Dalton Transactions

PAPER

View Article Online
View Journal | View Issue

Cite this: *Dalton Trans.*, 2014, **43**, 6689

Interaction of Cm(III) and Am(III) with human serum transferrin studied by time-resolved laser fluorescence and EXAFS spectroscopy

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Received 29th November 2013,
Accepted 16th February 2014

DOI: 10.1039/c3dt53371a

www.rsc.org/dalton

The complexation of Cm(III) with human serum transferrin was investigated in a pH range from 3.5 to 11.0 using time-resolved laser fluorescence spectroscopy (TRLFS). At pH ≥ 7.4 Cm(III) is incorporated at the Fe(III) binding site of transferrin whereas at lower pH a partially bound Cm(III) transferrin species is formed. At physiological temperature (310 K) at pH 7.4, about 70% of the partially bound and 30% of the incorporated Cm(III) transferrin species are present in solution. The Cm(III) results obtained by TRLFS are in very good agreement with Am(III) EXAFS results, confirming the incorporation of Am(III) at the Fe(III) binding site at pH 8.5.

Introduction

In the case of an accidental release of radionuclides to the environment, especially actinides cause a serious health risk upon incorporation into the body *e.g.* by wounds, ingestion or inhalation. In contrast to other metal ions, actinides have no essential function in the biochemistry of the human body.[1] Apart from chemical toxicity, their hazardousness depends on radiological toxicity. Availability and toxicity of the incorporated actinides in the body are mainly influenced by their concentration and speciation (chemical form and oxidation state).[2] Up to now, little is known about the chemical behavior and potential toxic effects of actinides in the human body. Hence, it is important to understand the mechanisms of relevant biochemical reactions with regard to the development of potential decontamination therapies.[3]

The chemistry of actinides in aqueous systems under physiological conditions (pH ~ 7.4) is dominated by the formation of complexes with available organic and inorganic ligands (OH^-, CO_3^{2-} *etc.*). Blood serum proteins have a high affinity to various metal ions and might be relevant for the biochemical behavior of incorporated actinides.[4] One representative of utmost importance is the iron carrier protein transferrin.

Human serum transferrin is a single-chain glycoprotein with a molecular mass of 79 570 kDa which consists of 679 amino acids.[5–7] Transferrin can bind to two Fe(III) ions and transport them to the cells where the transferrin metal complex is recognized by the receptor and taken up *via* endocytosis.[8,9] The ternary structure of transferrin is characterized by folding into two similar but not identical lobes which are joined by a short peptide chain.[10] Each lobe consists of α/β-subunits and is divided into two domains separated by a cleft housing the metal binding site. In both lobes the metal ion is coordinated by two tyrosines, one aspartate, one histidine and the synergistic anion in a distorted octahedral geometry.[11] The synergistic anion stabilizes the Fe(III) transferrin complex by coordinating as a bidentate bridging ligand to the protein and the metal ion. In living organisms the synergistic anion is carbonate, but other anions such as carboxylates can also play a role.[12–14] Although the coordination mode is the same at both binding sites, the C-terminal site is larger and has a higher affinity to Fe^{3+} than the smaller N-terminal site. At pH 7.4 the affinity of the binding site at the C-lobe to Fe(III) is about 20 times higher than that at the N-lobe.[15] The coordination of Fe(III) and the anion leads to a conformational change from an open to a closed form. This mechanism stabilizes the transferrin metal complex and is important for the recognition of the metal transferrin complex by the transferrin receptor (TfR).[5,16]

In regular blood serum only approximately 30% of the transferrin molecules are saturated with iron. Consequently, non-saturated transferrin is available for the complexation of other metal ions. Besides iron, about 30 other tri- and tetravalent metal ions have been identified to bind to transferrin, *e.g.* Ga(III),[17,18] Ru(III)[19,20] and lanthanide(III) ions like Nd(III).[21,22] Regarding the interaction of transferrin with actinides, a few studies have been performed with Th(IV), Pa(V), U(VI), Np(IV), Np(V), Pu(IV), Am(III) and Cm(III).[4,23–32] Most of them are phenomenological studies using methods like UV-visible spectroscopy or fluorescence spectroscopy of the protein with excitation of transferrin at 280 nm.[29] They give only semiquantitative results on the binding of transferrin to actinides and

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provide no information on the complexation mechanism, binding structure and number of species. The complexation of Np(IV), Pu(IV), Th(IV) and U(VI) with transferrin and nitrilotriacetic acid (NTA) as a synergistic anion was investigated using EXAFS spectroscopy.[25–27] Furthermore, the U(VI) transferrin system was studied by time-resolved laser fluorescence spectroscopy (TRLFS).[24] Stability constants were determined for U(VI) and Pu(IV).[24,33] The stability constants of Cm(III) and Am(III) were derived theoretically by using a mathematical correlation based on the first hydrolysis constants.[28,34] In a recent paper, the complexation of Cm(III) with transferrin was investigated at pH 8.6 using fluorescence spectroscopy with excitation of the protein at 280 nm.[29] Emission spectra of Cm_2Tf and Cm_CTf were obtained. The fluorescence lifetimes of Cm_CTf and Cm_NTf were estimated to be 220 and 206 μs, respectively, and correspond to about two water molecules and seven other ligands in the coordination environment of Cm(III) at both Fe(III) binding sites of transferrin. Furthermore, the stability constants for the N- and C-lobe of Cm_2Tf were determined experimentally to be log K_C = 8.8 ± 0.3 and log K_N = 7.0 ± 0.1 (pH 8.6), respectively.[29] K_C and K_N were derived from direct titration of transferrin with $CmCl_3$ and reverse NTA competition titrations using UV-visible and fluorescence spectroscopy with indirect and direct excitation of Cm(III) at 280 and 397 nm. Since there is no variation in pH the formation of further (non-specific) Cm(III) transferrin species cannot be excluded.

Time-resolved laser fluorescence spectroscopy (TRLFS) is a very sensitive method for determination of the speciation of lanthanides and actinides in submicromolar concentration ranges.[35] Because of its excellent fluorescence properties Cm(III) is used as a representative for trivalent actinides.[36] Fluorescence spectra of different Cm(III) species are characterized by the shape and position of the emission bands and provide information on the coordination environment of the metal ion. In contrast to the work of Sturzbecher-Hoehne *et al.* who studied the complexation of Cm(III) with transferrin at pH 8.6, the present work focuses on the pH dependency of the complexation reaction. Furthermore, the Cm(III) transferrin system was studied at physiological temperature. Fluorescence lifetimes of the pH dependent Cm(III) transferrin species were determined to obtain valuable information on the coordination structure.

In addition to the TRLFS measurements, extended X-ray absorption fine-structure (EXAFS) spectra of Am(III) transferrin at pH 7.2 and 8.5 were recorded to get detailed information on the coordination environment of the metal ion. These measurements were performed at low temperatures (77 K) to improve the signal-to-noise ratio.

