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Short communication

Influence of *trans*-1,2-diaminocyclohexane structure and mixed carboxylic/phosphonic group combinations on samarium-153 chelation capacity and stability

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Abstract

A simple procedure was developed to compare chelating agents for ^{153}Sm complexes as a preliminary step to synthesise bifunctional analogues. Several variables affecting the efficiency of complex stability were investigated, such as the pre-organisation concept, cavity size, and the nature of coordination sites. Four semi-rigid agents incorporating carboxylic and/or phosphonic groupings fixed at *trans*-1,2-diaminocyclohexane were evaluated for their ^{153}Sm chelation properties, and competition studies were performed. Data on the stability of the best chelating agent compound **3**: *trans*-cyclohexane-1,2-bis(aminomethylphosphonic)-*N,N′*-bis(ethyl-2-iminodiacetic acid) in human serum are presented.

Keywords: Radioimmunotherapy; Samarium-153; Chelating agent; Competition studies; Serum stability

1. Introduction

Radioimmunotherapy with radiolabelled antibodies should allow fairly specific targeting of certain cancers [1,2]. Iodine-131 is probably not the best isotope for tumour therapy because of its low beta-energy (182 keV), relatively long half-life (8.1 days) and excessive gamma ray energy [3]. Replacement of ^{131}I with isotopes providing better physical properties could improve therapeutic efficacy. Lanthanides have already been evaluated in experimental protocols using tumour xenograft model [4,5]. Samarium-153 is produced by neutron irradiation of isotopically enriched $^{152}Sm_2O_3$ at specific activities up to 30 GBq mg^{-1}. Samarium-153 could be an appropriate radiometal insofar as (i) its half-life (1.95 days) is adapted to the kinetics of whole immunoglobulins and F(ab′)$_2$ fragments, (ii) its β-emissions of 810 keV (maximum) to 290 keV (average) are suitable for treating small lesions while limiting irradiation of adjacent normal tissues, and (iii) its γ-ray energy (30% in the zone of 103 keV) facilitated gamma camera detection and the recording of biodistribution data may be appropriate for radioimmunotherapy. However, its application in nuclear medicine requires bifunctional chelators that can hold the radiometal on the vector with high stability under physiological conditions, thereby avoiding excessive radiation damage to non-target cells. A sufficiently stable radionuclide complex is needed to bind β-emitting particles tightly.

Most chelating agents used in nuclear medicine preclinical trials for radioimmunotherapy applications are linear polyaminocarboxylic acids. Animal studies performed with ^{153}Sm-DTPA or ^{153}Sm-CITC-DTPA (6-*p*-isothiocyanatobenzyl-diethylene-triamine penta-acetic acid) have shown that labelling stability, even though improved for the latter combination, is not yet satisfactory in a biological environment and might cause non-specific uptake in liver and bone tissues [4,6–8]. Thus, a chelating agent forming more stable ^{153}Sm complexes is needed before clinical trials can be performed in patients.

The stability constant can be increased either by modifying the molecular skeleton or the nature and size of the

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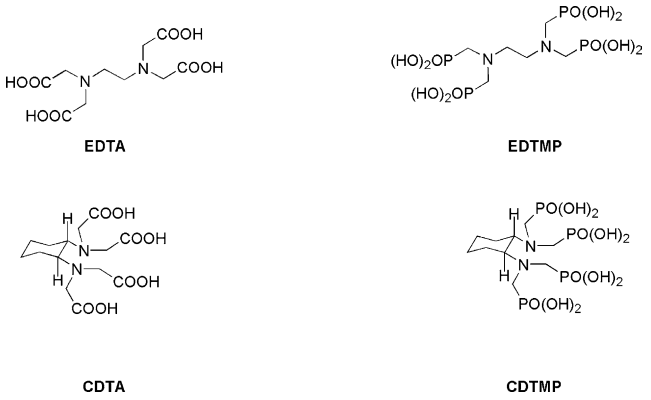

Fig. 1. Structures of EDTA, EDTMP, CDTA and CDTMP.

chelating group. Previous studies considered the benefit of the pre-organisation concept, which is based on the incorporation of ethylenediaminetetraacetic acid (EDTA) into a cyclohexane ring (CDTA) [9,10], and showed how this semi-rigid structure affects the stability of the resulting complexes. Phosphonic groups could provide an alternative to carboxylic functions. Different studies concerning ^{153}Sm-polyaminophosphonic acid complexes [11,12] have shown that ethylenediaminetetramethylphosphonic acid (EDTMP) derivatives allow stable quantitative ^{153}Sm chelation.

