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In the case of oral ingestion of radioactive contaminants, the first contact medium is saliva in the mouth. To gain a first insight into the interaction of radioactive contaminants in human saliva, the speciation of curium (Cm(III)) and europium (Eu(III)), *i.e.*, trivalent *f*-elements, was investigated in different salivary media with time-resolved laser-induced fluorescence spectroscopy (TRLFS). The results indicate that these metal cations are primarily complexed with carbonates and phosphates, forming the ternary complexes with a possible stoichiometry of 1:1:2 (M(III) : carbonate : phosphate). For charge compensation, calcium is also involved in these ternary complexes. In addition to these inorganic components, organic substances, namely α-amylase, show a significant contribution to the speciation of the trivalent *f*-elements in saliva. This protein is the major enzyme in saliva and catalyzes the hydrolysis of polysaccharides. In this context, the effect of Eu(III) on the activity of α-amylase was investigated to reveal the potential implication of these metal cations for the *in vivo* functions of saliva. The results indicate that the enzyme activity is strongly inhibited by the presence of Eu(III), which is suppressed by an excess of calcium.

Introduction

The accidental releases of radioactive materials from nuclear facilities, such as Chernobyl or Fukushima nuclear power plant accidents[1,2] or the dispersal events of so-called "dirty bombs"[3,4] could potentially cause large-scale radioactive contamination of the environment, which would eventually increase the risk for human to incorporate radionuclides via inhalation or ingestion.[1-4] In case of incorporation into the human body, radionuclides such as transuranium (TRU) elements (*e.g.*, neptunium, plutonium, americium, and curium) potentially represent serious health risks due to their radiotoxicity.[5] In addition to the contamination with plutonium from global fallout, etc., the accidental or occupational contamination with trivalent TRU elements (*e.g.*, Am(III)) have also been reported by several official agencies such as USTUR (U.S. Transuranium and Uranium Registries).[6-9] Based on these reports, the biokinetic models on An(III) and other TRU elements in the human body have been developed and published in several NCRP (National Council on Radiation Protection and Measurements) and ICRP (International Commission on Radiological Protection) recommendations.[5,9-15]

In addition to the potential release of An(III) into the environment and bio-sphere, the emerging technological and medical applications of trivalent lanthanides (Ln(III)), a series of chemical analogues to An(III), have drastically activated the relevant mining activities in the last decades.[16] This results in the release of a considerable amount of lanthanides into the environment, which would raise the risk of their accumulation into humans via the food chain.[17,18]

In order to assess the toxicological behavior of An(III) and Ln(III) (namely trivalent *f*-elements), it is fundamental to understand their chemical speciation *in vivo* on a molecular level, because the chemical speciation of an element significantly influences its *in vivo* behavior, such as bio-availability, carcinogenicity, mutagenicity, metabolism, and biokinetics.[11,19-21] Despite these facts, very little is known about the speciation of trivalent *f*-elements in body fluids.[12,22] In particular, the studies on An(III) are scarce due primarily to the inherent ethical and technical difficulties associated with the use of highly radioactive materials with living organisms. Consequently, the precedent studies reporting the speciation of An(III) in body fluids have been limited primarily to blood and urine systems *in vitro* or in animals,[11,19,22-26] although there are several studies on the speciation in some target organs such as liver and bone.[19,27-30]

Saliva is one of the most important body fluids in the digestive system, as it is the primary contact medium in the human body in case of oral ingestion. Thus far, the speciation of actinides in human saliva has been reported only for hexavalent uranium

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(U(VI)).[31] Webb and co-workers have simulated the speciation of An(III) and Ln(III) in saliva based on thermodynamic data, reporting different outcomes between An(III) and Ln(III) due to the limited availability of thermodynamic database, especially for An(III), at that time.[32] These results are not substantiated by experiments up to date.

To compensate for the lack of experimental information on the speciation of An(III) and Ln(III) in saliva and to substantiate the contradictory results from the thermodynamic calculations by Webb and co-workers, we herein report the first experimental study on the speciation of An(III) and Ln(III) in human saliva by means of time-resolved laser-induced fluorescence spectroscopy (TRLFS). TRLFS is a powerful tool to investigate the chemical speciation of luminescent substances at trace concentrations relevant to *in vivo* or environmental conditions.[33-41] In the present study, Cm(III) and Eu(III) have been employed as representatives of An(III) and Ln(III), respectively. An(III) and Ln(III) have similar ionic radii, showing chemical similarities. Both Cm(III) and Eu(III) exhibit unique luminescence properties, being suitable for the probe of TRLFS which can provide information about the comprehensive chemical speciation of the target ions from multi-dimensional data-set (*e.g.*, spectra and lifetimes).

Experimental

Collection of natural human saliva samples

Ten samples of human saliva were collected from healthy adult volunteers without any gustatory or masticatory stimulation. Before sampling, each individual took at least one hour of interval after the last intake of food or drink. The sampling was performed by spitting saliva in a dry plastic tube for 20 – 30 min. Approximately 25 mL saliva were collected from each individual between 10:00 and 11:30 a.m., and the TRLFS measurements were carried out no later than 5 hours after the sample collection (*i.e.*, on the same day of sampling). Given the fact that saliva is in constant contact with air in the human mouth, the sampling in ambient conditions and the 5 hours of interval after the sample collection are not expected to have a significant influence on the chemical conditions of the collected saliva samples (*e.g.*, pH or carbonate content). The collected saliva samples were centrifuged for 10 min with 4000 rpm before further experiments to remove food debris or other solids.[42] Some saliva samples which were not possible be measured on the same day of sampling were stored at 4 °C for up to 24 hours until the measurement.

Analysis of salivary composition

The inorganic composition of the collected saliva samples was determined by inductively coupled plasma mass spectrometry (ICP-MS, PerkinElmer ELAN 9000) and ion chromatography (IC-system 732/733, Metrohm). The total organic carbon (TOC) was measured with a TOC/TN_b analyzer (multi N/C 2100 S, Analytik Jena AG). The pH values were determined with a BlueLine 16 pH electrode supplied from Schott.

Preparation of artificial saliva solutions

Three different types of artificial saliva solutions were prepared for comparison with the natural human saliva. Stock solutions of inorganic (NaCl, KCl, $CaCl_2$, $NaHCO_3$, $KHCO_3$, Na_2HPO_4, NaH_2PO_4, Na_2SO_4, and KSCN from Merck or Roth) and organic components (Na-citrate (Cit) from Roth, Na-L-lactate (Lac) from Fluka, and urea and uric acid from Acros) were freshly prepared by dissolving the solids in deionized water. All these chemicals were p.a. grade with > 99% purity. The proteins (porcine pancreas α-amylase (Amy) from Sigma and mucin (Muc) from Roth) were added to the sample solutions as solids. The resultant solutions showed circumneutral pH, on which additional pH adjustments were performed using HCl or NaOH.

