Application note

Assessing Binding Equilibria through UV-Visible Absorption Techniques

Introductions

Binding processes are crucial in a myriad of chemical and biochemical reactions. For biological environments, the interactions between enzymes and ligands often involve the formation of a supramolecular complex which can allow specific reactions to proceed.[1-3] Consequently, the study of this binding phenomenon is needed in drug discovery work to better understand the considerations needed to optimize the product's efficacy. In chemical and materials environments, the adsorption of molecules to heterogeneous surfaces can play an important role in photocatalytic systems and wastewater treatment, among other applications.[4-6] Similarly, these processes are often studied to better understand the experimental conditions needed to improve production.

The simplest binding expression involves the reaction between one ligand (L) with one active site on a given compound (C) to form a complex (CL) as described in Eqn. 1.

$$C + L \overset{K_{eq}}{\leftrightarrow} CL$$

Equation 1.

Note, in many of these interactions, the binding is described as an equilibrium between the association and dissociation of a given complex. From thermodynamics, the equilibrium constant (K_{eq}) is expressed as the ratio between the concentration of the products and the concentration of the reactants, as shown through Eqn. 2.

$$K_{eq} = \frac{[products]}{[reactants]} = \frac{[CL]}{[C][L]}$$

Equation 2.

When considering reaction kinetics, K_{eq} can also be expressed as the ratio of the forward reaction (association) rate constant (K_a) and the reverse reaction (dissociation) rate constant (K_d) as outlined in Eqn. 3.

$$K_{eq} = \frac{K_a}{K_d}$$

Equation 3.

Considering both of these definitions, a value of $K_{eq} >> 1$ implies the reaction heavily favors the formation of the bound complex while a value of $K_{eq} << 1$ implies the dissociation of the ligand from the active site is favored at equilibrium. As such, it can be highly important to determine the equilibrium constant so as to better understand which reaction is favored and how the equilibrium can be shifted to optimize the production of a given substance.

Volume of 10 mM Fe^{3+} (µL)	Volume of 400 mM citrate (mL)	Volume of DI water (mL)
240	0	5.760
	0.045	5.715
	0.150	5.610
	0.375	5.385
	0.750	5.010
	1.125	4.635
	1.500	4.260
	1.875	3.885
	2.625	3.135
	3.000	2.760
0	3.000	3.000

Table 1. Sample preparation of Fe-citrate complexes.

Binding experiments often involve measuring multiple samples with varying concentration of one compound and a constant concentration of the other. The resulting concentration of the formed complex is then monitored to determine K_{eq}. There are many methods which can be used to follow the complex formation for this purpose, including chromatographic and spectroscopic techniques.[1,2] Specifically, UV-Visible absorption spectroscopy can be a highly useful analytical method for this type of study.

In this analytical method, light within the UV-Visible spectral range either passes through the sample or is absorbed by the analytes present. Light is absorbed when the energy of the incident photon is sufficient to induce transitions between the ground and excited states of electrons within the compound of interest. This response is specific to the analyte measured and is often used as a method of quantification through Beer's law (Eqn. 4),

$$A_\lambda = cl\varepsilon_\lambda$$

Equation 4.

where A_λ is the measured absorbance as a function of wavelength, c is the concentration of the analyte, l is the pathlength and ε_λ is the extinction coefficient. As outlined in this equation, the absorbance is directly proportional to the concentration of the analyte. Consequently, this method is ideal for monitoring the change in complex concentration as the reactant concentration changes.

Herein, binding experiments are demonstrated using the complexation reaction between iron and citrate as a model system. Absorption spectra of samples with various citrate concentrations were collected using a Thermo Scientific™ Evolution™ One Plus Spectrophotometer. From this data, binding curves were constructed, including a Scatchard plot, and the equilibrium constant was assessed.

