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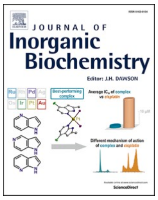

Complexation of ferrous ions by ferrozine, 2,2′-bipyridine and 1,10-phenanthroline: Implication for the quantification of iron in biological systems

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ABSTRACT

Iron is an essential nutrient for virtually all forms of life. Because of its redox properties and involvement in a wide range of biological processes, a number of qualitative and quantitative chemical tools have been developed to detect reduced (Fe^{2+}) and oxidized (Fe^{3+}) forms of iron in biomolecules. These types of measurements are not only important in detecting iron species in solution, but also in understanding iron distribution, accumulation, and role in physiological and pathological processes. Here, we use UV–vis spectrophotometry and three common chromogenic reagents, ferrozine, 2,2′-bipyridine, and 1,10-phenanthroline to detect and quantify the concentration of ferrous ions in aqueous solutions, owing to the unique absorption spectra, specific molar absorptivity, and characteristic colors of these Fe^{2+}-chelator complexes. Our results show that the kinetics of the formation of the {Fe^{2+}-(ferrozine)$_3$} complex, but not the{Fe^{2+}-(bipyridine)$_3$} or the {Fe(II)-(phenanthroline)$_3$} complexes depend on the concentration of the iron chelator, requiring up to 20 min to complete when close to stoichiometric ratios are employed. The molar absorptivity values of these complexes under excess chelator concentrations were ~ 10% to 15% higher than reported literature values (i.e. 31,500 ± 1500 M^{-1} cm^{-1} for ferrozine at 562 nm, 9950 ± 100 M^{-1} cm^{-1} for 2,2′-bipyridine at 522 nm, and 12,450 ± 370 M^{-1} cm^{-1} for 1,10-phenanthroline at 510 nm). Our results have important implications when quantifying iron in biological systems and reveal optimal experimental conditions that must be employed for the accurate measurements of ferrous ions, whether free in solution, or after reduction of protein-bound ferric ions.

1. Introduction

Iron is one of the most important elements for most forms of life and plays a significant role in various physiologically important functions, owing to its distinctive redox properties [1]. However, the high reactivity and interconversion between the multiple oxidation states of iron can be problematic and can cause cellular damage and apoptosis. One of the most harmful reactions in biology is the Fenton reaction where Fe^{2+} ions catalyze the generation of highly toxic reactive chemical species that can lead to pathological processes including cancer, and neurodegenerative diseases [1–5]. Many chemical tools and assays have been developed to qualitatively and quantitatively measure the redox status of iron and its distribution and accumulation in biological systems [6]. One of the most common and frequently used spectroscopic methods to quantify the concentration of metal ions in aqueous solutions involves the measurement of absorbance changes using chromogenic reagents. These metal ions-ligand complexes show unique absorption spectra with characteristic colors in the visible region of the electromagnetic spectrum and are due to either metal to ligand charge transfer (MLCT) or ligand to metal charge transfer bands. Because these metal-ions complexes often have high stability constants and specific molar absorptivity values, the color changes are directly proportional to the concentration of metal ions in solution, thus enabling accurate quantification.

Among the most stable and intensely colored ferrous ion complexes that are suitable for the quantitative determination of iron are 1,10-phenanthroline ($\varepsilon_{510\ nm}$ = 11,100 M^{-1} cm^{-1}) [7,8], 4,7-diphenyl-l,l0-phenanthroline (or bathophenanthroline, ε_{533nm} = 22,400 M^{-1} cm^{-1}) [9], 2, 2′ -bipyridine $\varepsilon_{522\ nm}$ = 8650 M^{-1} cm^{-1}) [10], 2,6-bis(2-pyridyl)-pyridine (also known as 2,2′,2″-terpyridyl, $\varepsilon_{552\ nm}$ = 11,500 M^{-1} cm^{-1}) [10], 2,4,6-tripyridyl-l,3,5-triazine (or TPTZ, $\varepsilon_{593\ nm}$ = 22,600 M^{-1} cm^{-1}) [11], and 3-(4-phenyl-2-pyridyl)-5,6-diphenyI-l,2,4-triazine (or PPDT, $\varepsilon_{561\ nm}$ = 28,700 M^{-1} cm^{-1}) [12]. All of these reagents contain a

