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Investigation of *para*-sulfonatocalix[n]arenes [n = 6, 8] as potential chelates for ^{230}U

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Abstract

Literature reports of the efficacy of *para*-sulfonatocalix[6]- and calix[8]-arenes as U(VI) complexants indicated that they might be useful for in vivo chelation of the novel therapeutic alhpa-emitter ^{230}U. We have studied the complexation of U(VI) with p*ara*-sulfonatocalix[6]arene and *para*-sulfonatocalix[8]arene by time resolved laser induced fluorescence spectroscopy and using competition methods with Chelex resin and 4-(2-pyridylazo)resorcinol in simplified and in biological media. New thermodynamic parameters describing the stability of U(VI)-*para*-sulfonatocalix[n]arene [n = 6, 8] complexes were obtained. Although the interactions are strong, the complexes do not exhibit sufficient stability to compete with carbonate ions and serum proteins for complexation of U(VI) under physiological conditions.

Introduction

The alpha emitter ^{230}U ($t_{1/2}$ = 20.8 d) is a promising novel therapeutic radionuclide for application in targeted alpha therapy (TAT) of cancer [1-3]. Its decay generates a highly cytotoxic cascade of 5 alpha particles with a cumulative energy of 33.4 MeV. 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 [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. Ideally, a suitable chelating agent should form uranium complexes of higher stability than ligands competing for uranium complexation under physiological conditions, such as carbonate and proteins [5]. Due to the relatively long half-live of ^{230}U of 20.8 days, the radioconjugate should show high stability over extended time periods.

The "uranophiles" *para*-sulfonatocalix[6]-arene (L6) [6] and *para*-sulfonatocalix[8]arene (L8) [7] seem promising to meet these requirements, as they have been reported to exhibit large stability constants for complexation of uranium(VI). In addition, calixarenes can easily be bi-functionalised at the upper rim [8] to be linked to biological carrier molecules without modifying the functional groups available at the lower rim for the complexation of the metal ion (Fig. 1). Unfortunately, the thermodynamic data on the stability of the

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complexes of L6 and L8 available in literature are few and vary significantly [6-7, 9-11].

The aim of the present work was to derive a set of reliable quantitative parameters describing the complexation of U(VI) with L6 and L8 and to assess their potential for application in TAT, *i.e.* whether they form uranyl complexes of sufficient stability in human blood serum, when the concentrations of both the metal ion and the bifunctional ligand are strongly diluted and competing ligands are present in high excess.

To address these questions, the complexation of uranium(VI) with L6 and L8 was investigated by time resolved laser induced fluorescence spectroscopy (TRLFS) and competition methods using absorption spectroscopy and sorption to Chelex resin. Based on the parameters derived for U(VI)/*para*-sulfonatocalixarene interaction, and using literature data on the interaction of U(VI) with serum components [5], the stability of U(VI)-*para*-sulfonatocalixarene complexes under blood serum conditions was modelled and compared with experimental data obtained in human blood serum using Chelex column chromatography.

Results and discussion

Interaction of U(VI) with the competing agents 4-(2-pyridylazo)resorcinol (PAR) and Chelex resin

Competition methods are ideal for the quantitative determination of the equilibrium constants of ligands with strong uranyl binding properties. However, the reliability of the obtained quantitative parameters depends on the constants describing the interaction between U(VI) and the competing agent. Therefore, interaction of U(VI) with PAR and the functional groups of the Chelex chelating resin was carefully studied.

U(VI) complexation with PAR

The method was used at physiological pH of 7.3 ± 0.1. In the UV/Vis absorption spectrum, the formation of the U(VI)-PAR complex is characterized by the appearance of an absorption band at λ_{max} = 520 nm (ε = 2.7 × 10^4 L mol^{-1} cm^{-1}) and by the decrease of the absorption band of the free ligand at λ_{max} = 413 nm (ε = 3.3 × 10^4 L mol^{-1} cm^{-1}) (Figs. 2A and 2B). Both bands were used to characterize the complexation reaction. The existence of an isosbestic point indicates that only one U(VI)-PAR complex needs to be considered in the modelling approach. U(VI) complexation with PAR was studied as a function of total U(VI) (Fig. 2C) or PAR (Fig. 2D) concentration. The experimental data could be well explained through the formation of complex between one U(VI) cation and one PAR molecule. According to the data reported for other +2 metal ions [12], the formation of an 1:1:0 complex was considered in the modeling, where 0 indicates that the deprotonated ligand interacts with U(VI) (see the "modelling approaches" part for a detailed explanation on how the equilibria are considered). The experimental data could be well described with log $\beta_{1:1:0}$ = 16.1 (Table 1). This value is significantly higher than the conditional constant reported by Blake *et al.* [13] (log β = 6.4) obtained under similar experimental conditions. The difference can be explained by the fact that the authors used a Langmuir-type model and did not take into account the speciation of U(VI) and PAR, i.e. the complexation of U(VI) with hydroxide and carbonate ions and the degree of deprotonation of PAR.

U(VI) interaction with Chelex 100

The influence of both pH and uranyl concenration on sorption to Chelex resin is shown in Fig. 3. The analysis of the sorption isotherm yields a site capacity of 0.4 meq/g of dry resin (Fig. 3B). The sorption was found to be strong in the pH range studied with K_d

values higher than 10^4 L/kg for trace U(VI) concentrations. Two species must be considered to describe the sorption behaviour; the 1:1:1 dominates the sorption below pH 4, while the 1:1:0 species is predominant between pH 4 and 7 (see species distributions in Fig. 3A). The decrease in K_d value above pH 6 can be explained by the competition between Chelex-100 resin and OH$^-$ for complexation of U(VI). The quantitative description was performed with the parameters given in Table 1. The data are in good agreement with a study made with the model sub-unit N-methyl iminodiacetic acid [14]: a 1:1:0 species was characterized in the pH range 4.5–6.5 with a complexation constant of $10^{10.55}$ (recalculated at zero ionic strength), compared to a value of $10^{10.85}$ for the Chelex-100 resin found in this study.

U(VI) complexation with L8

The formation of a binuclear complex 2:1 between U(VI) and L8 has been shown in solution by a continuous variation method [7,9-10] and has been characterized in the solid state by X-ray diffraction (XRD) with the *para-tert*-butyl derivative of L8 [15]. This stoichiometry was therefore assumed for our calculations. The number of protons involved in the equilibrium (z, see eq. (3)) was assessed by studying the pH dependence of K_d using the competition method with Chelex-100 (Figs. 4A and B). Assuming the existence of a 2:1:-3 species as reported by Sonada *et al.* [10] (Table 1), the prediction of the competition between L8 and Chelex-100 does not agree well with the experimental data (dashed lines). However, a good agreement is obtained when the formation of a 2:1:0 species [7,9] with a stability constant of $10^{31.4}$ is considered (solid lines). This constant also allows to describe the titration study performed at pH 5.7 (Fig 4C) and is in good agreement with the one obtained from the competition study using PAR ($10^{30.5}$, Fig. 4D, Table 1).

