An Investigation of the Interaction between Resazurin and Cd²⁺ and Zn²⁺ Ions in Aqueous Medium

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ABSTRACT

The voltammetric behaviour of resazurin in the presence of Cd²⁺ and Zn²⁺ ions at a hanging mercury drop electrode (HMDE) was investigated by using square wave voltammetry (SWV), cyclic voltammetry (CV) and adsorptive square wave voltammetry (AdsSWV). The interaction of resazurin with Cd²⁺ and Zn²⁺ starts above pH 4 and seems to be completed at pH 5.2 with Cd²⁺ ions and at pH 7.5 with Zn²⁺ ions. A new peak appears at –0.93 V in the presence of Cd²⁺ and at –1.07 V in the presence of Zn²⁺. These peaks may be attributed to M²⁺–resazurin complexes and the logarithm of their stability constant was determined where log $\beta_{1:1}$ = 4.22 for Cd²⁺-resazurin and log $\beta_{1:1}$ = 4.74 for Zn²⁺-resazurin. Cyclic voltammetry was used to characterize the redox mechanism and to calculate voltammetric data of M²⁺-resazurin complexes. The amount of adsorbed M²⁺-resazurin complexes on the mercury drop electrode surface (Γ) is calculated as 5.48 × 10⁻¹² mol cm⁻² for Cd²⁺-resazurin and 1.10 × 10⁻¹¹ mol cm⁻² for Zn²⁺-resazurin. The voltammetric data were compared with the data obtained using spectrophotometric methods and were observed to be in good agreement.

1. Introduction

Resazurin [7-hydroxy-10-oxido-phenoxazin-3-one ($C_{12}H_7NO_4$)] also known as Alamar blue (Scheme 1) is a oxidation-reduction dye that belongs to the phenoxazine family. The resazurin redox reaction, in aqueous solution, is reversible when reduced by electron-transfer to water-soluble fluorescent product resorufin. The resazurin has been successfully used to prepare modified electrode as mediator.[1,2]

The biological significance of resazurin is well known. Resazurin has been widely used in a clinical research setting for sensitivity testing of pathogenic microorganisms including many Gram-negative bacteria, enterococci and Gram-positive bacteria.[3,4]. Resazurin can also act as a ligand by means of N-oxide group for the crucial metal ions in biological processes. This group is known as a binding site to the metal centre.[4,5] Metal ions (such as Cd and Zn) are vital for biological systems. Knowledge of the complexation of a metal with biologically active ligands is of great importance in the understanding of metal bioavailability. The metal complexes are known as the accelerator agents of chemical reactions. On the other hand they can serve the clarification of clinical results.

Voltammetric techniques have been widely used to study the interaction between metal ions and ligands[6–14]. Electrochemical studies of uncomplexed N-oxides were described several years ago[15,16]. In our previous work, we reported voltammetric behaviour of resazurin in aqueous solutions[17]. No voltammetric studies on the interaction of resazurin with Cd²⁺ and Zn²⁺could be found in the literature. This study focused on the complexation of resazurin with some metals ions in aqueous medium Thus, in the present study, the voltammetric and spectroscopic behaviours of resazurin in the presence of Cd²⁺ and Zn²⁺ ions are reported.

This paper will represent an important methodological
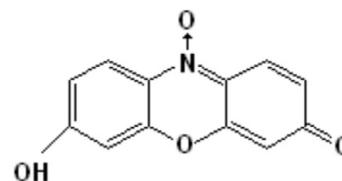
Scheme 1

contribution that will allow study of the complexation reactions of some metals with resazurin on the mercury drop electrode in aqueous medium. As working electrode, we chose the mercury electrode because its polarization range is well understood and the ease with which a fresh electrode surfaces can be obtained by using a new drop each time.

2. Experimental

2.1. Chemicals

All reagents were of analytical grade. Stock standard solutions were freshly prepared everyday and protected from light and air. Solutions with lower concentrations were prepared by dilution with deionized and triply distilled water and were used within a few hours. 0.1 M CH_3COONa solution was used as the supporting electrolyte for Cd²⁺-resazurin (pH 5.2) and the Zn²⁺-resazurin (pH 7.5) complexes.