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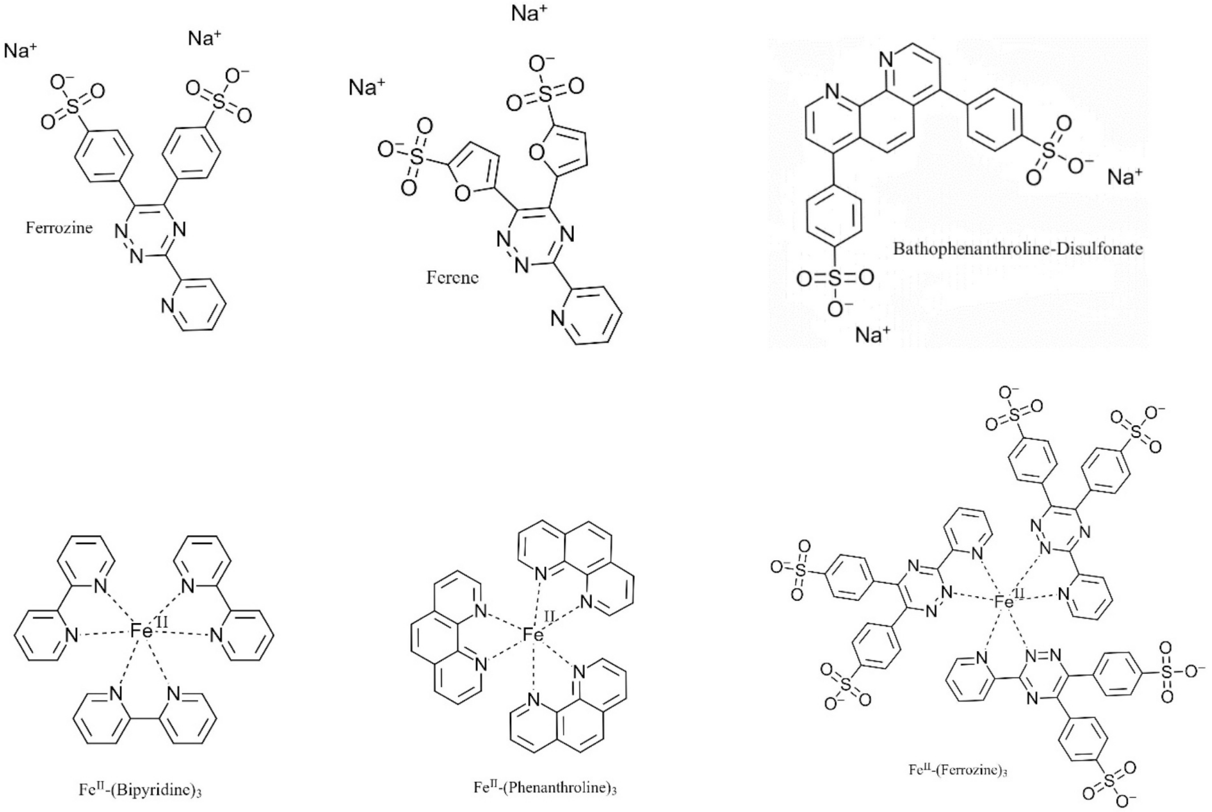

Scheme 1. Chemical structures of ferrozine, ferene, bathophenanthroline disulfonate, {Fe(II)-(Bipyridine)$_3$}, {Fe(II)-(Phenanthroline)$_3$} and {Fe(II)-(Ferrozine)$_3$}.

specific and cyclic atomic configuration N═C—C═N that binds metal ions, including Fe(II), to form colored complexes.

Perhaps one of the most common and frequently used ferrous iron chelators is the disodium salt of 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine, also referred to as ferrozine (FZ). Since its synthesis in the 1970 [7], FZ has been extensively used in a variety of applications to quantify ferrous ions [7,13]. One of the main advantages of FZ over other reagents is its high-water solubility, high sensitivity, and stability over a wide range of pH (i.e. pH 4 to 9) [7]. The ferrous-ferrozine complex exists in a 3:1 ratio of ferrozine to iron and is reported to have one of the highest molar absorptivity values (i.e. 27,900 M^{-1} cm^{-1} at 562 nm) among other commonly used iron complexes. Although PPDT has a slightly higher molar absorptivity, the problem with that reagent is its very poor water solubility and very slow kinetics with Fe (II) in dilute aqueous solutions [12].

The only other highly sensitive and water-soluble ferrous ion chelator that has similar characteristics to ferrozine is the disodium salt of 3-(2-pyridy1)-5,6-bis(2-(5-fury1 sulfonic acid))- 1,2,4-triazine (also known as ferene) ($\varepsilon_{593\text{ nm}} = 34{,}500\ M^{-1}\ cm^{-1}$) [14]. Structurally, the only difference between ferrozine and ferene is the presence of an oxygen atom in each of the furyl rings; the two phenyl sulfonate groups in ferrozine are substituted with sulfonyl furyl groups in ferene (Scheme 1). Although both molecules behave as bidentate chelating agents via the pyridyl and 2-triazine nitrogen atoms, the {Fe(II)-(ferene)$_3$}complex (logK = 14.9) forms a blue color at 593 nm whereas the {Fe(II)-(ferrozine)$_3$}complex (log K = 15.7) has a magenta color at 562 nm [7,13–15].

