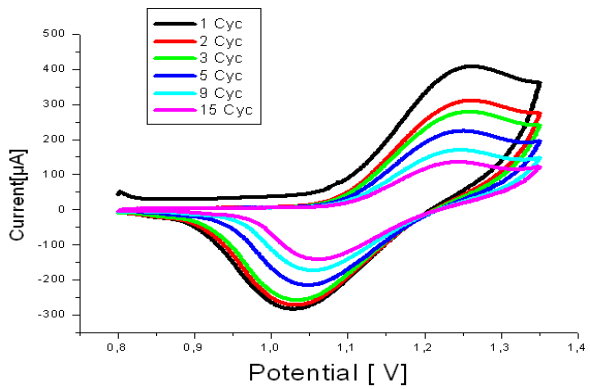


Figure 5: Volta mmogramon platinum H_2SO_4 environment (0.5 M) in the presence of pPD to 80 µg/mL with repetition of potentialcycles of 1 to15 cycles, at a potential scanning rate of 50 mV/S.

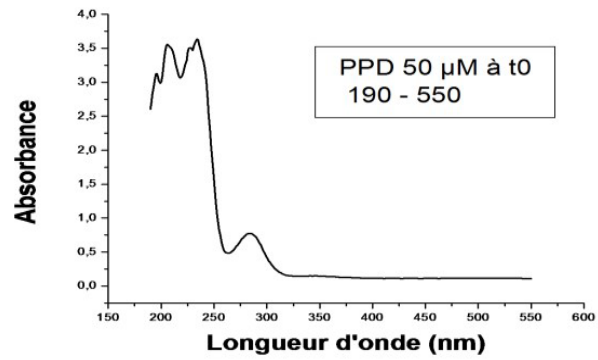
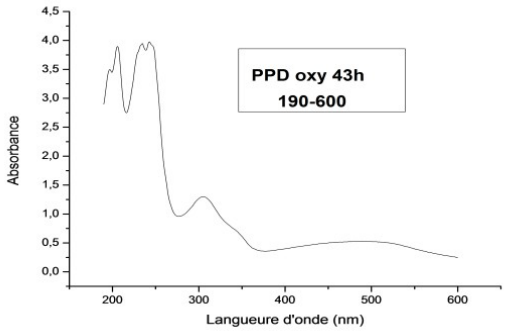


Figure 6a: The first spectrum shows no absorption in the visible but in ten sepeaks appear in the UV pointed around 200; 210; 240 and 270 nm. This absorption band is the pPD in the monomer state.



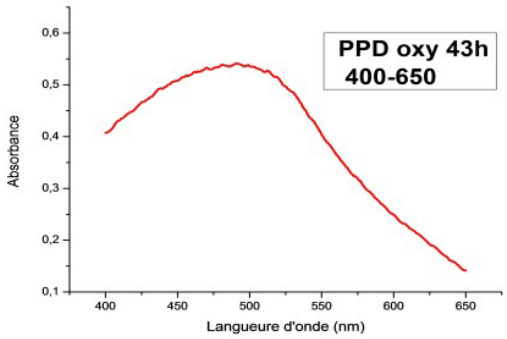


Figure 6b: UV-visible spectra of pP Data concentration of 50 µM in bi-distilled water at time t=43 h.

and bands related to the self-polymerization of pPD are not observed even after 5 days, demonstrating that the addition of GSH in pPD solution (50 µg/mL) completely inhibits the formation of poly-pPD (Figure 7).

On the contrary, in the presence of BME this band is absent after 43h but it appears after five days and the color of the solution changes to blue-violet. The spectra obtained by UV spectrophotometer showing that the antioxidants GSH and BME block the polymerization of pPD (Figures 7 and 8) with better performances for GSH.