2.2. Methods

2. 2. 1. *Instrumentation*

An EG & G PARC Model 303A HMDE three-electrode system (mercury working electrode, platinum counter electrode and Ag/AgCl (saturated KCl, reference electrode)) along with EG & G PAR 394B polarographic analyser was used for voltammetric measurements at room temperature. Electronic spectra were

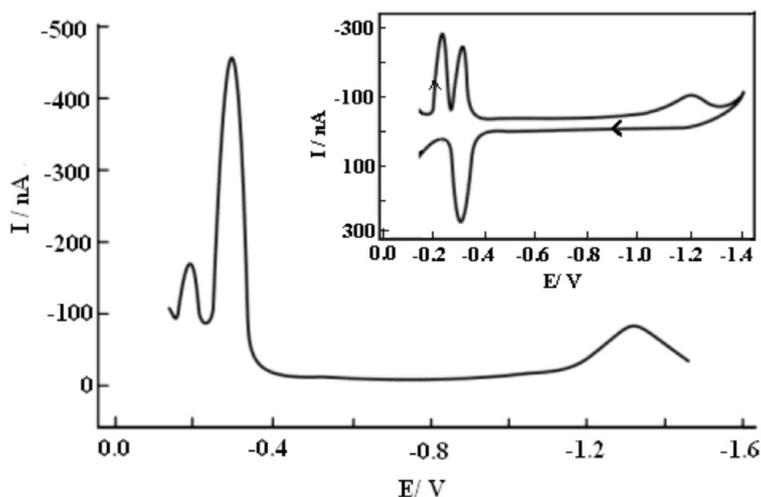* To whom correspondence should be addressed. E-mail: scakir@gazi.edu.tr

Figure 1 Square-wave voltammogram (SWV) of 1×10^{-6} mol L^{-1} resazurin in NaCH$_3$COO solution (pH 5.2). Inset: cyclic voltammogram (CV) of 1×10^{-6} mol L^{-1} resazurin Experimental conditions: pulse height, 20 mV; frequency, 50 Hz; drop size, large; scan increament, 2 mV ; equilibrium time, 5 s; scan rate, 200 mV s^{-1} for CV.

recorded on P general T80 Double beam UV-vis spectrophotometer in the 800–200 nm range at 1 cm cell length.

2. 2. 2. *Voltammetry*

Before each measurement the solutions were deaerated by a stream of pure nitrogen. A known volume of a standard solution of the metal ions was added to the voltammetric cell, which was closed, deaerated, and blanketed with oxygen-free nitrogen. The resazurin was added to the cell containing the metal ions and the voltammograms were recorded. The optimum experimental conditions were determined to observe the interaction of metals with resazurin. The potential scans were recorded using the square-wave and cyclic voltammetry modulations and the following optimum parameters (if not stated otherwise): pulse height, 20 mV; frequency, 50 Hz; drop size large and equilibrium time 5 s. Each measurement was carried out on a fresh mercury drop.

2.2.3. *Spectroscopy*

The spectra of mixtures with ambient mole ratio of both metal chlorides and resazurin aqueous solutions were recorded, following the changes in absorbance at the wavelength of maximum absorption.

3. Results and Discussion

3.1. Voltammetric Measurements

3.1.1. *Resazurin in the Absence of Metals Ions*

In previous work we reported the voltammetric behaviour of resazurin in Britton-Robinson buffer in the pH range 2–10.[17] We demonstrated that the electrochemical responses of resazurin were variable due to pH. The voltammogram of resazurin in the pH range 2.0–4.0 exhibited two cathodic reduction peaks while in the pH range 4.0–10.0 three cathodic reduction peaks were observed.[17] These voltammetric peaks were observed at –0.14 V (reversible) and –1.43 V (irreversible) at pH 3.2 which correspond to the reduction of resorufin to diydroresorufin and catalytical hydrogen wave, respectively.

In the pH range 4–10, additionally a new irreversible cathodic reduction peak was observed. This peak can be assigned to the reduction of protonated N-oxide group on the phenoxazin ring. This new irreversible peak is observed in the most positive

potential. The peak potentials of all peaks are shifted more negative with increasing pH from 2 to 10. The reduction of resazurin on the HMDE shows a partly adsorption controlled process.[17]

In this work, we focus on the effect of various metal ions on the reduction mechanism of resazurin. The possible complex formation reaction between the metal ions, e.g. Cd^{2+} and Zn^{2+} with resazurin was investigated. For this purpose, the optimum pH and supporting electrolyte were determined primarily to see complexation.

3.1.2. *Resazurin in the Presence of Metals Ions*

Preliminary experimental studies showed 0.1 M NaCH$_3$COO to be the most suitable, supporting electrolyte for Cd^{2+} and Zn^{2+} ions with resazurin complexation. The optimum pH for Cd^{2+} and Zn^{2+} complexes of resazurin was determined as 5.2 and 7.5, respectively.