The purpose of our preliminary study was to compare the ^{153}Sm chelation properties of linear EDTA and EDTMP with those of semi-rigid *trans*-1,2-cyclohexyldiaminetetraacetic acid (CDTA) and *trans*-1,2-cyclohexyldiaminetetramethylphosphonic acid (CDTMP) to investigate the benefit of carboxylic acid groups versus phosphonic acid ones and the influence of a semi-rigid structure on the stability of the resulting complexes. These chelating agents are described in Fig. 1. As no ^{153}Sm studies with these four chelating agents have been reported in the literature, it was necessary to determine the most appropriate combination for the design of new chelating agents.

On the basis of these preliminary results, four semi-rigid chelating agents (Fig. 2) carrying several reactive functions (carboxylic and/or phosphonic acid) were synthesised in our laboratory using a synthetic pathway from the commercial product, *trans*-1,2-diaminocyclohexane [13]. These four

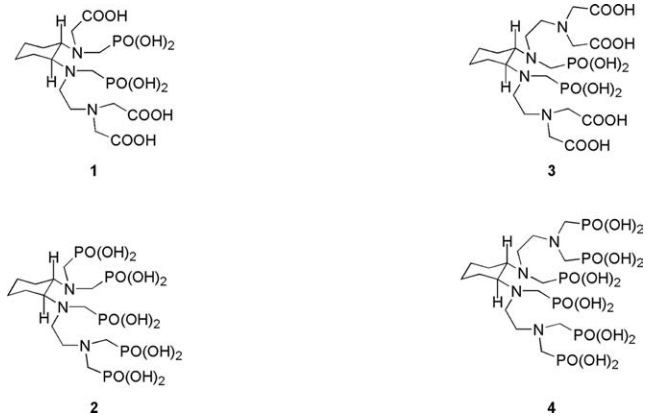

Fig. 2. Structures of four carboxylic, phosphonic or mixed chelating agents.

agents were evaluated for their chelation capacity and in competition studies to select the best candidate. Human serum stability studies performed on the selected compound confirmed that the structure of this chelating agent is of potential interest for radioimmunotherapy applications.

2. Results

2.1. Preliminary study

2.1.1. EDTA, CDTA, EDTMP, and CDTMP ratio evaluation

This preliminary study allowed us to define the best chelating agent/radionuclide ratio to achieve complete chelation and thereby avoid any interference of free ^{153}Sm. A **4–1** chelating agent-to-radionuclide ratio was needed for each agent, with 2 h of stirring at 37 °C, to reach maximum chelation efficiency in the same incubation conditions.

2.1.2. Competition study

The results obtained in the competition study are summarised in Fig. 3. For linear systems, carboxylic and phosphonic functions appeared equivalent, resulting in an equilibrium at 55% for ^{153}Sm EDTA when 4 equiv. of EDTMP were added, and at 42% for ^{153}Sm EDTMP when 4 equiv. of EDTA were added (EDTA/EDTMP ratio = 1).

In all cases, linear systems seemed to have better ^{153}Sm chelation properties than small semi-rigid systems. For EDTA/CDTMP, ^{153}Sm EDTA was 88% when 4 equiv. of CDTMP were added (CDTMP/EDTA ratio = 1), and 20% for ^{153}Sm CDTMP when 4 equiv. of EDTA were added (EDTA/CDTMP ratio = 1). For EDTMP/CDTA, ^{153}Sm CDTA was 0% when 3 equiv. of EDTMP were added (EDTMP/CDTA ratio = 0.75), and 60% for ^{153}Sm EDTMP when 3 equiv. of CDTA were added (CDTA/EDTMP ratio = 0.75). Finally, CDTA seemed to be better than that of CDTMP, giving 88% for ^{153}Sm CDTA when 3 equiv. of CDTMP were added (CDTMP/CDTA ratio = 0.75), and 37% for ^{153}Sm CDTMP when 3 equiv. of CDTA were added (CDTA/CDTMP ratio = 0.75).

