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ABSTRACT

The quantitative description of the interactions of uranium with blood serum components is of high relevance for a rational design of molecules suitable for *in vivo* chelation of uranium. We have determined the stability constants for the complexation of U(VI) with human serum transferrin and albumin by Time Resolved Laser Induced Fluorescence Spectroscopy and Difference Ultraviolet Spectroscopy. Both proteins interact strongly with U(VI), forming ternary complexes with carbonate acting as a synergistic anion. Together with literature data describing the interaction of U(VI) with low molecular weight inorganic and organic serum components, the speciation of U(VI) in blood serum was calculated. In agreement with published experimental data, the model calculation shows that complexation with proteins and carbonate ion governs U(VI) speciation; 35 % of U(VI) is bound to proteins and 65 % to carbonate. Among the protein pool, albumin is the main protein interacting with U(VI). In

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addition, the results show that Ca(II) must be considered in the model as a competitive metal ion with respect to U(VI) for binding to albumin surface sites. Based on these findings several promising molecules for *in vivo* chelation of ^{230}U could be identified.

KEYWORDS: ^{230}U, targeted alpha therapy, speciation, albumin, transferrin

1. Introduction

The principle of targeted alpha therapy (TAT) is based on the stable binding of alpha emitting radionuclides to cancer selective carrier molecules, such as antibodies or peptides, *via* bifunctional chelating agents (BCAs). Due to the short range (< 100μm) and the high linear energy transfer (≈100 keV/μm) of alpha radiation in human tissue, TAT allows to selectively deliver a highly cytotoxic radiation dose to targeted cells while sparing surrounding healthy tissue [1]. The alpha emitter ^{230}U ($t_{1/2}$ = 20.8 d) is a promising novel radionuclide for application in targeted alpha therapy of cancer [2-4]. For safe therapeutic application of ^{230}U in targeted therapy, a chelating agent is required to link the radiometal to biological carrier molecules in a stable manner, since release of the alpha emitter from the radioconjugate *in vivo* might cause toxicity to normal organs. Due to the relatively long half-live of ^{230}U of 20.8 days, the radioconjugate should show high stability over extended time periods. Ideally a suitable chelating agent should form uranium complexes of higher stability than ligands competing for uranium complexation under physiological conditions, such as carbonate, phosphate and proteins [5-7]. In order to evaluate the potential of candidate molecules for uranium chelation, in particular with respect to the stability of their uranium complexes *in vivo*, a full thermodynamic description of the interaction of uranium with competing ligands present in human body fluids, and in particular in blood serum, would be highly desirable. Certainly a quantitative speciation of uranium under physiological

conditions is also of high relevance for the understanding of toxicological effects of uranium as well as for the development of effective decorporation agents for uranium [5,8].

Hexavalent U(VI) is the most stable oxidation state of uranium under physiological conditions [8]. It has been reported that U(VI) in the blood stream is forming complexes with carbonate and serum proteins, in particular with albumin and transferrin [7,9-11]. The thermodynamic parameters describing the interaction between U(VI) and relevant low molecular weight inorganic and organic ligands have been extensively studied and are rather well known [12,13]. However, the interaction between U(VI) and serum proteins is still a subject of discussion with respect to the nature of the interacting proteins [11,14], the quantitative description of the interaction [5] and the coordination environment of U(VI) [15], in particular with respect to the possible presence of the carbonate ion in the coordination sphere [7,14,15].

Human serum albumin (HSA) is the most abundant protein in human blood plasma at a concentration of 30 to 50 g/L and comprises about half of blood serum protein. The interaction of uranium and HSA has been studied as early as 1948 by Guzman Barron *et al.* [16] using ultrafiltration. The authors report the reversibility of uranium binding to HSA and the influence of carbonate and citrate as competing ligands. No further thermodynamic analysis was performed. Chevari *et al.* [17] report a conditional stability constant of log K=10 for the binding of uranium to HSA at pH=6 using a Shubert-type method [18], while Duff *et al.* [19] have determined conditional stability constants of log K = $1.6*10^7$ M^{-1} and log K = $2.8*10^5$ M^{-1} for the binding to high-affinity and low-affinity sites at pH 5.5 using isothermal titration calorimetry.

Human serum transferrin (HSTF) is an iron binding single-chain glycoprotein with a molecular mass of 79570 Da, containing 679 amino acids and consisting of two structurally related lobes referred to as N and C lobe. Each lobe is further divided into two subdomains by

a cleft, with each cleft housing a metal binding site. Metal binding to HSTF is generally facilitated through concomitant binding of carbonate as a synergistic anion [20]. The concentration of HSTF in serum is ca. 2.5 g/L, where about 30% are saturated with ferric ion. HSTF is also the primary serum transport agent of a large number of tri- and tetravalent metal ions [21]. Stevens *et al.* [9] reported that U(VI) in blood plasma of beagles injected with uranium citrate was present as 60% carbonate complex and 40% protein bound, with most of the protein-bound fraction presumably being associated with transferrin. The binding of uranium(VI) to HSTF has been studied by Scapolan *et al.* [10] using time-resolved laser-induced fluorescence spectroscopy (TRLFS) at physiological pH. The formation of a 2:1 complex of U(VI):HSTF has been reported, with an overall conditional stability constant of log K = 16. Sun *et al.* [22] have developed a correlation of the overall metal-binding constant of HSTF with the first metal hydrolysis constant, leading to a value of log K = 14.7 for the U(VI)-HSTF complex. This correlation was further refined by Ansoborlo *et al.* [6] through a critical assessment of the metal ion hydrolysis constants and the consideration of two individual binding sites, predicting values of log K = 14.1 and 12.6 for the C and N lobe, respectively. Vidaud *et al.* [15] have studied the structural properties of HSTF complexes of U(VI), and propose the existence of two binding sites, where U(VI) is coordinated under participation of two tyrosine residues.