The square-wave voltammogram of resazurin in 0.1 M NaCH$_3$COO supporting electrolyte at pH 5.2 gave one reversible peak at –0.28 V and two irreversible peaks at –0.17 V and –1.32 V (Fig. 1). The square-wave voltammogram of resazurin in 0.1 M NaCH$_3$COO supporting electrolyte at pH 7.5 was similar at pH 5.2. But the peak potential of these peaks shifted to more negative potentials.

The voltammograms of 2×10^{-6} M Cd^{2+} ions and 1×10^{-6} M Zn^{2+} ions in the absence of resazurin gave a reversible peak due to the reduction of free Cd^{2+} and Zn^{2+} ions with two electron reaction at –0.68 V and –1.10 V, respectively, in 0.1 M NaCH$_3$COO (Fig. 2A and Fig. 2B). When resazurin was added to the cell containing Cd^{2+} or Zn^{2+} ions, the voltammograms of Cd^{2+} or Zn^{2+} considerably changed . Fig. 3A and Fig. 3B shows the effect of resazurin on the voltammogram of Cd^{2+} or Zn^{2+}

Gradually increasing the resazurin concentration from 2×10^{-6} M to 7×10^{-6} M in the cell containing 2×10^{-6} M Cd^{2+} result in both a decrease in the peak corresponding to the reduction of free Cd^{2+} and the appearance of a new peak at more negative potential (–0.93 V) . This new peak at –0.93 V may be attributed to the complexation of resazurin with Cd^{2+} ions in the solution at pH 5.2. Kompany and coworkers[18] have reported Cd^{2+} – 1, 10-phenanthroline complex in 0.01 M acetic acid acetate buffer at pH 5. In this study the reduction peak of Cd^{2+} – 1, 10-phenanthroline complex occurred at –0.85 V.[18]

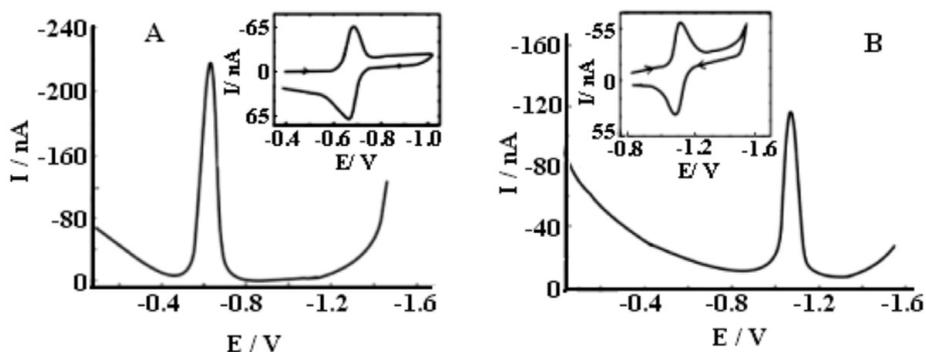A considerable change in the peak current of Zn^{2+} (1×10^{-6} M)

Figure 2 (**A**) Square wave voltammogram of the 2×10^{-6} mol L^{-1} Cd^{2+} ions in $NaCH_3COO$ solution (pH 5.20). Inset: cyclic voltammogram of 2×10^{-6} mol L^{-1} Cd^{2+} ions. (**B**) Ssquare wave voltammogram of the 1×10^{-6} mol L^{-1} Zn^{2+} ions in $NaCH_3COO$ solution (pH 7.50). Inset: cyclic voltammogram of 1×10^{-6} mol L^{-1} Zn^{2+} ions. Experimental conditions as in Fig. 1.
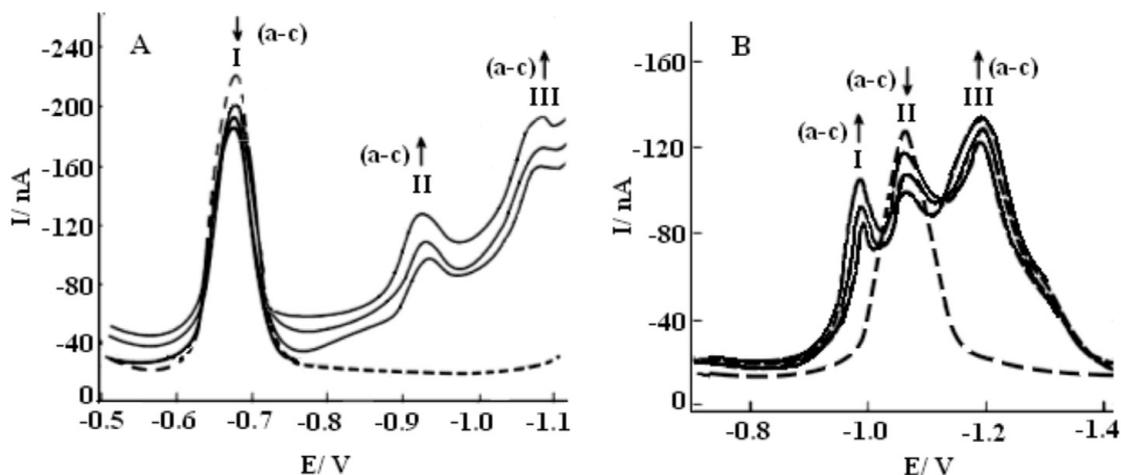