Artificial saliva samples were prepared according to three different composition models: Model A: 20 mM KCl, 15 mM $NaHCO_3$, 5.3 mM KSCN, 1.4 mM NaH_2PO_4, 10 mM Lac; pH = 6.7 (based on Tani and Zucchi[43], modified after Sanna et al.[44]),

Model B: 24 mM KCl, 15 mM $KHCO_3$, 15 mM NaH_2PO_4, 10 mM NaCl, 8.0 mM Na_2SO_4, 4.0 mM KSCN, 1.0 mM $CaCl_2$, 0.1 mM urea, 6.6 mM uric acid, 1.0 g/L Amy (M = 55 kDa; 1 g/L of Amy equals to 0.018 mM protein containing 0.9 mM carboxyl groups[45,46]), 0.5 g/L Muc; pH = 6.5 (based on the international Unified Bioaccessibility Method (UBM) by the Bioaccessibiltiy Research Group of Europe (BARGE),[47] modified after Rotard et al.,[48] Oomen et al.,[49] and Marques et al.[50]), and

Model C: 4.5 mM $KHCO_3$, 4.0 mM Na_2HPO_4, 2.0 mM NaCl, 1.0 mM $CaCl_2$, 0.015 mM Cit, 1.0 g/L Amy, 0.5 g/L Muc; pH = 7.0 (based on own analytic data from human saliva).

Speciation determination of europium and curium in natural and artificial saliva

A stock solution of Eu(III) was prepared by dissolving $EuCl_3 \cdot 6\,H_2O$ (Sigma, 99.9 %) into deionized water, while a stock solution of Cm(III) (^{248}Cm in 1 M $HClO_4$) was supplied from Oak Ridge National Laboratory, U.S. Department of Energy, Office of Basic Energy Science. The stock solutions were spiked into 3 ml of human or artificial saliva to give the final concentrations of $(1 – 3) \times 10^{-5}$ M and 3×10^{-7} M for Eu(III) and Cm(III), respectively. All experiments with Cm(III) were carried out in an inert glove box. The pH values were measured before and after the metal addition in saliva solutions, confirming similar values with variations below ± 0.1 in pH unit. Therefore, no pH adjustment was carried out after the metal addition. TRLFS measurements were performed between five minutes and one hour after spiking, as the static luminescence spectra showed no significant changes within this period.

Time-resolved laser-induced fluorescence spectroscopic measurements

TRLFS data were collected either at 25 ± 1 °C or at 37 ± 1 °C using a pulsed flash lamp pumped Nd:YAG-OPO laser system (Powerlite Precision II 9020 laser equipped with a Green PANTHER EX OPO, Continuum). The temperature was controlled using a stirred cuvette holder (Flash 300™, Quantum Northwest). The laser pulse energy (between 1 and

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2 mJ) was monitored using a photodiode. The fluorescence emission spectra were recorded using an optical multi-channel analyzer-system, consisting of an Oriel MS 257 monochromator, a spectrograph with a 300 or 1200 lines per mm grating, and an Andor iStar ICCD camera (Lot-Oriel Group). Emission spectra were recorded in the ranges of 440 - 780 nm (300 lines per mm grating) and 570 - 650 nm (1200 lines per mm grating) for Eu(III) and Cm(III), respectively. A constant time window of 1 ms was applied. The excitation wavelength for Eu(III) samples was 394 nm, while 396 nm was used for Cm(III) samples. For time-resolved measurements, 40 - 60 spectra were measured with delay time steps between 10 and 50 µs.

For Eu(III), the emission bands of the $^5D_0 \rightarrow {}^7F_0$ (at ~ 580 nm), $^5D_0 \rightarrow {}^7F_1$ (at ~ 590 nm), and $^5D_0 \rightarrow {}^7F_2$ transitions (at ~ 615 nm) are of special interest. The first one is a symmetry forbidden transition and, hence, it should appear only upon the rearrangement of the first coordination shell of Eu(III). The magnetic dipole transition $^5D_0 \rightarrow {}^7F_1$ is hardly influenced by complexation, while the relative intensity and the splitting pattern of the hypersensitive induced electric dipole transition $^5D_0 \rightarrow {}^7F_2$ reflects changes in the local coordination environment of Eu(III).[51] In contrast, the luminescence signal of the $^6D_{7/2} \rightarrow {}^8S_{7/2}$ transition of Cm(III) shows significant red shifts with changes of its coordination environment.[52] The luminescence lifetimes of both metal ions are also strongly affected by their coordination environment, particularly in the first coordination shell. Water molecules have a strong quenching effect and, hence, the replacement of these water molecules in the first coordination shell with other coordinative ligands usually results in an increase of the luminescence lifetimes.

The TRLF spectra were analyzed using OriginPro9.0 (OriginLab Corporation). For better comparison, all Cm(III) spectra were normalized to the overall peak area, while all Eu(III) spectra were normalized to the area of the $^5D_0 \rightarrow {}^7F_1$ transition (585 – 600 nm). The lifetimes of luminescent species were generally determined by the following equation:

$$E(t) = \sum_i E_i \times \exp\left(-\frac{t}{\tau_i}\right) \quad (1),$$

where E is the total luminescence intensity at the time t, E_i the luminescence intensity of the species i at $t = 0$, and τ_i the corresponding luminescence lifetime. The observed decay mode was either mono- or bi-exponential. If the luminescence lifetimes of two co-existing species are very closed to one another or if one species has a small contribution to the sum spectrum, it could be fitted with a subordinate exponential function, meaning that further species could not be detected.

With the luminescence lifetimes (in ms), the number of water molecules in the first coordination shell of the metal ions ($n(H_2O)$) can be estimated using the following empirical equations:[53,54]

$$n(H_2O) \pm 0.5 = 1.07 \times \tau^{-1} - 0.62 \text{ for Eu(III)} \quad (2),$$

and

$$n(H_2O) \pm 0.5 = 0.65 \times \tau^{-1} - 0.88 \text{ for Cm(III)} \quad (3).$$

The luminescence lifetime of the Eu(III) aquo ion in H_2O is 110 ± 4 µs, while that of the Cm(III) aquo ion is 65 ± 2 µs.[55] These values correspond to the coordination of each nine water molecules in the first coordination shell.

