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ARTICLE TYPE

pFe^{3+} Determination of Multidentate Ligands by a Fluorescence Assay

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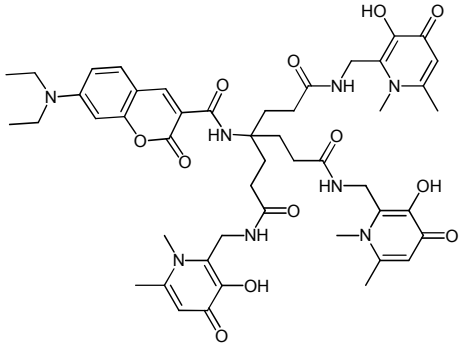
The fluorescence intensity of the iron-CP691 complex in the presence of a competing multidentate ligand is associated with pFe^{3+} of the competing ligand and the relative fluorescence has a linear correlation with the pFe^{3+} values. A correlation was also found to exist between the relative fluorescence and the ratio of a competing ligand to the probe CP691. Based on this assay, the pFe^{3+} value of a range of hexadentate ligands, dendrimers and polymers can be determined when they fall in the range 24.5-30.5. Only small quantities of chelators are required for this assay.

Iron is an essential element in all biological systems including microorganisms, plants and animals by virtue of its unique chemical properties[1]. It plays an important role in oxygen transport, energy transduction and the functioning of many enzymes[2]. The low solubility of Fe^{3+} has forced living systems to synthesize and secrete siderophores to scavenge iron from soil, water and living organisms and to carry it into the cell via specific high affinity uptake receptors[3, 4] . There are over 500 different siderophores in nature and over 270 been structurally characterized[5]. Siderophores are low-molecular-weight ligands (<1500 daltons) and possess a high affinity for Fe^{3+}.

Conventional spectrophotometric and potentiometric assays have been used to measure acid dissociation constants and stability constants of simple ligands, such as bidentate and tridentate chelators[6, 7]. However, these two procedures are relatively difficult to apply to multidentate ligands as the latter contain multiple acid dissociation constants which have close values. For example, the pK_a values of three COOH groups on each of the phytosiderophores DMA, MA, DMA and eHMA are reported to fall in the range 2.3-3.5[8]. The 3-hydroxy and 4-oxo groups from tri-hydroxypyridinone-based hexadentates possess pK_a values in the ranges of 8-10 and 2.5-4 respectively[9]. Furthermore it is reported that the pK_a value of the tertiary amine on the tripodal scaffold of the short chain hexadentate is not detectable by both spectrophotometric and potentiometric assays and the pK_a of the tertiary amine of the longer chain hexadentate can be only detected by potentiometric titration[9]. Only a few hydroxypyridinone-based hexadentates have been investigated using spectrophotometric or potentiometric assay, due to difficulties associated with their extremely high stability constants. However, it is important to establish the iron affinity constants of hexadentate and dendrimeric chelators, especially the pFe^{3+} value, which is a more reliable parameter than the stability constant under physiological conditions, since the pFe^{3+} value takes into account the effects of ligand protonation, denticity and metal hydrolysis[10, 11]. In a previous study, we developed a novel fluorescence assay for pFe^{3+} determination, which is based on the correlations between pFe^{3+} values of the ligands and the relative fluorescence in the presence of probe and iron[12]. We used the bidentate fluorescent probe CP645 for measuring the ligands which possess pFe^{3+} values in the range of 17-23. In this study, we have extended this application by introducing the fluorescent probe CP691[13], which contains a hexadentate hydroxypyridinone. The aim was to measure pFe^{3+} values of ligands which are greater than 23.

The ligands used in this study were either synthesized by previously published methods[13-16] or purchased from EMC microcollections GmbH (Tuebingen, Germany). They were stored under desiccation at room temperature. All consumables were washed with nitric acid (0.1%) to remove contaminating iron. Fluorescence of CP691 was monitored on a Perkin-Elmer spectrofluorometer (type LS 50B) at 435 nm for excitation using a scan speed at 120 nm min^{-1}. The maximum emission wavelength of iron free CP691 was found to be at 474 nm and it was not shifted or increased by the addition of iron or non-fluorescent competing ligands. In contrast, the fluorescence intensity of CP691 was quenched in the presence of iron and this fluorescence change can be recovered by an excess addition of strong non-fluorescent ligands.