Experimental section

Chemicals and sample preparation

The protein samples were prepared in TRIS or HEPES buffered solutions (10 mM, pH 7.4) with a physiological sodium chloride medium of 0.15 mM NaCl using ultrapure water (Millipore, Billerica, MA, USA; 18.2 MΩcm). Human serum apo-transferrin of high reagent grade (apo-transferrin human, 98%) was purchased from Sigma, Calbiochem and Applichem. Commercially available transferrin is obtained from human blood serum. Since about 30% of transferrin in the blood is complexed with Fe(III), a complexing agent *e.g.* EDTA or citrate salt is added to remove Fe(III) quantitatively.[37] Separation of the complexing agent with the bound Fe(III) from the transferrin using ultrafiltration yields Fe(III)-free transferrin. EDTA is a strong complexing ligand for trivalent actinides and the Cm(III) concentration used for the TRLFS measurements is quite low (c(Cm) = 1 × 10^{-7} M). Hence, it is necessary to purify the commercially available transferrin to remove the remaining traces of EDTA by size exclusion chromatography and filtration before use according to the protocol of Harris *et al.*[17,38] 100 mg of apo-transferrin were dissolved in 4 ml of 10 mM HEPES or TRIS buffer (pH 7.4) and passed through a 2 × 30 cm column (25 g Sephadex G-25 medium, GE Healthcare). After identification of the protein containing eluent fractions by TRLFS, the transferrin solution was passed through Amicon Centrifugal Filter Units (30 kDa) and washed five times each with 2 ml of a buffer solution. The protein concentration of the transferrin stock solution was determined by UV/Vis spectroscopy at λ = 280 nm using an extinction coefficient of ε = 93 000 M^{-1} cm^{-1}.[39]

The Cm(III) stock solution used for the TRLFS studies (c(Cm) = 3.33 × 10^{-6} M in 15.7 mM $HClO_4$) had an isotopic mass distribution of 89.7% Cm-248, 9.4% Cm-246, and ≤1% Cm-243, Cm-244, Cm-245 and Cm-247. The Cm(III) concentration of the TRLFS samples was fixed at 1.00 × 10^{-7} M by adding 30 μl of the Cm(III) stock solution to 970 μl of a buffered transferrin solution with a concentration of 4.85 × 10^{-6} M, resulting in a final transferrin concentration of 5.00 × 10^{-6} M. Complexation studies were carried out at varying pH values between 3.5 and 11.0. The pH was adjusted using NaOH and HCl solutions of different concentrations (1.0 M, 0.2 M, and 0.1 M). TRLFS measurements were performed at room temperature (296 K) and physiological temperature (310 K).

The concentration of the Am(III) stock solution used for the EXAFS measurements was c(Am-243) = 1.64 × 10^{-2} M in 0.5 M HNO_3. Two Am(III) transferrin samples were prepared with c(Am) = 5.00 × 10^{-4} M and c(Tf) = 2.40 × 10^{-3} M at pH 7.2 and 8.5. The Am(III) EXAFS measurements were performed at 70 K.

Analytical methods

Time-resolved laser fluorescence spectroscopy. TRLFS was performed using a Nd-YAG (Continuum Surelite Laser) pumped dye laser system (NARROWscan D-R Dye Laser) with a repetition rate of 10 Hz. For the excitation of Cm(III) a wavelength of 396.6 nm was used. Emission spectra were recorded in the range of 570 to 645 nm after a delay of 1 μs to discriminate short-lived fluorescence of organic compounds. After spectral decomposition by a spectrograph (Shamrock 303i) with a 900 lines mm^{-1} grating, the spectra were recorded with an ICCD camera (iStar Gen III, ANDOR) containing an

integrated delay controller. For better comparison all spectra are normalized to the same peak area.

For lifetime measurements the delay time between the laser pulse and the detection of the fluorescence emission was increased continuously with time intervals of Δt = 15 μs. The lifetime τ is obtained by fitting the fluorescence intensity I as a function of the delay time τ according to

$$I(\lambda) = I_0(\lambda) \cdot e^{-t/\tau} \quad (1)$$

with the initial intensity I_0 at t = 0.

EXAFS measurements. EXAFS measurements were performed at the INE-Beamline of the Angströmquelle Karlsruhe (ANKA, Karlsruhe, Germany). The beamline is equipped with a double-crystal monochromator (Si(111), Si(311), InSb(111), and Ge(422) crystal pairs available) and a mirror focusing system (Rh coated silicon mirrors). X-ray absorption spectra were collected in fluorescence mode using a 5 pixel high purity germanium solid state detector (Canberra Ultra-LEGe). The detector was positioned at an angle of 90° relative to the incoming beam. To obtain a better signal-to-noise ratio, the samples were cooled to 70 K using a liquid N_2 cryostat (OptistatDN, Oxford Instruments). A detailed description of the design of the INE-Beamline is given in Rothe *et al.*[40]

For energy calibration, a Zr foil was measured simultaneously with each sample. The data evaluation was performed using the software packages Athena 0.8.061 and EXAFSPAK.[41,42] Theoretical scattering phases and amplitudes were calculated with FEFF8.40 using the crystal structure of Fe-transferrin (RCSB protein database entry: 3QYT) and replacing Fe by Am.[43] The best theoretical model was used to fit the k^3-weighted raw Am L_{III}-edge EXAFS data using the Marquardt algorithm included in EXAFSPAK. The amplitude reduction factor S_0^2 was set to 0.9.

Results

Identification of transferrin impurities by TRLFS

The complexation of Cm(III) with commercially available apo-transferrins from different suppliers was investigated at pH 7.4 before and after purification using TRLFS. The Cm(III) spectra with non-purified transferrin show a sharp emission band at λ_{max} = 603.8 nm resulting from the formation of a Cm(III) EDTA complex (Fig. 1).[44] This confirms that commercially available transferrin is contaminated with EDTA. The intensity of the Cm(III) EDTA emission band at λ_{max} = 603.8 nm varies for different samples, indicating that transferrins from different suppliers contain varying amounts of EDTA (Fig. 2). Since the Cm(III) concentration used for TRLFS measurements is quite low (c(Cm) = 1 × 10^{-7} M) and EDTA is a strong complexing agent, transferrin has to be purified before usage in complexation studies. A reliable purification was performed using a combination of size exclusion chromatography followed by filtration. The quantitative separation of EDTA by size exclusion chromatography was monitored by addition of Cm(III) and by investigation of the eluent fractions with TRLFS.

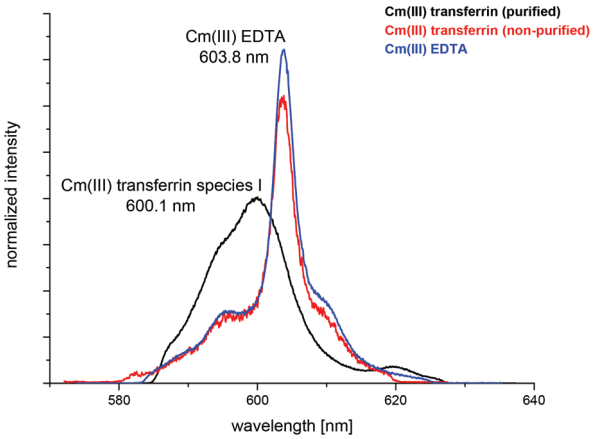

Fig. 1 Normalized fluorescence spectra of Cm(III) with purified and non-purified transferrin and EDTA at pH 7.4 in a TRIS buffered solution (10 mM); c(Cm) = 1.0 × 10^{-7} M, $c(Tf)_{purified}$ = 5 × 10^{-6} M, $c(Tf)_{non\text{-}purified}$ = 1.3 × 10^{-5} M, c(EDTA) = 1.7 × 10^{-5} M.