Speciation calculation

The speciation calculation was done with Hydra/Medusa.[56] The salivary composition used for thermodynamic modeling is based on model C (Table 1), and the stability constants used for modeling are listed in Table S1 in the Electronic Supplementary Information (ESI).[35,39,57-60]

Enzyme activity measurements

The activity of Amy was determined according to the method of Bernfeld.[61] This standard enzyme assay consists of the following procedure: (i) 1 mL of a 1 % (w/v) starch solution (in TRIS buffer) is mixed with 1 mL of a 0.1 % (w/v) enzyme solution and stirred for 3 min, (ii) 1 mL of a color reagent (96 mM 3,5-dinitrosalicylic acid in tartrate/NaOH solution) is added, capped, placed in a boiling water bath for 15 min, and cooled down to room temperature with ice, (iii) after the addition of 9 mL deionized water, the absorbance at 540 nm is recorded (CARY G5 UV-vis-NIR spectrometer, Varian Inc.). The blank solution was prepared in the same manner but without enzyme. To investigate the influence of metal ions on the enzyme activity, the enzyme solution was spiked either with only Eu(III) or a mixture of Eu(III) and Ca(II). The concentrations of Eu(III) and Ca(II) were $10^{-7} - 10^{-4}$ M and 10^{-3} M, respectively.

Results and discussion

Composition analysis of natural human saliva samples

The composition of the collected natural human saliva samples is summarized in Table 1. Given their common occurrence in body fluids, here we consider sodium, potassium, and calcium as major inorganic cations, while phosphate, carbonate, chloride, and organic carbons (as TOC) are taken into account as anionic species. The anionic species are of particular importance with regard to their ability to coordinate to An^{3+} and Ln^{3+}. Hereafter, we use the generic terms “phosphate” (or “phos”) for all $H_nPO_4^{(3-n)-}$ species and “carbonate” (or “carb”) for all $H_nCO_3^{(2-n)-}$ species for simplification.

The composition of the inorganic components measured in the collected human saliva samples was in good agreement with those reported in other studies on saliva.[31,62,63] The TOC concentration was determined to be in the range of 0.5 – 1.0 g/L. It is well-known that the main organic substances in human saliva are proteins with about 2 g/L.[62] The major protein, the digestive enzyme α-amylase (Amy; M = 55 kDa), is usually present in concentrations of about 1 – 1.5 g/L[62] ($(1.8 - 2.6) \times 10^{-5}$ M), which is equal to $9 \times 10^{-4} - 1.4 \times 10^{-3}$ M carboxyl groups, according to the amino acid composition calculated by ProtParam Tool on ExPASy.[45,46] Amy has a carbon

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Table 1. Composition of chemical compounds in natural and artificial human saliva

Inorganic (mM)[a]								Organic[b]	pH
Na^+	K^+	Ca^{2+}	Cl^-	Phos	Carb	SCN^-	SO_4^{2-}		
Natural saliva (range of 10 samples)									
3.0–9.4	3.4–21.5	0.7–1.5	10.4–11.9	4.1–5.8	3.1–8.0	n.d.	n.d.	0.5–1.0 g/L TOC	6.9–7.4
Literature values[42,62,63]									
2.0–34	6.4–37	0.5–2.8	10–28	1.0–23	1.0–11	0.2–1.7	0.1–0.2	2 g/L proteins (1–1.5 g/L Amy) 0.1–7 mM urea 0.04–0.4 mM uric acid 0.02-0.2 mM Lac 0.01–0.05 mM Cit	5.0–8.0
Artificial saliva									
Model A[43,44]									
16	25	–	20	1.4	15	5.3	–	10 mM Lac	6.7
Model B[47-49]									
41	43	1.0	36	15	15	4.0	8.0	1.0 g/L Amy 0.5 g/L Muc 6.6 mM uric acid 0.1 mM urea	6.5
Model C[c]									
10	4.5	1.0	4.0	4.0	4.5	–	–	1.0 g/L Amy 0.5 g/L Muc 0.015 mM Cit	7.0

[a] Phos stands for all $H_nPO_4^{(3-n)-}$ species in equilibrium, Carb stands for all $H_nCO_3^{(2-n)-}$ species in equilibrium. [b] TOC = total organic carbon, Amy = α-amylase, Cit = citrate, Lac = lactate, Muc = mucin. [c] based on average values of natural saliva samples + Amy, Muc and Cit. n.d. = not determined

content of 54 %,[45,46] indicating that Amy accounts for ~ 0.5 – 0.8 g/L of TOC in the investigated saliva samples. Minor contributions come from other proteins such as mucin (Muc), immunoglobulins, other enzymes and several small organic molecules.[62] Citrate (Cit) is a common organic substance found in many biofluids, often forming strong complexes with metal ions. The concentrations of other small organic molecules in saliva, such as lactate (Lac), acetate, glucose, urea, uric acid, and free amino acids are usually all below 1 mg/L[62] (see Table 1), accounting in sum for only ~ 0.1 g/L of TOC in the whole saliva. Furthermore, the complex formation constants of these small organic molecules with An(III) and Ln(III) are reported to be small,[64-67] indicating that their contribution to the speciation of An(III) and Ln(III) in saliva is expected to be insignificant. Given all these facts, we conclude that, among all the TOC in saliva, Amy would play the most important role in the speciation of An(III) and Ln(III) in saliva.

TRLFS measurements of europium and curium in natural human saliva

Ten and five natural saliva samples from different individuals were used for Eu(III)- and Cm(III)-TRLFS, respectively. Shown in Figure 1 are the luminescence spectra of Eu(III) (left) and Cm(III) (right) in natural human saliva. The Eu(III)- and Cm(III)-TRLFS spectra in different saliva from different individuals show similar spectral shapes for the same metal ion, suggesting a similar coordination environment of Eu(III) or Cm(III) in the different human saliva media. As an overall trend, the luminescence decay of Eu(III) and Cm(III) in human saliva samples was calculated to be mono- or bi-exponential (Tables 2 and 3). This indicates that Eu(III) and Cm(III) are forming at least one or two independent and co-existing species in natural human saliva.

Europium. As shown in Figure 1 (left), the relative intensity of the hypersensitive $^5D_0 \rightarrow {}^7F_2$ transition at ~ 615 nm is significantly enhanced in the saliva samples as compared to that for the Eu(III) aquo complex (black data). Furthermore, the $^5D_0 \rightarrow {}^7F_2$ transition and the $^5D_0 \rightarrow {}^7F_1$ transition at ~ 590 nm Laporte selection rule[51]) $^5D_0 \rightarrow {}^7F_0$ transition at ~ 580 nm arises. All of these spectral changes indicate the complexation of Eu(III) with saliva components. The spectral parameters of Eu(III) luminescence obtained in human saliva samples are summarized in Table 2.

Curium. The Cm(III) aquo complex shows a strong luminescence peak for the $^6D_{7/2} \rightarrow {}^8S_{7/2}$ transition at 593.8 nm (black data in Figure 1-right). The interaction of saliva components with Cm(III) causes a strong red shift of the luminescence peak maximum, resulting in a new peak

Table 2. Spectral parameters of Eu(III) luminescence in natural human and artificial saliva samples, as well as the luminescence with major single components of saliva.