Discussion

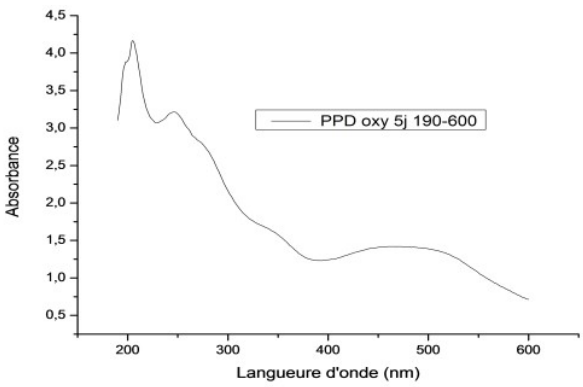
The cellular hemolysis is due to the call of molecules of water from the hypotonic extracellular environment to the hypertonic intracellular environment. The excess of water in RBC weakens its plasma membrane that bursts, releasing its rich hemoglobin content. The disruption of the cellular plasma membrane could occur following a disruption of the exchange of electrolytic ions or a change in pH of the environment.

Our study confirmed the temporary effect of stabilizing by pPD of the membrane of the human RBC in hypotonic environment. Adding pPD to the RBC liquid does not change neither the conductivity nor the pH of the environment, showing that the temporary stabilizer effect is due neither to the ionic effect of the molecule nor to the disturbance of the balance of H+ ions on either side of the plasma membrane of the RBC. However, hemolysis is complete after 2 h incubation regardless of the concentrations of NaCl and pPD. The precariousness of the stabilizing effect in hypotonic environment is probably related to the high chemical reactivity of pPD, demonstrated by our consistent results with those previously reported by Habti et al. [12]. Our hypothesis is that, once mixed, the molecules of the pPD preferentially react together to form a polymer poly-pPD, until exhaustion of monomers pPD. Nevertheless, a small number of these molecules react with the membrane components of the RBC. Thus, with low doses of pPD in the liquid, alteration of membrane components is minimal and the weaken effect is negligible. However, further researches shown that in the case of higher concentration of pPD monomeric PPD molecules remain free in solution and react with the membrane components to weaken the RBC.

To check if the poly-pPD polymer contributes to the stabilization of RBC in a hypotonic environment, we proceeded blocking the polymerization process by anti-oxidants. The stabilizing effect was then abolished. This effect has also disappeared when the polymer poly-pPD was fixed on a Pt electrode by preventing it from freely reacting with the constituents of the environment. These preliminary results suggest that poly-pPD contributes in preventing the penetration of water molecules within the RBC in hypotonic liquid, thus protecting them from hemolysis. The formation of poly-pPD solution by chemical oxidation has been described in the literature [8-12].

Furthermore, our results demonstrated that chemical anodic oxidation of the pPD leads to a thin and insulating polymer film on the electrode. And since pPD contains two acids in its molecular structure, it can be easily oxidized at a positive potential to produce cationic radicals which react with pPD monomers to form the poly-pPD film.

From the studies by Mann [1], Barnes [42] and Smith [43] on the electrochemical oxidation of primary amines and from previous



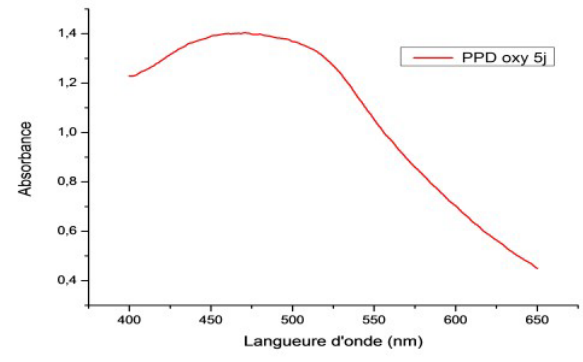


Figure 6c: UV-visible spectra of pP Data concentration of 50 µM in bi-distilled water at time=5 days.