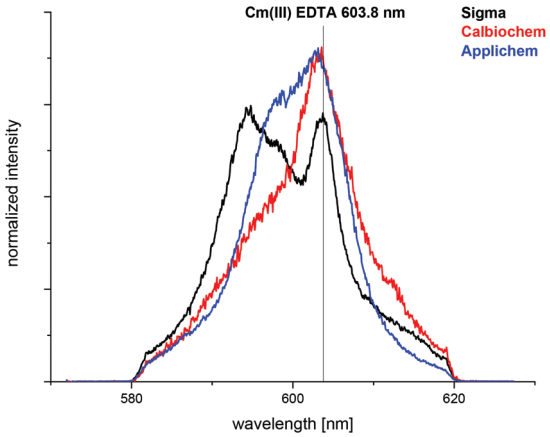

Fig. 2 Normalized fluorescence spectra of Cm(III) with non-purified transferrin from different suppliers at pH 7.4 in a HEPES buffered solution (10 mM); c(Cm) = 2.0 × 10^{-7} M, $c(Tf)_{Sigma}$ = 1.7 × 10^{-6} M, $c(Tf)_{Calbiochem}$ = 1.3 × 10^{-6} M, $c(Tf)_{Applichem}$ = 4.1 × 10^{-6} M.

Spectra of purified transferrin samples at pH 7.4 display an emission band at λ_{max} = 600.1 nm which can be attributed to the Cm(III) transferrin species I (see the next section).

TRLFS studies of Cm(III) transferrin complexation

Complexation studies of Cm(III) with transferrin at varying pH and room temperature. Complexation of Cm(III) with transferrin as a function of pH was investigated at room temperature (296 K) using two samples with an initial pH of 7.4 corresponding to physiological blood serum conditions and adjusting the pH of the samples stepwise with HCl or NaOH, respectively. At each pH step a fluorescence spectrum was recorded using an excitation wavelength of 396.6 nm. The normalized fluorescence spectra are shown in Fig. 3. TRLFS measurements in TRIS and HEPES buffered solutions yield identical spectra which proves that there is no interaction of Cm(III) with buffer molecules in the pH range from 3.5 to 11.0.

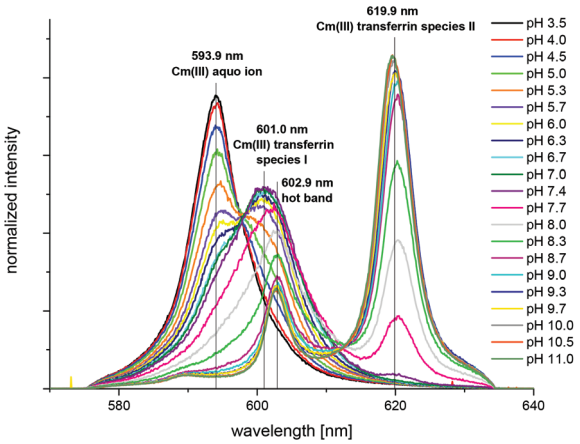

Fig. 3 Normalized fluorescence spectra of the Cm(III) transferrin complexation at room temperature in a TRIS buffered solution (10 mM) in the pH range between 3.5 and 11.0; c(Cm) = 1.0 × 10^{-7} M and c(Tf) = 5.1 × 10^{-6} M.

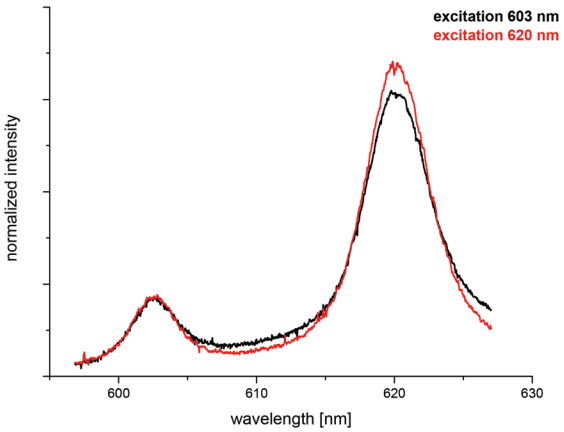

Fig. 4 Normalized fluorescence spectra of the Cm(III) transferrin species II upon excitation at 603 nm and 620 nm; c(Cm) = 1.0 × 10^{-6} M and c(Tf) = 5.0 × 10^{-5} M, pH 9.0.

The spectra reveal a strong pH dependency of the complexation reaction. Up to pH 5.3 the system is dominated by the Cm(III) aquo ion, displaying an emission maximum at λ_{max} = 593.9 nm.[44–46] With increasing pH the emission band at 601.0 nm increases. Comparison with blank solutions (without transferrin) in the same pH range proves that this emission band results from Cm(III) transferrin interaction (Cm(III) transferrin species I). This Cm(III) transferrin species dominates in the pH range between 6.3 and 7.7. At higher pH another Cm(III) transferrin species II is formed and becomes the dominating species for pH ≥ 8.3. The spectra display a sharp emission band with an emission maximum at λ_{max} = 620.3 nm. The bathochromic shift between the emission bands of the Cm(III) aquo ion and both Cm(III) transferrin species originates from an increased complexation of the metal ion in concert with a larger ligand field splitting upon bonding to the protein. For the emission band of the Cm(III) transferrin species II the bathochromic shift of 26 nm relative to the Cm(III) aquo ion is extraordinary and indicates incorporation of Cm(III) at the Fe(III) transferrin binding site.

The spectrum of the Cm(III) transferrin species II is further characterized by two hot bands with emission maxima at λ_{max} = 602.9 nm and 589.0 nm (very weak). To confirm that the emission band at 602.9 nm is a hot band and not the emission band of an additional Cm(III) transferrin species, direct excitation of the emitting state was performed using an excitation wavelength of 603 nm and 620 nm respectively (c(Cm) = 1.0 × 10^{-6} M and c(Tf) = 5.0 × 10^{-5} M, pH 9.0). As a result, excitation at 603 nm and 620 nm yields the same emission spectra confirming the existence of only one Cm(III) transferrin species at high pH (Fig. 4). The spectrum of the Cm(III) transferrin species II with the emission band at λ_{max} = 620.3 nm and two hot bands at λ_{max} = 602.9 nm and 589.0 nm corresponds to the results of Sturzbecher-Hoehne *et al.*[29] The authors determined identical emission spectra of Cm_2Tf and Cm_CTf at pH 8.6 using direct excitation of the protein at λ = 280 nm.

The fluorescence spectra of the pure components (Cm(III) aquo ion and the two Cm(III) transferrin species) were determined from the pH dependent fluorescence spectra and are shown in Fig. 5. The fractions of the three species at various pH values were determined by peak deconvolution of the emission spectra using the spectra of the pure components.