Sample	pH	I_2/I_1[a]	Exponential decay manner[b]	luminescence lifetime, estimated hydration number[c]			
				τ_1 (μs)	$n_1(H_2O)$	τ_2 (μs)	$n_2(H_2O)$
Eu^{3+}(aq)	4.0	0.6	mono	107 ± 3	9.4		
Natural human saliva							
S1	7.4	1.3	bi	409 ± 29	2.0	845 ± 95	0.6
S2	7.1	1.7	bi	294 ± 69	3.0	633 ± 40	1.1
S3	6.9	1.4	bi	125 ± 37	8.0	438 ± 12	1.8
S4	6.9	1.6	bi	212 ± 44	4.4	508 ± 25	1.5
S5	6.9	1.4	mono	372 ± 37	2.3		
S6	7.1	1.7	mono	515 ± 10	1.5		
S7	7.1	1.4	bi	302 ± 37	2.9	815 ± 33	0.7
S8	7.0	1.2	bi	302 ± 46	2.9	694 ± 65	0.9
S9	7.1	1.3	bi	246 ± 40	3.7	649 ± 68	1.0
S10	6.9	1.7	bi	156 ± 19	6.2	472 ± 10	1.6
Artificial saliva							
Model A	6.5	1.0	bi	128 ± 14	7.7	320 ± 22	2.7
Model B	6.7	1.4	bi	146 ± 18	6.7	470 ± 16	1.7
Model C	7.0	1.5	bi	259 ± 12	4.1	575 ± 21	1.2
Single and mixed components							
Eu/Carb	7.0	2.7	mono	102 ± 2	9.8		
Eu/Phos	7.0	1.8	mono	100 ± 2	10.0		
Eu/Carb/Phos(+Ca)	7.0	1.3	mono	445 ± 6	1.8		
Eu/Amy	7.2	2.2	bi	412 ± 21	2.0	794 ± 18	0.7
Eu/Muc	7.0	3.1	bi	267 ± 8	3.4	699 ± 7	0.9

[a] Intensity ratio of the transitions into the $^7F_2$ and $^7F_1$ ground states (intensity of the $^5D_0 \rightarrow {}^7F_1$ transition band is fixed as 1). [b] Corresponds to index *i* in eq. (1) [c] Number of water molecules ± 0.5 according to eq. (2).[54]

Table 3. Spectral parameters of Cm(III) luminescence in natural human and artificial saliva samples, as well as the luminescence in major single components of saliva.

Sample	pH	λ (nm)[a]	Exponential decay manner[b]	luminescence lifetime, estimated hydration number[c]			
				τ_1 (μs)	$n_1(H_2O)$[c]	τ_2 (μs)	$n_2(H_2O)$
Cm^{3+}(aq)	3.0	593.8	mono	68 ± 1	8.7		
Natural human saliva							
S3	6.9	605.1	bi	104 ± 29	5.4	500 ± 6	0.4
S4	6.9	605.4	bi	158 ± 19	3.2	484 ± 23	0.5
S5	6.9	605.1	bi	224 ± 40	2.0	513 ± 22	0.4
S6	7.1	605.1	bi	191 ± 23	2.5	561 ± 15	0.3
S7	7.1	605.2	bi	158 ± 16	3.2	563 ± 10	0.3
Artificial saliva							
Model B	6.4	603.6	bi	98 ± 15	5.7	346 ± 18	1.0
Model C	7.0	603.8	bi	112 ± 8	4.9	502 ± 27	0.4
Single and mixed components							
Cm/Carb	7.1	600.6	mono	134 ± 2	4.0		
Cm/Carb/Phos	6.9	601.4	mono	135 ± 2	3.9		
Cm/Carb/Phos(+Ca)	6.9	602.0	mono	163 ± 3	3.1		
Cm/Amy	7.2	603.0	bi	123 ± 6	4.4	326 ± 15	1.1
Cm/Muc	6.0	603.5	bi	80 ± 3	7.2	259 ± 5	1.6

[a] Main emission wavelength ± 0.1 nm. [b] Corresponds to index *i* in eq. (1) [c] Number of water molecules ± 0.5 according to eq. (3)[54].

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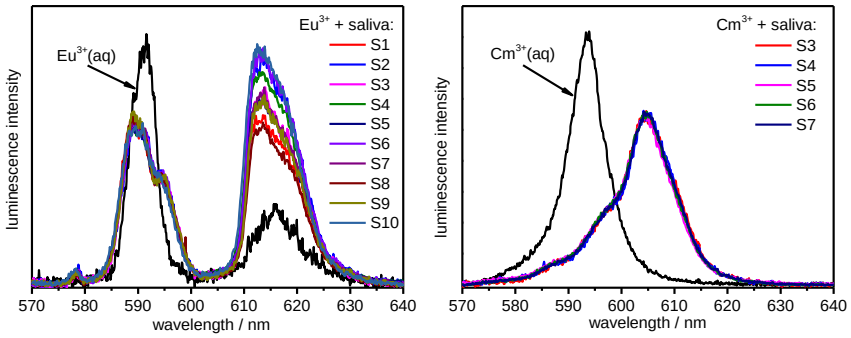

Figure 1. Luminescence spectra of Eu(III) (left) and Cm(III) (right) in natural human saliva at 25 °C.

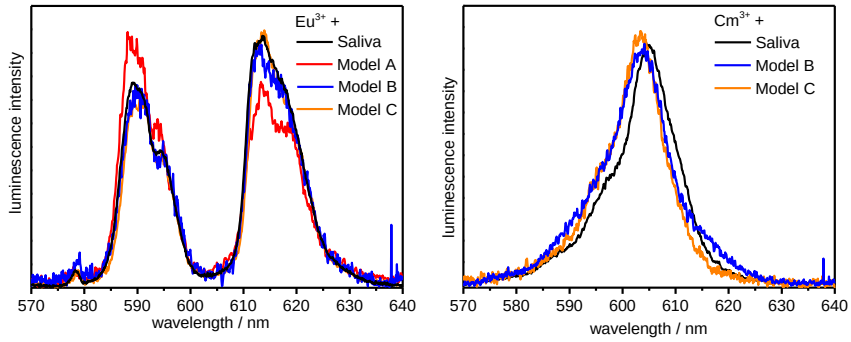

Figure 2. Luminescence spectra of Eu(III) (left) and Cm(III) (right) in artificial saliva solutions (Models A, B and C) at 25 °C, along with the spectra in natural saliva (black data, average of ten and five samples for Eu(III) and Cm(III), respectively).

at 605 nm (Figure 1-right) with peak-broadening. This would be a hint that more than one species with similar peak maxima contribute to the sum spectra. The spectral parameters of Cm(III) luminescence obtained in natural human saliva samples are also summarized in Table 3.