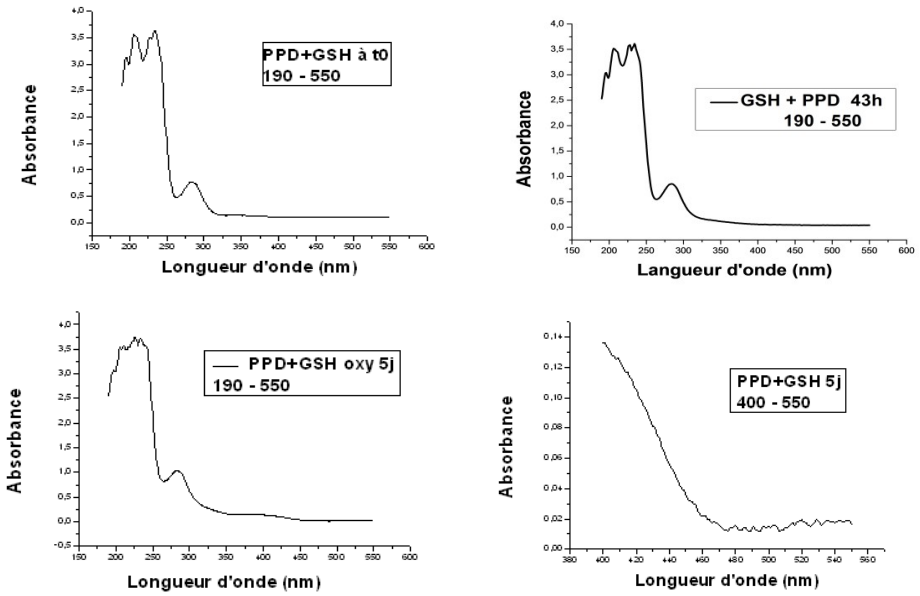


Figure 7: Spectra UV-visible pP Data concentration of 50 µM in the presence of GSH at a 1 mM Concentration.

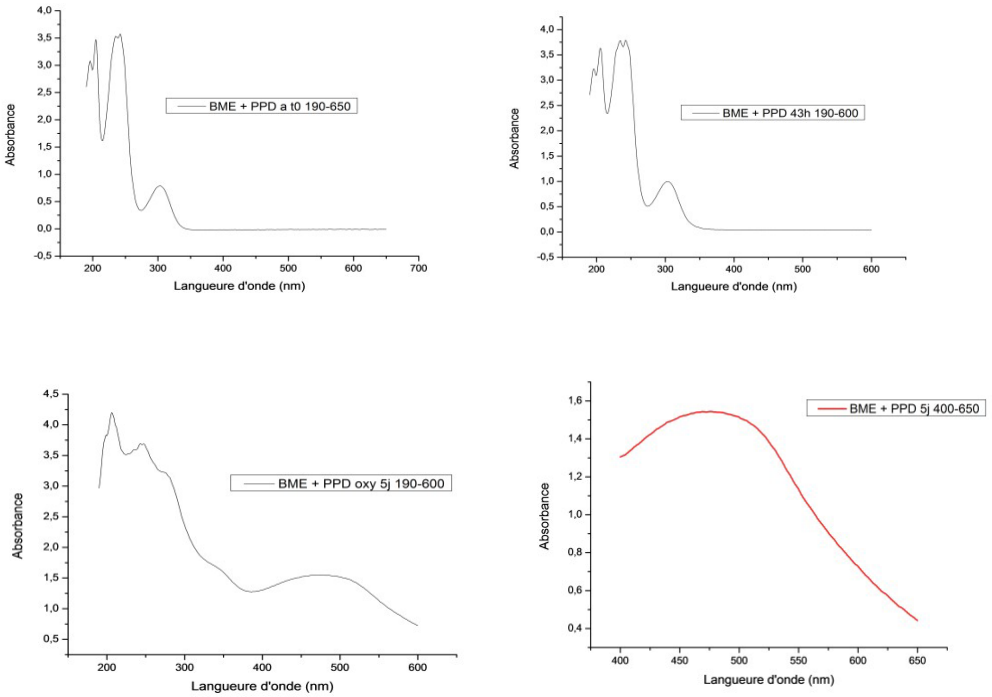


Figure 8: Spectra UV-visible pP Data concentration of 50 µM in the presence of BME at a 1 mM concentration.