Experimental

Sample Preparation

131.1 mg of iron (III) chloride hexahydrate was dissolved in 48.5 mL of DI water to prepare a 10 mM stock Fe^{3+} solution. A 400 mM sodium citrate stock solution was prepared by dissolving 3.053 g of disodium citrate sesquihydrate in 30 mL of DI water. Subsequent solutions were prepared as outlined in Table 1. This procedure was based on a similar experiment outlined by Vukosav et al.[7]

Instrument Parameters

An Evolution One Plus spectrophotometer was used to measure the absorption spectra of samples outlined previously. Spectra were collected between 250 and 800 nm using a 1.0 nm data interval, 1.0 nm spectral bandwidth and 0.1 s integration time. A 1.0 cm quartz cuvette was used for all samples. The background was collected using DI water as the blank material. Using the extinction coefficient for the Fe-Citrate complex,[8] the concentration of the Fe-Citrate complex was determined.

Results/Discussion

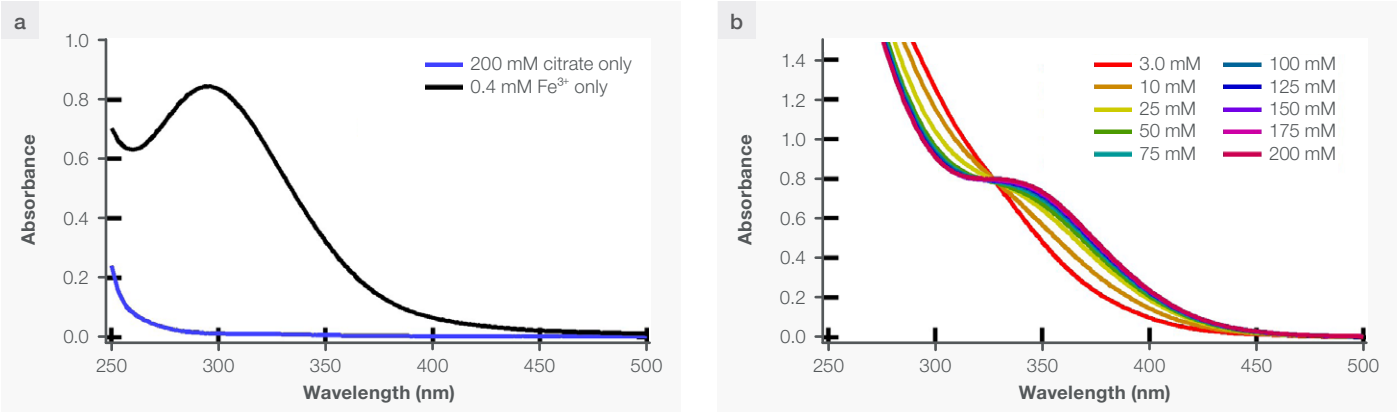

Figure 1. (a) UV-Visible spectra of 0.4 mM $FeCl_3$ (black) and 200 mM citrate (blue). (b) UV-Visible spectra of 0.4 mM $FeCl_3$ with varying concentrations of sodium citrate. All samples measured in a 1.0 cm pathlength.

Figure 1a includes the UV-Visible spectrum of 0.4 mM $FeCl_3$ which has a maximum absorbance at 295 nm. With the addition of citrate (Figure 1b), an absorption maximum at 350 nm is shown to grow in with increasing ligand concentration while a concomitant decrease in absorbance at wavelengths shorter than 327 nm. According to literature, the Fe-citrate complex absorption maximum is 345 nm,[8] suggesting the absorption feature observed growing is from the formation of Fe-citrate. The absorbance at 327 nm does not change with citrate concentration. For spectroscopic measurements, a wavelength at which the absorbance does not change with either time, for a reaction, or sample concentration, for binding experiments, is referred to as an isosbestic point. This feature occurs when the extinction coefficients of two absorptive species in a sample are equivalent. In binding studies, this typically indicates only two species are involved in the equilibrium.

Given a clear absorption feature for the Fe-citrate complex can be found in the collected UV-Visible spectra, this absorbance can be used to determine the concentration of the formed complex, and consequently K_{eq}. In this circumstance, the concentration of the complex as a function of citrate concentration was monitored. As is shown in Figure 2a, the absorbance changes more significantly at ligand concentrations below ~50 mM and less at higher citrate concentrations.