Speciation determination of europium and curium in natural human saliva

In order to further interpret the results obtained for Cm(III) and Eu(III) in natural human saliva samples, additional TRLFS measurements were performed with three different artificial saliva solutions (Models A – C). These model solutions were composed of major inorganic and organic components of saliva (see Table 1). Model A[43,44] is a very simple artificial saliva solution with only Lac as an organic component and is often applied in dental research.[44] Model B[47-50] follows an international unified bioaccessibility method (UBM).[47] It contains more variety of organic substances (urea, uric acid, Muc, and Amy) than Model A and, hence, it is more appropriate to simulate the conditions of body fluids. Model C is based on the average values of inorganic contents obtained for the natural human saliva samples investigated in this study with the addition of Cit, Muc and Amy according to literature values.[42,50,62] For each model, the spectral data were collected for the single components and different mixtures consisting of (i) inorganic components, (ii) all inorganic and one single organic components, and (iii) a complete mixture of all inorganic and all organic components, in order to elucidate which components significantly contribute to the Eu(III)/Cm(III)-TRLFS spectra (namely their speciation). The obtained luminescence spectra and spectral parameters are summarized in Figure 2 as well as Tables 2 and 3 (plus Figures S1 – S5 and Table S2 in ESI).

Europium. Figure 2 (left) shows the results of the TRLFS measurements for Eu(III) in different artificial saliva solutions. The corresponding spectral parameters are summarized in Table 2.

For Model A, neither the luminescence spectrum nor the spectral parameters (*e.g.*, luminescence lifetimes, intensity ratio I_2/I_1) are comparable to those of natural saliva samples. This clearly indicates that Model A is not suitable for simulating the conditions of human saliva and, hence, suggests

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that organic salivary constituents excluded in this model might play an important role. In contrast, Models B and C appear to successfully reproduce the spectra in human saliva and the obtained spectral parameters are also comparable. Between these Models, Model C reproduces the human saliva data more reasonably than Model B (Table 2).

The luminescence spectra of Eu(III) with single inorganic or organic components do not reproduce the spectra of the natural saliva samples in all cases (Tables 2 and S2, Figures S1 – S3, ESI). The luminescence decay of Eu(III) with single inorganic components (carbonate and phosphate) is mono-exponential with short lifetimes comparable to that of the Eu(III) aquo ion (Table 2). The decay with the single organic components (Amy and Muc) is bi-exponential which results in a longer and a shorter luminescence lifetime, which are both longer than that of the aquo Eu(III) species (see Table 2). Among several combinations of inorganic and organic components, the sample solution containing anionic carbonate and phosphate with Ca^{2+} can reproduce well the Eu(III) spectrum in the human saliva. The luminescence decay of this inorganic mixture is mono-exponential with a luminescence lifetime of 445 µs, which corresponds to two water molecules remaining in the first coordination shell. This is in line with the formation of a ternary complex (named Eu/carb/phos(+Ca) hereafter). The binary complexes of Eu/carb and Eu/phos would have resulted in one (or two) shorter lifetime(s) of approximately 100 µs (see Table 2). The lifetime of the ternary complex is similar to that of $Eu(CO_3)_3^{3-}$ in solution, where carbonates coordinate bidentate (440 µs).[68] This suggests that three bidentate ligands (carbonate and phosphate) are coordinating to the metal ion in the ternary complex. This points to a 1:1:2 or 1:2:1 (Eu : carb : phos) stoichiometry. Ca^{2+} is probably involved in the outer coordination shell for charge compensation as observed for the calcium-uranyl(VI) carbonate complex in aqueous solution.[69,70]

The addition of the proteins Amy and/or Muc to this inorganic mixture has no significant effect on the shape of the single emission spectrum of Eu(III) but provokes a bi-exponential luminescence decay with lifetimes comparable to those of Models B and C as well as in human saliva (see Tables 2 and S2).

Further attempts were carried out to reproduce the Eu(III) luminescence spectra for human saliva by the linear combination fitting (LCF) based on the reference spectra of single inorganic and organic components as well as selected mixtures. As shown in Figure 3, the LCF based on the combination of the ternary inorganic complex Eu/carb/phos(+Ca) with the addition of Amy and Muc provides the best fitting results. The addition of Cit in the LCF analysis does not further improve the fitting results. The LCF analysis only with the binary inorganic complexes (Eu/carb and Eu/phos) or with the addition of organic species (Amy, Muc and Cit) results in higher residuals (see Figure S6, ESI). The LCF results for all the human saliva spectra are summarized in Table 4. The ternary inorganic complex Eu/carb/phos(+Ca) always appears to be the dominant component. Additionally,

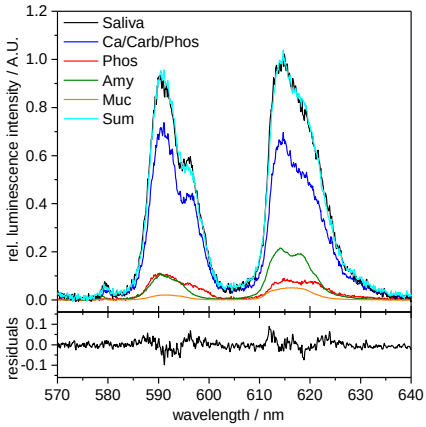

Figure 3. Linear combination fitting on the luminescence spectrum of Eu(III) in human saliva (sample S9) based on the reference spectra of inorganic and organic species.

binary inorganic (carbonate or phosphate) and organic (Amy and Muc) Eu(III) species appear as minor components. On average, the fractions of the inorganic Eu(III) species are ranging between ~60 and 95 % and the organic Eu(III) species represent ~5 – 40 % in human saliva (see Table 4). This indicates that, in natural human saliva, Eu(III) is likely to form ternary complexes like Eu/carb/phos(+Ca), rather than binary complexes with a single anionic species. It is also obvious that bio-macromolecules such as Amy (or Muc to a minor extent) play an important role in the Eu(III) speciation in saliva. For comparison, the LCF analysis of Model C resulted in 70 % inorganic (ternary complex) and 30 % organic species (mainly Amy), whereas only inorganic complexes were found in Model B (see Table 4). This further supports the TRLFS data (Table 2) indicating that Model C fits the human saliva samples more reasonably than Model B.

The LCF results on the human saliva samples suggest at least three independent species present in the samples, while their luminescence decay were either mono- or bi-exponential. Hence, the lifetimes obtained should be rather considered as an average value of multiple species.

