Journal of Inorganic Biochemistry 198 (2019) 110753

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

The solution thermodynamic stability of desferrioxamine B (DFO) with Zr (IV)

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ARTICLE INFO

Keywords:
Desferrioxamine B (DFO)
Zr(IV) chelation
Thermodynamic solution studies

ABSTRACT

Desferrioxamine B (DFO, $[H_4L]^+$, ligand) is currently the preferred chelator for ^{89}Zr(IV), however the biological studies suggest that it releases the metal ion *in vivo*. Herein, we present the solution thermodynamics of complexes formed between Zr(IV) and this hexadentate chelating agent, the data surprisingly not yet available in the literature. Several techniques including electrospray ionization mass spectrometry (ESI-MS), potentiometry, UV–Vis spectroscopy and isothermal titration calorimetry (ITC) were used to determine the stoichiometry and thermodynamic stability of complexes formed in solution over pH range 1–11, overcoming all the difficulties with the characterisation of the aqueous solution chemistry of Zr(IV) complexes, like strong hydrolysis and lack of spectral information. A model containing only mononuclear complexes, *i.e.* $[ZrHL]^{2+}$ $[ZrL]^+$, $[ZrLH_{-1}]$ throughout the entire measured pH range is proposed. The stability constants and pM (Zr(IV)) value determined for Zr(IV)-DFO system, place DFO among good Zr(IV) chelators, however the formation of 6-coordinate unsaturated complexes (*i.e.* with coordination sphere of 8-coordinate Zr(IV) completed by water molecules), together with the susceptibility of coordinated water molecule to deprotonation, are suggested to be the reason of *in vivo* instability of ^{89}Zr(IV)-DFO complexes.

1. Introduction

^{89}Zr(IV) is radioactive isotope that can be harnessed for application in the development of positron emission tomography (PET) which provides sensitive, quantitative, and non-invasive images of a variety of molecular processes and targets. The recent, big attention to ^{89}Zr(IV) ion is due to its favourable decay characteristics (^{89}Zr: $t_{1/2}$ = 78.4 h, β^+ = 23%, $E_{\beta+max}$ = 90 MeV; EC: 77%, E_γ = 909 keV), that makes the isotope nearly ideal for use with biological vectors that have long circulation times such as antibodies and nanoparticles [1–5]. In order to apply ^{89}Zr(IV) to PET, the metal must be sequestered by efficient chelators to obviate metal hydrolysis and transchelation. Ligands used for this application are usually covalently linked to a biologically active targeting molecule (biovector), creating an active radiopharmaceutical agent.

As an early transition metal with relatively low effective nuclear charge, zirconium can exists in various oxidation states, such as Zr(II), Zr(III) and Zr(IV), with the latter dominating the aqueous chemistry of zirconium [6,7]. The large charge density of Zr(IV) is highly polarising leading to dramatic enhancement in acidity of the coordinated aqua ligands and strong tendency to form oxides and hydroxides, which undergo hydrolytic polymerisation to give polyoxometallate species even in acidic solutions [6,8]. Being an extremely hard cation according to Pearson theory, the tetravalent Zr(IV) is exhibiting a strong preference for polyanionic hard donor chelators, with which it can achieve the maximum coordination number of 8 with complicated geometry of dodecahedron or square antiprism [9,10].

To develop ligands for ^{89}Zr(IV) chelation, chemists got inspiration in nature, and Mejis et al. performed the first evaluation of desferrioxamine B (DFO) (Fig. 1) as a ^{89}Zr(IV) chelator, revealing its high stability in human serum [11]. DFO, a natural tris-hydroxamate siderophore and a growth-promoting agent secreted by *Streptomyces pilosus* to acquire ferric ions from the environment, is commercially produced and is for a long time a drug approved and used in the clinic for the treatment of Fe(III) overload [12,13]. As a hexadentate ligand with hard base donor atoms, the DFO binds Fe(III) strongly over a large pH range (1–11) [14], and, given Zr's preference for hard, anionic donor groups, and its ability to form complexes with monohydroxamates [15,16], was predicted to efficiently bind also Zr(IV) ions. Since the first trials, there has been significant progress in the development of DFO–based bifunctional ^{89}Zr chelators, with many derivatives prepared to facilitate bioconjugation to antibodies. This group is by far the most investigated in both pre-clinical and clinical context, among all the bifunctional chelators bound to antibodies [16]. The progress in ligand

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https://doi.org/10.1016/j.jinorgbio.2019.110753
Received 13 March 2019; Received in revised form 10 June 2019; Accepted 14 June 2019
Available online 15 June 2019

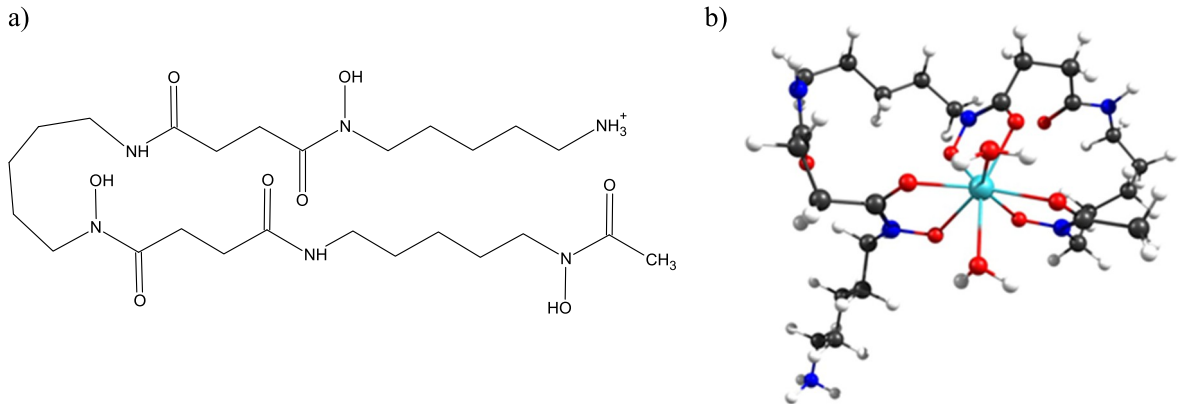

Fig. 1. a) Structure of DFO ligand in fully protonated form, $[H_4L]^+$. b) DFT calculation for Zr(IV)-DFO complex [3] (b). This research was originally published in the Journal of Nuclear Medicine (see ref. 3). Copyright 2010 Society of Nuclear Medicine and Molecular Imaging, Inc.

development and current status of clinical trials of DFO–based radiopharmaceuticals are summarised in recent reviews [16–18].