U(VI) complexation with L6

According to the literature [6,9], U(VI) and L6 form complexes with a stochiometry of 1:1; therefore x and y were set to 1 in our calculations. Data given by Shinkai *et al*. obtained at pH 10.4 were considered in the data analysis (Fig. 5A). The competition between PAR and L6 for U(VI) was studied at pH 7.4 (Fig. 5B). The effect of pH was studied by Time Resolved Laser Induced Fluorescence Spectroscopy (TRLFS) in the pH range 4–8 (Fig. 5C).

First, the model of Shinkai *et al*. was tested, considering the formation of a 1:1:0 species with a stability constant of $10^{19.7}$. However, the modelling results do not agree with our experimental data. The complexation of U(VI) with L6 is systematically overestimated (dotted lines).

Secondly, the model of Sonoda *et al*. was tested: the 1:1:-3 complex is the dominant species at pH above 10, 1:1:-2 and 1:1:-3 complexes coexist in the pH range 6-10 and 1:1:-1 and 1:1:0 complexes are formed below pH 6 (supplementary information, Fig. SM-1A). The competition between carbonate ions and L6 for complexation of U(VI) studied at pH 10.4 by spectrophotometry in the work of Shinkai *et al*. is therefore a direct evidence of the formation of the 1:1:-3 complex. The quantitative analysis of the data gives a constant in good agreement with the one determined by potentiometric titration by Sonoda *et al*. (see Table 1). Considering the different methodologies used, this confirmed the reliability of the obtained constant. The 1:1:-3 complex contributes to the speciation at physiological pH, but at the same time the 1:1:-2 complex is present (see Fig. SM-1A). The constant characterizing the formation of the 1:1:-2 complex has therefore been deduced from the data obtained at pH 7.4 using the competition method with PAR (Fig. 5B) while fixing $\log\beta_{11-3}$ to that determined above. A good agreement between the calculation and the experimental data is found provided that $\log\beta_{11-2}$ is increased with

respect to the data given by Sonoda *et al.* (see Table 1). The two constants previously determined (for 1:1:-2 and 1:1:-3 complexes) were then used together with those given by Sonoda *et al.* [11] for 1:1:0 and 1:1:-1 species to predict the pH dependence studied by TRLFS between pH 4 and 7. The results presented in Fig. 5C show that the agreement between the experimental data and the prediction (solid line) is satisfactory. In the conditions of the present study and using the constant determined from Fig. 5B for 1:1:-2 complex, 1:1:0 and 1:1:-1 species do not significantly contribute to the complexation of uranyl ion (less than 3 %, see Fig. SM-2 in the supplementary information).

Stability of U(VI) complexes with L6 and L8 under blood serum conditions

In order to evaluate the stability of U(VI)-*para*-sulfonatocalixarene complexes under blood serum conditions, an experimental approach is required that allows to distinguish between the fraction of U(VI) complexed with L6 or L8 and the fraction of U(VI) that has been released from the *para*-sulfonatocalixarene complex and is bound to carbonates and/or the protein pool, which are the major competing components for uranium binding in blood serum [5]. In the present work, the distinction was achieved based on the differences in lability of the complexes using Chelex column chromatography. Under blood serum conditions, U(VI) is mainly complexed by carbonate, human serum albumin and transferrin [5]. It was found that these complexes are not stable enough to compete with a high excess of Chelex resin, i.e. when passing a solution of uranyl ion equilibrated in blood serum through a Chelex-100 column, only 13 ± 4% (n=9) of U(VI) were eluted through the column, while the remaining major fraction of U(VI) was sorbed to the resin. However, when U(VI) is complexed with L6 or L8, typically less than 20% of U(VI) are sorbed on the resin (Table 2) whereas the calculation based on thermodynamic data predicts a complete sorption. The complex is therefore sufficiently kinetically stable over the time of the experiment (a few minutes) to

avoid any significant decomplexation, probably because of macrocyclic structure of the ligand [7].

After complexation of U(VI) with L6 or L8 in HEPES buffer at physiological pH, the stability of the formed complexes was followed after dilution in 0.9 % NaCl or human blood serum. In agreement with the model calculations, while no significant decomplexation is expected in 0.9 % NaCl in equilibrium with atmospheric CO_2, U(VI)-L6/U(VI)-L8 complexes were found to be rapidly dissociated in blood serum at carbonate concentrations of 25 mmol/L.

Discussion

We report a set of parameters describing the complexation of U(VI) with *para*-sulfonatocalix[6]arene based on the model of Sonoda *et al.* [11]. These parameters enable the results obtained using different methods over a wide range of pH (3.5−11) to be explained. Assuming the commonly observed pseudoplanar hexa- or penta-dendate coordination geometry for uranyl ion, and considering that the sulfonato groups are not involved in the interaction, the metal ion would be bound by two deprotonated –OH groups and four or three water molecules, the latter undergoing progressive deprotonation with increase in pH. This is consistent with the form of the 3:3 U(VI)-L6 complex characterized by XRD, where the calixarene acts as a bidentate ligand [16]. However, it cannot be excluded that the proton release observed at increasing pH is due to the dissociation of phenolic hydroxyl groups (presenting no initial acid-base properties in the pH range investigated) arising from a metal ion-induced calixarene reorganization [11]. In agreement with this model, a four-fold deprotonated calixarene moiety was characterized by XRD in the case of the 2:2 complex providing each uranyl ion with a close-to-octahedral coordination [17]. The significant difference between our findings and the model of Sonoda *et al.* [11] is the increase of the equilibrium constants for the 1:1:-3 and 1:1:-2

complexes, which are the species governing U(VI) speciation at physiological pH. The evaluation of the data of Sonoda *et al.* [11] shows some inconsistencies. According to the paper, the authors do not take into account the possible formation of hydroxo species in the data evaluation. This would be a viable assumption only if the complexation strength of L6 was strong enough to avoid hydrolysis of U(VI). The data were reevaluated using the thermodynamic database of Guillaumont *et al.* [18] (supplementary information, Fig. SM-1). If the assumption is valid above pH 6, where the species 1:1:-3 and 1:1:-2 are present, this is not the case in the pH range 3−6 where 1:1:0, 1:1:-1 and 1:1:-2 species prevail, *i.e.* as much as 17 % of U(VI) is predicted to exist as polynuclear hydroxo species. They account for the proton mass balance and the constants of 1:1:0, 1:1:-1 and 1:1:-2 complexes obtained by potentiometric titration are therefore underestimated. Another reason which would explain this underestimation is related to the slow kinetics characterizing U(VI)/L6 interaction, which has also been observed in the current work. As pointed by out by Shinkai *et al.* [6], this limits the applicability of the potentiometric titration method as equilibrium conditions may not be reached, but this point was not discussed in the work of Sonoda *et al.* [11].

The model of Shinkai *et al.* significantly overestimates the stability of the 1:1:0 complex. Assuming the stability constant reported by Shinkai *et al.,* U(VI) should be quantitatively complexed by L6 under blood serum conditions (data in parenthesis in Table 2), while our experimental tests show a rapid complete dissociation of the complex upon introduction into serum media. In line with the report of Harrowfield [16], the ideal preorganisation of L6 −OH sites for U(VI) postulated by Shinkai *et al.* [6] to explain this high stability constant is not likely: L6 is unable to adopt a conformation in which it encircles the linear uranyl ion to get the preferred pseudoplanar hexa- or pentadendate coordination geometry. As discussed above, additional ligands (H_2O, OH^-) are rather involved to complete the

coordination sphere.