Another series of experiments were performed at physiological temperature of 37 °C based on Model B. The results are summarized in Figure S4 and Table S2 in the ESI. The obtained results are all comparable to those obtained at room temperature, suggesting that no significant change is expected for the Eu(III) speciation between ambient and physiological temperature.

Curium. Based on the results obtained for Eu(III), the TRLFS measurements for Cm(III) were performed with the dominant inorganic and organic components, *i.e.*, carbonate, phosphate, calcium, Amy, and Muc. Concentrations of the inorganic components and the proteins were based on Models B and C (Table 1). The representative luminescence spectra are given in Figure 2 (right) and the obtained spectral parameters are summarized in Table 3. Other collected spectra are shown in the ESI (Figure S5).

In contrast to the results of Eu(III), the luminescence spectra of Cm(III) in the artificial saliva Models B and C are not well comparable to the spectrum in natural saliva.

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Table 4. Fractions of dominant Eu(III) and Cm(III) species in human saliva. The fractions were determined by linear combination fitting with reference spectra.

Sample	Carb/Phos(+Ca)	Phos	Carb	Amy	Muc	Σ inorganic	Σ organic
Eu(III)	*Fractions of the Eu(III) complexes (%)*						
S1	58	30	0	12	0	88	12
S2	73	0	9	11	7	82	18
S3	77	11	0	3	9	88	12
S4	56	18	0	17	9	74	26
S5	57	25	0	17	0	82	18
S6	73	0	5	13	9	78	22
S7	71	15	0	12	2	86	14
S8	67	27	0	5	0	95	5
S9	76	12	0	10	2	88	12
S10	44	17	0	34	5	61	39
Range	**44 - 77**	**0 - 25**	**0 - 9**	**3 - 34**	**0 - 9**	**61 - 95**	**5 - 39**
Model B	85	10	5	0	0	100	0
Model C	70	0	0	28	2	70	30
Cm(III)	*Fractions of the Cm(III) complexes (%)*						
S3	31	0	0	65	0	31	65
S4	26	0	0	70	0	26	70
S5	38	0	0	58	0	38	58
S6	34	0	0	63	0	34	63
S7	30	0	0	67	0	30	67
Range	**26 - 38**			**58 - 70**		**26 - 38**	**58 - 70**

The peak maximum observed in artificial saliva samples is slightly blue-shifted (603 – 604 nm) as compared to that in natural saliva (605 nm), which is in line with the results previously reported for human urine at near neutral pH.[22] The luminescence decay in the artificial saliva samples was, however, found to be bi-exponential, reproducing the lifetimes of the natural saliva samples (Figure 2-left, and Table 3).

As observed for Eu(III), the luminescence spectra of Cm(III) in natural saliva are not reproducible when we only consider single inorganic and organic components (Table 3 and Figure S5, ESI). The luminescence decay with the inorganic components (carbonate and phosphate) is mono-exponential, while the decay with the organic components (Amy and Muc) is bi-exponential (Table 3). In contrast to Eu(III), the luminescence spectrum of Cm(III) in a mixture of carbonate, phosphate, and Ca, representing the ternary complex Cm/carb/phos(+Ca), does not well reproduce the spectral features of the natural saliva samples. The peak maximum observed for the ternary complex is slightly blue-shifted (602 nm) as compared to that for the natural saliva (605 nm). The luminescence decay of this ternary complex is again mono-exponential with a luminescence lifetime of 163 µs. This corresponds to three water molecules remaining in the first coordination shell, which further implies the presence of three bidentate ligands (*i.e.*, carbonate or phosphate) in the first coordination shell. This lifetime is longer than those of the binary complexes Cm/carb (134 µs) (see Table 3) and Cm/phos (118 µs)[39] but shorter than that of Cm(III)-phosphate colloids (220 µs) formed at near neutral pH.[39] Given these facts, we can assume a similar stoichiometry for the ternary complex Cm/carb/phos(+Ca) as observed for the corresponding Eu(III) complex.

The LCF analysis on the Cm(III) saliva spectra based on the reference spectra of the inorganic and organic components (Figure S7, ESI) did not provide as reasonable results as those observed for Eu(III). This is probably because the spectral feature of Cm(III) luminescence does not change drastically with different species as compared with that for Eu(III) luminescence. Cm(III) exhibits only small peak shifts on a modification of the coordination mode, which is, at least at room temperature and in aqueous solution, hardly detectable and results only in a peak-broadening. In contrast, Eu(III) shows a broad variety of intensity ratios and splitting patterns of the $^5D_0 \rightarrow {}^7F_1$ and $^5D_0 \rightarrow {}^7F_2$ transitions (see Figures S1 – S3), which is more suitable for speciation determination. This also suggests that the TRLFS of Cm(III) is less sensitive to the speciation change than that of Eu(III), which could be a possible reason why the speciation results for Cm(III) are different from those for Eu(III), despite their chemical similarity. Nevertheless, the determined spectral data combined with LCF results (see Table 4 and Figure S7, ESI) indicate that the complex formation of Cm(III) in natural saliva is comparable to that of Eu(III). Like for Eu(III), the mixed inorganic ternary complex Cm/carb/phosph(+Ca) and the organic complex with Amy are the dominating species, even though, contrary to Eu(III), the Amy species seems to be more pronounced than the inorganic ternary complex species (see Table 4).

In summary, the results obtained from the TRLFS measurements of Eu(III) and Cm(III) suggest that a dominant species of Eu(III) and Cm(III) in salivary media is a ternary inorganic complex composed of carbonate and phosphate as coordinative ligands and calcium for charge compensation. Similar Eu(III) and Cm(III) species were identified in our

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previous study in natural human urine at neutral pH.[22] It has been also reported that some divalent cations (Mg, Mn, Fe, Co, Ni, Cu) are forming ternary carbonate-phosphate complexes.[71,72] Binary complex species with inorganic (carbonate or phosphate) and organic (Amy or Muc) components are also present as minor species in the Eu(III) speciation, whereas the Amy species is more pronounced in Cm(III) speciation in salivary media. Other saliva constituents, such as chloride, sulfate, thiocyanate, and minor organic components (urea, uric acid and Lac) are not expected to contribute to the Eu(III)/Cm(III) speciation, based on their weaker coordination abilities to the metal cations in the relevant conditions.[64,65,67] Citrate could possibly interact with the metal cations,[35] although the present TRLFS measurements do not detect any contribution of the citrate-related species, probably due to its low concentration in natural saliva and Model C. Furthermore, the concentrations of the metal ions of interest and potential concurrent bulk metal ions play an important role for interactions with the different ligands, which could eventually influence the speciation.