studies we made on the electrochemical oxidation of aliphatic diamines [24,25], we took the anodic oxidation of pPD the mechanism described in Figure 9. The first step is the adsorption of pPD on the surface electrode. Then this monomer is oxidized with the loss of an electron and the formation of a cation radical. This step is followed by the cleavage of the C–N bond with formation of a primary carbocation, which attacks another molecule of PPD. After the expulsion of the proton from the protonated amine, an additional loss of an electron and C–N bond cleavage take place. Therefore poly PPD, – $(C_6H_4–NH)_n–$, grows progressively on the electrode surface.

Following the analysis with UV-Visible spectrometry in the presence of antioxidants, the spectra obtained by UV-Visible spectrophotometry pPD show that the formation of poly-pPD after oxidation of pPD is absent in the presence of these antioxidants.

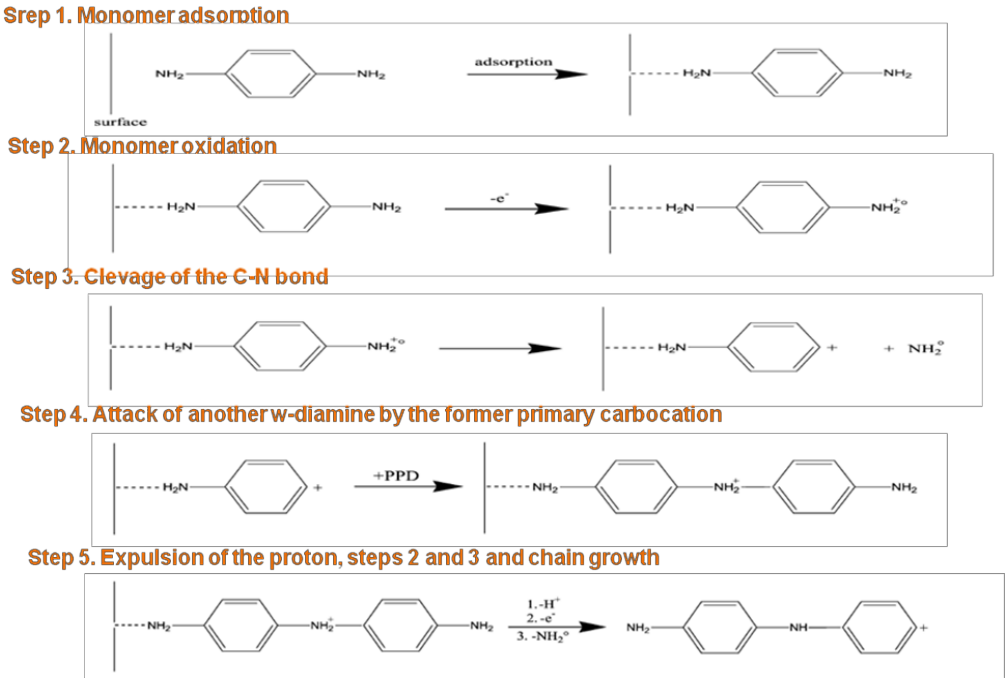


Figure 9: Anodic electro polymerisation of paraphenylenedia mine, 2003 [19].

Prospects

To strengthen the confirmation of our hypothesis that once put in solution the molecules preferentially pPD react together to form a poly-pPD polymer. The poly-pPD polymer contributes to the stabilization of RBC in hypotonic environment, so we proceeded to identify the poly-pPD structure formed in the solution of RBC by GC-MS, spectrometry or FTIR and NMR physicochemical technics. In the future, it is recommended to use poly-pPD previously prepared and isolated by fractional precipitation or purchased on the market in order to remove the effect of a monomer pPD that persists in the liquid after the auto-polymerization blend that could be the cause of the disappearance of the polymeric stabilizing effect beyond 2 h.

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