In the case of U(VI) complexation by L8, the pH dependence shows the existence of the 2:1:0 complex instead of the 2:2:-3 proposed by Sonoda *et al.* [10]. This is in agreement with the crystallographic structure obtained with the *p-tert*-butylcalix[8]arene where the ligands acts as a tetraanion [15]. The stability constant obtained by two independent experimental methods is lower than the one proposed by Archimbaud *et al.* [9] and Nagasaki *et al.* [7]. A detailed examination of the data of Nagasaki *et al.* does not confirm the reported constant. The authors considered in the modeling only the presence of CO_3^{2-}, whereas a significant amount of HCO_3^- exists (about 40 %) under their experimental conditions. The given $\log\beta_{210}$ is thus overestimated and a value of $\log\beta_{210}= 37.1$ (at zero ionic strength) was recalculated. This correction however does not significantly change the constant and the former remains six order of magnitudes higher than the one determined in our work using two different methodologies in a wide range of experimental conditions. Furthermore, similarly as for L6, our model confirmed that L8 cannot compete with proteins and carbonate for complexation of U(VI) in serum media as shown by *in vitro* tests, whereas the model of Nagasaki *et al.* predicts a significant *in vitro* stability of U(VI)-L8 complex (see data in parenthesis in Table 2).

Experimental

Reagents and conditions

The stock solution of natural uranium(VI) 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 uranium concentration of 1×10^{-3} M at pH ~ 3. For radiotracer experiments, either ^{230}U ($t_{1/2}= 20.8$ d) or ^{237}U ($t_{1/2}= 6.75$ d) were used.

^{230}U was produced *via* proton irradiation of natural ^{232}Th at the cyclotron of JRC-IHCP, Ispra, according to the reaction ^{232}Th(p,3n)^{230}Pa(β$^-$)^{230}U [3] and separated from irradiated targets using a combination of extraction chromatography and sorption to silica gel. ^{237}U formed from alpha decay of ^{241}Pu ($t_{1/2}$= 14.35 y) was separated from a plutonium stock solution using extraction chromatography as described in [19]. Radioactivity measurements of ^{230}U and ^{237}U were performed by high resolution gamma spectrometry (HRGS) using a calibrated HpGe well detector connected to a 92X spectrum master multi-channel analyzer (EG&G Ortec). ^{237}U was analysed *via* the 59.5 keV gamma emission (34% emission probability). Activity of ^{230}U was analysed using the 111 keV gamma emission (3.2% emission probability) of its daughter nuclide ^{226}Th after radiochemical equilibrium was reached, typically after 1 day.

A stock solution of 8.4 × 10^4 M PAR (4-(2-pyridylazo)resorcinol, p.a., Fluka) was prepared by dissolution of PAR in 0.05 M TRIS (tris(hydroxymethyl)aminomethane) buffer. A stock solution of 10^{-2} M L6 (*para*-sulfonatocalix[6]arene hydrate, 95%, Acros Organics) was prepared by dissolution of L6 in 0.9% NaCl, filtered through 0.22 μm filter cartridges (Whatman, Spartan-HPLC Syringe filters) and used for further dilutions. A stock solution of 0.12 M L8 (*para*-sulfonatocalix[8]arene hydrate, containing 20% water, TCI Europe) was prepared by dissolution of L8 in 0.9% NaCl and filtered as described above.

Chelex-100 extraction resin (sodium form, 50-100 mesh (dry), Sigma) was washed several times with Milli-Q water until neutral conditions and conditioned with 0.9% NaCl prior to use. For serum stability studies, 0.5 mL of wet resin was loaded in polypropylene chromatographic columns (Bio-Spin, Bio-Rad Laboratories, Inc.) and covered with quartz wool.

For the preparation of human blood serum, blood samples were collected from healthy

volunteers into Vacutainers containing EDTA as anticoagulant (0.2 % in weight). A simulation made with the parameters quantifying U(VI)/EDTA[28] and U(VI)/serum components[5] interaction showed no effect of the presence of the chelating agent on U(VI) speciation. Serum was separated from blood cells by centrifugation, stored at -20 °C, and brought to room temperature immediately before use. All other chemicals were reagent grade and all solutions were prepared in Milli-Q water. Experiments were performed at room temperature (23 ± 3 °C) in the pH range 4–11 at an ionic strength of 0.1 (NaCl or NaClO$_4$) under normal atmosphere.

Investigation of interactions of U(VI) with *para*-sulfonatocalixarenes

Considering the high complexation strength of L6 and L8 with U(VI), competition methods were used to study the complex formation. The species distribution of U(VI) was determined based on sorption to Chelex chelating resin followed by phase separation (CM-Chelex) or by spectrophotometric detection in the presence of PAR as competing ligand (CM-PAR). In the case of L6, TRLFS was used as a third independent method. All solutions/suspensions used for the sorption/complexation studies were prepared well in advance to allow for equilibration with atmospheric CO$_2$.

Competition method with Chelex-100 resin (CM-Chelex)

Chelex-100 is a strong chelating resin containing iminodiacetic acid groups as active goups. Experiments were performed based on a classical batch method. A pre-equilibration of solid and liquid phases was performed before addition of U(VI) (*i.e.* the solution composition is expected not to vary in the presence of the solid during the adsorption experiments). After addition of U(VI), solutions were left to equilibrate (< 12 h), followed by addition of the competing ligand. The system re-equilibration was found

to be relatively slow, *i.e.* at least 2 days and 12 hours were necessary for L6 and L8, respectively. The separation between liquid and solid phases was performed by centrifugation. The sorption of U(VI) on Chelex was expressed by Eq. (1):

$$K_d = \frac{A_{tot} - A_L}{A_L} \cdot \frac{L}{S}$$

(1)

where K_d is the distribution coefficient, A_{tot} is the overall activity added to the system (Bq), A_L is the equilibrium activity in the liquid phase (Bq), and S/L the solid to liquid ratio in kg/L. To exclude effects of adsorption of the metal ion to the walls of the vials, A_{tot} was determined by analysing the uranium activity of an aliquot of the suspension. Preliminary experiments showed that the sampling of the suspension was homogeneous.

Competition method with PAR (CM-PAR)

Competition experiments with PAR were performed at the pH of human blood serum (pH 7.3 ± 0.1). Solutions containing U(VI) and PAR were equilibrated for 1 min before addition of the competing ligand. The re-equilibration time was shown to be fast (less than 15 min). Spectra were recorded on a Ultrospec™ 2100 *pro* UV/Visible spectrophotometer in the wavelength range from 300-600 nm.