Speciation calculation of europium and curium in human saliva

The precedent calculations on the speciation of Ln(III) and An(III) in human saliva by Webb *et al*.[32] predicted a predominant complexation of Cit (nearly 80 %) with minor contributions of hydrogen phosphate (20 %) for Am(III), while 100 % complexation of bicarbonate was estimated for Eu(III). This is contradictive to the experimental results obtained in this study. However, the reliability of speciation calculations depends largely on the stability constants available at the time. Thus far, no stability constants have been reported either for the ternary inorganic complexes with carbonate and phosphate or for the bio-macromolecular complexes (*i.e*., Amy and Muc) of Ln(III) and An(III). To supplement this significant lack of data with the objective of improving the speciation calculation, we have recently determined the complex stability constants of Eu(III) and Cm(III) with Amy,[73] because this protein is considered to be one of the major binding partners for An(III)/Ln(III) in salivary media (Table 4). This now enables us to perform more reliable speciation calculations of these metal ions in human saliva. The interaction of Eu(III) and Cm(III) with the carboxylate groups of Amy can be described according to the equations (4) and (5).[73]

$$M^{3+} + \text{Amy}{-}COO^{-} \leftrightarrows M(\text{Amy}{-}COO)^{2+} \quad (4)$$

$$M^{3+} + 3\ \text{Amy}{-}COO^{-} \leftrightarrows M(\text{Amy}{-}COO)_3 \quad (5)$$

Consequently, Eu(III) and Cm(III) are forming complexes with 1:1 and 1:3 stoichiometries with the carboxyl groups of the protein. We have also demonstrated that at least 3 metal ions can bind to one amylase molecule.[73]

Figure 4 shows the species distribution for Eu(III) (left) and Cm(III) (right) at pH 7 in artificial saliva (Model C) without Amy (top) and with Amy but without consideration of the ternary species (middle). The whole speciation distribution with Amy as a function of pH is given in Figure S8 in the ESI. Even the speciation without Amy, calculated with current available thermodynamic data of the saliva components with Eu(III) and Cm(III)[35,39,57-60] (see Table S1, ESI), differs drastically from that made by Webb *et al*.[32] We found for both metals mainly phosphate complexation (about 85 – 90 %) and minor contribution of citrate (about 10 %), and only 1 – 2 % carbonate complexation (*cf*. Webb *et al*.: 100 % bicarbonate for Eu(III), about 80 % Cit and 20 % hydrogen phosphate for Am(III)[32]). With Amy, the total inorganic and organic species account for approximately 50 % of the total fraction, respectively. Among the inorganic components, the phosphate species dominate the speciation and the carbonate species play only a minor role, whereas Amy represents the dominant organic component. This result is in good agreement with our TRLFS results (Table 4 and Figure 4, bottom).

The speciation calculation can further help specify the composition of the ternary complex M(III)/carb/phos(+Ca) (with M = Eu or Cm). From the TRLFS measurements, we concluded the formation of a 1:1:2 or 1:2:1 complex (M(III) : carb : phos). The speciation calculation shows that the amount on phosphate species is higher than that of the carbonate species (Figure 4, top and middle). This suggests that the 1:1:2 stoichiometry would be more preferable in the ternary complex than the 1:2:1 one. Furthermore, the carbonate species are calculated to be a completely deprotonated form (*i.e*., carbonate species (CO_3^{2-})), whereas the phosphate species are calculated to be partly deprotonated and partly protonated. Therefore, the carbonate ligand in the ternary complex would be in the carbonate form (CO_3^{2-}), whereas several different forms (*e.g*., HPO_4^{2-} and PO_4^{3-}) could be considered for the phosphate ligands in the ternary complex.

In the case of the organic species, the contribution of Cit is also expected in the calculation, even in the presence of Amy. The TRLFS results indicated, however, no significant fraction of Cit species but the complexation with Muc instead. Since there are no stability constants available for the Ln(III)/An(III) complexes with Muc and their ternary inorganic complexes thus far, these species are not able to be included in the present speciation calculation. This could explain the contradiction between the TRLFS results and the obtained speciation calculation suggesting the presence of Cit species. In fact, the LCF analysis on the TRLFS data for Model C indicates no significant fraction of Cit species under the same Cit concentration used for the speciation calculation, while the Muc species was detected. This suggests that Muc could have a stronger coordination ability towards M(III) than Cit.

The complex stability constants of Eu(III)/Cm(III) with Cit are much larger than those with Amy (see Table S1 in the ESI). In this context, the current results indicating the complex formation between M(III) and Amy (and Muc), not Cit, would sound contradictive. However, it is difficult to make a direct comparison of stability constants between a small and simple molecule (*e.g*., Cit) and a huge molecule with many different functional sites (*e.g*., Amy and Muc). This study demonstrates experimentally that Amy and Muc have larger apparent stability constants with Eu(III)/Cm(III) than Cit, highlighting the importance to develop appropriate theory and methodology

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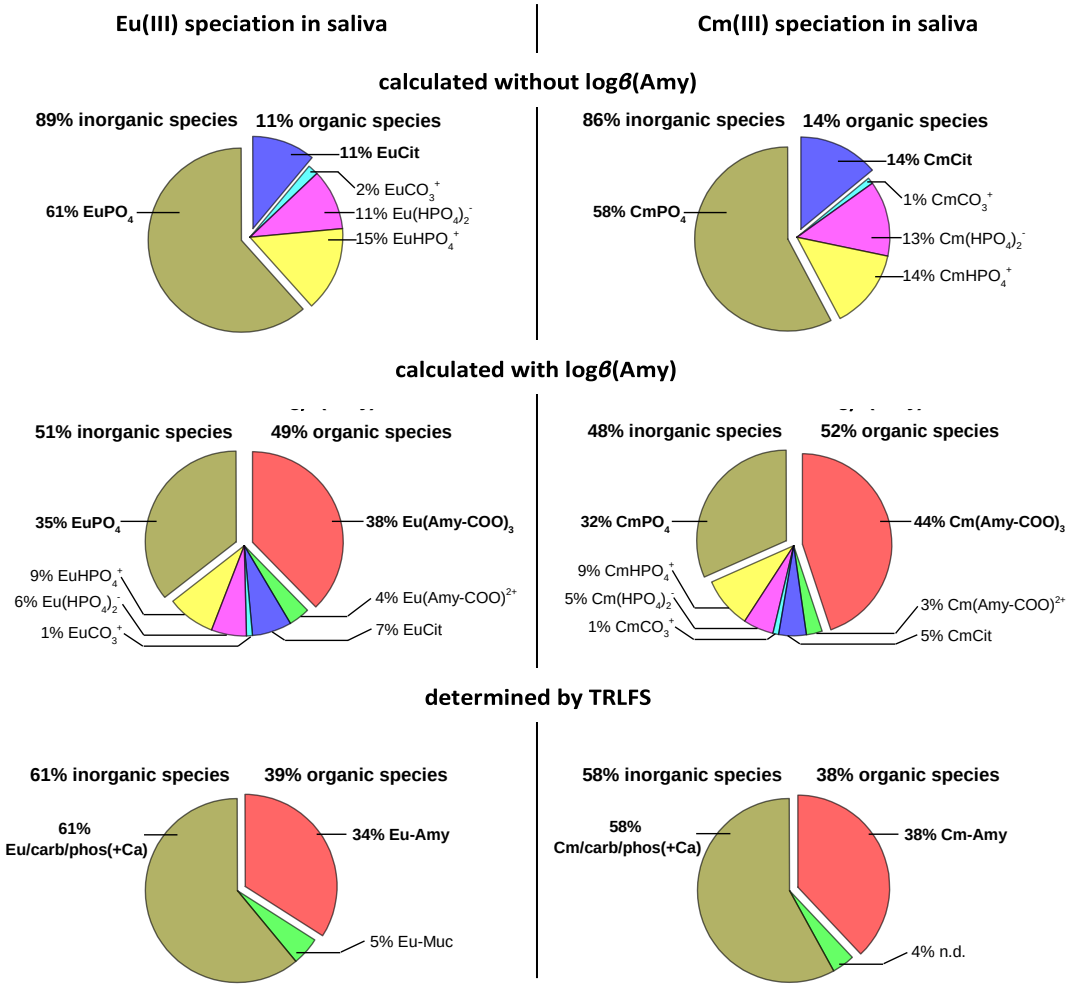