Although no X-ray crystal structure of the Zr(IV)-DFO has been determined, a density functional theory (DFT) calculations predicted the lowest energy structure with three hydroxamate groups and two water molecules located in *cis*-positions in Zr(IV) coordination sphere, all arranged in a distorted square antiprismatic geometry [2]. The potential lability of the two monodentate water molecules, and a possibility of their easy replacement in the complex, are suggested to be an initial stage of *in vivo* DFO demetallation, followed by deposition of radioactive ^{89}Zr(IV) isotope in the bone (up to 15% of the injected dose) [2,3,19–22]. The reports of limited stability of DFO have stimulated a search for DFO derivatives with enhanced stabilities (mostly by adding another binding group to the DFO skeleton) [23,24], however, to the best of our knowledge, till now the solution thermodynamic stability of DFO with Zr(IV) has not been characterized, and the corresponding constants are unknown [16,19]. To gain an understanding of the solution behaviour of Zr(IV)-DFO system, and to fully realize the drawbacks of DFO chelator, in this paper we report for the first time the experimental determination of the speciation and stability constants of Zr(IV) complexes with DFO.

2. Material and methods

2.1. Reagents

All reagents and solvents used for the experiments were purchased from commercial suppliers and were used without further purification. The ligand, desferrioxamine (DFO) was used as mesylate salt (Sigma Aldrich, ≤ 92.5% purity). All solutions were prepared in doubly distilled water. Aqueous stock solution of DFO ligand was freshly prepared by direct dissolution of a weighed portion of ligand in water prior to each set of experiments. A stock solution of Fe(III) was prepared immediately before use from $Fe(ClO_4)_3{\cdot}XH_2O$ (Sigma Aldrich, chlorides < 0.005%) in 0.01 M $HClO_4$ (ChemPur, 70%) and standardized by inductively coupled plasma - optical emission spectrometer (ICP–OES) (iCAP 7400 Duo ICP-OES) along with spectrophotometric determination, based on molar extinction coefficient $\varepsilon = 4160\,M^{-1}\,cm^{-1}$ at 240 nm [25,26]. A Zr(IV) stock solution was prepared from $ZrCl_4$ anhydrous (Sigma-Aldrich, 99.99%) in known amount of $HClO_4$ (0.1 M) and standardized by ICP–OES along with direct titration in 1 M HNO_3 (ChemPur, 70%) with ethylenediaminetetraacetic acid (EDTA) (Fluka, ≥ 99.0%) and xylenol orange (POCH, 99.0%) used as the indicator [27]. The $HClO_4$ and HNO_3 solutions were titrated by standardized NaOH (0.1 N, Titripur). Carbonate-free NaOH solution was standardized by titration with potassium hydrogen phthalate (Merck, 99.95%). All stock solutions were prepared using a R200D Sartorius analytical balance (with precision 0.01 mg).

2.2. Electrospray ionization mass spectrometry (ESI–MS)

ESI–MS data were recorded on a Bruker Q-FTMS spectrometer. The instrumental parameters were: scan range, *m/z* 200–1600; dry gas, nitrogen; temperature, 170 °C; capillary voltage, 4500 V; ion energy, 5 eV. The capillary voltage was optimized to the highest signal-to-noise ratio. The spectra were recorded in the positive mode. The compound was dissolved in MeOH/H_2O solution (80/20 by weight); the same solvent mixture was used to dilute the matrix solutions to the concentration range 0.01 mM. The Zr(IV) and Fe(III) stock solutions were prepared as described previously and added to ligand in the 1:1, 1:2 and 1:5 metal-to-ligand molar ratios, pH was adjusted to 3, 5 and 8 by using acetic acid and ammonium hydroxide. The free hydrogen ions concentration was measured with the combined glass electrode Metler Tolledo InLab Semi-Micro filed with NaCl (Fluka, p.a.) in MeOH/H_2O (80/20 by weight). Potential differences were given by a Beckman ϕ72 pH meter, standardized according to the classical methods with buffers prepared according to published procedures in MeOH/H_2O solvent (80/20 by weight) [28,29].

2.3. Potentiometric titrations

The potentiometric titrations of ligand and its complexes were carried out using an automatic titrator system Titrando 905 (Methrom), equipped with a combined glass electrode (Mettler Toledo, InLab Semi-Micro, with XEROLYT® EXTRA Polymer filling) and a dosing system 800 Dosino, equipped with a 2 ml micro burette. The ionic strength was fixed at $I = 1.0$ M with $NaClO_4$ (Alfa Aesar, 99.0%) The electrode was daily calibrated in terms of hydrogen ion concentration using $HClO_4$ (0.1 M) with CO_2-free NaOH solutions (0.1 M) [30]. A stream of high purity grade argon, pre-saturated with water vapour, passed over the surface of the solution cell, filled with 50 ml of studied solution, and thermostated at 25.0 ± 0.2 °C. At least four titrations were performed for each system, *i.e.* DFO, Zr(IV)-DFO and Fe(III)-DFO, with a starting concentration of the ligand of 1 mM, and 1:1.2, 1:2 and 1:3 metal-to-ligand molar ratios in the pH range 2–11. The purity and exact concentration of the ligand solution was determined using the Gran method [31]. Special care was taken to ensure that complete equilibration was attained. The titrations curves were checked carefully, and did not display any pH fluctuations which often accompany precipitation of metal hydroxides. The potentiometric data (about 140 points collected over the pH range 2–11) were refined with the SUPERQUAD [32] or HYPERQUAD [33] programs, which use nonlinear least-square methods. The successive protonation constants of the ligand were calculated from the cumulative constants determined with the program

Table 1
Protonation ($\log\beta^H$) and stability ($\log\beta$) constants of Fe(III) and Zr(IV) complexes with DFO in aqueous solution[a].