To our knowledge, thermodynamic data describing the complexation of uranium by HSA or HSTF applicable to a wide variety of body fluid compositions have not been reported to date. In this work we have determined the stability constants for the complexes of U(VI) with HSA and HSTF using TRLFS and Difference Ultraviolet Spectroscopy (DUS). Based on these parameters and including literature data of thermodynamic constants of U(VI) complexation with low molecular weight organic and inorganic ligands, a model calculation was performed to describe U(VI) interactions in human blood serum in a bottom-up approach.

The reliability of the simulation was assessed by comparison with published experimental *in vitro* speciation results [9,11,17]. To identify promising molecules for *in vivo* chelation of ^{230}U, the serum stability of several U(VI) chelate complexes was simulated.

2. Materials and methods

2.1. Reagents

Human serum transferrin (apo-transferrin human ≥ 98 %, Sigma) was used as received or purified by size-exclusion chromatography on a PD-10 column using 0.1 M NaCl/0.05 M HEPES at pH 7.4 as an eluent. No difference in complexation was observed between purified and non-purified HSTF and no distinction was made in the following. HSTF concentration was determined spectrophotometrically at λ = 280 nm using 93,000 cm^{-1} M^{-1} as a molar extinction coefficient [23]. Human serum albumin (albumin from human serum ≥ 96 %, Sigma, mol wt 66,478 Da by calculation) was used as received. U(VI) stock solution was prepared by dilution of a standard solution (SPEX CertiPrep® Single-element Solution Standard, [U] = 1000 μg/mL, 2 % HNO_3) with addition of sodium hydroxide to obtain a final concentration of 1×10^{-3} M at pH ~ 3. All other chemicals were reagent grade. HSTF, HSA and carbonate stock solutions were freshly prepared before use. Solutions were prepared with ultrapure water (MilliQ, 18 MΩ·cm) and pH measurements were performed using a Lab850 pH meter combined with a micro electrode (Schott, Germany).

2.2. Sample preparation

All experiments were performed in solution of pH 7.4 ± 0.1 and ionic strength 0.1 (NaCl) buffered with 2×10^{-3} M HEPES at room temperature (23 ± 2 °C). Experiments in the absence of carbonate were performed in a glove box under 100% nitrogen atmosphere. Samples in the carbonate system were freshly prepared and kept closed to prevent equilibration with

atmospheric CO_2. Irrespective of the system studied, equilibrium was shown to be established rapidly, i.e. in less than 20 minutes. During this time period, the concentration of carbonate in the closed system is considered to be constant. The experimental conditions are summarized in Table 1.

2.3. Time Resolved Laser induced Fluorescence Spectroscopy (TRLFS)

Details concerning the spectroscopic device as well as details on how spectroscopic data were obtained are described in [24]. U(VI) was excited at 430 nm with a laser intensity of about 3 mJ. As shown in Figure 1A, complexation of U(VI) by HSTF reaches equilibrium in less than 20 minutes and leads to a complete extinction of the fluorescence signal. Under given experimental conditions, the percentage of U(VI) not bound to HSTF can thus be determined according to Eq. (1):

$$\%(\mathrm{U(VI)}) = \frac{\mathrm{FI}}{\mathrm{FI_{tot}}} \quad (1)$$

where FI_{tot} and FI correspond to the fluorescence intensity measured before and after addition of HSTF, respectively. The applicability of the method is limited in the presence of high carbonate concentrations, since carbonate complexes of U(VI) also do not emit fluorescence [10].

2.4. Difference ultraviolet spectroscopy (DUS)

DUS is the most widely used technique to evaluate metal binding to HSTF [20]. UV-spectra were recorded using an Ultrospec™ 2100 *pro* UV/Visible spectrophotometer. Metal complexation was evaluated from the absorbance measurements after addition of the metal using the difference spectra of the metal-protein complexes vs. unmetallated apo-protein. Either the peak height or the peak area in the range 235-271 nm were used to describe and quantify U(VI)-HSTF interaction. The main advantage of the method is that is the absorbance

signal of HSTF is not influenced by the presence of other ligands which may interact with U(VI) (e.g. carbonate). The interaction of HSA with U(VI) was studied using HSTF as a competitive agent [25].

2.5. Modelling the binding between U(VI) and proteins

Several models can be used to describe the interactions between U(VI) and proteins. The simplest model (model 1) considers the metal ion interaction with the protein as a global reaction, without considering the possibility that several binding sites of different affinity may exist. Furthermore, the metal ion speciation in solution, i.e. the species distribution between U(VI) and low molecular weight ligands is not taken into account. Equilibrium constants derived from this model represent conditional constants and can only be applied to the experimental conditions from which they have been obtained (i.e. pH, medium composition, protein concentration range). This model was used by Scapolan *et al.* [10] to describe U(VI) interaction with HSTF according to Eq.(2):

$$2\mathrm{U} + \mathrm{HSTF} \rightleftarrows \mathrm{U_2HSTF} \qquad (2)$$

The obtained constant cannot be extrapolated to blood serum conditions, since the constant was determined in the presence of a lower concentration of carbonate (2×10^{-4} M) compared to the carbonate concentration of blood serum (2.5×10^{-2} M). This simple model was also used by Chevari *et al.* [17] to describe the interaction of U(VI) and HSA considering the formation of a 1:1 complex at pH 6, consequently also not allowing to apply the constant to serum conditions.

A more complex model (model 2) takes into account the metal ion speciation and the possible presence of different binding sites at the protein surface:

$$\mathrm{U} + \mathrm{protein(site\ i)} \rightleftarrows \mathrm{U\text{-}protein(site\ i)} \qquad (3)$$

The description of the interaction remains however incomplete as the functional groups implied in the complexation are not explicitly differentiated. Given that the pH and ionic strength are kept constant, the model can be used as a predictive tool to describe the interaction in different media. This is the model generally used to describe metal-HSTF interactions. Metal ion speciation in solution is often simplified and taken into account in the calculation by introducing a strong complexing agent having a known equilibrium complexation constant for U(VI) (see [20] and quoted references).