12 Non-fluorescent ligands with pFe^{3+} values at the range of 20-31 were selected for the correlation study between the pFe^{3+} values and the relative fluorescence (Table 1). The fluorescence intensity of iron-free CP691 (6 μM) was set at 100% and that of iron-CP691 complex (6 μM, 1:1 ratio) at 0%. Based on this setting, the relative fluorescence intensity of CP691 in the presence of iron and the competing ligand could be calculated.



CP691

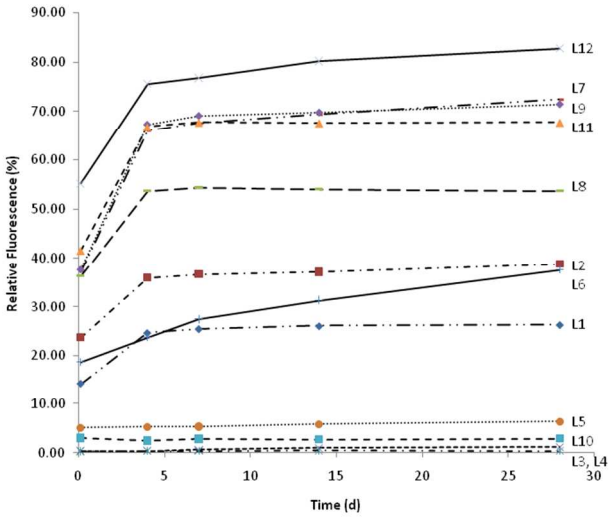


Figure 1: Time course for the fluorescence intensity change of CP691 (6 μM) in the presence of iron (6 μM) and various competing ligands (concentrations are dependent on the iron binding site numbers and is equivalent to 6 μM / (number of iron binding sites)). CP691 (6 μM) was incubated with iron-NTA (6 μM based on iron concentration) for 10 min before adding a competing ligand. The solution was then sealed and incubated in the dark. Relative fluorescence (λ_{ex} = 435nm, λ_{em} = 474 nm) of CP691 in MOPS buffer (50 mM, pH 7.4) was recorded at 3h, 4d, 7d, 14d and 28d respectively.

Due to the high affinity of hexadentate ligands for Fe^{3+}, the dissociation rate of iron from the formed iron-ligand complex is exceedingly low at neutral pH[17]. The exchange rate of iron between two hexadentate-based ligands is also extremely slow, the half-time of iron exchange between DFO-B and ferrichrome A for instance is 220 h[18]. However, based on thermodynamic considerations, ligands possessing higher pFe^{3+} values should be able to mobilize iron from the iron-CP691 complex and thus lead to greater fluorescence recovery. When relatively weak ligands such as, rhodotorulic acid, EDTA and rhizoferrin (L3-L4, L10) were used as the competing ligand at an equivalent amount to CP691, then less than 5% of the fluorescence was recovered (Figure 1). Due to this insensitivity, they were excluded from the competing ligand group for the standard curve. On the other hand, the strongest ligand in the selected group, HBED (L6, pFe^{3+}=31), is also excluded due to its kinetically slow competition property when competing iron from the iron-CP691 complex. Even after 28-day incubation, the fluorescence of the mixture was still increasing (Figure 1). The relative fluorescence of the remaining 8 ligands, which stabilised after 28 days, was plotted against the literature pFe^{3+} values resulting with a linear curve (Figure 2).

Based on the equation presented in Figure 2, the pFe^{3+} values can be calculated back from the relative fluorescence of iron-CP691 complex in the presence of the competing ligands. The results are presented in Table 1. There is a small difference between the pFe^{3+} values reported in the literature and those obtained by the standard curve, the differences all falling in the range of 0-0.7.