Figure 3 (**A**) Square wave voltammogram of the 2×10^{-6} M Cd^{2+} solution (- - -) containing a) 2×10^{-6} M b) 4×10^{-6} M c) 7×10^{-6} M resazurin (—) (pH 5.2). (**B**) Square wave voltammogram of the 1×10^{-6} M Zn^{2+} solution (- - -) containing a) 6×10^{-6} M, b) 7×10^{-6} M, c) 8×10^{-6} M resazurin (—) (pH 7.5). Experimental conditions as in Fig. 1.

was not observed upon adding resazurin (1×10^{-6} M). On the square-wave voltammogram (Fig. 3B) of 1×10^{-6} M Zn^{2+}, in the presence of excess resazurin, (from 6×10^{-6} M to 8×10^{-6} M) a new peak clearly appeared at more positive potential (–1.07 V) than the reduction peak of free Zn^{2+} (–1.10 V). Similarly the reduction peak for Zn^{2+}-guanine complex was observed at more positive potential (30 mV) than the reduction peak of free Zn^{2+} (–1.10 V) (vs. Ag/AgCl) on the mercury drop electrode.[19]

In the presence of M^{2+} ions, the peak potentials of the peaks of resazurin shifted to more positive potential values (about 50–80 mV) than that of resazurin alone. The third peak (III) showed a large positive shift (150–220 mV). The third peak (III) in

Fig. 3 can be attributed to the catalytic hydrogen wave of resazurin in the M^{2+}-resazurin complexes. The positive shifts in the reduction peaks are consistent with the complexation of resazurin to the Zn^{2+} and Cd^{2+} ions.[20–22]

Cyclic voltammetry was used to characterize the redox mechanism and to calculate voltammetric data for the Cd^{2+}-resazurin and Zn^{2+}-resazurin complexes. Cyclic voltammograms of Cd^{2+} or Zn^{2+} alone, Cd^{2+}-resazurin or Zn^{2+}-resazurin are shown in Fig. 2A,B (inset) and Fig. 4A,B, respectively. As can be seen, in Fig. 4A and Fig. 4B, peak I and peak II have an anodic counterpart, while peak III is not given a peak in the anodic branch. In Fig. 4A the reversible peak II and in Fig. 4B reversible peak I may
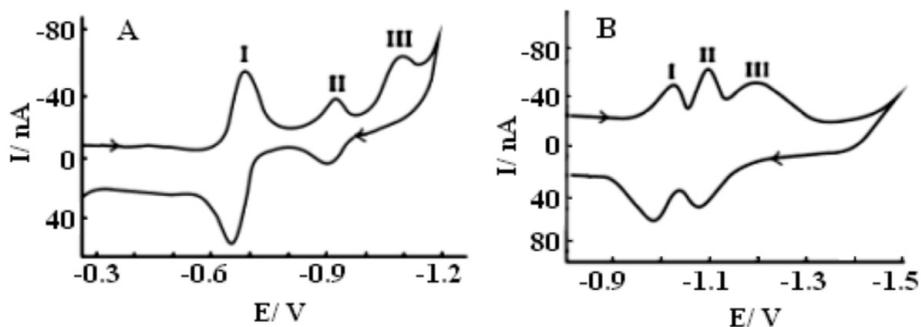