When a microscopic model (model 3) is used, interactions at the molecular level are converted to reactions according to the law of mass action and thermodynamic parameters can be derived. To our knowledge such a model has never been used to describe metal protein interactions considering the lack of spectroscopic data characterizing the interaction and considering the complexity to describe metal ion interactions with organic polyelectrolytes (e.g., see the case of natural organic matter [26]).

For the purpose of our study, only models 2 and 3 were applicable and were used to describe the interaction of U(VI) with HSA and HSTF, respectively. The interaction of U(VI) with proteins was described using a surface complexation model in analogy to studies investigating the interactions of metal ions with natural organic polyelectrolytes (see e.g. [27]) as detailed below.

2.5.1. Modelling the binding of U(VI) to HSTF

HSTF was shown to display two structurally related but slightly different binding sites ($HSTF\equiv {}_1S$ and $HSTF\equiv {}_2S$), each binding site interacting with U(VI) under the participation of two tyrosine groups [15]. The binding sites react with water according to Equation (4):

$$\begin{aligned} HSTF \equiv_{1,2} SH_2 + H_2O \rightleftarrows HSTF \equiv_{1,2} SH^- + H_3O^+ \\ HSTF \equiv_{1,2} SH^- + H_2O \rightleftarrows HSTF \equiv_{1,2} S^{-2} + H_3O^+ \end{aligned} \quad (4)$$

The pKa value associated to the Tyr188 residue was recently measured by 2D NMR-pH titration as 6.9 (0.5 M KCl) [28]. Based on the Davies equation we recalculated a value of 7.2 for zero ionic strength [29] for introduction into the calculation code. For the other tyrosine residue Tyr95, no data is available and a pKa value of 10 (10.2 at zero ionic strength), associated with a "normal" tyrosine residue, was used [30]. We assume that the species interacting with the binding sites in the absence of carbonate is UO_2^{2+}, i.e. there are no ternary complexes formed with other anions present in the medium (OH^-, Cl^-, NO_3^-). The reaction is then described by Eq.(5):

$$HSTF \equiv_{1,2} S^{-2} + UO_2^{2+} \underset{\leftarrow}{\rightarrow} HSTF \equiv_{1,2} SUO_2 \qquad (5)$$

Each lobe of transferrin contains two tyrosine Fe(III) binding sites that are remarkably similar ([20] and quoted references). This was also observed to a certain extent with U(VI): Vidaud *et al.* demonstrated that both tyrosines at the iron binding site are involved in uranium binding [15]. Irrespective of the metal ion considered [20], the stability constants for interaction with the binding sites differ by approximately one unit in their log K value, the difference being probably related to outer-sphere effects [25]. This ratio of 10 was set as a fixed parameter in the fitting of our experimental data. In the presence of carbonate, a ternary complex may be formed [7,15] according to Eq.(6):

$$HSTF \equiv_{1,2} S^{-2} + UO_2^{2+} + (CO_3^{2-})_n \underset{\leftarrow}{\rightarrow} HSTF \equiv_{1,2} SUO_2(CO_3)_n^{-2n} \qquad (6)$$

No electrostatic effects were considered in the calculation and the parameters must be considered as operational.

2.5.2. Modelling the binding of U(VI) to HSA

Metal binding to HSA is of complex nature, characterized by multiple binding sites whose affinity and binding capacity are varying (e.g. [31-33]). The microscopic model 3 is therefore not applicable and model 2 was used instead. To describe the interaction of U(VI) with HSA

in blood, Chevari *et al.* [34] proposed an exchange process between Ca(II) and U(VI). According to [35], about 70% of calcium in the blood serum is complexed with low molecular weight molecules, while the rest is bound to proteins. The protein bound fraction of calcium can be considered as attached mainly to HSA: Ca is known to interact with HSA [31,32] and Ca(II) interaction with HSTF is expected to be negligible; its interaction is weak [20] and protein-bound Ca concentration represents about 8 % of the total HSTF concentration. Based on a typical serum composition [6], this leads to an average of 0.7 Ca atoms bound per HSA molecule. The total number of binding sites (N_{tot}) reported in the literature for Ca on HSA varies [32] with the highest value reported as 30 [31]. The value depends on the HSA/metal ratio: for a given data set, N_{tot} reflects the total molar binding ratio of the predominant calcium binding class and not the maximal molar binding ratio [32]. Anderson showed, however, that Ca interaction under physiological conditions is governed by the interaction with a strong site [31]. A site of stronger affinity was as well considered for other divalent metal ions like VO^{2+} [33] and Ni^{2+} [36]. Glennon & Sarkar showed that both Cu^{2+} and Ni^{2+}, although presenting different coordination properties, interact with the same HSA binding site [36]. Based on these considerations, our model considers the existence of only one binding site at the HSA surface which interacts with both U(VI) and Ca(II) cations, according to Eq.(7):

$$\begin{aligned} &HSA \equiv S + Ca^{2+} \overrightarrow{\leftarrow} HSA \equiv SCa^{2+} \\ &HSA \equiv S + UO_2^{2+} + (CO_3^{2-})_n \overrightarrow{\leftarrow} HSA \equiv SUO_2(CO_3)_n^{2-2n} \end{aligned} \qquad (7)$$

Contrary to the equilibrium generally considered in model 2 (Eq. (3)), the possible formation of ternary complexes of U(VI) with HSA and carbonate must be considered under serum conditions (see comments above). All calculations were performed using the simulation code PHREEQC, a geochemical modelling code for aqueous systems [37]. Stability constants for metal ion complexation in solution were taken from NEA-OECD [12] (complexation of

U(VI) with inorganic ligands), NIST [13] (U(VI) and citrate) and Llnl.dat [37] (Ca complexation) thermodynamic databases.