Using the equation for the correlation of pFe^{3+} and the relative fluorescence, the pFe^{3+} values of a series of complicated hexadentate ligands, dendrimers and polymers were determined from their relative fluorescence values, when competing with the

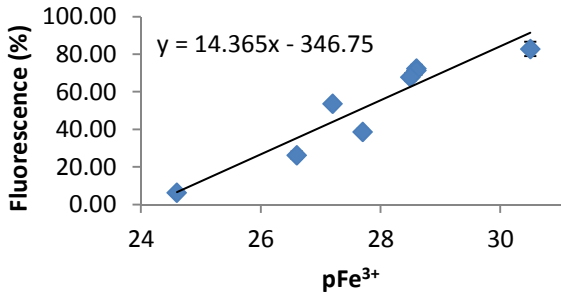


Figure 2: Correlation between previously reported pFe^{3+} values of 8 hexadentate ligands and the relative fluorescence of iron-CP691 complex incubating with the ligands for 28 days. The fluorescence of iron-free CP691 (6 μM) in MOPS buffer (50 mM, pH 7.4) was set up at 100% and iron-CP691 (6 μM: 6 μM) at 0%. The pre-incubated iron-CP691 (6 μM: 6 μM) was then incubated with the 8 non-fluorescent ligands respectively at a concentration, dependent on the iron binding site numbers; it equals to 6 μM / (number of the iron binding site). The relative fluorescence was calculated based on this setting

iron-CP691 complex (Table 2). As L13-L24 contain similar iron chelating moieties, the pFe^{3+} values of the group were assumed to be in a same range. In fact, the relative fluorescence of the group only varies in the range 78.5-88.6% and their pFe^{3+} values fall within the range 29.6-30.3. This finding indicates that neither the substituent on N1 of the chelating moieties of the hexadentate ligands nor the nature of core greatly influence the pFe^{3+} values. Furthermore, the incorporation of such chelating units in dendrimers and polymers also has only a minor influence on pFe^{3+} values.

Ligand L25 contains two hydroxamate groups and one hydroxyphenyloxazoline group and was determined to possess a pFe^{3+} value of 25.2 (Table 2). Ligand L26 contains three hydroxypyranone groups and consequently was predicted to possess a lower pFe^{3+} value than that of the corresponding hydroxypyridinone-based ligands as the pFe^{3+} values of bidentate analogues are close to 15 compared to those of 19 for bidentate

Table 1 Literature pFe^{3+} values and relative fluorescence intensities of 12 selected ligands.

Entry	$pFe^{3+(a)}$	Fluorescence (%)[(b)]	$pFe^{3+(c)}$
L1 (DFO B)	25[5]; 26.6[22]	26.3±1.9	26.0
L2 (DFO E)	26.6[5]; 27.7[22]	38.7±1.5	26.8
L3 (Rhodotorulic acid)	22[5]	1.2±0.1	--
L4 (EDTA)	23.4[21]	0.4±0.1	--
L5 (DTPA)	24.6[23]	6.4±1.5	24.6
L6 (HBED)	31[21]	37.5±5.4	--
L7	28.6[14]	72.4±1.9	29.2
L8	27.2[15]	53.7±1.5	27.9
L9	28.6[14]	71.4±2.4	29.1
L10 (Rhizoferrin)	20[5]	2.9±0.2	--
L11	28.5[14]	67.8±2.1	28.9
L12	30.5[21, 24]	82.8±3.9	29.9

[a]literature reported values; [b]relative fluorescence intensity was calculated after 28 days incubation of the iron-CP691 complex with the competing ligands; [c]pFe^{3+} was calculated based on the correlation curve in Figure 2.

Table 2 Relative fluorescence of iron-CP691 complex in the presence of various hexadentate and dendrimer ligands and the calculated pFe^{3+} values of the ligands.

entry	Fluorescence (%)[a]	pFe^{3+} [b]
L13	86.2	30.1
L14	83.2	29.9
L15	87.4	30.2
L16	79.1	29.7
L17	86.4	30.2
L18	78.5	29.6
L19	87.0	30.2
L20	79.5	29.7
L21	84.6	30.0
L22	88.0	30.3
L23	79.4	29.7
L24	80.6	29.8
L25	15.2	25.2
L26	13.7	25.1

[a]relative fluorescence intensity was calculated after 28 days incubation of the iron-CP691 complex with the competing ligands. [b]pFe^{3+} was calculated based on the correlation curve in Figure 2.

hydroxypyridinones[10]. In this study, the pFe^{3+} value of L26 was calculated at 25.1 (Table 2).