2.2. ^{153}Sm chelation properties of the four chelating agents ***1–4***

2.2.1. Effect of chelator concentration on radiolabelling efficiency

Chelation efficiency is dependent on several important factors, including chelate concentration, duration of chelation, and incubation temperature. The first ^{153}Sm chelation study allowed us to evaluate our four chelating agents **1–4** in terms of chelation abilities. All of these agents reached 100% of ^{153}Sm chelation for a maximum of 10 equiv. chelating agent excess but further data on the thermodynamic stability of the resulting complexes could not be obtained in this manner.

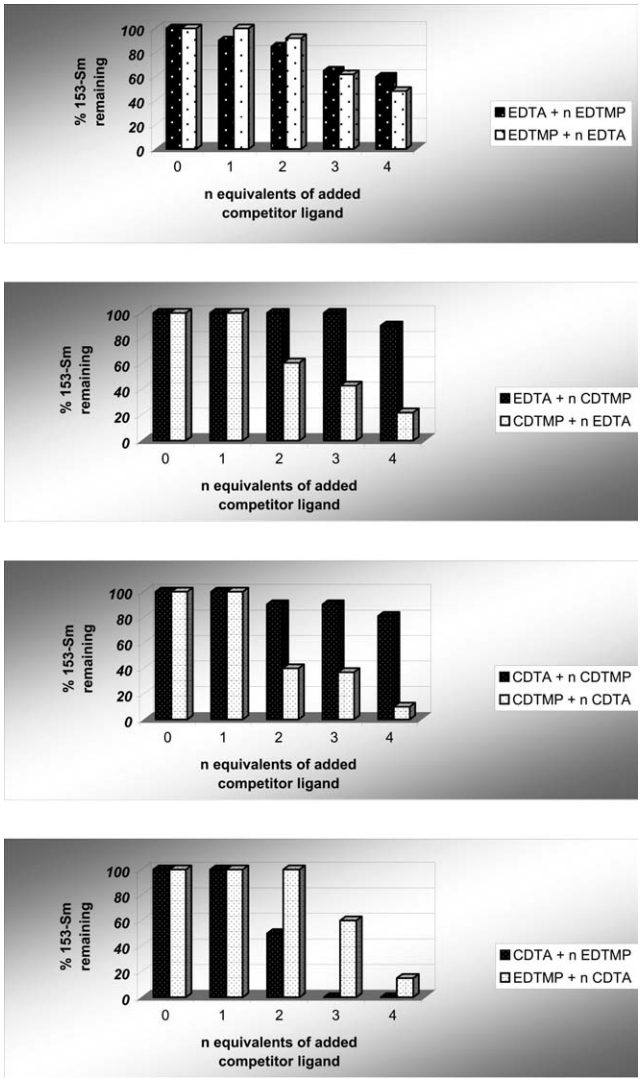

Fig. 3. EDTA, CDTA, EDTMP and CDTMP competition studies.

2.2.2. Competition with EDTMP

In these high dilution conditions, a large excess of chelating agents (50 equiv.) was used to ensure complete chelation of all agents in the subsequent competition study.

No significant metal transchelation exchanges were observed at early times. As rates of exchange were relatively slow, experiments were conducted at 72 h to allow complete exchange reactions. The results are expressed as a percentage of the ^{153}Sm that remained chelated in the four agents after 72 h of incubation (Fig. 4).

Compound **4** showed dramatic instability, with complete release of the radionuclide towards the formation of ^{153}Sm-EDTMP, whereas 15% remained in compound **2**. Better results were obtained with compound **1** (67% of retained activity), while compound **3** gave excellent results (100% of ^{153}Sm remained chelated).