Figure 4. Species distributions of Eu(III) (left) and Cm(III) (right) based on the thermodynamic data in artificial saliva (Model C) at pH 7 without Amy (top), with Amy (middle) and the TRLFS results (bottom; Eu = sample S10 and Cm = sample S5). Ternary inorganic complex species and Muc species are not taken into account on the calculation because of the lack of their thermodynamic data. Concentrations: [Eu(III)] = 3×10^{-5} M, [Cm(III)] = 3×10^{-7} M. Concentrations of inorganic and organic components are given in Table 1. Other data required for speciation calculation are given in Table S1 in the ESI. "n.d." = not defined

to compare thermodynamic properties of small and huge molecules on the same basis.

Minor differences in Eu(III) and Cm(III) speciation in natural human saliva may be caused by the two orders of magnitude of difference in the metal concentrations employed for the TRLFS experiments and the speciation calculation (10^{-5} M Eu(III) vs. 10^{-7} M Cm(III)).

Effect of trivalent *f*-elements on the enzyme activity of α-amylase

Our TRLFS results suggest a strong interaction between the enzyme Amy and Eu(III)/Cm(III), which implies that the presence of these metal cations could potentially affect the enzyme activity of Amy. This fact further motivated us to perform another study to investigate the effect of Eu(III) on the relative enzyme activity of Amy. The results are summarized in Figure 5. When 10^{-6} M of Eu(III) was added, the relative enzyme activity of Amy was drastically reduced down to less than 50 %. This can be explained by our recent findings that Eu(III) can replace the Ca(II) which is initially contained in Amy as a result of the complexation between Eu(III) and Amy.[73] Calcium is known to be essential for the enzyme stability and activity[74] and, hence, removal of Ca(II) from the enzyme leads to a decrease either in thermochemical stability or in enzymatic activity, or both.[75] Hence, the replacement of Ca(II) with Eu(III) could consequently reduce the enzyme activity.

This is further substantiated by the fact that the effect of Eu(III) on the enzyme activity became ignorable when an excess amount of Ca(II) was simultaneously added to the sample in order to block Eu(III) from pushing out the Ca(II) on Amy. In this case, however, Eu(III) could still interact with the protein via many other functional groups on Amy, not influencing the enzyme activity.

Metal ions can affect the Amy activity in different ways. Some divalent metal ions, such as Ca, Ba, Mg or Co, are known to be able to enhance the enzyme activity,[76,77] while other divalent (*e.g.*, Hg) and trivalent metal ions (*e.g.*, Al or Nd) reduce the activity of Amy.[77,78] This study demonstrates that even a small amount of Eu(III) has a significant inhibiting effect on the enzyme activity of Amy, which is, however, easily restrained with an excess of calcium. The amount of calcium used in this study is well comparable to the average amount in natural

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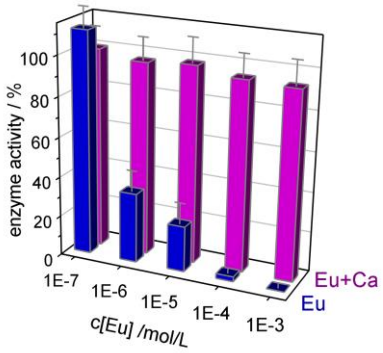

Figure 5. Enzyme activity of Amy in the presence of Eu(III) and that with an excess of Ca(II).

human saliva (*cf*. Table 1) and, hence, it is still relevant to the actual physiological conditions. This also suggests that the amount of calcium in the human body would be potentially sufficient to restrain the chemo-toxicity of Ln(III)/An(III) associated with the interaction with Amy upon oral ingestion.

Conclusions

The speciation of Eu(III) and Cm(III), as representatives of trivalent *f*-elements, respectively, was investigated in natural human salivary media by time-resolved laser-induced fluorescence spectroscopy. Ternary inorganic complexes containing carbonate and phosphate with a possible stoichiometry of 1:1:2 (M(III) : carb : phos, plus Ca(II)) and the organic species with the proteins Amy and Muc were identified as the major binding partners of the Eu(III) and Cm(III) species formed in the salivary media. The obtained experimental results are in good agreement with the speciation calculations based on the newly reported thermodynamic data on Amy,[73] although additional thermodynamic data on ternary inorganic complexes and complexes with bio-macromolecular ligands like Muc are still required for more reliable calculations for the metal speciation in body fluids. This study demonstrates that TRLFS is a powerful analytical method to investigate the metal speciation in biologically relevant conditions, particularly for Eu(III), and underlines the significant lack of thermodynamic data of the metal speciation associated with the biological systems, which is crucial for the reliable assessment of their bio-availability and toxicity. This study also reveals that the saliva model based on the international Unified Bioaccessibility Method (UBM) by the Bioaccessibiltiy Research Group of Europe (BARGE)[47] would contain significantly high concentrations of inorganics, especially carbonate and phosphate, as compared to natural human saliva, which would potentially lead to uncertain outcomes of digestive model experiments.

The presence of trivalent *f*-elements in the salivary media could reduce the enzyme activity of Amy. This effect, however, can be restrained by an appropriate excessive amount of Ca(II), which is relevant to the physiological conditions.

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