Although ferrozine is not a physiologically relevant ligand, the ferrozine assay has been widely used in the detection of ferrous ions in a variety of samples, including in mineral and biological systems. The accurate quantification of Fe^{2+} ions is affected by several factors including pH, temperature, concentration of reagents, incubation time, anions such as oxalate, cyanide and nitrite, and also the presence of Fe^{3+} cations [7,13–20]. Because of the rather high binding affinity of ferrozine for Fe^{2+} ions [15], many studies have used stoichiometric amounts of ferrozine, or a slight excess of chelator, to make sure that Fe^{2+} ions are fully bound. Surprisingly, while investigating the reductive mobilization of iron from the major iron storage protein ferritin, we obtained conflicting results and anomalous absorbance values that were inconsistent with literature reports and the published molar absorptivity for the {Fe(II)-(ferrozine)$_3$} complex. This work was therefore undertaken to examine the complexation reaction of Fe(II) by ferrozine under conditions close to physiological, and the effect of experimental factors such as pH, concentration of reagents, buffer, and incubation time on complex formation. Furthermore, we investigated the complexation kinetics of two other frequently used ferrous ion chelators (i.e. 2,2′-bipyridine and 1,10-phenanthroline) to explore whether the peculiar behavior observed with ferrozine is also prominent with these other strong Fe(II) chelators. Given the importance of iron in biology, chemistry, and the environment, our results underscore the significance of proper experimental conditions to accurately detect and quantify the presence of iron species in aqueous media.

2. Materials and methods

2.1. Chemicals

All chemicals employed in this study were reagent grade and used without further purification. MOPS [3-(N-morpholino)propanesulfonic acid] buffer and 2,2′-bipyridine were purchased from Sigma-Aldrich, tris-(hydroxymethyl)aminomethanehydrochloride (Tris-HCl) from G-Biosciences, 1,10 phenanthroline from TCI Chemicals, sodium chloride and acetic acid from Thermo Fisher Scientific, ferrous sulfate heptahydrate and sodium acetate was from J. T. Baker Chemical, and ferrozine (5,6-diphenyl-3-(2-pyridyl)-1,2,4-triazine-4,4′-disulfonic acid disodium salt hydrate, > 97% purity) from Alfa Aesar. Fe(II) stock solutions were freshly prepared immediately before each experiment using pH 2.0

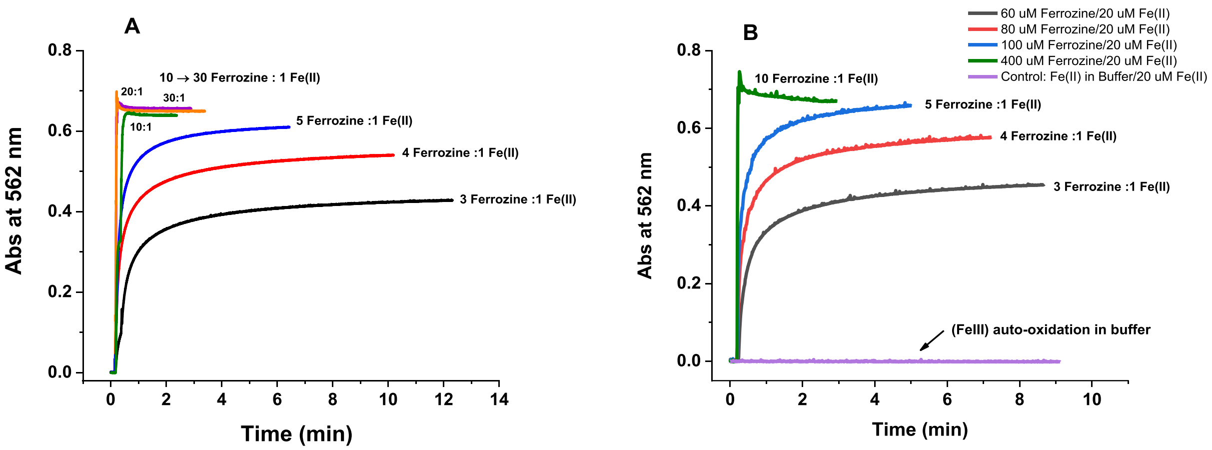

Fig. 1. (A) Kinetics of Fe(II) complexation with ferrozine in 50 mM Mops, 100 mM NaCl, pH 7.4. Conditions: 20 μM Fe(II), 60–600 μM ferrozine. (B) The experiment was repeated under strict anaerobic conditions using degassed solutions with pure argon gas.

deionized water. Ferrozine stock solutions were prepared in either DI water or in buffer. Stock solutions of 2,2′-bipyridine and 1,10-phenanthroline were prepared in a mixture of 50:50 water:ethanol.

2.2. Absorption spectroscopy and ultraviolet–visible (UV–vis) kinetics

UV–vis absorption spectroscopy was performed on a Varian Cary 50 Bio (or a Cary 60) spectrophotometer from Agilent Technologies. Unless otherwise noted, all experiments were conducted at 25.00 °C in 50 mM MOPS and 100 mM NaCl (pH 7.4). For the absorbance measurements, multiple samples were prepared as indicated in the figure captions and were left on a rocker, wrapped in aluminum foil, for 30–50 min. For the UV–vis titrations, multiple microliter injections of a ferrozine stock solution were added to a {Fe(II)-(ferrozine)$_3$} complex prepared in 132