Time Resolved Laser Fluorescence Spectroscopy (TRLFS)

Details concerning the spectroscopic device as well as details on how spectroscopic data were obtained are described in.[20] U(VI) was excited at a wavelength of 430 nm with a laser intensity of about 3 mJ. U(VI) complexation by L6 leads to a complete extinction of the fluorescence signal. This extinction cannot result from absorption processes, as negligible absorption coefficients at the excitation (430 nm) and the fluorescence emission (460-600 nm) wavelengths were observed for L6. In given experimental conditions, and considering a static

quenching phenomenon, the percentage of U(VI) not bound to L6 can be determined according to Eq. (2):

$$\%(\text{U(VI)}) = \frac{\text{FI}}{\text{FI}_{\text{tot}}} \qquad\qquad (2)$$

FI_{tot} and FI correspond to the fluorescence intensity measured before and after addition of L6, respectively.


Serum stability studies

The stability of U(VI)-L6 and -L8 complexes in human blood serum was studied using a Chelex-100 column method. For the separation of U(VI)-*para*-sulfonatocalixarene complexes from uranium species not bound to *para*-sulfonatocalixarene (i.e. uranium bound to carbonate or serum proteins), an aliquot of sample solution was loaded on the column and subsequently the column was washed with 3 mL 0.9% NaCl to elute U(VI)-*para*-sulfonatocalixarene complexes (fraction 1). "Non-calixarene-complexed" uranium was retained on the column and was subsequently eluted from the column with 3 mL 1 M HCl (fraction 2). Both fractions were measured by HRGS and activity of [230]U in each fraction was expressed as % of total activity. Blank runs were performed to determine the recovery of "calixarene-bound" and "non-calixarene-bound" uranium in fractions 1 and 2, respectively. For serum stability studies, complexation of [230]U with L6 or L8 was performed in HEPES buffer by stepwise addition of the minimum amount of ligand required to achieve > 80% complexation after equilibrium was reached. Subsequently, the sample was divided and diluted with 0.9% NaCl (served as a reference) or human blood serum (see conditions in Table 2) and the fraction of *para*-sulfonatocalixarene-bound uranium was analysed at regular time intervals as described above.

Modelling approaches

Complexation in solution

The interaction between the metal ion (M) and the ligand (L) is described by the following equilibrium as presented in Eq.(3):

$$xM^{m+} + yL^{n-} + zH^+ \rightleftarrows M_xL_yH_z^{(mx-ny+z)+} \tag{3}$$
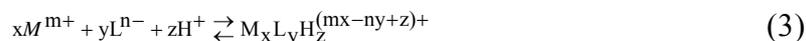
with n corresponding to the number of acid protons presenting acid-base properties, z the number of protons involved in the reaction, x the number of metal ions complexed and y the number of ligands complexing M. The associated stability constant is defined by Eq.(4):

$$\beta_{xyz} = \frac{(M_xL_yH_z^{(mx-ny+z)+})}{(M^{m+})^x (L^{n-})^y (H^+)^z} \tag{4}$$

Where (i) represents the activity of species i. All ionic strength corrections were done using the Davies equation [21].

In the case of PAR, z = 2 with pK_a values of 5.56 and 11.98 (0.1 M ionic strength) [12]. Based on the XRD structures obtained with the para-t-butylcalixarenes and U(VI) [15-17], we assume that U(VI) interacts with the hydroxyl groups of the ligands and that the sulfonato groups are not involved in the complexation. L6 has two ionizable OH groups [22,11] in the pH range generally considered for complexation studies (pH 3–12). pK_a values were taken from [11], *i.e.* 3.28 and 4.86 (0.1 M KNO$_3$, 25 °C). L8 can be viewed as two tetrameric units originating from the pinched conformation as fairly independent from one another [22]. For each tetrameric unit, two types of hydroxyl groups were observed: one acidic type and one which dissociates in the alkaline region. pK_a values of 3.73, 4.39, 8.07 and 10.1 (0.1 M KNO$_3$, 25 °C) were taken from Sonoda *et al.* [10] When the mass balance of the reaction indicates that more protons are produced than consumed [10,11], we consider that they arise from the complexation of uranyl hydroxo species as presented in Eq.(5):

$$xM^{m+} + yL^{n-} + zH_2O \rightleftarrows M_xL_yOH_z^{(mx-ny-z)+} + zH^+ \tag{5}$$
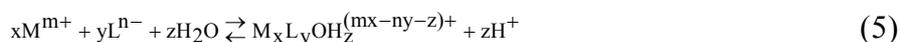
The equilibrium constants describing the interaction between U(VI) and inorganic ligands (chloride, carbonate, hydroxide) were taken from Guillaumont *et al.* [18]. Carbonate concentration, unless fixed, was calculated considering an equilibrium with atmospheric CO_2 (p_{CO_2} = -3.5). All the calculations were done using the simulation code PHREEQC, a modelling code for aqueous systems [23].

U(VI) sorption on Chelex

Chelex resin is a styrene divinylbenzene copolymer containing iminodiacetic acid chelating groups. The resin is considered to act as a "solid complexing" agent, *i.e.* the reactions characterizing the interaction in solution (acid properties, complexation reaction) are supposed to occur at the resin surface. The surface complexation model developed for surfaces of oxides was used [24] and Eq. (3–5) are therefore applicable. The most important difference between complexation of U(VI) with ligands in solution in comparison to complexation with Chelex resin is that in the case of the resin, the ligand is bound on a solid support and the dependence of the complexation strength on the ligand concentration is not the same. Furthermore, the activity coefficient correction is different if the ligand is free or bound. When the ligand is bound, an electrostatic effect should be considered because of the surface charge [25], e.g. using one of the models developed for oxides available in the PHREEQC code. However, the most significant difference between Chelex resin and sorption on the oxides is that Chelex resin swells as the pH increases (protons are replaced by Na^+ ions) [26]. This leads to a change of the specific surface and does not allow a simple correction for the electrostatic term to be made. The electrostatic effects were therefore neglected in this study, *i.e.* the activity coefficient of the bound species is equal to 1. Acid-base properties of the resins were investigated previously [25, 27]. The authors came to the conclusion that the pK_a values are in agreement with those

reported for the two model ligands, the benzyl- and methyl-iminodiacetic acids. In the present study, published pK_a values of N-benzyl-iminodiacetic acid were used (2.21 and 8.9, 0.1 M ionic strength, 25 °C) [28].

Conclusions

A new set of parameters is reported to describe U(VI) interaction with L6 and L8. Contrary to what is stated in the literature, L6 cannot be considered as a super-uranophile as its structure is not ideally pre-organized for U(VI) complexation. L8 can accommodate two uranyl ions without other synergistic anions in the first coordination sphere. Both simulation and experimental data show that L6 and L8 are not suited for TAT applications due to the low stability of their U(VI) complexes under blood serum conditions where the expected ligand concentrations would be less than 10^{-8} M.

Acknowledgements

Abbreviations

CM   competition method

HEPES   4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

L6   *para*-sulfonatocalix[6]arene

L8   *par*a-sulfonatocalix[8]arene

PAR   4-(2-pyridylazo)resorcinol

TRIS   tris(hydroxymethyl)aminomethane

TRLFS   time-resolved laser-induced fluorescence spectroscopy

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Table 1. Quantitative description of the complexation of U(VI) with PAR, Chelex resin, L6 and L8.