These results indicate the great advantage of using mixed carboxylic/phosphonic chelating agents rather than pure phosphonic chelating agents. In this respect, a nice comparison can be made between compounds **3** and **4**. Although both

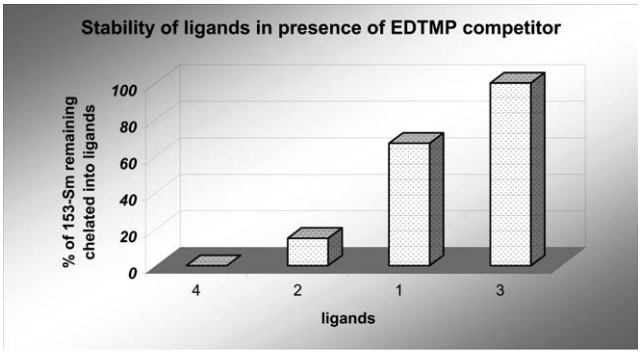

Fig. 4. Transchelation effect of Sm from ligand **3** to EDTMP Competitor[a]. [a] Number of experiments: two.

carry four nitrogens, the presence of two carboxylic groups has a considerable effect on the stability of the resulting chelating agent (0% and 100% of activity remained chelated, respectively, for compounds **4** and **3**). The same comparison can be made between compounds **2** and **1**, for which, respectively, 15% and 67% of the activity remained chelated.

At this stage, increasing the number of phosphonic acid functions in an eight coordinate semi-rigid ligand, as well as in a 10 coordinate semi-rigid ligand, reduced the stability of the resulting complex. A 10 coordinate semi-rigid chelating agent would in any case be more appropriate for stable ^{153}Sm chelation.

2.3. Serum stability

This experiment was conducted for 10 days, and no changes were noted in the results. The ^{153}Sm-**3** complex remained unchanged and moved up to R_f 0.7 on ITLC plate controls. Compound **3** showed excellent stability under physiological conditions, with no release of ^{153}Sm.

3. Discussion

The synthesis and evaluation of new bifunctional chelating agents able to bind radionuclides such as ^{153}Sm have been described in the literature [14,15]. Some of these agents are acyclic, e.g. nitro-CHX-A-DTPA, nitro-MX-DTPA and DTPA, or macrocyclic, e.g. nitro-DOTA and nitro-PADOTA, but all carry carboxylic acids for ionic chelation with the radionuclide. However, previous studies suggested that EDTMP carrying phosphonic acid chelating groups could be a good agent for ^{153}Sm chelation because of its high stability constant (log K = 22.4) and the fact that its complex, known as QUADRAMET@, is a radiopharmaceutical used in treating metastatic bone cancer [16]. No studies have compared the ^{153}Sm chelating properties of carboxylic chelating agents with those of their phosphonic analogues. Moreover, slow chelation kinetics have been reported for macrocyclic-chelating agents, whereas chelation reaction with acyclic chelating agents is instantaneous. This could lead to certain difficulties in chelate formation with macrocyclic deriva-

tives, or to very rapid release of the radionuclide with acyclic ligands. Our study proposes an alternative (semi-rigid chelating agents) between those two concepts, which would seem to provide rapid kinetics and high stability. The study of Stimmel and Kull [14] and Stimmel et al. [15] supports our point of view concerning the better stability of ^{153}Sm-nitro-CHX-DTPA as compared to ^{153}Sm-nitro-MX-DTPA.

The present study attempted to determine whether it would be useful to synthesise stable phosphonic and/or carboxylic semi-rigid chelating agents for ^{153}Sm chelation applications in cancer radiotherapy. A reliable procedure was established to select a promising candidate chelating agent prior to undertaking the long procedure involved in synthesising a bifunctional analogue. Although the conditions of in vitro procedures can never duplicate those of a real in vivo test, they are useful for selecting among the candidate agents. If the agents are ineffective in this selection process, they will certainly not be stable in vivo.

We first attempted to determine the equivalent of chelating agent needed to form chelates with ^{153}Sm in our conditions. The results showed that a chelator-to-radionuclide ratio of 1:2 was sufficient for most cases. However, to ensure maximum chelation efficiency and avoid free ^{153}Sm, a 4:1 ratio was used for 2 h of stirring at 37 °C.