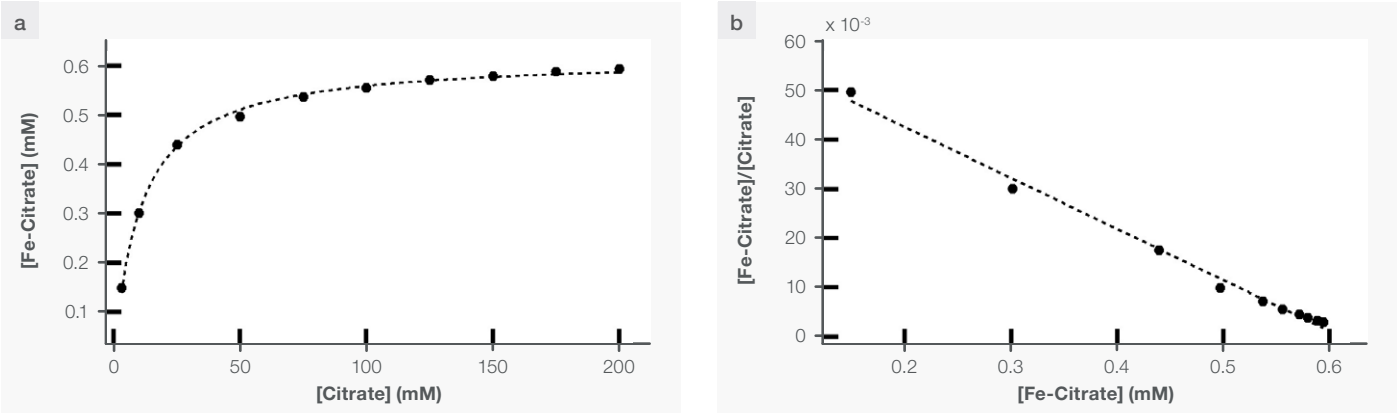

Figure 2. (a) Fe-Citrate concentration versus citrate concentration. (b) Scatchard plot of binding between Fe^{3+} and citrate. Dashed lines indicate fits to the reported data.

To determine K_{eq}, the relationship outlined in Equation 2 (where the concentration of the products and reactants is correlated to K_{eq}) is needed. In the context of the experiments described herein, this expression can be further simplified to Equation 5,

$$[Fe - Citrate] = \frac{n*K_{eq}\,[Citrate]}{1+K_{eq}\,[Citrate]}$$

Equation 5.

where *[Citrate]* is the concentration of citrate added, *n* is the number of binding sites, and *[Fe-Citrate]* is the concentration of Fe-citrate.[4] This equation is only applicable assuming the binding sites are equivalent. For the Fe-citrate system included herein, this is a fair assumption. Using this method, K_{eq} was found to be 0.097 ± 0.004 while *n* was found to be 0.617 ± 0.004. As $K_{eq} < 1$, it is implied that the reverse reaction is favored.

While Equation 5 fits the data well, it can often be easier to fit data to a linear function. For binding equilibrium, this is accomplished by linearizing Equation 5, as shown here (Eqn. 6).

$$\frac{[Fe\text{-}Citrate]}{[Citrate]} = nK_{eq} - K_{eq}\,[Fe - Citrate]$$

Equation 6.

By plotting the ratio between the bound (Fe-Citrate) and free (citrate) species as a function of the bound species, a Scatchard plot can be developed. As shown in Equation 6, the slope of the best fit line is equivalent to K_{eq}. Figure 2b includes the Scatchard plot for the data collected herein. Using the Scatchard equation, K_{eq} = 0.104 ± 0.003 and n = 0.611 ± 0.006, in agreement with the previous fits. While this result demonstrates the consistency of the two methods, it should be noted that greater variation is often reported when using the Scatchard method for equilibrium analysis.

Conclusions

As shown by the binding experiments described herein, UV-Visible absorption spectroscopy is a highly useful technique for monitoring binding. Through Beer's law, the change in concentration of the reactants and/or products can be determined. This data can then be used to quickly determine K_{eq} through similar methods as shown herein, as well as other non-ideal binding equilibria. In addition to the quantitative capabilities, the non-destructive nature allows for bound systems to be further analyzed through additional methods if needed.

References

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