	Number of complexes	complex x:y:z	log β_{xyz}	Note	Conditions	Method	Ref
PAR	1	1:1:0 †	16.1 ± 0.3	–	0.1 M NaCl, pH 7.3±0.1	Spectrophotometric determination	this work
Chelex-100	2	1:1:1	6.0 ± 0.2	–	0.1 M NaCl, pH 1.5–7.8	batch experiments	this work
		1:1:0	10.8 ± 0.3				
L6		1:1:0 †	*19.7 ± 0.1*	re-evaluated at 19.2	0.01 M carbonate, pH 10.4, I~0.025 M	competition method with carbonate	6
	4§	1:1:0	*4.33*	–	0.1 M KNO$_3$, pH 4–11, 25°C	potentiometric titration method	11
		1:1:-1	*0.39*				
		1:1:-2	*-4.6*				
		1:1:-3	*-12.7*				
	4	1:1:0	4.33	fixed in the fitting	–	–	this work
		1:1:-1	0.39	fixed in the fitting	–	–	
		1:1:-2	-3.4 ± 0.2	log $\beta_{11\text{-}3}$ fixed at -12.2	0.1 M NaCl, pH 7.4	competition method with PAR	
		1:1:-3	-12.2 ± 0.1	Analysis of the data from ref. 6	0.01 M carbonate, pH 10.4, I~0.025 M	competition method with carbonate	
		–	–	all parameters fixed (log$\beta_{11\text{-}2}$ and log$\beta_{11\text{-}3}$ fixed to -3.4 and -12.2, respectively)	0.1 M NaClO$_4$, pH 3.5–7	TRLFS	
L8	1	2:1:0 †	40.8	–	not given	not given	9
	1	2:1:0 †	*37.6*	re-evaluated as 37.1	0.01 M carbonate, pH 10.4, I~0.025 M	competition method with carbonate	7
	1§	2:1:-3	*13.4*	–	0.1 M KNO$_3$, pH 4–11 25°C	potentiometric titration method	10
	1	2:1:0	31.4 ± 0.4	–	0.1 M NaCl, pH 3–7	Competition method with Chelex-100	this work
	1		30.5 ± 0.3	–	0.1 M NaCl, pH 7.3±0.1	Competition method with PAR	

§ number of species determined in the pH range of interest of the study (4-11);
† z=0 is an assumption based on literature data; data in italic: log K values recalculated at zero ionic strength