Since the total fluorescence intensity changed with varying pH, fluorescence intensity factors (f_i factors) of the different Cm(III) transferrin species had to be determined and taken into account for the calculation of the concentration ratios. f_i factors describe the decrease or increase of the fluorescence intensity relative to a reference species, in this case the Cm(III) aquo ion. They are determined from the overall fluorescence intensity of the spectra normalized to the concentration of Cm(III) in the sample and the energy of the laser beam as a function of pH according to eqn (2).[47,48]

$$x_{aq} f_i(aq) + x_{tf1} f_i(tf1) + x_{tf2} f_i(tf2) = I \quad (2)$$

x_{aq}, x_{tf1} and x_{tf2} are the peak areas of the three species determined by peak deconvolution, f_i(aq), f_i(tf1) and f_i(tf2) the

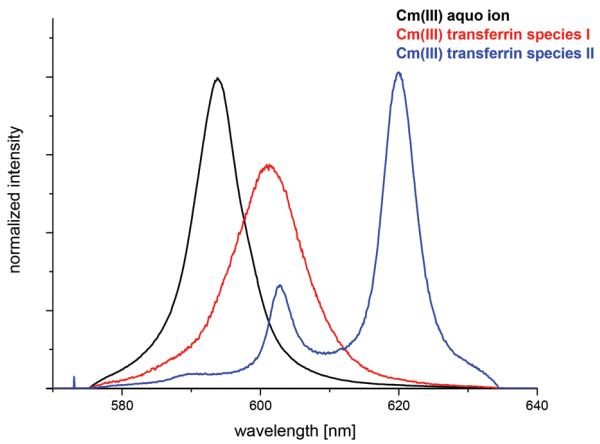

Fig. 5 Normalized fluorescence spectra of the Cm(III) aquo ion, the Cm(III) transferrin species I and the Cm(III) transferrin species II.

fluorescence intensity factors and I the overall fluorescence intensity. Defining a f_i factor of 1 for the Cm(III) aquo ion, the f_i factors of the two transferrin species were derived to be 0.44 for Cm(III) transferrin species I and 0.73 for Cm(III) transferrin species II. Fig. 6 shows the measured and calculated fluorescence intensity using eqn (2) and the f_i factors of the Cm(III) transferrin species I and II. The error in the fluorescence intensity is mainly caused by fluctuations of the laser energy and is estimated to be 10%. The experimentally determined values are in very good agreement with the fitted line.

Generally, complexation with organic ligands results in an increase of f_i factors. Depending on the ligands, f_i factors of up to 165 have been reported.[49] An increase in fluorescence intensity is based on intramolecular ligand-to-metal energy transfer.[50] This requires an overlap between the metal and ligand orbitals.[51] The fluorescence intensity factors observed for the Cm(III) transferrin species I and II excited at 396.6 nm were determined to be <1. They are comparable to f_i factors found for Cm(III) sorption processes on mineral surfaces or the formation of colloid-born Cm(III) species.[52,53] The small f_i factors of both Cm(III) transferrin species indicate that no energy transfer from the ligand to the metal occurs upon excitation at 396.6 nm as the absorption coefficient of transferrin at this wavelength is rather low. In contrast to this, Sturzbecher-Hoehne *et al.* estimated the fluorescence emitted from the Cm(III) transferrin species II excited at 280 nm (absorption maximum of the protein) to be at least 10^7 times more intense than that observed upon excitation at 397 nm (excitation of Cm(III)), indicating an excellent energy transfer from the protein to the metal.[29]

The fluorescence intensity factors of the three Cm(III) species were used for the calculation of the species concentrations as a function of pH (species distribution, Fig. 7). The Cm(III) aquo ion dominates the speciation up to pH 4.7, although the concentration decreases with increasing pH. The Cm(III) transferrin species I is formed in the pH range from 3.5 to 9.7 and becomes the dominating species between pH 5.0 and 8.2. Above pH 7.0 the concentration of the Cm(III) transferrin species II increases continuously until this species is solely present in solution above pH 9.7.

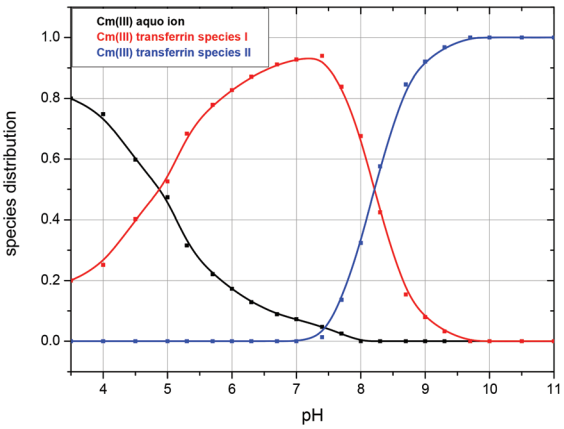

Fig. 7 Speciation of Cm(III) with transferrin as a function of pH in a 10 mM TRIS buffered solution at room temperature (296 K); c(Cm) = 1.0×10^{-7} M and c(Tf) = 5.1×10^{-6} M.

In order to characterize the different complex species, fluorescence lifetimes of the Cm(III) transferrin complexes were determined. The fluorescence lifetime of the Cm(III) transferrin species II was measured at high pH where this species is formed as a pure component. As an example, Fig. 8 shows the decay of the fluorescence emission of Cm(III) as a function of the delay time at pH 9.7. The fluorescence decay is identical for both, the emission bands at 620.3 nm and 602.9 nm, confirming the existence of only one Cm(III) transferrin II complex with the emission band at 602.9 nm being a hot band. With $\tau = 1/k_{obs}$ the fluorescence lifetime is calculated from the slope (k_{obs} = 0.00453 μs^{-1}) of the linear plot. This yields an average fluorescence lifetime of τ = 221 ± 5 μs for the Cm(III) transferrin species II resulting from several measurements.

The determination of the fluorescence lifetime of the Cm(III) transferrin species I is more complicated as it does not exist as a pure component at any pH value. For several samples

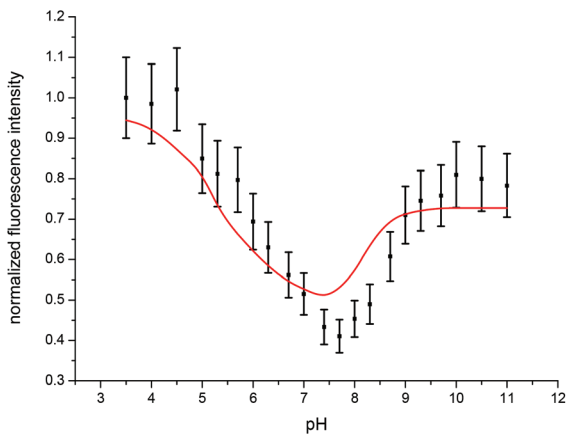

Fig. 6 Experimentally determined (black) and calculated (red) fluorescence intensities of the emission spectra of the Cm(III) transferrin complexation as a function of pH at 296 K.