Figure 4 (**A**) Cyclic voltammogram of the 2×10^{-6} M Cd^{2+} + 5×10^{-6} M resazurin (I: Cd^{2+}, II: Cd^{2+}-resazurin complex, III: catalytic hydrogen wave of Cd^{2+}-resazurin complex). (**B**) The CV voltammogram of the 1×10^{-6} M Zn^{2+} + 1×10^{-5} M resazurin (I: Zn^{2+}-resazurin complex, II: Zn^{2+}, III: catalytic hydrogen wave of Zn^{2+}-resazurin complex). Experimental conditions as in Fig. 1.
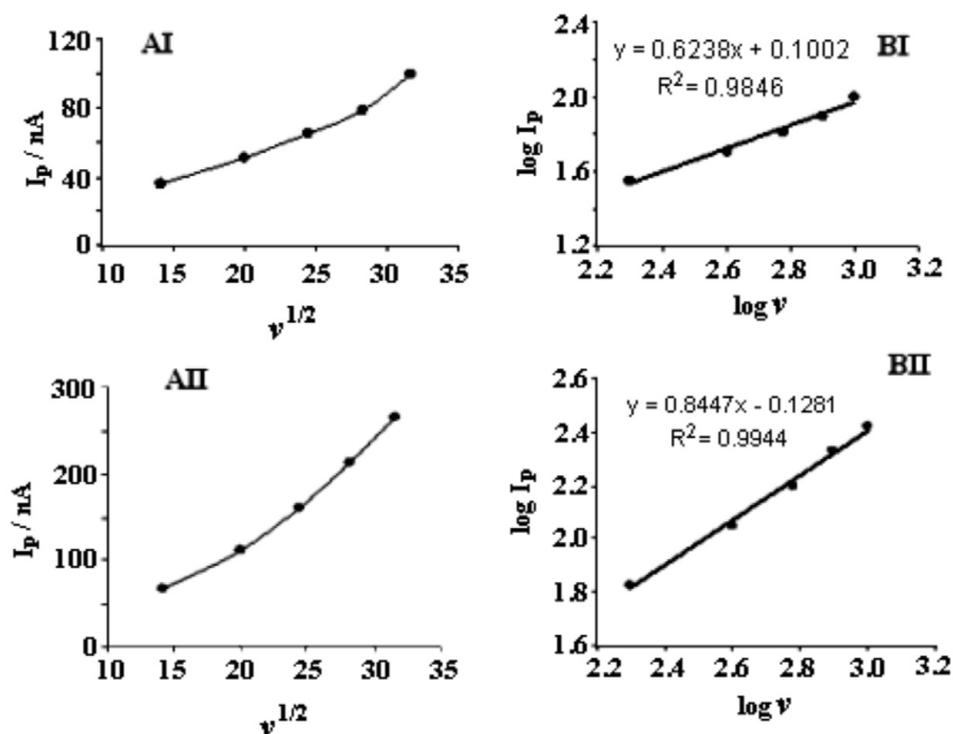
Figure 5 Dependence of peak intensity (I_{pc}) of the reduction of M^{2+}-resazurin complex with square root of scan rate ($v^{1/2}$) **AI**: Cd^{2+}-resazurin complex; **AII**: Zn^{2+}-resazurin complex. Dependence of the logarithm of peak intensity (log I_{pc}) of the reduction of M^{2+}-resazurin complex with logarithm of scan rate (log v) **BI**: Cd^{2+}-resazurin complex; **BII**: Zn^{2+}-resazurin complex.

Table 1 Voltammetric data for Cd^{2+} and Zn^{2+}-resazurin complexes.

Complex	M:L	Log$\beta_{1:1}$	m	n	W$_{1/2}$/mV	α	Γ/mol cm^{-2}	E°/mV
Cd^{2+}-resazurin	1:1	4.22	1	2	59.09	0.77	5.48×10^{-12}	915
Zn^{2+}-resazurin	1:1	4.74	1	2	42.86	1.06	1.10×10^{-11}	1055

be assigned to the reduction of Cd^{2+}-resazurin complex and Zn^{2+}-resazurin complex, respectively. The peak-to-peak separation ($\Delta Ep = Epa - Epc$) for the new (peak II and peak I) peak is about 30 mV, at a rate of 200 mV s–1 (Fig. 4A and Fig. 4B). This result suggests that the electrode process for M^{2+}-resazurin complexes are two-electron transfer reactions.

The formal potential, E°, for a reversible redox couple is easily determined as the average of the two peak potentials, $E° = (Epa + Epc)/2$. Formal potentials for Cd^{2+}-resazurin complex and Zn^{2+}-resazurin complexes were calculated to be 915 mV and 1055 mV, respectively (Table 1).

The dependence of the peak current (I_{pc}) of Cd^{2+}-resazurin complex and Zn^{2+}-resazurin complexes with square root of the scan rate ($v^{1/2}$) was completely nonlinear (Fig. 5 AI and AII). A plot of the logarithm of the peak current (log I_{pc}) *versus* the logarithm of the scan rate (log v) gave a straight line with a slope of 0.624 for Cd^{2+}-resazurin and 0.845 for Zn^{2+}-resazurin. These values may be considered close to 1.0, which is expected for ideal adsorption behaviour and so partial adsorption of Cd^{2+}-resazurin and Zn^{2+}-resazurin complexes on the HMDE can be suggested (Fig. 5 BI and BII).