Table 2. Serum stability of U(VI)-*para*-sulfonatocalixarene complexes; comparison between experiment and calculation. The data in parenthesis are calculated with the parameters given in the literature [6,7]. HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Ligand	Conditions, $[U(VI)]_{tot} \sim 10^{-8}$ M	time	% exp	% calc	conditions	time	% exp	% calc
		Complex formation			**dissociation**			
L6	4.7×10^{-5} M L6 pH 7 0.1 M HEPES	3.8 h	82	100 (100)	dilution 1:10 in 0.9 % NaCl. pH 7.6, 37°C	15 h	85	99 (100)
	10^{-5} M L6, pH 7 0.1 M HEPES	5.8 h	80.5	99 (100)	dilution 1:10. serum, 37 °C	2 h	15	0 (100)
L8	2.1×10^{-5} M L8 pH 7.6 1 M HEPES	10 min	91	99 (100)	dilution 1:10 in 0.9 % NaCl. pH 7.6, 37°C	200 h	90	97 (100)
	2.5×10^{-5} M L8 pH 7.6 0.8 M HEPES	30 min	86.4	99 (100)	dilution 2/9. serum, 37 °C	10 h	0	0 (39)
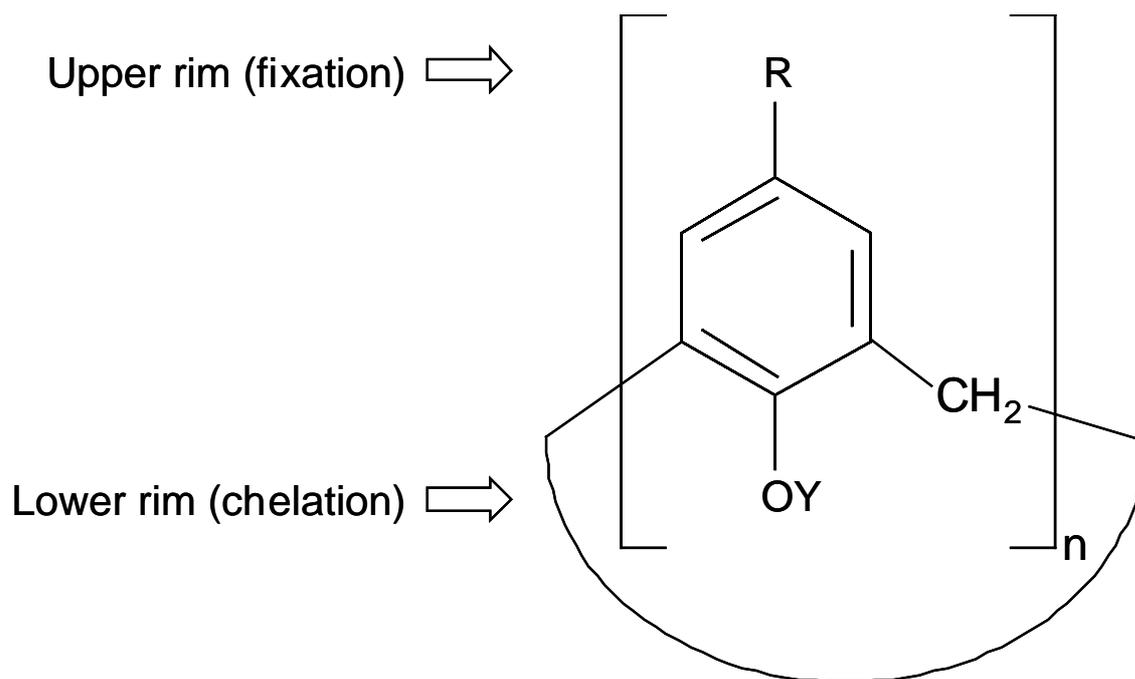
Figure 1: Calixarene family and selected calixarenes: L6 (n=6, R=-SO$_3$Na; Y=H) and L8 (n=8, R=-SO$_3$Na; Y=H).
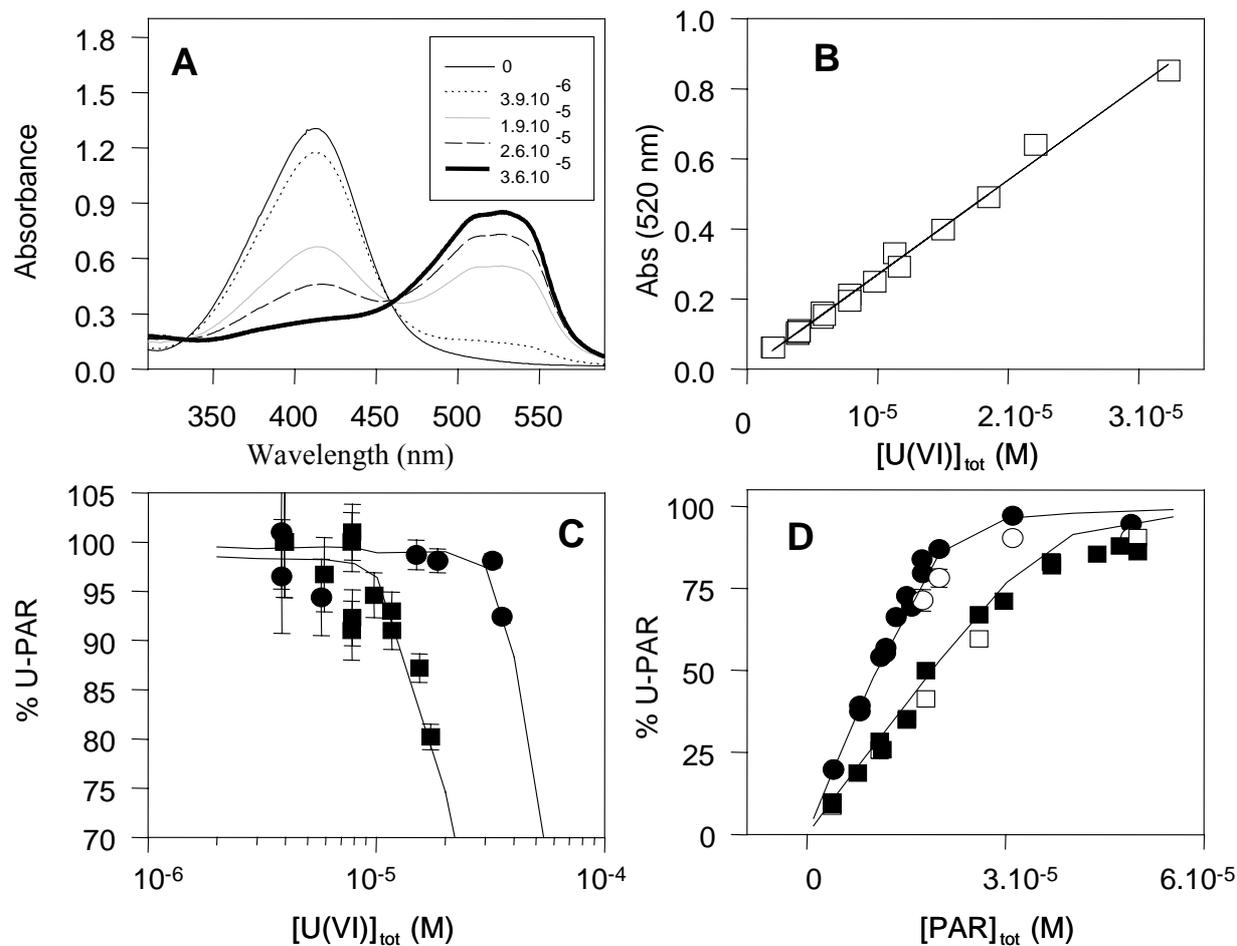
Figure 2: U(VI) interaction with PAR, pH 7.3 ± 0.1, 0.1 M NaCl, 2 × 10^{-3} M TRIS. (A) effect of U(VI) concentration on PAR absorption spectra. (B) Absorption of the complex at 520 nm for as a function of U(VI) concentration. The line is calculated with $\varepsilon = 2.7 \times 10^4$ L mol^{-1} cm^{-1} (U(VI)-PAR) at $\lambda_{max} = 