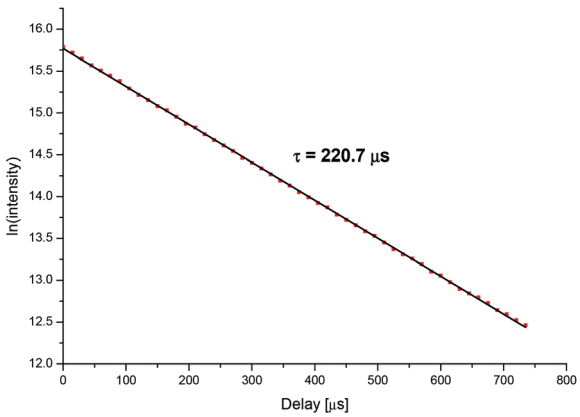

Fig. 8 Decay of the fluorescence intensity of the Cm(III) transferrin species II as a function of the delay time, pH 9.7; c(Cm) = 1.0×10^{-7} M and c(Tf) = 5.1×10^{-6} M.

containing Cm(III) and transferrin at pH 6.0 and 7.0 the decay of the fluorescence emission of Cm(III) as a function of delay time was fitted using a biexponential decay function. The fluorescence lifetime of the short-lived species was fixed at τ = 65 μs (lifetime of the Cm(III) aquo ion).[46] Biexponential fitting of the results of several lifetime measurements provides an average fluorescence lifetime of τ = 129 ± 20 μs for the Cm(III) transferrin species I. The increased error is explained by the biexponential fitting procedure in comparison to the monoexponential decay of the fluorescence of the Cm(III) transferrin species II.

Kinetic studies of the Cm(III) transferrin complexation reaction. Many bioligands like DOTA or other chelating ligands show slow complexation kinetics.[54–57] Since the effective ionic radius of Cm(III) is about two times larger than that of Fe(III) it is expected not to fit into the Fe(III) binding site of transferrin which might result in slow complexation kinetics.[58] The kinetics of the complexation reaction of Cm(III) with transferrin was analyzed at different pH values using TRLFS in the time range from 0 to 96 hours. Fig. 9 depicts the normalized fluorescence spectra of two Cm(III) transferrin samples at pH 7.4 and 9.0 at increasing time up to 96 hours. The spectra show that the emission bands of the Cm(III) transferrin species I at pH 7.4 and II at pH 9.0 do not change with time which indicates extremely fast kinetics of the complex formation of the transferrin species. At both pH values, equilibrium was already reached after five minutes. Therefore, measurements without long equilibration periods are legitimate for all studies on the interaction of Cm(III) with transferrin.

Complexation studies of Cm(III) with transferrin at varying pH and physiological temperature. In addition to the results shown above, the complexation of Cm(III) with transferrin at body temperature (310 K) was investigated using two samples at pH 7.4 corresponding to the physiological pH value in blood serum and adjusting the pH of the samples stepwise with HCl or NaOH. The normalized fluorescence spectra in the pH range from 3.5 to 11.0 are shown in Fig. 10. The spectra correspond to those measured at room temperature and display the emission bands of the Cm(III) aquo ion at λ_{max} = 594.0 nm, the Cm(III) transferrin species I at λ_{max} = 600.6 nm and the Cm(III) transferrin species II at λ_{max} = 620.3 nm with the hot band at λ_{max} = 602.9 nm.

In accordance with the measurements at room temperature the total fluorescence intensity changes with varying pH. Due to the temperature dependency of f_i factors, it is necessary to determine the fluorescence intensity factors of the Cm(III) transferrin species at 310 K using eqn (2). The f_i factor of the Cm(III) aquo ion was set to 1 which results in f_i factors of 0.58 for the Cm(III) transferrin species I and 1.04 for the Cm(III) transferrin species II. Comparison with the fluorescence intensity factors at room temperature ($f_{i(\text{Cm-Tf I})}$ = 0.44, $f_{i(\text{Cm-Tf II})}$ = 0.73) shows an increase of 0.14 and 0.31, respectively, and confirms the temperature dependency of the f_i factors. Fig. 11 shows the measured and calculated fluorescence intensities according to eqn (2) using the determined f_i factors. The fluorescence intensity factors of the three Cm(III) species were used for the calculation of the species concentrations as a function of pH (species distribution, Fig. 12). In comparison to the results at room temperature, the formation of the Cm(III)

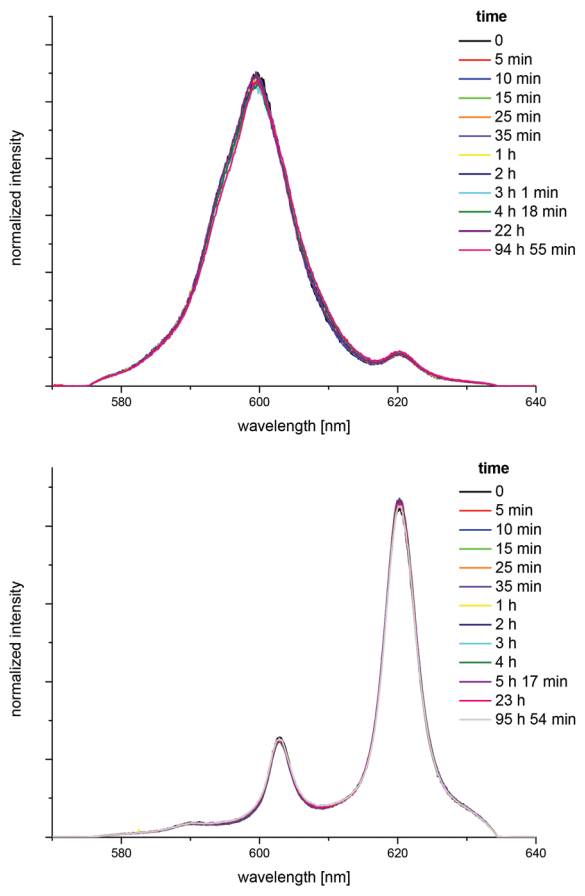

Fig. 9 Normalized fluorescence spectra showing the kinetics of the Cm(III) transferrin complexation at pH 7.4 (top) and pH 9.0 (bottom) in HEPES buffered solution (10 mM); c(Cm) = 1.0 × 10^{-7} M and c(Tf) = 5.1 × 10^{-6} M, time range 0–96 h.

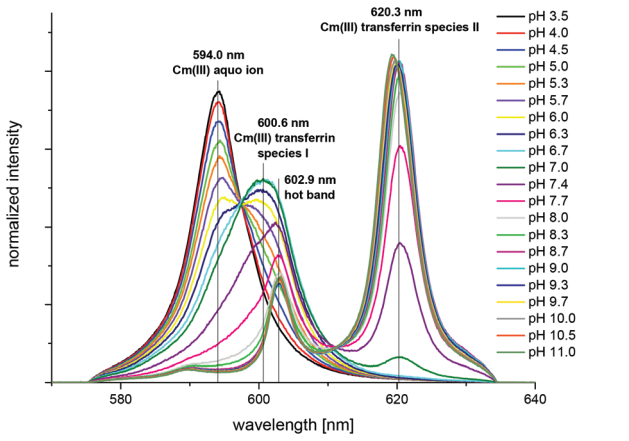

Fig. 10 Normalized fluorescence spectra of the Cm(III) transferrin complexation at 310 K in a TRIS buffered solution (10 mM) in the pH range between 3.5 and 11.0; c(Cm) = 1.0 × 10^{-7} M and c(Tf) = 5.1 × 10^{-6} M.