As reported by Stimmel and Kull [14], the dissociation of ^{153}Sm in vivo caused bone toxicity and limited clinical efficacy. These authors described a test based on stability at different pH values for the evaluation of macrocyclic and acyclic carboxylic chelating agents. Comparison with serum stability showed different results for one compound, ^{153}Sm-nitro-CHX-DTPA, which had exceptional stability at low pH (even better than ^{153}Sm-nitro-DOTA and ^{153}Sm-nitro-PADOTA), but then proved less stable in human serum. As results are difficult to predict, our complex stability properties were evaluated by performing in vitro competition tests with another chelating agent (Fig. 4). Actually, this method can only be used if a chromatographic system or other analytical method allowing the ^{153}Sm-chelating agent to be distinguished from its competitor can be found. In our experiments EDTMP was chosen as a reference for competition studies because its ^{153}Sm-complex is used in nuclear medicine. A previous study [12], which provided the ^{153}Sm-stability constants of EDTMP, EDTA, DTPMP, DTPA, and DOTA, showed that DTPA had a higher constant than DTPMP and nearly the same constant as DOTA (log K = 22.3, 20.7 and 23, respectively), whereas EDTMP is greater than EDTA (log K = 22.4 versus 17.1). Thus, there appears to be a relation between size and coordinate functions. In our preliminary study reported here, the ^{153}Sm chelation properties of EDTA, EDTMP, CDTA, and CDTMP were compared in order to evaluate the benefit of carboxylic acid groups over phosphonic acid groups and to determine the influence of semi-rigid structure on the stability of the resulting complexes.

As no similar studies had been performed with ^{153}Sm on these chelating agents, this preliminary work was conducted to choose the most appropriate combination for the design of new chelating agents.

Linear systems seem to be more adapted to ^{153}Sm chelation, possibly because of their flexibility, which provides faster kinetics. No dramatic differences between the ability of phosphonic and carboxylic acids to complex ^{153}Sm have been observed, except in the case of CDTA. As linear systems are more stable than small semi-rigid complexes (EDTA > CDTMP and EDTMP > CDTA), they appear to adapt more easily to ^{153}Sm. On the basis of its chromatographic profile, CDTA seems to be a better chelate than CDTMP, owing to its great ability to extract ^{153}Sm from its phosphonic analogue complex as well as its high capacity for retaining the radionuclide in the cage. It is possible that the phosphonic acid function is greater than the carboxylic acid function, which could lead to a strained complex, especially in the case of a medium-sized semi-rigid chelate. These results are in agreement with those obtained by Volkert et al. [12]. Phosphonic groups present a steric bulk, which can either be favourable (in case of long linear systems) or unfavourable (in case of small cages). These preliminary results already indicate that cavity size should be adapted to ^{153}Sm size to benefit from the semi-rigid structure, and that it would be premature to conclude that carboxylic chelation functions are better than phosphonic ones.

To obtain thermodynamically stable complexes, chelating groups can be changed or the cavity size of the molecule increased. The use of a rapid method for obtaining semi-rigid chelating agents allowed us to test four compounds with differences in chelating functions, but also in cavity size. They possessed three or four nitrogens, carboxylic or phosphonic functions. Although the four chelates were effective in the first complexation test, chelation ability does not mean high stability. Therefore, these four chelating agents were challenged with EDTMP to measure their capability to retain chelated ^{153}Sm.

To eliminate differences in chelation capabilities, a 50 equiv. excess of the different chelating agents was used. Fifty equivalents of EDTMP were added to a solution of compounds **1–4** previously chelated with ^{153}Sm, which was then stirred for 3 h at 37 °C. The stability of ^{153}Sm-chelating agent complexes was measured at 3, 6, 24, 48 and 72 h. Although the results obtained at 72 h were quite the same as at 6 h, the period of 72 h allowed us to establish a reliable classification of the four agents (Fig. 4). Compound **4** (4N, 6P) and compound **2** (3N, 5P) showed no or only slight capacity to retain ^{153}Sm (0% and 15%, respectively), whereas compound **1** (3N, 2P, 3C) and compound **3** (4N, 2P, 4C) gave favourable results (67% and 100%, respectively). A comparison of compounds **3** and **4** with compounds **1** and **2** made it easy to conclude that pure phosphonic semi-rigid chelating agents are not suitable for in vivo use. When the results for compound **2** (3N, 5P, eight coordination sites) and compound **1** (3N, 2P, 3C, eight coordination sites) were compared, i.e. respectively, 15% and 67%, the influence of carboxylic functions was evident. The same observation was