To obtain the coordination number (m) and stability constant (β) from the voltammetric data of Cd^{2+}-resazurin and Zn^{2+}-resazurin complexes the following equations can be used (Eq. 1)[23]

$$1/I_p = 1/I_{p, max} + 1/\{\beta\, I_{p, max}\, [C_{res}]^m\} \qquad (1)$$

where Ip stands for the peak current, $I_{p, max}$ the peak current when all the metal ion forms the complex and [C_{res}] the concentration of resazurin. The curve of –log [$I_p/(I_{p, max} - I_p)$] *versus* –log [C_{res}] is linear with a slope of *m*. The values *m* and log $\beta_{1:1}$ of M^{2+}-resazurin complexes are given in Table 1. The stability constant (logβ) of Cd^{2+}-resazurin and Zn^{2+}-resazurin complexes were calculated to be 4.22 and 4.74, respectively. Assuming $n = 2$ the charge transfer coefficient can be evaluated as 0.77 for Cd^{2+}-resazurin and 1.06 for Zn^{2+}-resazurin.

Other voltammetric data of M^{2+}-resazurin complexes are collected in Table 1. The stociometry of M^{2+}-complexes were calculated as 1:1 from the following Lingane equation (Eq. 2)[24]

$$\Delta E_p = (E_p)_s - (E_p)_c$$
$$= (2.303RT/nF) \log\beta + j\, (2.303RT/nF) \log [L] \qquad (2)$$

where $(E_p)_s$ is the peak potential of the simple, uncomplexed metal ion, M^{2+} ; $(E_p)_c$ is the peak potential of the metal in the presence of the ligand concentration [L]; *n* is the number of electrons; and (2.303 RT/n) is a constant having the value of 0.059 at 25 °C.

Adsorption properties of the complexes of resazurin with Cd^{2+} and Zn^{2+} ions on the mercury electrode were studied. The effect of the accumulation time (10–300 s) on the reduction peak intensity (I_{pc}) of Cd^{2+}-resazurin complex and Zn^{2+}-resazurin complex were evaluated for 2 × 10^{-6} M Cd^{2+} or 1 × 10^{-6} M Zn^{2+} in the presence of 2 × 10^{-6} M resazurin solution. The peak currents of Cd^{2+}-resazurin and Zn^{2+}-resazurin complexes increase linearly with accumulation times 0–150 s and 0–100 s, respectively and then remained constant, due to saturation of the electrode
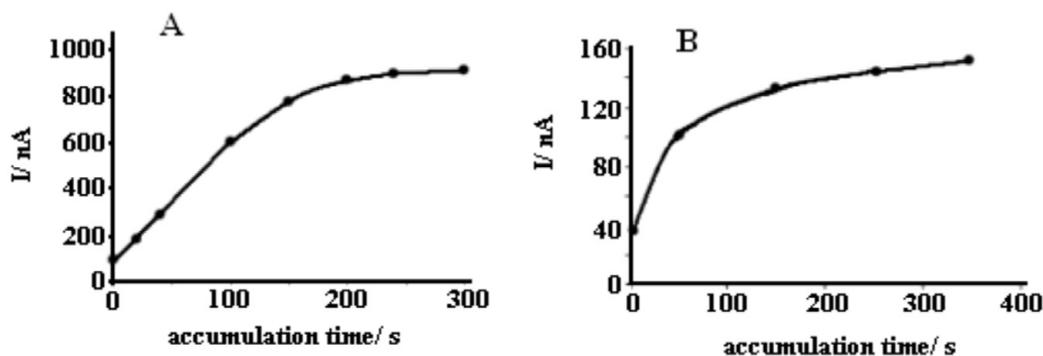
Figure 6 Effect of accumulation time on the reduction peak intensity of Cd^{2+}-resazurin complex for 2×10^{-6} M Cd^{2+}+2×10^{-6} M resazurin (**A**) and Zn^{2+}-resazurin complex for 1×10^{-6} M Zn^{2+}+2×10^{-6} M resazurin (**B**).

Table 2 UV-vis spectrophotometric characteristics of the M^{2+}-resazurin complexes.