3. Results and discussion

3.1. Binding of U(VI) to proteins

3.1.1.Binding of U(VI) to HSTF.

TRLFS experiments performed in the absence of carbonate show a significant complexation of U(VI) by HSTF at pH 7.4, i.e. 76 % of U(VI) were found to be bound to HSTF in the presence of a 4-fold excess of HSTF over U(VI). The stability constants describing the interaction (Eq. (5)) were found to be log K = 12.4 and log K = 11.4 (I=0) for the high-affinity and low-affinity site at zero ionic strength, respectively (Table 2). This set of constants was used to calibrate the UV-spectroscopy method, i.e. to relate the area of the absorption peak at 242 nm with the amount of the complexed species U(VI)-HSTF. As this absorption band is linked to the interaction of the metal ions to the phenolic groups of the tyrosine residues [10,20], we assume that the extinction coefficient is not affected by the nature of other ligands coordinated in the first coordination sphere of U(VI), i.e. the calibration parameters are applicable also when the synergistic carbonate ion is bound (Eq. (6)).

The experimental data obtained in the presence of carbonate are presented in Figure 1B. The values obtained by TRLFS and DUS are in good agreement. The fraction of U(VI) bound to HSTF increases with increasing carbonate concentration up to a total carbonate concentration of ca. 2×10^{-4} M, indicating the formation of a ternary complex in solution, with carbonate acting as synergistic anion according to Eq. (6). At higher concentrations of total carbonate, the fraction of HSTF-bound U(VI) decreases, due to increasing competition of carbonate with HSTF for U(VI) complexation. The complete set of experimental data could

be well described considering one carbonate ion bound to U(VI) with stability constants of log K = 20.8 and log K = 19.8 (I=0) for the ternary complexes formed at the high-affinity and low-affinity site (Table 2). The data could not be fitted without considering the formation of ternary complexes. Under blood serum conditions, the ternary complexes will be the dominant species, i.e. the binary U(VI)-HSTF complex formed according to Eq.(5) will not be formed.

The results of a titration of HSTF with U(VI) are presented in Figure 2A. Increasing metallation of HSTF leads to an increase of absorbance signal that could be extrapolated by non-linear regression to a plateau at $\Delta\varepsilon = (30 \pm 2) \times 10^3$ M^{-1} cm^{-1} at 242 nm associated with the formation of a 2:1 U(VI):HSTF complex. This value is in excellent agreement with the values previously reported for U(VI) [15] and for other metal ions [20,38]. The increase of absorption observed above the theoretical saturation level (2 U(VI) for one HSTF molecule, r=2) is due to the presence of uncomplexed U(VI) as the chosen experimental conditions do not allow for complete complexation of U(VI) by HSTF.

The reliability of the determined stability constants describing the formation of the ternary complexes, derived from the experimental data presented in Figure 1B, relies on the reliability of the constants characterizing the carbonate complexation of U(VI) taken from [gui03]. Therefore, to test the obtained constants, they were used to calculate U(VI) speciation in a simplified system simulating blood serum, i.e. containing HSTF and carbonate ion (4×10^{-4} M) as a function of concentration of citrate, a well known strong complexing agent for U(VI) [6] and the calculated results were compared to experimental data. Considering the relatively large size of the citrate molecule, we assume that the synergistic carbonate ion bound to U(VI) with HSTF will not be exchanged by citrate. The experimental data, representing the percentage of U(VI) bound to HSTF determined by spectrophotometry as a function of citrate concentration, are presented in Figure 2B. As shown by the solid line, a good agreement

between the prediction and the experiment is obtained showing the reliability of our stability constants for HSTF complexation of U(VI).

According to the literature, there is a good correlation between the strength of metal binding to transferrin and the stability constant for hydroxide binding to the same metal ion [6,20]. Compared to the values of log K = 14.1 and 12.6 derived from this correlation for strong and weak sites, respectively [6], the values determined in this work are approximately one order of magnitude lower (see Table 2). This might be explained by the fact that the binding sites of HSTF are ideally pre-organized to complex spherical cations in three dimensions like Fe(III), whereas complexation of the linear uranyl cation must account for its preferred equatorial coordination. This is in agreement with the recent work of Vidaud *et al.* [15] who showed that the coordination with U(VI) cannot be compared with that of Fe(III) and that the participation of the His249 ligand in the coordination sphere of uranyl can be excluded.

3.1.2.Binding of U(VI) to HSA.

The binding between U(VI) and HSA was studied using a displacement method in the presence of HSTF [25]. This method is applicable because no significant absorption of the U(VI)-HSA complex occurs in the HSA concentration range explored ($< 4\times10^{-5}$ M) that could interfere with the measurement of the absorbance signal of the U(VI)-HSTF complex. This observation indicates that HSA does not interact with U(VI) *via* tyrosine groups, in agreement with the nature of the interaction of HSA with Cu(II) and Ni(II) at the strong binding site [36]. The system with HSTF and HSA competing for U(VI) complexation is also a good representation of the situation in blood serum. The concentration of carbonate ion in the system was varied to assess its role for the coordination of U(VI) with HSA. It was found that the data at equilibrium are not affected by the addition order of the components, indicating

that the interaction of U(VI) with the two proteins is reversible. The experimental results are shown in Figure 3. In order to obtain a good fitting of the experimental data, the formation of a ternary HSA-U(VI)-carbonate complex needs to be taken into account at higher concentrations of carbonate (Fig. 3B). The stability constants were determined as log K = 10.8 (I = 0) for U(VI)-HSA and log K = 17.7 (I = 0) for U(VI)-HSA-carbonate as summarized in Table 2.