	$\log\beta$	$\log K$
DFO		
$\log\beta_1^H$	10.97 (1)[b]	10.97
$\log\beta_2^H$	20.91 (2)[b]	9.94
$\log\beta_3^H$	30.12 (2)[b]	9.21
$\log\beta_4^H$	38.83 (3)[b]	8.71
	$\log\beta$	pK
Fe(III)		
$\log\beta_{FeH_2L}$	42.82 (4)[c]	
$\log\beta_{FeHL}$	41.80 (5)[c]	1.02
$\log\beta_{FeL}$	31.10 (3)[b]	10.70
Zr(IV)		
$\log\beta_{ZrHL}$	47.7 (2)[d]	
	47.7 (1)[e]	
	47.3 (6)[f]	
	46.4 (1)[b]	
$\log\beta_{ZrHL(OH)}$	40.04 (3)[b]	6.36
$\log\beta_{ZrL(OH)}$	29.15 (9)[b]	10.89

[a] I = 1.0 M $NaClO_4$, T = 25 °C.
[b] Calculated from potentiometric titrations.
[c] Calculated from pH dependent UV–Vis titrations (SI).
[d] Calculated from competition batch UV–Vis titration Fe(III)-DFO + Zr(IV).
[e] Calculated from competition batch UV–Vis titration Zr(IV)-DFO + Fe(III).
[f] Calculated from competition batch UV–Vis titration Zr(IV)-Fe(III) + DFO; charges are omitted for clarity.

and defined by Eqs. (1) and (2) (charges are omitted for clarity).

$$H_{n-1}L + H \leftrightarrows H_nL \quad (1)$$

$$K_n^H = \frac{[H_nL]}{[H_{n-1}L][H]} \quad (2)$$

The stability constants calculated for metal complexes are defined by Eqs. (3) and (4):

$$mM + hH + lL \leftrightarrows M_mH_hL_l \quad (3)$$

$$\beta_{M_pH_qL_r} = \frac{[M_pH_qL_r]}{[M]^p[H]^q[L]^r} \quad (4)$$

In the calculations of complex stability constants, the protonation constants of free ligand (Table 1) and the constants related to hydrolytic Zr(IV) [8]: $Zr(OH)^{3+}$ $\log\beta_{ZrH_{-1}} = -0.87$, $Zr(OH)_2{}^{2+}$ $\log\beta_{ZrH_{-2}} = -2.10$, $Zr(OH)_3{}^{+}$ $\log\beta_{ZrH_{-3}} = -4.00$, $Zr(OH)_4$ $\log\beta_{ZrH_{-4}} = -6.7$, $Zr_2(OH)_6{}^{2+}$ $\log\beta_{Zr_2H_{-6}} = -2.42$, $Zr_3(OH)_4{}^{8+}$ $\log\beta_{Zr_3H_{-4}} = 4.1$, $Zr_4(OH)_8{}^{8+}$ $\log\beta_{Zr_4H_{-8}} = 5.2$, and Fe(III) [34]: $Fe(OH)^{2+}$ $\log\beta_{FeH_{-1}} = -2.66$, $Fe(OH)_2{}^{+}$ $\log\beta_{FeH_{-2}} = -7.0$, $Fe(OH)_3$ $\log\beta_{FeH_{-3}} = -12.5$, $Fe(OH)_4{}^{-}$ $\log\beta_{FeH_{-4}} = -20.6$, $Fe(OH)_5{}^{2-}$ $\log\beta_{FeH_{-5}} = -30.8$, $Fe(OH)_6{}^{3-}$ $\log\beta_{FeH_{-6}} = -43.4$, species were taken into account. The pK_w used in the calculation in the 1 M $NaClO_4$ ionic strength was −13.76 [35].

The uncertainties in the $\log K$ values correspond to the added standard deviations in the cumulative constants. The species distribution diagrams were computed with the HYSS program [33]. The data were processed using Origin 7.0.

2.4. Metal competition batch UV–Vis titrations

The competition titrations for Zr(IV)-Fe(III)-DFO systems were carried out as a function of concentration with a Varian Cary 300 Bio spectrophotometer in the 250–700 nm range (with 1 nm precision) using a Hellma quartz optical cells with 1 cm path length. Spectrophotometric titrations were performed on samples with concentration of ligand at 0.15 mM, I = 1 M (completed by adding $NaClO_4$), at 25.0 ± 0.1 °C, pH 2.0 was adjusted by adding proper volume of $HClO_4$ and checked with a Mettler Toledo Super Easy pH meter with accuracy of ± 0.01.

In order to determine the stability constant of $[ZrHL]^{2+}$ three different series of competition experiments were performed, all carried out at pH 2: (i) Fe(III)-DFO + Zr(IV), (ii) Zr(IV)-DFO + Fe(III), and (iii) Zr(IV)-Fe(III) + DFO. The following procedure was used (i) fifteen samples with a constant concentration of Fe(III) ions and DFO (0.16 mM for both) titrated by Zr(IV) ions (starting from 0 equiv. up to 3 equiv.); (ii) eighteen samples with a constant concentration of Zr(IV) ions and DFO (0.15 mM for both) titrated by Fe(III) ions (starting from 0 equiv. up to 300 equiv.); (iii) seventeen samples with a constant concentration Zr(IV) and Fe(III) ions (0.18 mM for both) titrated by DFO (starting from 0 equiv. up to 4 equiv.). After preparation, each solution was allowed to equilibrate for about 1 h, and then its UV–Vis spectrum was recorded. The vials were kept in the dark and absorbance was measured again after 24, 48 and 72 h; changes were observed between spectra collected after 1 h and 24 h; spectra collected during the next few days were the same as after 24 h.