Compound	λ_1/nm	λ_2/nm	λ_3/nm	λ_4/nm	λ_5/nm	λ_6/nm	M:L	Log$\beta_{1:1}$
Resazurin	–	229	284	377	600	–	–	–
Zn^{2+}	200	–	–	–	–	–	–	–
Cd^{2+}	201	–	–	–	–	–	–	–
Cd^{2+}–resazurin complex	198	226	280	378	607	212	1:1	5.10
Zn^{2+}–resazurin complex	194	228	285	380	611	210	1:1	5.80

surface (Fig. 6A and Fig. 6B). It was observed that the process of electrode reduction of the complexes was partially controlled by adsorption. Equation 3 below gives the amount of M^{2+}-resazurin complexes adsorbed on the mercury drop electrode surface;

$$\Gamma = 4RT\, I_p/n^2F^2Av \tag{3}$$

Γ is calculated as 5.48×10^{-12} mol cm^{-2} for Cd^{2+}-resazurin and 1.10×10^{-11} mol cm^{-2} for Zn^{2+}-resazurin. The amount of adsorbed Zn^{2+}-resazurin complex is bigger than Cd^{2+}-resazurin complex (Table 1).

3.2. Spectroscopic Measurements

UV-vis absorption spectrum of resazurin in aqueous solutions (1×10^{-4} M) exhibits four absorption bands at 229, 284, 377 and 600 nm (Table 2). The Uv-vis spectrum of resazurin showed $\pi \rightarrow \pi^*$ transitions related to the benzene ring at 229 nm and 284 nm. The two bands at 602 nm and 377 nm are assigned to the $\pi-\pi^*$ transition of the phenoxazin-3-one and to the weak n-π^* transitions of the N-oxide, respectively.[25,26] At 602 nm the absorption intensity increases linearly.

To compare the voltammetric data of the complexes of resazurin with Cd^{2+} and Zn^{2+} ions, the electronic spectra studies were carried out at pH 5 and 7, respectively. Absorption spectra of 1×10^{-4} mol L^{-1} resazurin in the presence of 1×10^{-4} mol L^{-1} Cd^{2+} or 1×10^{-4} mol L^{-1} Zn^{2+} ions showed changes. As shown in Table 2, Fig. 7 and Fig. 8, Cd^{2+} and Zn^{2+} ions produces a red shifts in the absorption maxima from 600 to 610 nm for resazurin. The observed red shifts of the maxima are probably due to formation of a complex. On the other hand the intensity of the absorbance decreased with increasing metal concentration. In addition, a
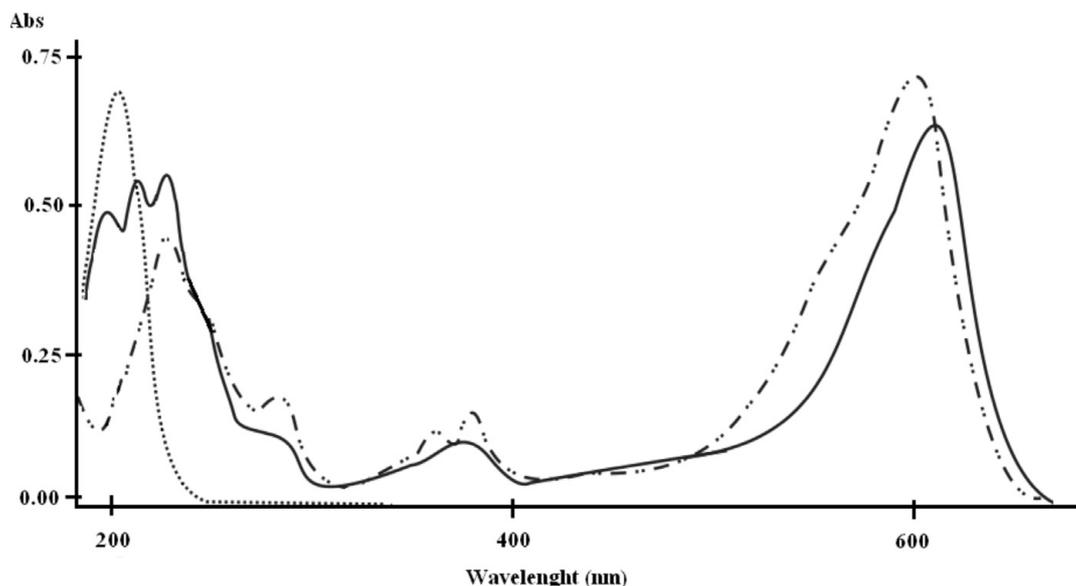