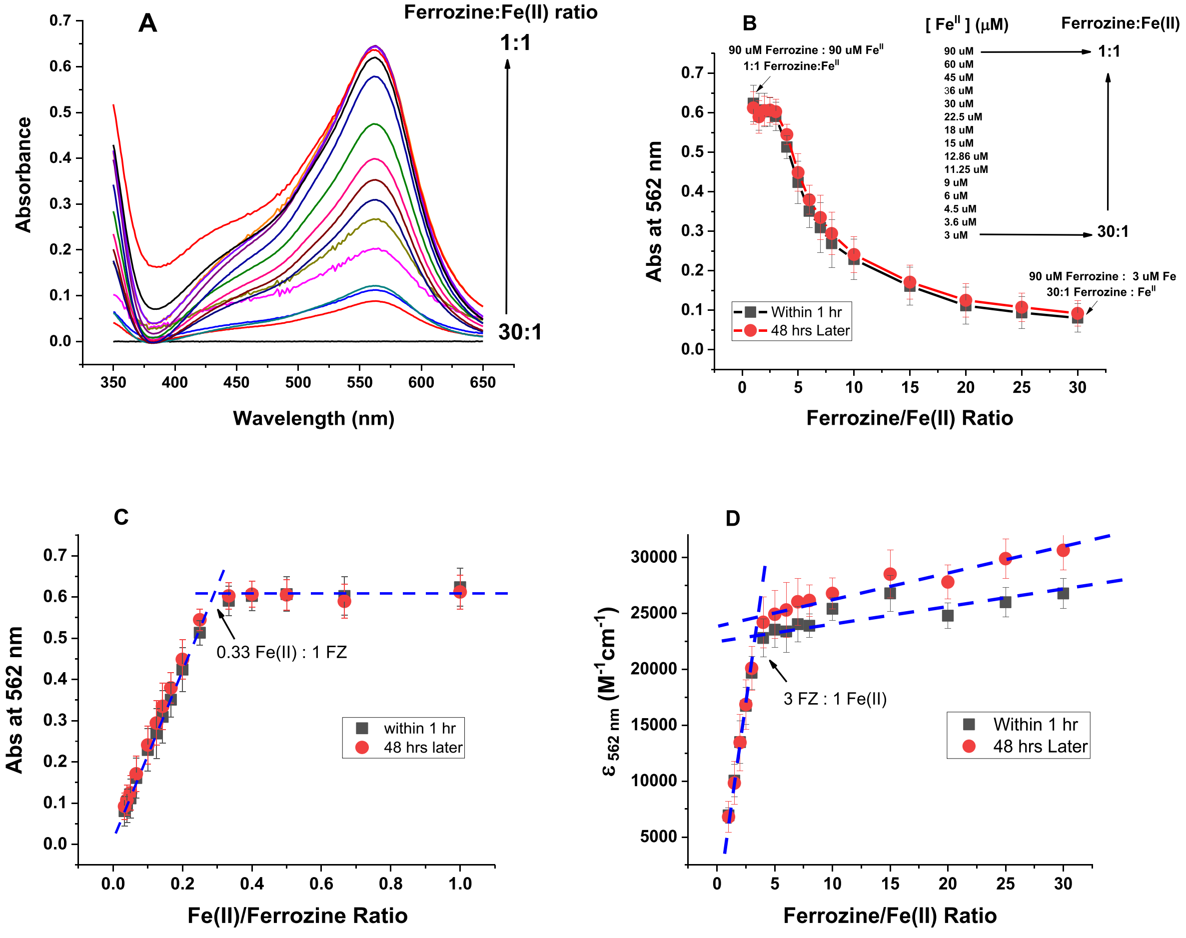

Fig. 2. (A) UV–vis absorption spectra of different ferrozine-Fe(II) solutions; (B) Plots of the absorbance change at 562 nm vs. ferrozine/Fe(II) and (C) vs. Fe(II)/ferrozine; (D) Plot of the molar absorptivity (ε) change at 562 nm as a function of ferrozine/Fe(II). Conditions: 90 μM ferrozine in 50 mM Mops, 100 mM NaCl, pH pH 7.4 (or in 132 mM Tris, pH 7.4) with changing Fe(II) concentrations (3–90 μM), at 1 h and 48 h following the preparation of the ferrozine-Fe(II) complexes. The error bars represent the standard deviation from 5 separate experiments.