520$ nm, [PAR]$_{tot} = 4 \times 10^{-5}$ M. Complexation of U(VI) with PAR as a function of uranium concentration (C; circles: [PAR]$_{tot} = 3.8 \times 10^{-5}$ M, squares: [PAR]$_{tot} = 1.6 \times 10^{-5}$ M) and PAR concenration (D; circles: [U(VI)]$_{tot}$=1.9 × 10^{-5} M; squares: [U(VI)]$_{tot} = 3.6 \times 10^{-5}$ M) concentrations. The filled and open symbols are obtained from the absorbance at 520 nm and 413 nm, respectively. The lines are calculated with the parameters given in Table 1.
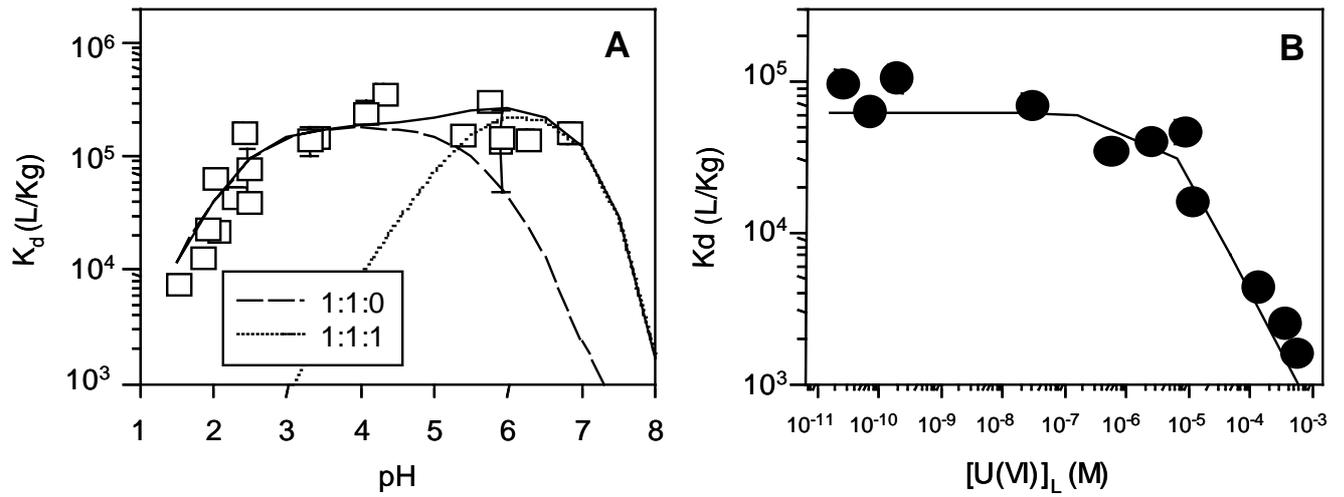
Figure 3. U(VI) sorption on Chelex-100 in 0.1 M NaCl (S/L=0.05-1 g/L) as a function of pH ($[U]_{sol} < 10^{-7}$ M), (A) and uranium concentration (pH 2.2) (B). The lines are calculated with the parameters given in Table 1.
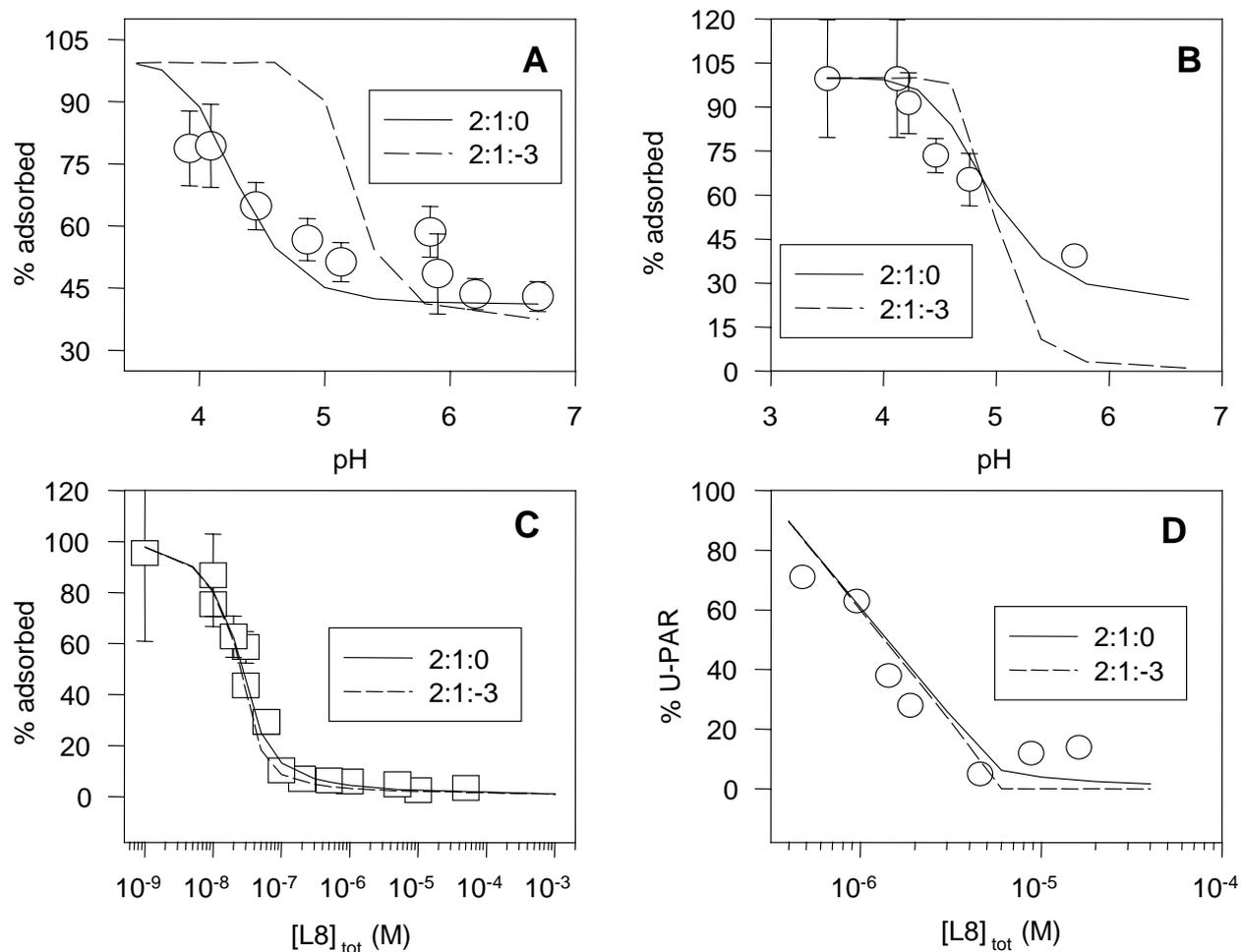
Figure 4. Complexation study between L8 and U(VI) using CM-Chelex (A, B, C) and CM-PAR (D) methodologies. (A) S/L=0.14 g/L, $[U(VI)]_{tot}=10^{-7}$ M, $[L8]_{tot} =3 \times 10^{-8}$ M. (B) S/L=0.5 g/L, $[U(VI)]_{tot}=10^{-7}$ M, $[L8]_{tot}=6 \times 10^{-6}$ M. (C) S/L=0.14 g/L, $[U(VI)]_{tot}=10^{-7}$ M, pH 5.7. (D) $[PAR]=1.6 \times 10^{-5}$ M, pH 7.3, $[U(VI)]_{tot}=7.9 \times 10^{-6}$ M. All experiments were performed in 0.1 M NaCl. The dotted lines are the predictions made with the parameters of Sonoda *et al.* [11]. The solid lines are calculated with the model presented in this work (Table 1).