Figure 7 UV-vis spectrum of 1×10^{-4} M Cd^{2+} (········), 1×10^{-4} M resazurin (–··–··–) and Cd^{2+} – resazurin mixed solution mole ratio 1:1 (——) (pH 5).
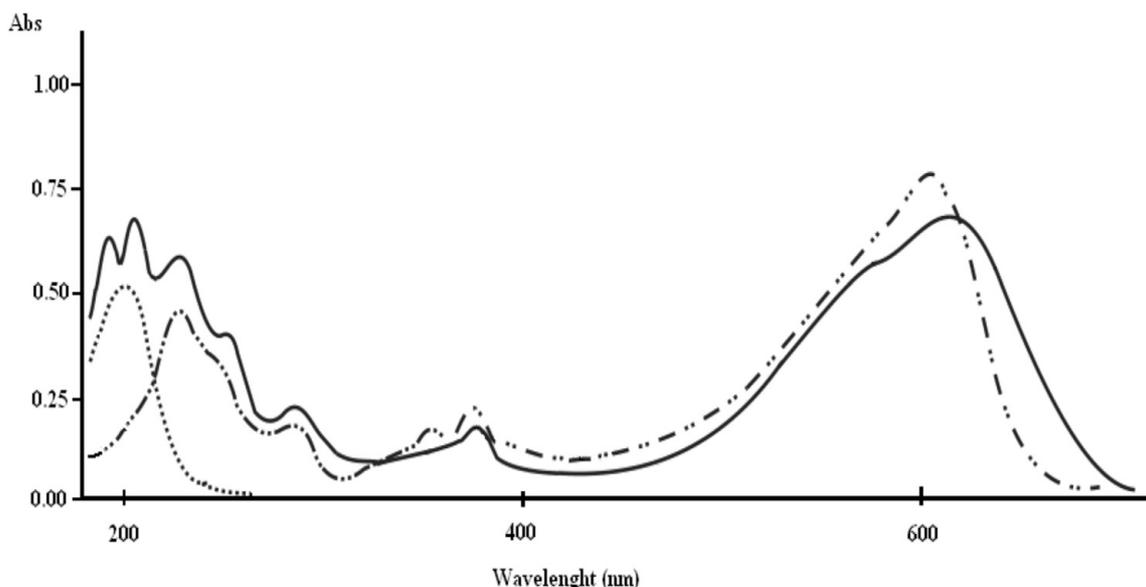
Figure 8 UV-vis spectrum of 1×10^{-4} M Zn^{2+} (.......), 1×10^{-4} M resazurin (–··–··–) and Zn^{2+} – resazurin mixed solution mole ratio 1:1 (——) (pH 7).

new absorption band was observed for the Cd^{2+} and Zn^{2+} complexes at 212 nm and 210 nm, respectively. The UV absorptions bands of the complexes can be assigned as ligand to metal charge transfer bands (LMCT). The absorption bands of Cd^{2+}-resazurin (212 nm) and Zn^{2+}-resazurin (210 nm) complexes are similar to absorption bands of $[Cd_3(SR)_6(tacn^{iPr})_2]$, ($SR=SCH_2CF_3$; $tacn^{iPr}$ = 1,4,7-triisopropyl-1,4,7-triazacyclononane) and $[Zn(SR)(tacn^{iPr})](ClO_4)$, ($SR=SC_6H_{11}$; $tacn^{iPr}$ = 1,4,7-triisopropyl-1,4,7-triazacyclononane) complexes.[27]

The stability constants and stoichiometries of the complexes were determined by Job and mole ratio methods. The values are given in Table 2. A stoichiometry of 1:1 (metal : ligand) in aqueous medium was found, which is the same as that found by voltammetry.

The literature indicates that the metal binding site of phenoxazin derivatives is through the phenoxazine ring nitrogen while for aromatic nitroxides the N-oxide group may be coordinated to the metal.[28–31]

4. Conclusions

Water-soluble heterocyclic N-oxide dyes can be used as either donor or acceptor for the study of biological materials. Resazurin is a phenoxazin-3-one dye widely used for testing various biological materials such as biochemical antioxidants and clinical research. The complexation of metals with biologically active ligands is important in the understanding of their bioavailability. Therefore, it is very important, to investigate interactions of heterocyclic N-oxide dyes such as resazurin with some metals in biological processes. When used as a medicine, possible interaction between metal and resazurin may affect pharmacological activity.

The knowledge of the complexation mechanism of resazurin with metals can give a useful clue in elucidation of the mechanism of resazurin interaction with living cells. In this paper we have reported formation of complex Cd^{2+} and Zn^{2+} of resazurin in aqueous media by voltammetric and spectroscopic studies.

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