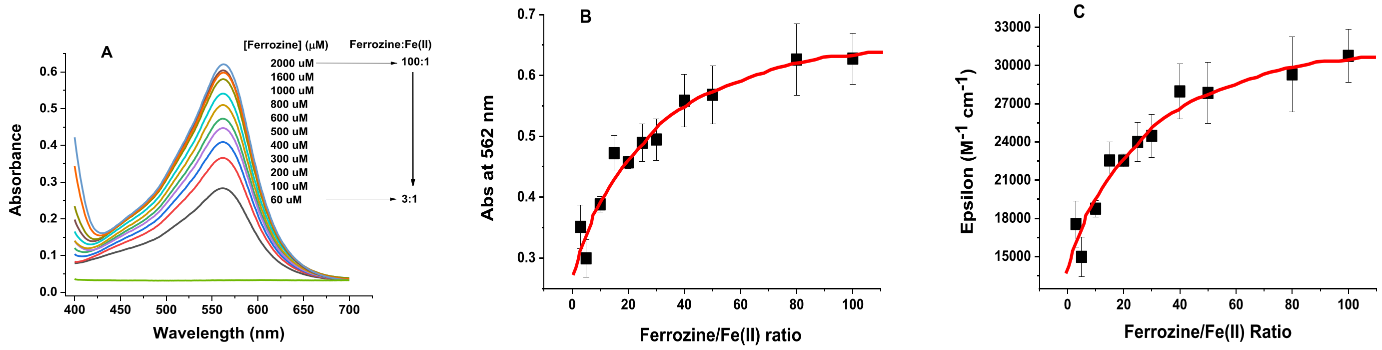

Fig. 3. (A) UV–vis absorption spectra of different ferrozine-Fe(II) solutions; (B) Plot of the absorbance change at 562 nm vs. ferrozine/Fe(II) and (C) Plot of the molar absorptivity (ε) change at 562 nm as a function of ferrozine/Fe(II). Conditions: 20 μM Fe(II) with changing ferrozine concentrations (60–2000 μM). The ferrozine solution was prepared in 50 mM Mops, 100 mM NaCl, pH 7.4 and the Fe(II) stock solution was prepared in DI water pH 2.0. The error bars represent the standard deviation from 7 separate experiments.

mM Tris buffer, pH 7.4. The acetate buffer (0.1 M, pH 5.0) was prepared by dissolving 0.5772 g of sodium acetate in 100 mL of DI water, and then adding a 171 μL of glacial acetic and adjusting the pH to 5.0 using 10 M HCl. Final reagent concentrations for each experiment are given in the figure captions.

The kinetics of Fe(II) complexation with ferrozine were followed at 562 nm where the {Fe(II)-(ferrozine)$_3$} species absorbs. To ensure reproducibility, all experiments were repeated 3 to 10 times, using freshly prepared solutions. The instrument is zeroed using the ferrozine-buffered solution as the blank, and the experiment is typically initiated with the addition of a few microliters of a ferrous sulfate solution, prepared in deionized H_2O (pH 2), into the 1 mL ferrozine solution, with a spin bar rapidly stirring under the conditions stated in the figure captions. Similar experiments were repeated with 2,2′-bipyridine and 1,10 phenanthroline. Time-dependent absorbance kinetic traces were analyzed with OriginLab version 8.0 (OriginLab Corp.).

Because a rigorous quantification of Fe(II) ions in solution is critical to validating molar absorptivity values of all Fe(II)-complexes studied here, the concentrations of working Fe(II) solutions and the amount of Fe(II) ions added to solutions of various ligands have been checked by atomic absorbance spectroscopy (AAS). The AAS data showed that the concentrations of Fe(II) ions in solution matched the predicted Fe(II) concentration with >98% accuracy of the expected Fe(II) concentration.

3. Results and discussion

3.1. Fe(II)-ferrozine complexation kinetics

The kinetics of Fe(II) complexation with ferrozine has been studied earlier [15] using rapid stopped-flow mixing and visible spectrophotometry at 562 nm in acidic pH. The rate constants for the formation and dissociation of the Fe(II)-ferrozine complex were on the order of 10^{11} M^{-3} s^{-1} and 10^{-5} s^{-1} at 25.00 °C, respectively, with an equilibrium association constant of $\sim 5 \times 10^{15}$ M^{-3}. These kinetic experiments were performed under conditions of excess ferrozine (i.e. 100 times more than Fe(II)). We thought to examine the kinetics of complexation at pH 7.4 starting at the stoichiometric ratio of 3:1 ferrozine:Fe(II) and up to 10 times higher ferrozine concentration (i.e. 30 ferrozine: 1 Fe(II)). Under conditions close to stoichiometric ratios (i.e. 3 to 5 ferrozine molecules per Fe(II) ion), the kinetics of complexation were slow requiring at least 10 min for completion (Fig. 1). At higher ratios (>10 ferrozine:Fe(II)), the kinetics were instantaneous and exhibited higher absorbance values, suggesting that excess ferrozine is required for the formation of a stable {Fe(II)-(ferrozine)$_3$} complex. To make sure that Fe(II) auto-oxidation is not a factor affecting the absorbance of the slow kinetics, the experiment was repeated under strictly anerobic conditions, by degassing all solutions for at least 1 h using pure argon gas. The kinetics were then performed in a tightly sealed UV-cuvette to prevent diffusion of atmospheric oxygen. As can be clearly seen in Fig. 1B, there is virtually no effect from the presence of O_2 in solution on the complexation kinetics or the absorbance values of the {Fe(II)-(ferrozine)$_3$} complex, under our experimental conditions.