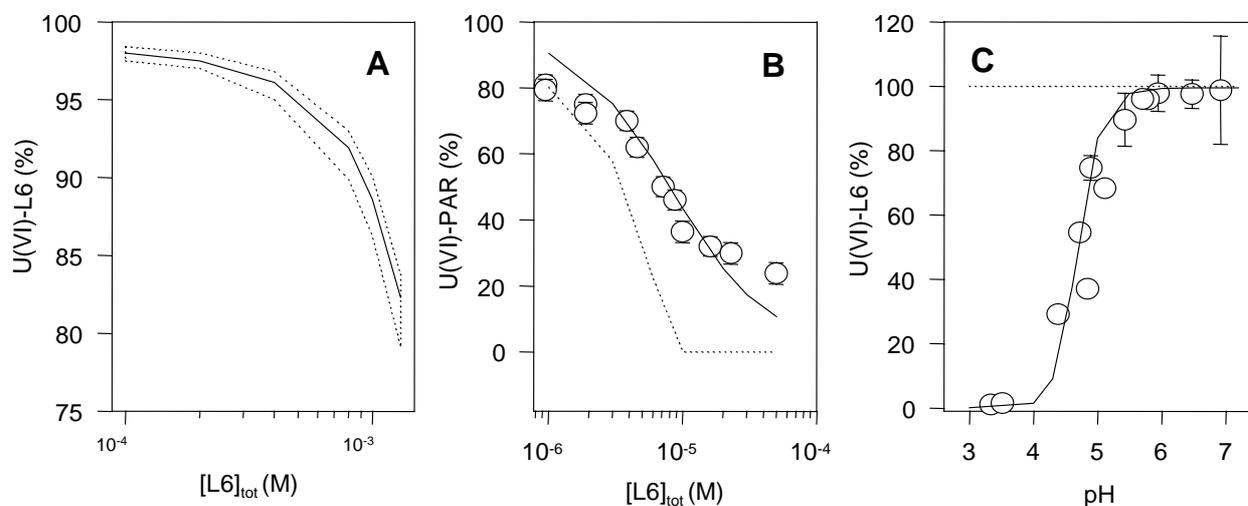
Figure 5: U(VI) Complexation by L6. (A) Data range (dotted lines) calculated based on the data of Shinkai *et al.* [6] (the two lines account for the error associated to the published constant) pH 10.4, $[K_4UO_2(CO_3)_3]=1.51 \times 10^{-3}$ M, 0.01 M carbonates. (B) Competition method with PAR. $[PAR]_{tot}=1.5 \times 10^{-5}$ M, pH 7.4 and $[U(VI)]_{tot}=7.9 \times 10^{-6}$ M; $[NaCl]=0.1$ M. (C) Study as a function of the pH by TRLFS; $[NaClO_4]=0.1$ M, $[U(VI)]_{tot}=1.5 \times 10^{-6}$ M and $[L6]=10^{-5}$ M. The dashed lines are calculated based on the model of Shinkai *et al.* [6] .The solid lines correspond to our model (Table 1).
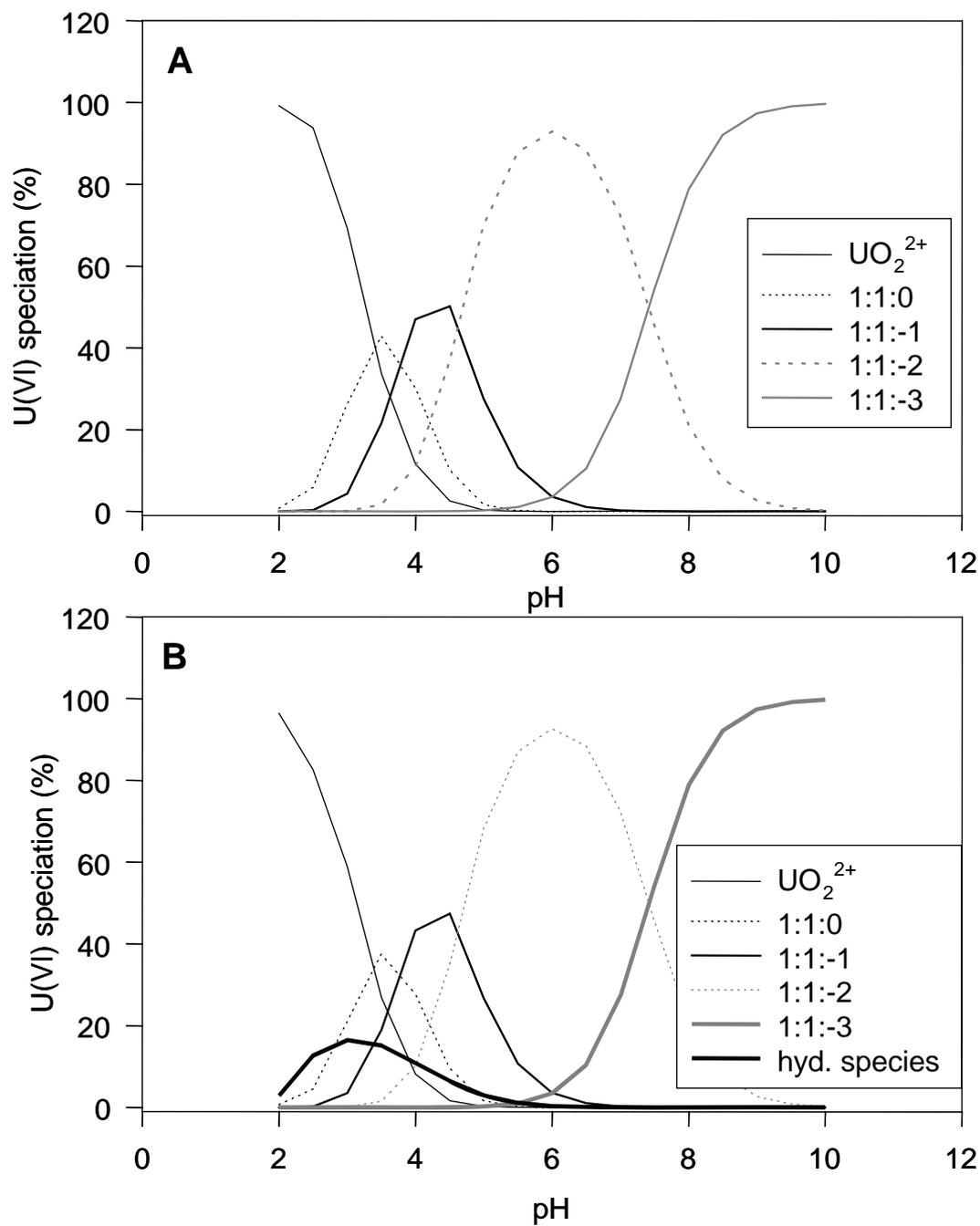
Figure SM-1: Complexation of U(VI) by L6. Speciation diagram as a function of pH according to the work of Sonoda *et al.* [11], considering (B) or not considering (A) the formation of hydrolysed species (hyd. Species; notably $(UO_2)_2OH^{3+}$ and $(UO_2)_2(OH)_2^{2+}$).
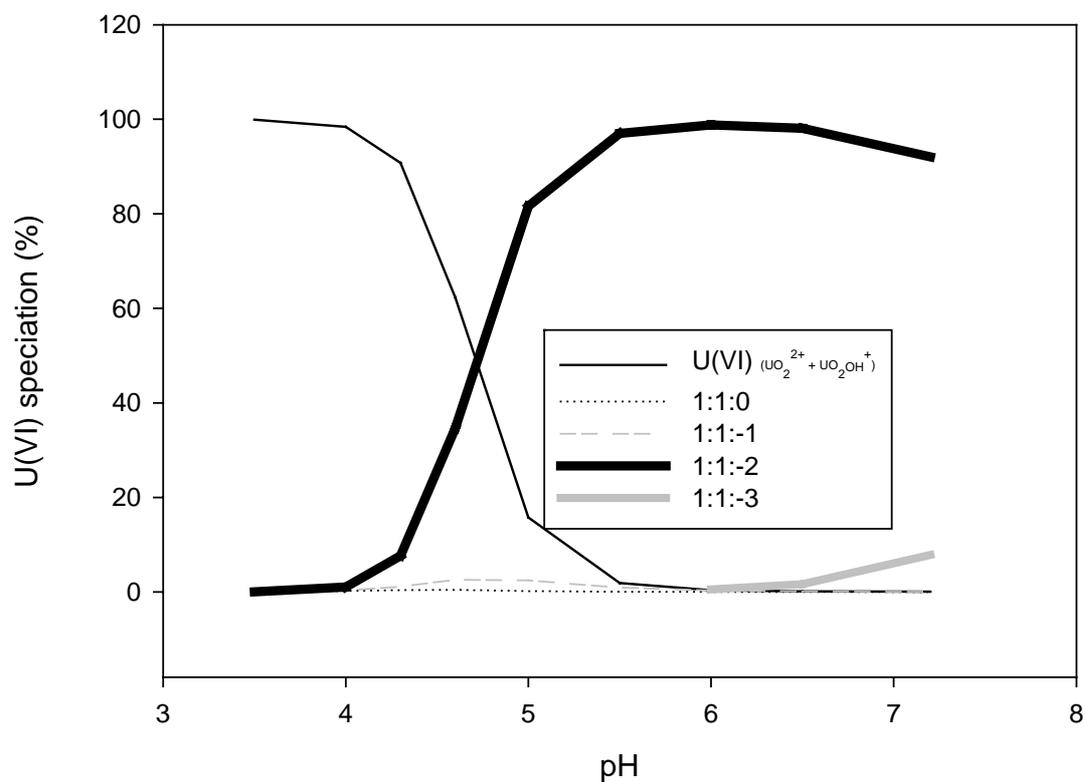
Figure SM-2: Complexation of U(VI) by L6. Speciation diagram calculated with the parameters determined in this work (Table 1) as a function of the pH in the conditions of Figure 5C, i.e. $[NaClO_4]=0.1$ M, $[U(VI)]_{tot}=1.5 \times 10^{-6}$ M and $[L6]=10^{-5}$ M.