The competition data were refined to obtain the overall Zr(IV)-DFO binding constant ($\log\beta_{ZrHL}$) using SPECFIT/32 software [36–38] which adjusts the absorptivity and the stability constants of the species formed at equilibrium. Specfit uses factor analysis to reduce the absorbance matrix and to extract the eigenvalues prior to the multiwavelength fit of the reduced data set according to the Marquardt algorithm [36–38]. Uncertainties in $\log\beta$ were calculated from the standard deviation. The protonation constants of DFO and formation constants for Fe(III)-DFO complexes (Table 1) were used as fixed parameters during data analysis. The concentration of $[FeH_2L]^{2+}$ and $[FeHL]^{+}$ complexes was calculated from the absorbance spectra (collected in 250–700 nm range, Fig. S3). Hydrolytic forms of ferric ion at studied pH range are characterized by absorption band with λ_{max} below 300 nm, and therefore they are beyond the experimental wavelength window. However, the spectrum of Fe(III) in solution at pH 2 was fixed in the calculations [39,40].

The competition equilibrium is described by Eqs. (5) and (6):

$$FeDFO + Zr(IV) \leftrightarrows ZrDFO + Fe(III) \quad (5)$$

$$K = \frac{[ZrDFO][Fe(III)]}{[FeDFO][Zr(IV)]} = \frac{\beta_{ZrDFO}}{\beta_{FeDFO}} \quad (6)$$

The molecular charges are omitted for clarity. The data were processed using Origin 7.0.

2.5. Isothermal titration calorimetry (ITC)

ITC experiments were carried out using a Nano ITC calorimeter (TA Instruments) with a standard volume of 1.0 ml cell at 25 °C. Titrations mode of ligand solution being an analyte and metal solution being a titrant was applied. The solution of ligand (at pH 1, 0.1 M $HClO_4$, ionic strength was completed to 1 M by adding $NaClO_4$) was placed in the cell and the solution of Zr(VI) (8 mM, at pH 1, 0.1 M $HClO_4$, ionic strength was completed to 1 M by adding $NaClO_4$) was taken up in a 250 µl injection syringe. Each sample was degassed prior the titration for 30 min. The total number of 25 injections, 10 µl each were added after the calorimeter finalized the primary equilibration, with 350 s apart. The stirring rate was 400 rpm. The calorimeter was operated using the Nano ITC Run software and all the data obtained were analyzed with the NanoAnalyze v. 3.1.2 program. 'Independent' model was used to evaluate the results obtained and the control experiments were performed in each case; the enthalpies of reagents dilution were subtracted from the enthalpies of binding processes. Each ITC data was collected by two independent measurements and reproducible data was employed.

3. Results and discussion

3.1. Stoichiometry of Zr(IV)-DFO complexes

ESI-MS spectrometry is frequently used as the first step for the determination of metal complexes' stoichiometry and has been employed by us for the purpose of metal-hydroxamate ligands interactions on previous occasions [26,41,42]. Although ESI–MS is not able to distinguish the ionizable protons in the species, this method can be successfully applied to determine the metal-to-ligand stoichiometry directly from the *m/z* values. The spectra collected for Zr(IV)-DFO solutions in metal-to-ligand molar ratios of 1:1, 1:2 and 1:3, all at pH 3, 5 and 8, were only slightly different by intensity, and characterized by the presence of a single peak corresponding to mononuclear $\{ZrL\}^+$ complex (*m/z* 647.24) (Fig. S1). The ligand itself is characterized by the signal at *m/z* = 561.36, corresponding to $\{LH_4\}^+$ form.

3.2. Binding properties and overall complex stability

The aqueous solution chemistry of Zr(IV) complexes is rather difficult to characterise by standard protocols using direct potentiometric and spectroscopic titrations, due to the hydrolytic and optical properties of Zr(IV). The strong hydrolysis of Zr(IV) requires the complexation experiments to be performed in a very acidic media to prevent hydrolytic polimerization, however, potentiometry is not accurate in the pH range lower than 2 due to error of the glass electrode [6,8,21]. Moreover, Zr(IV) is expected to form strong complexes with DFO, fully formed at acidic pH, and therefore one may only relay on the competition between the ligand and hydroxyl ions for Zr(IV) binding (occurring at high pH). Additionally, as Zr(IV) is a transition metal with a d^0-electron count, its complexes are UV–Vis silent. In such a case, a competition of two metal ions for a ligand, where one of the metal chelates has a strong absorption band either in the visible or ultraviolet region of the spectrum, with an extinction coefficient much different from that of the free metal ion, and performed under acidic conditions, becomes an alternative. A further requirement for accuracy is that the equilibrium constant for the competition reaction must not be very small or very large. These requirements are fulfilled in competition experiments between Zr(IV) and Fe(III) for the DFO ligand. Zr(IV)-DFO complexes do not absorb light in the visible region, while Fe(III)-DFO complexes possess a broad ligand to metal charge transfer (LMCT) band with a maximum at 470 and 430 nm, corresponding to bi- and tri-hydroxamate species, respectively [43]. The Zr(IV)-Fe(III) competition titrations have been previously used to characterise the stability constants of Zr(IV)-EDTA/Nitrilotriacetic acid (NTA)/Tiron complexes [44–46], and Zr(IV)-Ce(IV) for hydroxypyridinone-based chelator [47,48], being rare examples of polydentate ligands, for which the Zr (IV) stability constants were determined till far.