Overall our experimental data on the interaction of U(VI) with HSTF and HSA obtained in the presence of carbonates could be well described by considering the formation of ternary complexes between the protein, one carbonate ion and U(VI). The occurrence of ternary complexes is in agreement with general understanding of metal binding to HSTF [20,21], and agrees well with the reports of Van Horn *et al.* and Huang *et al.* [7,14] on the binding of U(VI) to proteins. However, they propose the involvement of two carbonate ions in the U(VI) coordination sphere when bound to proteins or peptides. To the best of our knowledge, there is no experimental proof to support this statement, but it is rather an assumption based on the presence of the U(VI)-bis-carbonato complexes in blood serum. Our data show that both HSA and HSTF have a strong but similar affinity for U(VI). Indeed, if the experimental data are analysed based on model 2 (Eq. (3)), the stability constants obtained for U(VI) complexation are identical for both proteins (see Table 2).

3.2. Simulation of U(VI) speciation in the blood serum and comparison with in vitro speciation results

3.2.1. Serum composition.

The aim of our model calculation was to simulate the speciation of trace concentrations of U(VI) under serum conditions, where U(VI) may interact with all types of complexing agents present in the serum, i.e. inorganic ligands, organic ligands and proteins. Considering the

complexity of the biological medium, simplifications were made considering that the interaction strength of given serum components with U(VI) will depend on their concentrations as well as the equilibrium complexation constants. All relevant inorganic ligands were taken into account (hydroxide, carbonate, phosphate, chloride, sulphate) using the constants given in [12]. Concerning low molecular weight organic components, only the citrate ion was considered as it is present in human serum at a relatively high concentration (1.6×10^{-4} M) and has a relatively high stability constant for U(VI) complexation [6]. Although recent work of Vidaud *et al.* showed that a number of proteins can bind U(VI) [11], only HSA and HSTF were considered in the present study as they constitute the predominant metallo-proteins present in high concentrations in human blood serum [6].

In addition, a possible competition with other metal ions present in blood serum has to be taken into consideration. Also in this case simplifications are necessary. Given the high difference in log K values (about 10 orders of magnitudes), we can safely consider that trace concentrations of U(VI) can not compete with Fe(III) for HSTF binding sites. The number of available HSTF binding sites was therefore set to 70 % of the total capacity, corresponding to the fraction of HSTF not loaded with ferric ion [20]. Another cation that has to be taken into account is Ca(II) as it is present in blood serum in relatively high concentrations [35] and it is known to strongly interact with HSA [32].

3.2.2. Ca interaction with HSA

In previous studies, model 1 was used to describe the interaction of calcium with HSA [32]. As discussed in the modelling part, this model is not applicable for the purpose of the present study, i.e. the competition between Ca(II) and U(VI) for the strong site must be explicitly described. For this purpose, experimental data obtained by Besarab *et al.* [32] in conditions similar to those found in blood serum were re-evaluated according to Eq.(7). To

characterize Ca(II) interaction with the strong site on HSA, only the experimental data where less than one Ca(II) is bound per HSA molecule were taken into account, resulting in a value for log K = 4.2 (see SM-1). Based on this stability constant together with thermodynamic data for calcium complexation taken from Llnl.dat [37], the species distribution of calcium in blood serum [6] was calculated. The calculation results in 34 % of Ca bound to HSA and 66 % "non-protein bound", i.e. 49 % in the ionic form, 8 % complexed with citrate, 5 % as a carbonato complex, 2 % bound to phosphates and the remaining 2 % being distributed between other species. This indicates that the stability constant for Ca(II)-HSA complexation is reliable since the calculation is in agreement with experimental data reported in [35], where 67 ± 2 % of Ca is in ionic form or complexed with low molecular weight organic and inorganic molecules, while the remaining part is bound to the protein pool.

3.2.3. Simulation of U(VI) speciation in human serum

Based on the stability constants for U(VI) complexation by HSTF and HSA determined in this study, together with known thermodynamic parameters for complexation of U(VI) with low molecular weight organic and inorganic ligands, the species distribution of U(VI) in human blood serum was calculated. The model calculation shows that U(VI) speciation is governed by both the carbonate ion and the proteins. 65 % of U(VI) are complexed with CO_3^{2-} as the bis- (3 %) and tris-carbonato (59 %) complexes, while the remaining fraction is bound to the protein pool, 28 % and 7 % with HSA and HSTF, respectively. Calcium has a significant effect on U(VI) speciation. Without considering the competition of Ca(II) for binding sites on HSA, approximately 70 % of U(VI) would be predicted to be bound to the protein fraction. In agreement with what is generally stated in the literature [6,7], both proteins (HSTF and HSA) and carbonate govern U(VI) speciation in blood serum at equilibrium. This is also supported by the experimental data reported by Chevari *et al.* and

Stevens *et al.* showing a ratio of carbonate vs. protein bound U(VI) in plasma of 64:36 and of 60:40, respectively [9,34]. The agreement is less satisfactory when comparing our model calculation with the data obtained by Vidaud *et al.* [11]. From *in vitro* experiments they concluded that about 20 % of uranyl in serum is associated with the protein pool, whereas our simulation leads to a value of 35 %.

In agreement with Chevari *et al.* [34], our simulation showed that the role of HSTF is minor and that binding of U(VI) to HSA is predominant. As both proteins display a similar strength for binding of U(VI), but HSA concentration in serum is one order of magnitude higher than HSTF concentration, 80% of protein bound U(VI) is associated with HSA compared to 20% of U(VI)-HSTF. This is in contrast to the predominance of HSTF binding of U(VI) reported in [9], however, the authors do not provide detailed support of their statement.