3.2. Ferrozine – Fe(II) stoichiometry

In order to determine the binding stoichiometry of Fe(II) to ferrozine, multiple 1 mL samples were prepared using a constant ferrozine concentration (90 μM) and different concentrations of Fe(II) (3 μM to 90 μM), such that the ratio of ferrozine to Fe(II) varied between 1 and 30. The samples were wrapped in aluminum foil and placed on a rocker to mix gently for a period of 30–50 min before absorbance measurements were performed. Fig. 2 shows the absorption spectra of 15 different

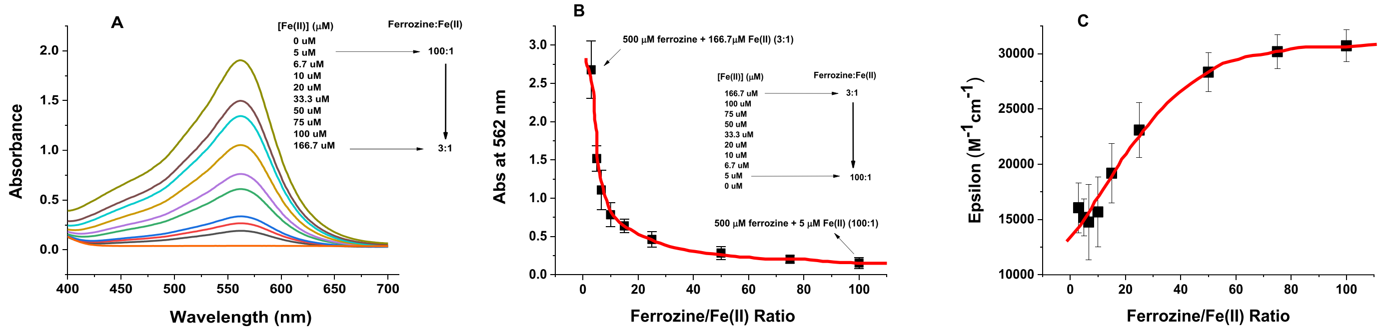

Fig. 4. (A) UV–vis absorption spectra of different ferrozine-Fe(II) solutions; (B) Plot of the absorbance change at 562 nm vs. ferrozine/Fe(II) and (C) Plot of the molar absorptivity (ε) change at 562 nm as a function of ferrozine/Fe(II). Conditions: 500 μM ferrozine in 50 mM Mops, 100 mM NaCl, pH 7.4 with changing Fe(II) concentrations (5–166.7 μM). The error bars represent the standard deviation from 4 separate experiments.