Potentiometric titrations for Zr(IV)-DFO system were carried out at pH range 2.3 to 11.0, at the metal-to-ligand molar ratios 1:1.2, 1:2 and 1:3. Prior to these experiments, the acid-base properties of the free DFO ligand, H_4L^+, were determined (Table 1), with the $\log K_4$ assigned to the protonation of amino group, and $\log K_2 - \log K_4$ to the protonation of the three hydroxamate groups. Allowing for the change in ionic strength, the protonation constants of DFO determined in the present work are in a very good agreement with those published earlier [49]. A satisfying fit of the potentiometric Zr(IV) – DFO titrations was obtained using a model containing only mononuclear complexes throughout the entire measured pH range, *i.e.* $[ZrHL]^{2+}$ $[ZrL]^+$, $[ZrLH_{-1}]$ (Table 1, Fig. 2). As already explained, the competition between DFO and hydroxyl ions for Zr(IV) binding was used to measure the stability of the Zr(IV)-DFO complexes, and therefore the known stability constants of Zr(IV) hydroxocomplexes (given in experimental section) were included in the calculations. In the first species, with the stability constant $\log\beta_{ZrHL} = 46.4(1)$, the three hydroxamate groups are assumed already bound to Zr(IV); the proton associated with the complex is on the free amine of the ligand which is not (apparently) displaced by Zr(IV). The $[ZrHL]^{2+}$ complex dominates the solution from pH 2 up to pH ~ 6.4. The deprotonation of $[ZrHL]^{2+}$ to $[ZrL]^+$ ($\log\beta_{ZrL} = 40.04(3)$) occurs with p*K* 6.36, which could be attributed to the deprotonation of a water molecule from the coordination sphere of Zr(IV). Analogous behaviour was observed for Zr(IV)-EDTA system, where the p*K* of the deprotonation of water was reported as 6.2 [45]. If this interpretation is correct, the $[ZrL]^+$ species should be rather noted as $[ZrHLH_{-1}]^+$ or even $[ZrHL(OH)]^+$. With further increase of pH, the last deprotonation step was observed, leading to $[ZrLH_{-1}]$ (or [ZrL(OH)]) complex ($\log\beta_{ZrLH_{-1}} = 29.15(9)$). The p*K* = 10.87 could be assigned to the deprotonation of the amino group of DFO ligand; the value is very close to the p*K* of free amino group (10.97, Table 1), and to the p*K* of amino group dissociation in Fe(III) (10.70, Table 1) and Ga(III) (10.14) [50] complexes, where it did not participate in the metal binding. The two above discussed dissociation steps were also observed for Th(IV) complexation to DFO, however, the pK_a were ascribed in reverse, *i.e.* 7.7 to the amino group dissociation and 10.3 to metal hydroxide formation [51].

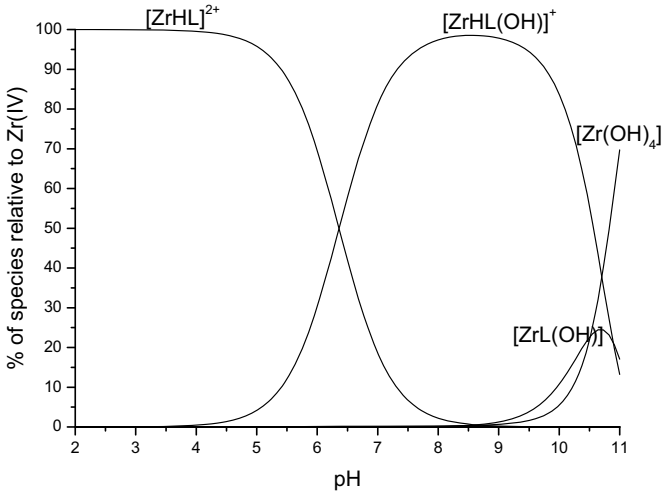

Fig. 2. Species distribution plot for Zr(IV)-DFO system at pH range 2–11, $c_{Zr(IV)} = 1$ mM, $c_{DFO} = 1$ mM, $I = 1$ M $NaClO_4$, $T = 25$ °C. Calculated from potentiometric titrations.

It has to be mentioned, that there might be an alternative interpretation of p*K* of 6.36. Due to a very strong tendency of Zr(IV) to hydrolysis, one cannot exclude a possibility of the formation of mixed Zr(IV) – DFO – OH complex even at acidic conditions. If this would be the case, p*K* of 6.36 might correspond to the substitution of an amide group (probably an amide oxygen) by the OH^- ion in the inner-sphere of Zr(IV) ion, suggesting for $[ZrHL]^{2+}$ a different coordination environment with respect to the structure proposed by DFT calculations [3]. Even if, to the best of our knowledge, there are some examples showing Zr(IV) binding to the oxygen from C(O)NH group [52,53] only when a stable 5-member ring with strong amine donor was created, and in the current Zr(IV)-DFO complex the formation of 7-member tensioned ring seems improbable, one cannot exclude such a possibility. Unfortunately, the ^{13}C NMR experiments performed to clarify the possibility of amide binding could not be conclusive due to a precipitation of Zr(IV)-DFO from the solution at pH 7.5 (due to high concentration of the ligand and complex = 3.2 mM, and long waiting time, *i.e.* up to 20 h, required to collect reasonable ^{13}C NMR spectra). All the stability constants determined for Zr(IV)-DFO system are collected in Table 1 and the species distribution diagram is shown in Fig. 2.

As the stability constants were determined by competition with hydroxide at high pH, and it is known that slight changes in pH are difficult to extrapolate in the high pH region of the titration curve corresponding to the formation of $Zr(OH)_4$ and the low pH region,

where only $[ZrHL]^{2+}$ complex was dominating, and estimation of the free Zr(IV) ions concentration by the difference of Zr(IV) total – Zr(IV) bound is not reliable, additional, UV–Vis spectrophotometric experiments were carried out. First, the UV–Vis titrations of Zr(IV)-DFO equimolar solution at pH range from 0.1 to 4.8 were performed (Fig. S5), in order to analyse the spectral changes corresponding to hydroxamate groups protonation state [43]. The UV–Vis spectra show a well-defined absorbance band in the 200–300 nm range (with λ_{max} = 225 nm), which do not reveal any significant changes at studied pH range. This behaviour confirms the results obtained from potentiometric data, and indicates that the three hydroxamate groups are dissociated, and therefore most probably bound to Zr(IV) ions, already at very acidic pH. The disappearance of the band with max at 230 nm was earlier clearly seen in acidic range for Ga(III)-DFO (and was associated with two protonation constants, pK_1 = 1.10 and pK_2 = 0.78) [50] and Th(IV)-DFO (with pK_1 = 1.9) [50] complexes.