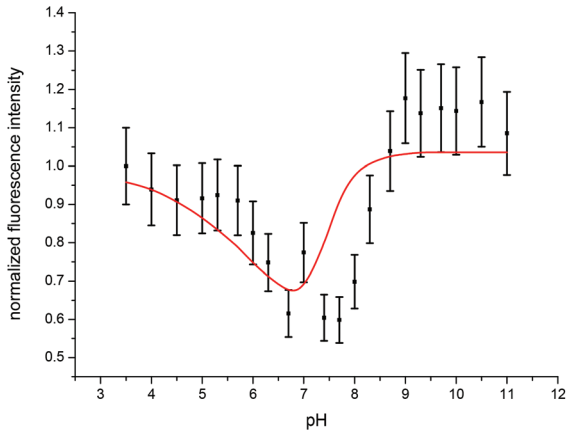

Fig. 11 Experimentally determined (black) and calculated (red) fluorescence intensities of the emission spectra of the Cm(III) transferrin complexation as a function of pH at 310 K.

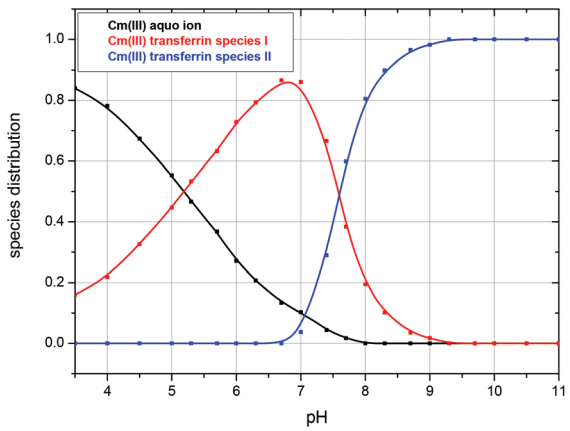

Fig. 12 Speciation of Cm(III) with transferrin as a function of pH in a 10 mM TRIS buffered solution at physiological temperature (310 K); c(Cm) = 1.0 × 10^{-7} M and c(Tf) = 5.1 × 10^{-6} M.

transferrin species II starts at lower pH values. The Cm(III) transferrin species I dominates in the pH range between 6.0 and 7.4 whereas the Cm(III) transferrin species II becomes the dominating species above pH 7.5. Comparison at the physiologically relevant pH value of 7.4 shows almost exclusive formation of the Cm(III) transferrin species I at room temperature whereas at elevated temperature 70% of the Cm(III) transferrin species I and 30% of the Cm(III) transferrin species II are formed (see the Discussion section).

EXAFS measurements of Am(III) transferrin

To complement the Cm(III) TRLFS results, the molecular structure of 0.5 mM Am(III) in the presence of 2.4 mM transferrin has been investigated by EXAFS at low temperature (77 K) at pH 7.2 and 8.5. The background corrected k^3-weighted Am L_{III}-edge EXAFS spectra are presented together with the related Fourier transforms in Fig. 13.

The recorded EXAFS spectra at different pH values (Fig. 13, left) show clear differences indicating significant structural differences of the Am(III) species. The EXAFS-oscillations are visibly shifted (highlighted by the vertical lines in Fig. 13) and their amplitude is reduced when the pH is increased from 7.2 to 8.5. Comparison of the related Fourier transforms (Fig. 13, right) shows a slight but visible shift of the main peak to lower distance with increasing pH (again highlighted by a vertical line). The results of the fits of both spectra are summarized in Table 1. The spectrum at pH 7.2 is fitted by 8–9 oxygen atoms at a distance of 2.47 Å which is in good agreement with literature data for the aquo species of Am(III) and other trivalent actinides.[59] Also the formation of a weak binding of Am(III) to transferrin resulting in an unspecific or partially bound species (comparable to the Cm(III) transferrin species I) cannot be excluded. The spectrum at pH 8.5 has been fitted with one, two or three shells. In all cases, about 9 nearest neighbors are present in the first coordination sphere at a lower distance of 2.38 Å as expected in the case of strong multidentate coordination of Am(III) at the transferrin binding cleft. Unspecific binding to functional groups of the protein surface would

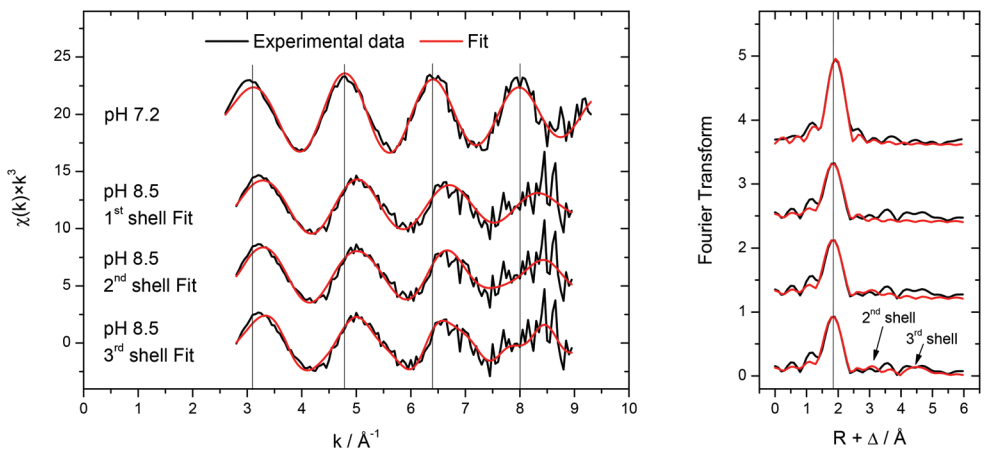

Fig. 13 k^3-weighted Am L_{III}-edge EXAFS spectra (left) and related Fourier transforms (right) for 0.5 mM Am(III) in the presence of 2.4 mM transferrin at pH 7.2 and 8.5 recorded at T = 77 K. For the spectrum at pH = 8.5, three different fits are shown (fit parameters, see Table 1).