3.3. Chelating agents for application of ^{230}U in targeted alpha therapy

The modelling of the speciation of U(VI) in blood serum can be used to make predictions on the stability of potential U(VI)-chelate complexes under serum conditions and to select promising ligands for further testing and experimental validation of their stability *in vitro* and *in vivo*. For a simplified estimation, assuming the injection of 1 mg of ^{230}U labelled antibody carrying one chelate per antibody molecule, the concentration of chelate in the blood pool will be initially in the range of 10^{-9} M. Taking into account the concentrations and stability constants of the competing ligands carbonate, HSA and HSTF, the model calculation shows that a chelate suitable for stable binding of ^{230}U(VI) under serum conditions is required to have a stability constant for UO_2^{2+} of 10^{19} M^{-1} at physiological pH if >90 % of U(VI) should remain bound at equilibrium. However, chelating agents with lower stability constants could be useful in case their U(VI) complexes display a sufficiently high kinetic stability against

dissociation under serum conditions within the residence time of the radioconjugate *in vivo*. A large number of potential complexing agents for U(VI) have been reported in literature for a variety of applications, including the development of decorporation agents [8] and antibody based assays [39]. Based on their high stability constants for complexation of U(VI), the calixarene family [40], in particular calix[6]- and calix[8]-arene [41,42], appear promising. Calixarenes can easily be bi-functionalised [43] to establish the link to biological carrier molecules without modifying the functional groups available for the complexation. According to the work of Mullen *et al.* [44], the siderophore desferrioxamine B (DFO), that is already used for nuclear imaging when labelled with ^{89}Zr(IV) [45], may as well be a good candidate. The authors report an equilibrium constant between UO_2^{2+} and the deprotonated ligand of $10^{17.1}$ M^{-1} for an ionic strength of 0.1 M. Another promising molecule is the versatile ligand 1,10-phenanthroline-2,9-dicarboylic acid (DCP) that has been shown to efficiently complex U(VI) in biological media [39]. The synthesis of a bifunctional derivative has been reported and a complexation constant between the deprotonated ligand and UO_2^{2+} exceeding 13 has been reported [46].

4. Conclusions

The stability constants determined in this work for the complexation of U(VI) by HSA and HSTF allow to predict the speciation of U(VI) in human body fluids. The model calculations could be validated through experimental data obtained in blood serum and provide a realistic, qualitative and quantitative description of U(VI) behaviour. The model initially composed of 43 equilibrium equations could be simplified to only a few, describing the formation of bis- and tris- carbonato complexes of U(VI), the complexation of U(VI) with HSA and HSTF and taking into account the competition between Ca and U(VI) for binding to the strong site of HSA.

5. Abbreviations

Targeted alpha therapy	TAT
Bifunctional chelating agents	BCAs
Human Serum TransFerrin	HSTF
Human Serum Albumin;	HSA
Time Resolved Laser Fluorescence Spectroscopy	TRLFS
Difference Ultraviolet Spectroscopy	DUS
N-(2-HydroxyEthyl)Piperazine-*N'*-2-EthaneSulfonic acid	HEPES

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Table 1: Interaction between U(VI) and proteins; methods and experimental conditions.

Method	Principle	U(VI)	HSTF	carbonate concentration	experiment	Figure
TRLFS	see Eq.(1)	$5x10^{-6}$ M	$2x10^{-5}$ M	0	kinetic	1A
		$2x10^{-5}$ M		0 - $2x10^{-3}$ M	carbonate titration	1B
DUS	absorption of U(VI)-HSTF complex at 242 nm			0 - $3x10^{-2}$ M		1B
				$2x10^{-4}$ M	U(VI) titration	2A
				$4x10^{-4}$ M	competition with citrate ion ($2x10^{-4}$-$4x10^{-2}$ M)	2B
				$2x10^{-4}$ M	competition with HSA (3.10^{-6} - 5.10^{-5} M)	3A
				$2x10^{-3}$ M		3B

Table 2: Quantitative description of U(VI) with HSTF and HSA and comparison with published data.

Protein	Equation	Model	Site / interacting group	log K	Ref.	Remarks
HSTF	Eq. (3)	2	Weak site	12	this work	carbonate concentration at 25 mM
			strong site	13		
				14.3*	[22]	
				14.1*	[6]	
			Weak site	12.6*		
	Eq. (2)	1		16	[10]	carbonate concentration at 0.1 mM
	Eq. (4)	3	Tyr188 (both sites)	-7.2	[28]	see experimental conditions in Table 1
			Tyr95 (both sites)	-10.2	[30]	
	Eq. (5)		strong site	12.4	this work	
			weak site	11.4		
	Eq. (6)		strong site, n=1 (CO_3^{2-})	20.8		
			weak site, n=1 (CO_3^{2-})	19.8		
HSA	Eq. (4)	3	strong site	7.2	[19]	pH 5.5, normal CO_2 atmosphere
			weak site	5.4		
	Eq. (2)	1	_	10	[17]	pH=6, ionic strength fixed at 0.1 M
	Eq. (3)	2	_	13	this work	carbonate concentration at 25 mM
	Eq. (7)		n=0 (CO_3^{2-})	10.8		see experimental conditions in Table 1
			n=1 (CO_3^{2-})	17.7		

* extrapolated values

Figures captions

Figure 1: Complexation of U(VI) by HSTF. Experimental conditions are summarized in Table 1. (**A**) Relative U(VI) fluorescence intensity measured as a function of the time after HSTF addition. (B) Complexation of U(VI) by HSTF as a function of carbonate concentration. The lines are calculated with the parameters given in Table 2 considering (solid line) or not-considering (dashed line) the formation of a ternary complex (Eq.(6)).

Figure 2: Complexation of U(VI) by HSTF as a function of U(VI) **(A)** and citrate **(B)** concentrations. In Figure 2A, r represents the ratio between total U(VI) and HSTF concentrations. $\Delta\varepsilon$ is calculated from the absorbance of U(VI)-HSTF complex measured at 240 nm divided by HSTF concentration. The lines are calculated with the parameters given in Table 2. For Figure 2B, an extinction coefficient of 3×10^4 $M^{-1}cm^{-1}$ characterizing the 2:1 U(VI):HSTF complex is used. Experimental conditions are summarized in Table 1.