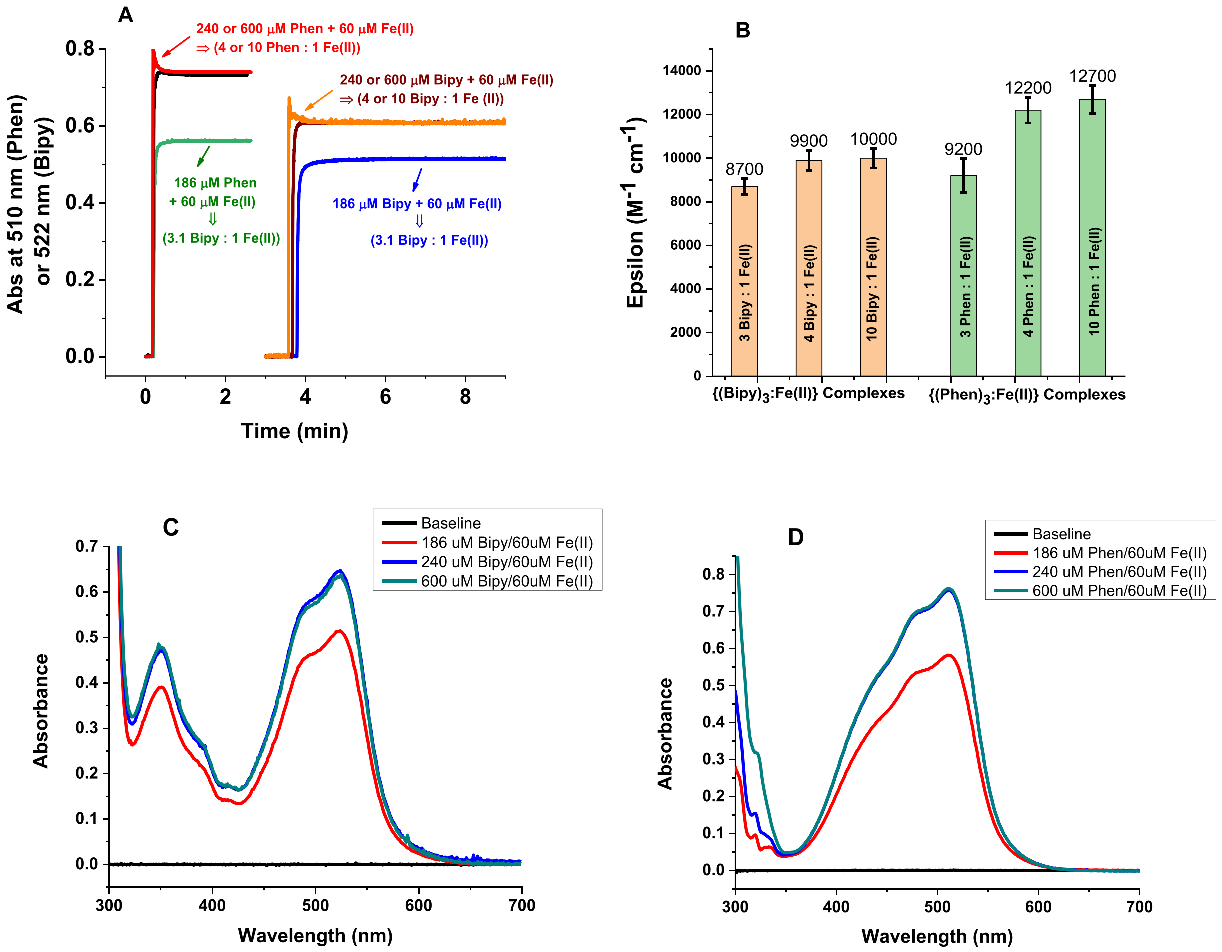

Fig. 5. (A) Kinetics of Fe(II) complexation with 2,2′-bipyridine at 522 nm and 1,10-phenanthroline at 510 nm. (B) Plot of the molar absorptivity (ε) change as a function of ligand/Fe(II) ratio. (C, D) Absorbance change of {Fe(II)-(bipyridine)$_3$} and {Fe(II)-(phenanthroline)$_3$} as a function of ligands concentration. Conditions: 60 μM Fe(II) and 186, 240, and 600 μM 2,2′-bipyridine or 1,10-phenanthroline for. The error bars in (B) represent the standard deviation from 9 separate experiments.

samples and their corresponding plots as a function of ferrozine/Fe(II) or Fe(II)/ferrozine ratios. A clear break at 3:1 ferrozine:Fe(II) is observed with a molar absorptivity value of 22,500 M^{-1} cm^{-1} that slightly increases to ~27,000 M^{-1} cm^{-1} at excess ferrozine concentration (i.e. 30 ferrozine:1 Fe(II)).

To examine the stability of the Fe(II)-ferrozine complexes formed under the conditions of Fig. 1, the samples were allowed to sit in the dark for 48 h before the absorbance changes at 562 nm were recorded again. A similar pattern with a slight increase in the final absorbance over a period of 2 days was observed, suggesting that under the conditions of our experiment, the Fe(II)-ferrozine complex is a stable entity that does not undergo major changes.

3.3. *Effect of ferrozine and Fe(II) on the molar absorptivity of the Fe(II)-ferrozine complex*

To test how the ferrozine concentration affects the molar absorptivity of the ferrozine–Fe(II) complex, a fixed 20 μM Fe(II) concentration was added to different ferrozine samples with concentrations ranging between 60 μM and 2 mM. The data of this experiment are displayed in Fig. 3 and show saturation at higher ferrozine concentrations. The absorbance exhibits a plateau at >50:1 ferrozine:Fe(II) ratio with a molar absorptivity value approaching 31,500 M^{-1} cm^{-1}(Fig. 3C). The experiment was then repeated at a fixed ferrozine concentration of 500 μM and varying concentration of Fe(II) (from 5 to 166.7 μM) such that the ratio of ferrozine to Fe(II) ranges between 100 and 3. A similar pattern emerged (Fig. 4) with molar absorptivity (ε) values ranging between a low ~15,000 M^{-1} cm^{-1} at low ferrozine:Fe(II) ratios, to a saturating value of ~31,000 M^{-1} cm^{-1} at ferrozine:Fe(II) ratios >50:1. These results are consistent with those presented earlier showing that stable {Fe(II)-(ferrozine)$_3$} complexes are formed in the presence of excess ferrozine.

Given the structural similarities between ferrozine, ferene, and bathophenanthroline-disulfonate (BPhen) (i.e. 3 molecules of ligands bind to 1 Fe(II) ion, and the net charge of – 4 on the {Fe(II)-(BPhen)$_3$} or the {Fe(II)-(ferene)$_3$} complexes, the repulsion between the negatively charged BPhen and ferene molecules, due to the six sulfonic groups, will likely lead to slower kinetics, similarly to what is observed here with ferrozine.

3.4. *The kinetics of Fe(II) complexation by 2,2′-bipyridine and 1,10-phenanthroline*

To examine the kinetics of the two other most commonly used Fe(II) chelators (2,2′-bipyridine and 1,10 phenanthroline), and the effects of ligand concentration on the Fe(II)-complexes, we designed UV–vis experiments under conditions similar to those employed with ferrozine. Fig. 5 shows the kinetic traces of Fe(II) complexation with 2,2′-bipyridine and 1,10-phenanthroline at 522 nm and 510 nm, respectively. Both ligands displayed very rapid kinetics with Fe(II) ions, irrespective of ligand concentration, although a lower absorbance was observed when stoichiometric ratios of ~3:1 bipyridine:Fe(II) or phenanthroline:

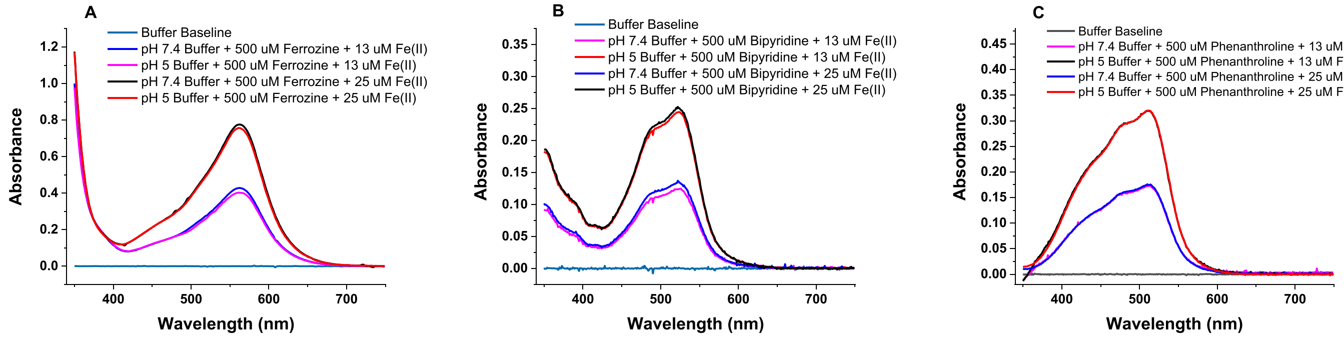

Fig. 7. UV–vis absorption spectra of different Fe(II) complexes of ferrozine, bipyridine and phenanthroline (at 38:1 and 20:1 chelator:Fe(II) ratios) in either 132 mM Tris, pH 7.4, or in 100 mM acetate buffer, pH 5.0. The baselines consist of buffer plus the chelator at either pH.

Fe(II) were used (Fig. 5A). Under these conditions, lower molar absorptivity values were calculated (Fig. 5B), suggesting that the complexes have not fully formed. When >3:1 ratios were employed, thermodynamically stable complexes of Fe(II) and bipyridine or phenanthroline are observed, with average molar absorptivity values of (9950 ± 100 M^{-1} cm^{-1} for {Fe(II)-(bipyridine)$_3$} at 522 nm and (12,450 ± 370 M^{-1} cm^{-1}) for {Fe(II)-(phenanthroline)$_3$} at 510 nm (Fig. 5B, C, D). When larger excess of 2,2′-bipyridine and 1,10-phenanthroline were employed (up to 100 times molar ratio), no further changes in absorbance was observed, suggesting that {Fe(II)-(bipy)$_3$} and {Fe(II)-(phenanthroline)$_3$} have formed stable complexes, consistent with their high formation constants of ~1.2 × 10^{17} M^{-3} and ~ 2.5 × 10^{21} M^{-3}, respectively. Notably, these formation constants are ~22-fold and 5 × 10^5-fold higher than that of the {Fe(II)-(ferrozine)$_3$} complex [15].

3.5. *Effect of pH*

Although the formation of the Fe(II)-ferrozine complex is not affected by changes in pH [7,15,20], it is unclear whether the formation of the Fe(II)-bipyridine and Fe(II)-phenanthroline complexes depends on pH variations. Fig. 7 shows the absorption spectra of 4 different solutions of Fe(II)-ferrozine, Fe(II)-bipyridine, and Fe(II)-phenanthroline complexes prepared at different chelator:Fe(II) ratios (20:1 and 38:1), in acetate buffer pH 5.0 and in Tris buffer pH 7.4. The data show that, similarly to the {Fe(II)-(ferrozine)$_3$} complex, neither {Fe(II)-(bipyridine)$_3$} nor {Fe(II)-(phenanthroline)$_3$} is affected by pH, in the range of 5.0 to 7.4.

4. Conclusions

Here, we have shown that the kinetics of Fe(II) complexation by ferrozine, but not by 2,2′-bipyridine or 1,10-phenanthroline depend on the concentration of the ligand, or the ligand:Fe(II) ratio. For ferrozine, it is recommended that a molar ratio of ~100:1 ferrozine:Fe(II) be employed with a corresponding molar absorptivity of 31,500 M^{-1} cm^{-1} at 562 nm, which is ~10% higher than the published value. Given the much higher formation constants of the {Fe(II)-(bipyridine)$_3$} and {Fe (II)-(phenanthroline)$_3$} complexes, molar ratios of >10:1 ligand:Fe(II) are required, although higher molar ratios are recommended. The experimental molar absorptivity values for {Fe(II)-(bipyridine)$_3$} and {Fe(II)-(phenanthroline)$_3$} are 9950 M^{-1} cm^{-1} and 12,450 M^{-1} cm^{-1} at 522 nm and 510 nm, respectively, and are ~12–15% higher than published values. Our results have important implications in the quantification of iron in biological systems, particularly when ferrozine is employed, since sometimes less than optimal concentrations, or incorrect molar absorptivity values have been used, leading to inaccurate determination of iron concentrations. The fact that a wide range of pH (5 to 7.4) can be employed with these ligands without an effect on the optical properties of these ligands make them much more attractive ligands for a variety of applications.

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Gideon L. Smith: Formal analysis, Investigation, Methodology. **Aliaksandra A. Reutovich:** Formal analysis, Investigation, Methodology. **Ayush K. Srivastava:** Formal analysis, Investigation, Methodology. **Ruth E. Reichard:** Formal analysis, Investigation, Methodology. **Cass H. Welsh:** Formal analysis, Investigation, Methodology. **Artem Melman:** Conceptualization, Formal analysis, Investigation, Methodology, Writing - review & editing. **Fadi Bou-Abdallah:** Formal analysis, Conceptualization, Funding acquisition, Investigation, Methodology, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

None.

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