Table 1 Structural parameters of 0.5 mM Am(III) in the presence of 2.4 mM transferrin at pH 7.2 and 8.5 recorded at T = 70 K (errors are given in brackets). The spectrum at pH 8.5 has been fitted with one, two or three shells. S_0^2 was set to 0.9

	1st shell			2nd shell			3rd shell				
pH	N	R/Å	σ/Å^2	N	R/Å	σ/Å^2	N	R/Å	σ/Å^2	ΔE_0	Red. Er.
7.2	8.7 (0.5)	2.47 (1)	0.007 (1)	—	—	—	—	—	—	−6.2 (0.6)	0.41
8.5	8.8 (1.0)	2.38 (1)	0.013 (2)	—	—	—	—	—	—	−3.8 (1.0)	0.67
	8.6 (1.0)	2.38 (1)	0.012 (2)	3.0 (1.0)	3.73 (3)	0.003[a]	—	—	—	−3.8 (0.9)	0.63
	8.8 (1.0)	2.38 (1)	0.013 (2)	2.8 (1.0)	3.72 (3)	0.003[a]	6.8 (2.3)	5.19 (3)	0.003[a]	−4.0 (0.9)	0.59

[a] Held constant during fit.

result in longer distances and can therefore be excluded. Comparing this Am(III) transferrin distance to the iron–ligand distances of human serum transferrin calculated by DFT, the present value of 2.38 Å is higher than the reported average distances of Fe(III) transferrin between 1.90 and 2.30 Å.[60] The differences are related to the two times larger effective ionic radius of Am(III) compared to Fe(III) ($IR_{Fe(III)}$ = 55 pm, $IR_{Am(III)}$ = 97.5 pm; the values are valid for a coordination number of six and Fe(III) low spin configuration).[58] Furthermore, the coordination number has to be considered. The determined coordination number of ≈9 exceeds the expected value of 6 resulting from an octahedral coordination at the Fe(III) binding site of the transferrin molecule.

The inclusion of a second and a third coordination sphere with 2.8–3.0 neighbors at 3.72–3.73 Å and 6.8 neighbors at 5.19 Å in the fit leads to a slight but visible improvement of the fit as the reduced error decreases from 0.67 to 0.59 (Table 1). Due to the small difference in atomic number and the highly complex structure of transferrin, it is not possible to determine whether the different coordination shells consist of carbon, nitrogen, oxygen atoms or a mixture of them. Nevertheless, the presence of coordination shells at distances up to ~5 Å in combination with the short oxygen distance in the first coordination sphere clearly indicates the presence of Am(III) in a complex but well-defined bonding environment within the transferrin molecule at pH = 8.5.

Discussion

Whereas in the literature the formation of only one Cm(III) transferrin species has been reported, it was possible to identify unambiguously different Cm(III) transferrin species (Cm(III) transferrin species I and II) as a function of pH for the first time using TRLFS. The spectra show a strong pH dependency of the complexation reaction of Cm(III) with transferrin at room temperature. The different Cm(III) transferrin species dominate in the pH ranges from 5.0 to 8.0 and 8.3 to 11.0, respectively. The spectrum of the Cm(III) transferrin species II with the emission band at λ_{max} = 620 nm and two hot bands with emission maxima at 602.9 nm and 589.0 nm corresponds to the spectrum observed by Sturzbecher-Hoehne *et al.* upon excitation of transferrin at 280 nm and energy transfer to Cm(III) at pH 8.6.[29] The shift of the emission band of a complex species relative to the emission band of the Cm(III) aquo ion is a measure of the ligand field splitting and is related to the complexation strength of the ligands. The emission band of the Cm(III) transferrin species I at 601.0 nm is shifted 7.2 nm relative to the aquo ion which is in the usual range of Cm(III) complexes with organic ligands.[57,61–63] In contrast to this, an extraordinarily large shift as observed for the emission band of the Cm(III) transferrin species II (λ_{max} = 620 nm) of 26 nm has so far only been reported for Cm(III) incorporated *e.g.* into calcite or Ca-montmorillonite.[64–67] Furthermore, the emission band at 620.0 nm is extremely sharp which is characteristic of complexes with chelating ligands (*e.g.* EDTA, Fig. 1) whereas the emission band of the Cm(III) transferrin species I is significantly broader as it was observed for various Cm(III) sorption species.[67–70] This indicates incorporation of Cm(III) into the Fe(III) binding site of transferrin above pH 8.3 and the formation of a species with partial complexation of Cm(III) to the protein in the pH range from 5.0 to 8.0. Between pH 7.4 and 9.0 both Cm(III) transferrin species are present.

Although the binding mode is the same at both binding sites of transferrin they are not kinetically and thermodynamically equivalent. The C-terminal site is larger and has a higher affinity to the complexation of metal ions than the smaller N-terminal site. The log K values for the complexation of Cm (III) to the N- and C-lobe at pH 8.6 have been determined experimentally to be log K_C = 8.8 ± 0.3 and log K_N = 7.0 ± 0.1.[29] They differ by almost two orders of magnitude which underlines the inequality of the binding sites. We therefore used a high excess of transferrin for the TRLFS measurements to ensure exclusive coordination of Cm(III) at the C-terminal binding site and the formation of a Cm(III) transferrin complex with a 1 : 1 stoichiometry.

Furthermore, the fluorescence lifetimes of the different Cm(III) species were determined. According to the empirical Kimura equation

$$n_{H_2O} = 0.65k_{obs} - 0.88, \quad k_{obs} = 1/\tau \qquad (3)$$

the fluorescence decay constant k_{obs} is correlated with the number of water molecules in the first coordination sphere.[71,72] The fluorescence lifetime of the Cm(III) transferrin species II of τ = 221 ± 5 μs is in good agreement with the lifetimes of Cm_CTf (220 μs) and Cm_NTf (206 μs) reported previously supporting the assumption that Cm(III) is incorporated

at the C-terminal binding site.[29] The fluorescence lifetime corresponds to two quenching water molecules in the first coordination sphere. According to solid state structural investigations, at the metal binding sites of transferrin Fe(III) is coordinated by four amino acid residues (two tyrosine, one histidine and aspartic acid) and a synergistic carbonate anion occupying the fifth and sixth coordination sites.[11] Assuming an overall coordination number of nine for Cm(III), the TRLFS results show that Cm(III) is coordinated by four amino acids of apo-transferrin, two H_2O molecules and three other ligands. In principle, OH^-, CO_3^{2-} and HCO_3^- which are present in solution are capable of acting as a synergistic anion. The proposed structure of the Cm(III) transferrin species II is displayed in Fig. 14.

The fluorescence lifetime of the Cm(III) transferrin species I was determined to be $\tau = 129 \pm 20$ μs which corresponds to 4.2 ± 1.0 coordinated water molecules. This suggests that Cm(III) is coordinated by four to five ligands (amino acids of the protein and synergistic anions like OH^-, CO_3^{2-} and HCO_3^-). The broad emission band in combination with the bathochromic shift of 7.2 nm indicates the formation of a Cm(III) species partially bound to transferrin indicating slight variation in the inner coordination sphere of Cm(III). This binding is supposed to occur presumably at the binding site although complexation at a nonspecific site cannot be excluded.

Since there is no literature data of Cm(III) complexed with transferrin available the presented results will be compared to literature data of Gd(III) transferrin. Gd is the lanthanide homolog of Cm. Both trivalent ions are characterized by similar physical and chemical properties and comparable effective ionic radii (nine-fold coordination: $IR_{Gd(III)} = 110.7$ pm, $IR_{Cm(III)} = 114.6$ pm) resulting in similar complex formation with transferrin.[58,73] UV/Vis, ESR and NMRD measurements show that specific and stoichiometric complexation of Gd(III) to the binding sites of transferrin occurs predominantly at pH ≥ 8.[74] At pH ≤ 7 Gd(III) is bound to nonspecific sites of transferrin. This leads to a complex coordination chemistry in the physiologically relevant pH range involving the formation of Gd(III) transferrin complexes at the specific (Fe(III) binding site) and nonspecific binding sites. These results are in good agreement with the formation of a Cm(III) transferrin species incorporated at the Fe(III) binding site above pH 7 and a partially coordinated Cm(III) transferrin species (possibly at a nonspecific site) at lower pH values.