Figure 3: Competition between HSTF and HSA for U(VI) in the presence of 2×10^{-4} M **(A)** and 2×10^{-3} M **(B)** of total carbonate. The system is pre-equilibrated before addition of HSTF (circle) or HSA (squares). Experimental conditions are summarized in Table 1. The lines are calculated with the parameters given in Table 2 considering (solid line) or not-considering (dotted lines) the formation of a ternary complex (Eq.(7)).

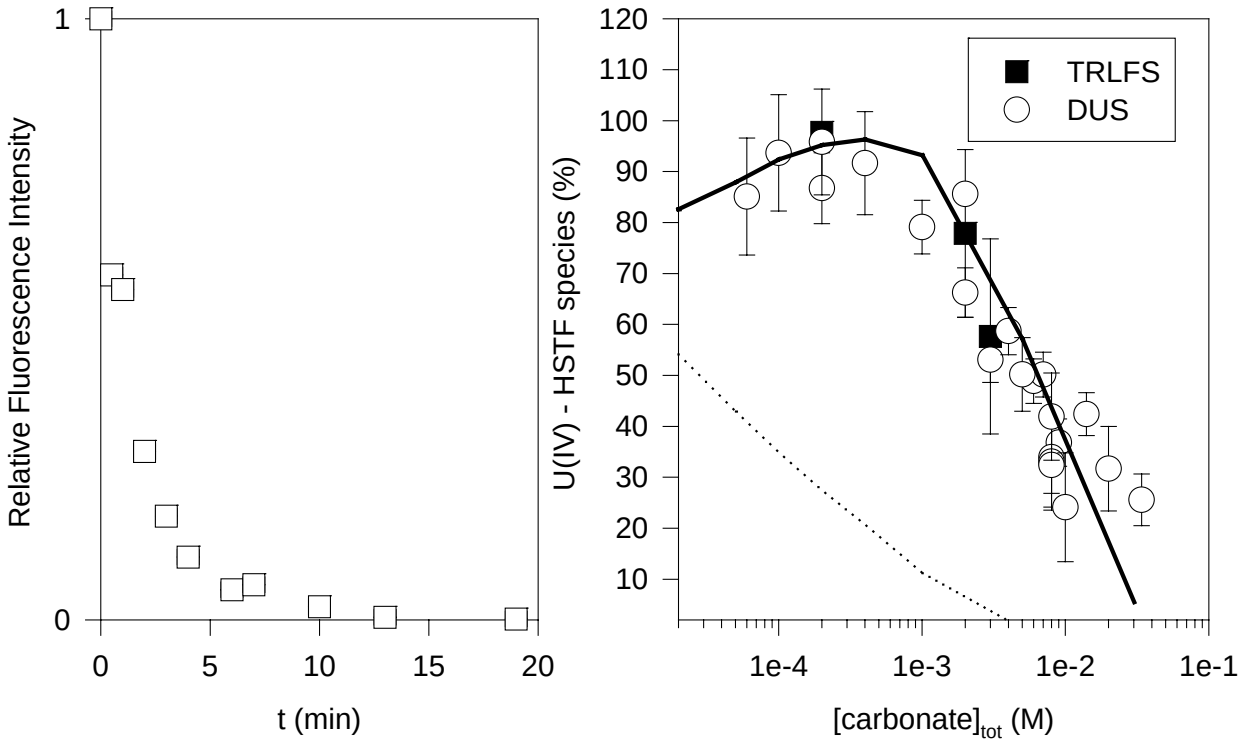

Relative Fluorescence Intensity
1
0
0
5
10
15
20
t (min)
U(IV) - HSTF species (%)
120
110
100
90
80
70
60
50
40
30
20
10
TRLFS
DUS
1e-4
1e-3
1e-2
1e-1
[carbonate]tot (M)