Assuming the formation of only one, monomeric $[ZrHL]^{2+}$ complex at low pH range, the stability of Zr(IV)-DFO complexes was confirmed using a UV–Vis competition batch experiments. The competition titration was performed in perchloric media at pH 2, to prevent hydrolysis of the metal ions and to avoid the decomposition of DFO, which is known to occur under acidic conditions [50]. The metal competition titration, using Zr(IV) as a competing metal is demonstrated in Fig. 3a, and reflects the changes in UV–Vis absorbance upon the addition of up to 3 equiv. of Zr(IV) to a solution of Fe(III)-DFO; the large LMCT band centred at 430 nm characteristic of trihydroxamate $[FeHL]^{+}$ species decreased gradually.[1] The refinement of the titration data, using the Fe(III)-DFO stability constants (Table 1), together with the Fe(III) and Zr(IV) hydrolysis constants, yielded a $\log\beta_{ZrHL}$ value of 47.7(2) for $[ZrHL]^{2+}$. To confirm that the Fe(III)-DFO + Zr(IV) (Fig. 3a) exchange experiments gave a true equilibrium constant, additional experiments were performed, approaching the same equilibrium position by another metal competition, *i.e.* titrating Zr(IV)-DFO by Fe(III) (Fig. 3b), as well as titrating a solution with Fe(III) and Zr(IV) equimolar mixture by DFO (Fig. 3c), both performed at pH = 2. Although the spectra in Fig. 3b are dominated by absorbance of ferric species present in high excess (up to 300 equiv), the refinement of the spectral data led to identical $\log\beta_{111}$ value, 47.7(1) for $[ZrHL]^{2+}$. $\text{Log}\beta_{ZrHL}$ from spectral data given in Fig. 3c, was calculated as 47.3(6). Taking all three experiments together, an average value of $\log\beta_{ZrHL}$ = 47.6(3) was calculated.

3.3. pM of zirconium complexes

In order to compare the Zr(IV) binding ability of DFO with other potential PET chelators, pM values ($pM(Zr(IV)) = -\log[Zr(IV)_{free}]$, c_L = 10 μM, $c_{Zr(IV)}$ = 1 μM) were calculated at pH of 7.4 (Table 2). Higher pM value indicates stronger chelating ability of the ligand. The data clearly show, that even if pM(Zr(IV)) = 32.2 of DFO remains within the range for effective zirconium chelators, like diethylenetriaminepentaacetic acid (DTPA) (pM(Zr(IV)) = 33.9) [44,47,54], its efficacy is lower than the most efficient octadentate chelating ligand 3,4,3-LI(HOPO), for which the pM(Zr(IV)) = 44.0 is significantly higher [55]. The comparison of the pM(Zr(IV)) vales calculated for zirconium complexes of 3,4,3-LI(HOPO) and its monomeric chelating unit 1,2-HOPO-NH*Pr* [56] show about 10 orders of magnitude increase when the four binding units are joint in one molecule. For DFO, the pM(Zr(IV)) is only about four orders higher when compared to the Zr(IV) complexes of acetohydroxamic acid (AHA) [57], revealing the lower binding power of hexadentate chelator for 8-coordinate metal ion. All the data confirm that DFO is indeed not the best PET chelator, and the lower stability of complexes, together with the susceptibility of coordinated water molecule to deprotonation, must be the reason of *in vivo* instability of ^{89}Zr(IV) complexes leading to transchelation and