MECAM (L27) has been reported to possess a pFe^{3+} value at 29.1[5]. However, in our competition study, the result shows that MECAM did not recover the fluorescence of iron-CP691 complex. A possible reason for this observation is that after a long incubation, some of the MECAM catechol groups were oxidised[19] and therefore lost their chelating capability. The same phenomena were found for another two catechol-based multidentate ligands L28 and L29. We attempted to avoid this phenomenon by undertaking the competition study in the presence of a sacrificial oxidant, namely phenol (100 mM)[20]. However, no improvement resulted.

In theory, weaker ligands should mobilize iron from the iron-CP691 complex when used in excess. To check this possibility, DFO was selected at various concentrations for investigation. The concentration of iron-CP691 complex was fixed at 6 μM and the concentration of DFO ranged from 6 μM to 4 mM. The relative fluorescence was plotted against the molar ratio of DFO : CP691 (Figure 3). By increasing the molar ratio of the two ligands, the relative fluorescence was correspondingly increased in an exponential manner. This curve is amenable to further analysis. Thus hexadentate ligands have simple equilibrium constants as indicated in eqns. 1 and 2 and the competition between hexadentate ligands for iron is shown in eqn. 3. As indicated in the supplementary information, the pFe value of CP691 can be independently calculated from any point on the exponential curve presented in Figure 3. In fact, at the point of 50% fluorescence,

$$Fe + L_1 \overset{K_1}{\rightleftharpoons} FeL_1 \qquad \text{Eq.1}$$

$$Fe + L_2 \overset{K_2}{\rightleftharpoons} FeL_2 \qquad \text{Eq.2}$$

$$FeL_1 + L_2 \overset{K}{\rightleftharpoons} L_1 + FeL_2 \qquad \text{Eq.3}$$

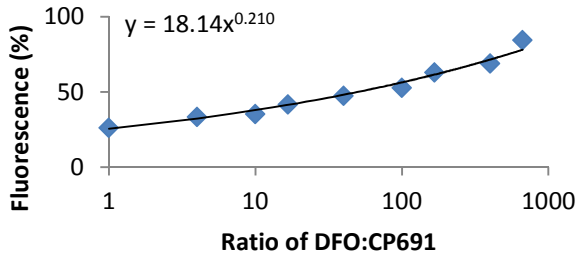


Figure 3: Correlation between the ratio of DFO / CP691 and its relative fluorescence intensity in the presence of iron. The iron-CP691 complex (6 μM) in MOPS buffer (50 mM, pH 7.4) was incubated with various concentrations of DFO from 6 μM up to 4 mM for 28 days. The relative fluorescence was calculated based on the previous setting (see Figure 2).

the pFe value of CP691 was calculated at 29.0 based on the pFe value of DFO-B at 26.6. The average pFe value of CP691 calculated from the experimental ratio points is 28.8.

In conclusion, this is a related study to a previously reported fluorescence method for the determination of pFe^{3+} values between the range 17-23 using CP645[12]. In this study, pFe^{3+} values in the range of 24.5-30.5 were found to achieve a linear fluorescence response using the stronger iron affinity probe CP691. In contrast to bidentate ligands which may form a range of iron complexes when two bidentate ligands compete for iron, hexadentate ligands will form simple iron complexes which bind iron at 1 : 1 ratio. They are unlikely to form mixed complexes[21]. Based on this concept, the pFe^{3+} values of new hexadentate ligands can be calculated from the pFe^{3+} value of the probe and its relative fluorescence intensity in the presence of iron and the competing ligand. Due to the necessity of the long incubation period this method is not applicable to catechol-containing complexes. In principle, this concept can also be applied to the determination of pM values for other metals.

Notes and references

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pFe^{3+} Determination of Multidentate Ligands by a Fluorescence Assay

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pFe value of a multidentate ligand is related to the fluorescence of an added probe in the presence of iron.