Figure 1

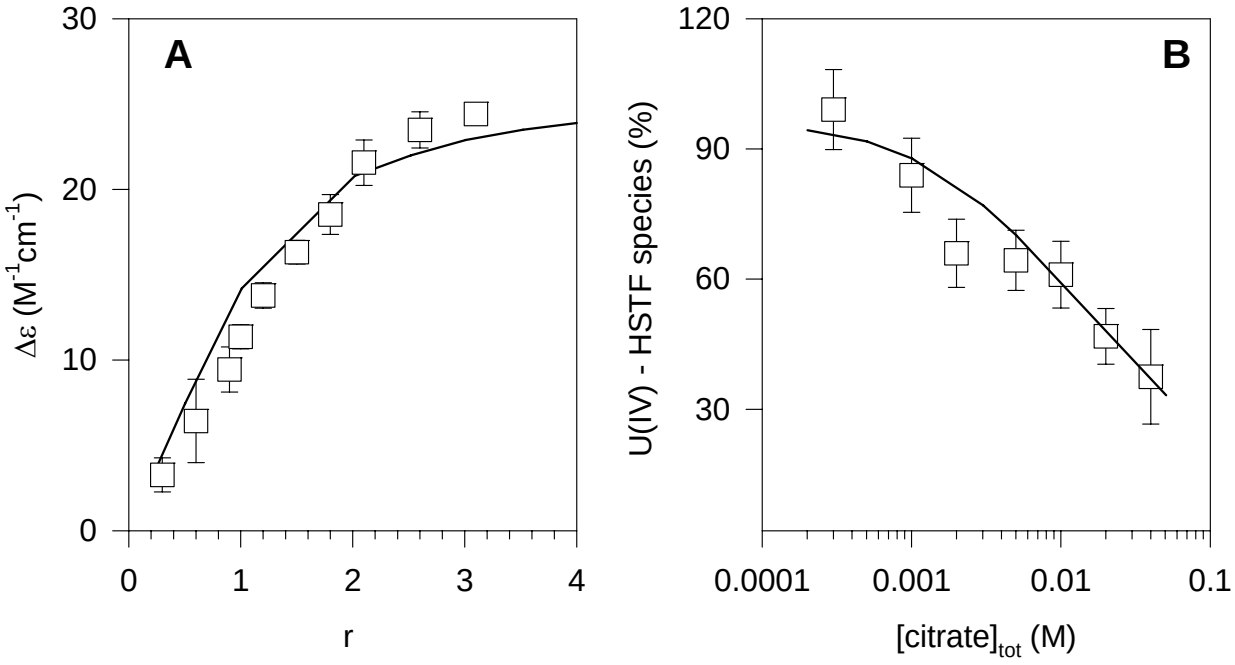

A
B
Δε (M-1cm-1)
U(IV) - HSTF species (%)
r
[citrate]tot (M)
0
10
20
30
0
1
2
3
4
30
60
90
120
0.0001
0.001
0.01
0.1

Figure 2

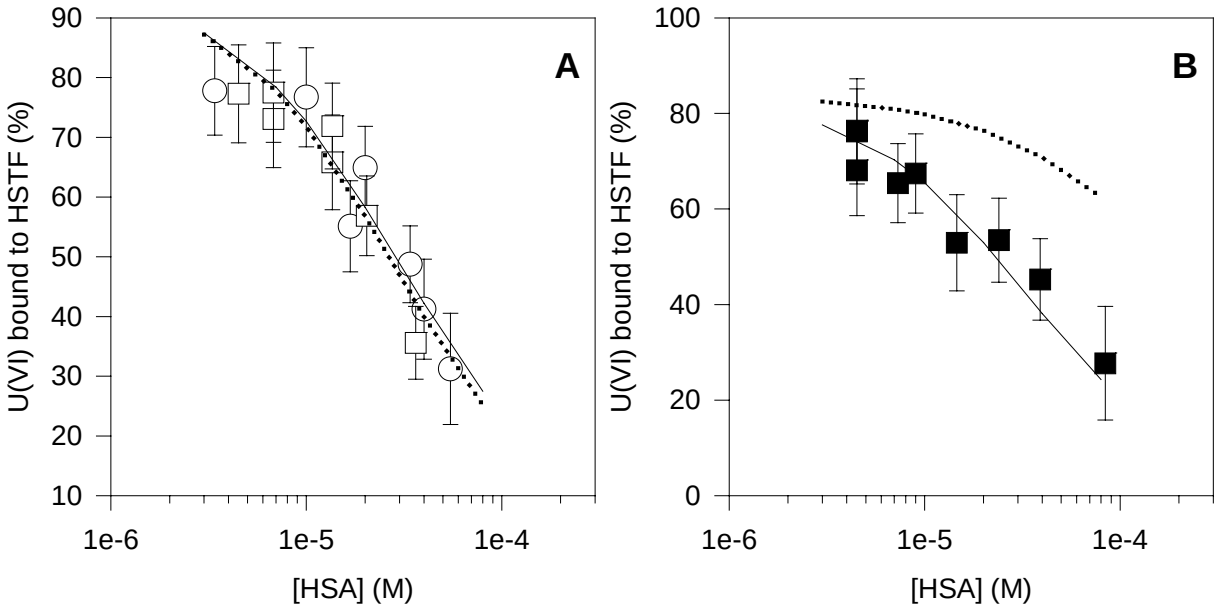
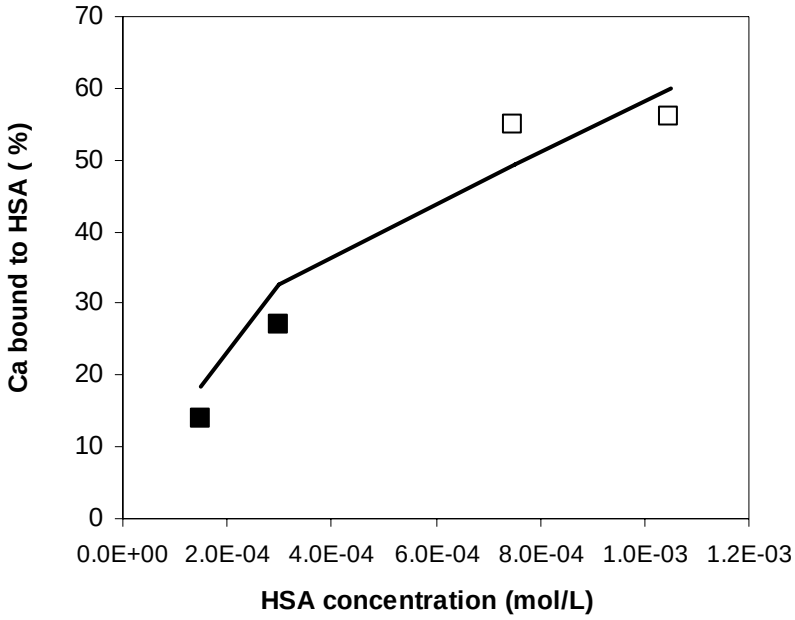

A
B
U(VI) bound to HSTF (%)
[HSA] (M)
90
80
70
60
50
40
30
20
10
100
0
1e-6
1e-5
1e-4

Figure 3

SUPPLEMENTARY MATERIAL

SM-1:

The Figure reports the experimental data given in [32] used to determine log K of Ca-HSA. They include the range of albumin concentration found in the blood serum and reflect the interaction of Ca with the strong site, where at equilibrium less than one Ca is bound per albumin molecule. The line was calculated using the parameters given in the main text.



(■) $[Ca]_{tot}$ =(4.2± 0.2) × 10^{-4} M; (□) $[Ca]_{tot}$ = (1.05 ± 0.05) × 10^{-3} M