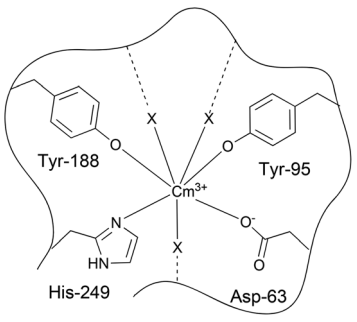

Fig. 14 Proposed structure of the Cm(III) transferrin species II; X represents additional ligands, such as carbonate, bicarbonate and/or hydroxide (the two coordinating water molecules are not shown).

In comparison with TRLFS results at room temperature the complexation of Cm(III) with transferrin at physiologically relevant temperature was investigated. The spectra show the formation of the same Cm(III) transferrin species with emission bands at 600.6 nm and 620.3 nm respectively and a "hot band" at 602.9 nm as obtained at room temperature. The Cm(III) transferrin species II is formed at pH values about 0.5 units lower compared to room temperature conditions. At physiological temperature (310 K) at pH 7.4 about 70% of the Cm(III) transferrin species I and 30% of the Cm(III) transferrin species II are formed whereas the species distribution at room temperature is dominated by more than 90% of the Cm(III) transferrin species I. This change in the speciation of the Cm(III) transferrin species with increasing temperature involves an increase of the stability constants of the Cm(III) transferrin species of 1.3 orders of magnitude.

The Cm(III) TRLFS results are compared to EXAFS results of the Am(III) transferrin system at high pH (pH 8.5). The average distance of 2.38 Å as well as the presence of several shells at higher distances indicate the complexation of Am(III) at the Fe(III) binding cleft which is in accordance with the formation of the Cm(III) transferrin species II. The speciation of Cm(III) at pH 7.0 is dominated by the Cm(III) transferrin species I, whereas the EXAFS results show the formation of more Am(III) aquo ion at pH 7.2. Since a weak binding of Am(III) to transferrin might result in only slightly smaller distances compared to the Am(III) aquo ion, the formation of a certain amount of an unspecific or partially bound Am(III) transferrin species (comparable to the Cm(III) transferrin species I) cannot be excluded. Am(III) and Cm(III) have comparable physical and chemical properties ($IR_{Cm(III)} = 98.0$ pm, $IR_{Am(III)} = 97.5$ pm; the values are valid for a coordination number of six).[75] Furthermore, the theoretically derived log K values for the complexation of both metal ions to the C- and N-lobe of transferrin are almost identical (Cm(III): log $K_C = 10.3$, log $K_N = 8.7$; Am(III): log $K_C = 10.1$, log $K_N = 8.5$).[28] Slight discrepancy in the speciation of Am(III) and Cm(III) with transferrin cannot be attributed to a different complexation behaviour but might be due to significant differences in the ligand to metal ion concentration ratios. Furthermore, the Am(III) EXAFS measurements were performed at low temperature (77 K) while the complexation of Cm(III) with transferrin was studied at room and body temperatures. Our TRLFS results on Cm(III) transferrin show a strong temperature dependency of the complexation reaction which contributes to the differences in the speciation of Cm(III) and Am(III) with transferrin.

The average distance of the 9 nearest neighbors (2.38 Å) of Am(III) transferrin at pH 8.6 is slightly higher compared to the distances found for the Pu(IV)/Np(IV)-NTA-Tf system at pH 8.0 (4 O/N at 2.21–2.23 Å and 5 O/N at 2.38–2.39 Å).[27] This can be explained by the modified coordination environment of the metal ion at the binding site of transferrin when NTA is used as an additional anion.

Under physiological conditions Fe(III) is incorporated at the binding site and forms a stable complex with transferrin until the pH is lowered to 5.3 during endocytosis. In contrast to this, the incorporated Cm(III) transferrin species II is formed above pH 7.0 and becomes the dominant species above pH 7.7. At pH 7.4, both Cm(III) transferrin species (I and II) exist. The differences in the complexation behavior of Cm(III) and Fe(III) to transferrin are attributed to the two times larger effective ionic radius of Cm(III) relative to that of Fe(III) ($IR_{Fe(III)}$ = 55 pm, $IR_{Cm(III)}$ = 97 pm; the values are valid for a coordination number of six and Fe(III) low spin configuration).[58] This means that Cm(III) does not fit optimally into the binding cleft which leads to a reduced incorporation of Cm(III) requiring higher pH values than for Fe(III). Coordination of Fe(III) at the binding site results in a domain closure which is important for the recognition of the metal transferrin complex by the receptor.[5,16] Since a certain amount of Cm(III) is also incorporated at the transferrin binding site at physiological temperature the important question arises whether the Cm(III) transferrin complex might be recognized by the receptor as well and brought into cells *via* endocytosis.[29] First studies by Sturzbecher-Hoehne *et al.* show that the shape of the emission spectrum and the fluorescence lifetime of Cm_2Tf remain unaltered upon receptor binding indicating that the coordination environment of Cm(III) does not change when the protein is coordinated to the receptor.[29] Deblonde *et al.* determined stability constants of the TfR:$(M_xTf)_y$ adducts which follow the order Fe(III) ≫ Th(IV) ∼ U(VI) ∼ Cm(III) > Ln(III) ∼ Ga(III) ⋙ Yb(III) ∼ Pu(IV) using a high performance liquid chromatography-based method.[76] The thermodynamic data show that Cm(III) might follow the receptor mediated iron acquisition pathway after incorporation.

Conclusions

In the present study two Cm(III) transferrin species were identified for the first time and characterized spectroscopically. At pH 7.4 about 70% of the partially bound (to the Fe(III) binding site or unspecific sites) Cm(III) transferrin species and 30% of the Cm(III) transferrin species with Cm(III) incorporated at the Fe(III) binding site are formed at physiological temperature (310 K). The incorporation of Cm(III) at the transferrin binding site is an important requirement for the recognition of the Cm(III) transferrin complex by the receptor followed by endocytosis which might be a possible pathway for the distribution of Cm(III) in the human body.

The results presented in this study will contribute to a better understanding of relevant biochemical reactions of actinides upon incorporation and can be of major importance for the future development of potential decontamination therapies. We showed that TRLFS is a powerful tool for the investigation of the complexation of Cm(III) with transferrin. This method will be applied further to determine thermodynamic data of the complexation reactions and to investigate the influence of the synergistic anion. Additional work regarding the interaction of Eu(III) with transferrin using TRLFS is in progress.

Acknowledgements

The synchrotron-based experiments were performed at the INE beamline of the ANKA synchrotron light source in Karlsruhe, Germany. The authors thank J. Rothe and K. Dardenne for their support with the EXAFS measurements.

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