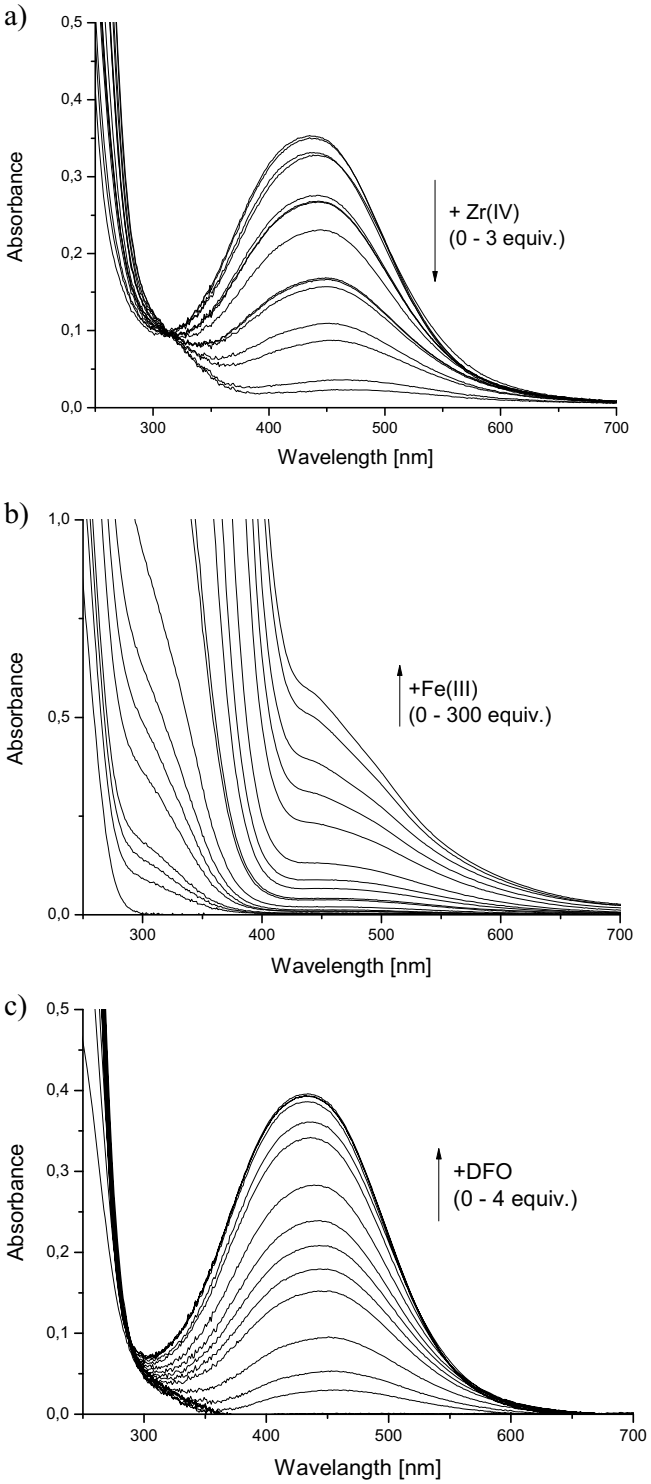

Fig. 3. Three types of competition titration experiments: a) Fe(III)-DFO complex titrated by Zr(IV) stock solution (starting from 0 equiv. up to 3 equiv.), $c_{Fe(III)} = c_{DFO}$ = 0.16 mM; b) Zr(IV)-DFO complex titrated by Fe(III) stock solution (starting from 0 equiv. up to 300 equiv.), $c_{Zr(IV)} = c_{DFO}$ = 0.15 mM; c) Zr(IV)-Fe(III) mix metals solution titrated by DFO stock solution (starting from 0 equiv. up to 4 equiv.) $c_{Zr(IV)} = c_{Fe(III)}$ = 0.18 mM; I = 1 M $NaClO_4$, T = 25 °C, pH 2.0.

[1] Although the Fe(III)-DFO speciation is already well characterized [49], in order to use the stability constants and spectral characteristics of appropriate complexes in competition experiments with Zr(IV), they all were redetermined under experimental conditions of this work (1 M $NaClO_4$); the experimental protocol and the results are given in Table 1 and SI.

Table 2
pM(Zr(IV)) values[a] with various synthetic and biological chelators.

Ligand	Donor set		pM(Zr(IV))
DFO	O_6 (3 hydroxamates)	This work	32.2
AHA	O_8 (4 hydroxamates)	[57]	28.9
DTPA	N_3O_5 (4 carboxylates, 3 amino groups)	[44,47,54]	33.9
3,4,3-LI(1,2-HOPO)	O_8 (4 1-hydroxy-2-pyridinonates)	[47,63]	44.0
1,2-HOPO-NH*Pr*	O_8 (4 1-hydroxy-2-pyridinonates)	[56]	33.5

[a] Values calculated at pH = 7.4 with $c_{Zr(IV)}$ = 1 μM and c_{DFO} = 10 μM, based on the constants calculated from potentiometric titrations and given in Table 1; hydrolysis constants of cations set to the values found in literature [8].

eventual accumulation of osteophilic ^{89}Zr(IV) in bone [16,58]. As shown on several examples, an additional chelating group included in DFO structure improved the *in vivo* stability of Zr(IV) complexes compared to DFO [24,59,60]. Of importance, thermodynamic stability is not the only parameter to be considered when discussing about *in vivo* stability of an imaging probe. Also, and most importantly, high kinetic inertness is the key factor that allows *in vivo* stability of the probe. To address the challenge of improving kinetic stability, a cyclisation of chelating compounds might be a choice. As an example, fusarinine C, a natural siderophore having three hydroxamate groups providing six oxygen donors for metal binding, arranged in cyclic structure, showed indeed superior resistance to transchelation and therefore superior stability in comparison to DFO [61]. In another approach, cyclen and cyclam were successfully used as macrocyclic scaffolds for the attachment of either three or four hydroxamate-based arms, again showing improved stability [62]. Unfortunately, the solution thermodynamic stability constants for these ligands were not determined and the order of increase cannot be quantified.

3.4. Isothermal titration calorimetry studies

The ITC method is a useful tool for the investigation of the metal-ligand interactions in solution and was used herein as an additional technique to prove the stoichiometry of the complex formed in solution of Zr(IV) and DFO at pH 1. This low pH was chosen in order to avoid hydrolysis of Zr(IV), which was used as a titrant at 8 mM concentration. The results in the form of binding isotherms, that depend on stoichiometry (n), binding constant (K_{ITC}) and change on enthalpy (ΔH^o_{ITC}), are shown in Fig. 4 with details of calculated fitting curves. The peak for the first injection of Zr(IV) stock solution into DFO solution was lower

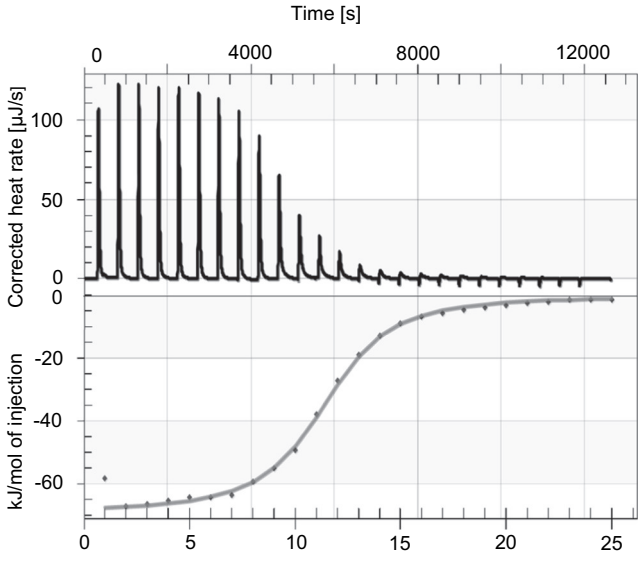

Fig. 4. The ITC experiments of the titration of DFO stock solution by Zr(IV), at pH 1.0; raw data (top) and calculated model (bottom).

Table 3
Data of ITC calculations[a].

Model	Independent
K_a	$4.64 \cdot 10^4$
ΔH^o_{ITC}	−69.19 kJ/mol
n	1.004
K_d	$2.16 \cdot 10^{-5}$
ΔS^o_{ITC}	−0.143 kJ/mol·K
ΔG^o	−26.64 kJ/mol

[a] $c_{Zr(IV)}$ = 8 mM, c_{DFO} = 1 mM, I = 1 M $NaClO_4$, T = 25 °C, pH 1.0.

than that of the second one which is a common phenomenon of dilution effect in ITC experiments. For this reason, the energetic effects of diluting DFO solutions as well as the effects of titration of Zr(IV) stock solutions with the solvents investigated were subtracted from the energetic effects of the titration measurements. Estimated values of K_{ITC}, n, ΔH^o_{ITC} and ΔS^o_{ITC} together with calculated Gibbs free energy ΔG_{ITC}, are given in Fig. 4, Table 3.The isotherm obtained for studied system could be fitted with 'independent site' model due to only one exothermic inflection point ($\Delta H^o_{ITC} < 0$) showed. Many classical interactions between ligand and metal ions are described by 'independent' model used to match the resulting binding isotherms that is based on 1 for 1 approach [64,65].

The inflection point in the titration curve, showing the enthalpy *vs.* metal-to-ligand molar ratio, clearly indicates that the stoichiometry of the complex formed at pH 1 is 1, which strongly supports the formation of equimolar complexes in Zr(IV)-DFO system, shown earlier by potentiometric calculations and ESI-MS data. The data reveals also that the complexation process is exothermic, and occurs with favourable enthalpy changes ($\Delta H < 0$). The binding between the metal and ligand is enthalpy driven and spontaneous process ($|\Delta H| > |T\Delta S|$, $\Delta G^o < 0$). The results of ITC measurements ($\Delta G^o < 0$, $\Delta H < 0$, $-T\Delta S > 0$) suggest unfavourable conformational changes in the complex.

Indeed, it is valuable when the results of two different methods, like ITC and potentiometry can lead to matching results. To relate the stability of Zr(IV)-DFO complexes obtained from potentiometric and ITC data, we have used Hyss program to calculate an apparent stability constant at pH = 1, including stability constants determined by potentiometry (Table 1) and concentration ranges used in ITC ($c_{Zr(IV)}$ from 0.08 up to 2 mM, c_{DFO} = 1 mM). The estimated $\log K_{app}$ = 10.51 seems to be definitely higher than $\log K_{ITC}$ = 4.67. However, it has to be underlined that titration calorimetry measures the sum of the heat, ΔH_{ITC}, associated with all processes occurring upon addition of aliquots of the titrant (here Zr(IV) stock solution), to the solution of ligand [64]. Although the heat of dilution of the titrant was corrected, the heat associated with other possible coupled processes, like the hydrolysis reactions of Zr(IV) ion or proton displacement from the DFO ligand upon metal binding, were not. As already indicated, the hydrolysis of Zr(IV) ions may be particularly insidious as it depends on the concentration and there is a significant dilution of the metal when it is injected into the DFO solution in the cell; still to minimize the hydrolysis influence we have chosen to perform the experiments at pH = 1. On the other

hand, under these experimental conditions the hydroxamate groups of DFO are protonated (pKa1 = 8.26, pKa2 = 9.21, pKa3 = 9.94, Table 1) and the coordination of Zr(IV) ions has to force the release of three protons. As it was previously shown for Ca^{2+} binding to EDTA, the ionization enthalpy is always unfavourable, and dramatically decreases metal affinity by reduction of the enthalpy term of the stability function [66]. Here, due to a very narrow experimental window caused by Zr(IV) hydrolysis problem on the one hand, and instability of DFO under very acidic conditions on the other hand, we could not determine the deprotonation contributions to ΔH_{ITC} and K_{ITC}. Other factors that may influence the ΔH_{ITC} and K_{ITC} are solvation effects that represent interactions between the metal ion/ligand and water molecules which are displaced when the complex is formed.

4. Conclusions

This work addresses itself to investigate the thermodynamic stability of Zr(IV)-DFO complexes, the data missing in the literature even if the DFO is currently the gold standard for ^{89}Zr(IV) chelation. Addressing the difficulties to characterise the aqueous solution chemistry of Zr(IV) complexes, in this paper we have used a fruitful combination of ESI–MS, potentiometric, spectroscopic and ITC physico-chemical techniques to determine the stoichiometry and thermodynamic stability of complexes formed in solution over pH range 1–11. As turned out, a model containing only mononuclear complexes, *i.e.* $[ZrHL]^{2+}$ $[ZrL]^{+}$, $[ZrLH_{-1}]$ throughout the entire measured pH range is proposed. The deprotonation of $[ZrHL]^{2+}$ to $[ZrL]^{+}$ ($\log\beta_{ZrL}$ = 40.04(3)) occurs with p*K* of 6.36, which could be attributed to the deprotonation of a water molecule from the coordination sphere of Zr(IV). Although DFO remains within the range for effective zirconium chelators (pM(Zr(IV)) = 32.2), the lower stability of unsaturated complexes, together with the susceptibility of coordinated water molecule to deprotonation, must be the reason of *in vivo* instability of ^{89}Zr(IV) complexes leading to transchelation and eventual accumulation of osteophilic ^{89}Zr(IV) in bone. We strongly believe that the results presented herein complete the knowledge on the Zr(IV)-DFO interactions, providing the speciation of complexes together with their stabilities, and might be useful when designing more effective PET chelators.

Acknowledgements

We thank Andrea Baldi (Faculty of Chemistry, University of Ferrara, Italy) for his skilful assistance in solution studies, and are grateful to Antonio Bianchi and Matteo Savastano (University of Florence, Italy) for fruitful discussions on equilibria possible in Zr(IV)-DFO system. Polish National Science Centre (NCN, UMO 2015/19/B/ST5/00413) is acknowledged for financial support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinorgbio.2019.110753.

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