true for the comparison of compound **4** (4N, 6P, 10 coordination sites) and compound **3** (4N, 2P, 4C, 10 coordination sites). Nevertheless, the best results were obtained with compound **3** (100% of ^{153}Sm remained chelated), indicating that a 10 coordination site compound is essential for the formation of a stable ^{153}Sm-semi-rigid chelate.

However, before it could be determined whether compound **3** was suitable for in vivo applications, it was necessary to confirm this potential by demonstrating human serum stability under physiological conditions.

^{153}Sm-**3**, when incubated in human serum at 37 °C, showed no loss of radioactivity. In fact, similar results have been obtained with nitro-DOTA and nitro-PADOTA in the same conditions [14]. These authors found that the eight coordinate ligand ^{153}Sm-nitro-CHX-DTPA was less stable in human serum than the nine coordinate ligand ^{153}Sm-nitro-MX-DTPA, which may be partly explained by the difference of one coordination site (5C, 3N and 6C, 3N, respectively). Compound **3** is a nitro-CHX-DTPA-like chelating agent, but has the advantage of possessing 10 coordination sites (carboxylic and phosphonic) associated with a semi-rigid structure, which makes it potentially suitable for in vivo applications.

4. Conclusion

The use of ^{153}Sm in cancer radioimmunotherapy protocols would be possible only if stable ^{153}Sm radioimmunoconjugates were formed. Stability can be increased by modifying different parameters, such as the number and nature of coordination sites, chelation cavity size, and the chelating agent skeleton. As the synthesis of bifunctional chelating agents is always very long and with no guarantee of success, a reliable methodology should be defined first to make a selection among several chelating agents and then synthesise the most promising bifunctional analogue. The method described here for this purpose was based on chelation efficiency, challenge with a competitor, and serum stability. Among the four original chelating agents, the potential of compound **3** was confirmed in vitro by stability trial in serum medium over a 10-day-period.

Although the complex ^{153}Sm-**3**, could possibly be directly evaluated for metastatic bone cancer therapy, the synthesis of a bifunctional analogue is in progress and will be reported in a future publication.

5. Experimental protocol

EDTA, EDTMP, CDTA were purchased from Sigma-Aldrich and CDTMP was synthesised in our laboratory according to a previously described method [17]. Various quantities of each chelating agent (5, 0.5, or 0.05 mg ml^{-1}) were dissolved in 0.1 M sodium acetate buffer, and final pH was adjusted to 5.8 for each solution. A stock solution of ^{153}Sm in 0.04 M HCl (200 μl; volumetric activity 5.2 GBq ml^{-1}, specific activity 40 GBq mg^{-1}) was produced by neutron irradiation of isotopically enriched $^{152}Sm_2O_3$ provided by CIS-BIO-International. Thin-layer chromatography (TLC) controls were performed on ITLC-SG Gelmann plates (Gelman Sciences) or 0.1 mm cellulose plates (Merck 5552/0025) and measured using a Phosphorimager 445SI.

5.1. Preliminary study

5.1.1. ^{153}Sm/chelating agent ratio

A first experiment was performed to determine the ratio of chelating agent to radionuclide needed to achieve maximum ^{153}Sm chelation efficiency and avoid free ^{153}Sm contamination in solutions in subsequent competition studies performed with EDTA, CDTA, EDTMP, and CDTMP. To form ^{153}Sm chelating agent complexes, a fixed quantity (2 μl = 10.4 MBq) of ^{153}Sm stock solution was added to a range of 0, 1, 2, 5, 10, 20 and 50 equiv. of each chelating agent. The volume of each sample was adjusted to 100 μl with 0.1 M sodium acetate, and the solutions were incubated at 37 °C for 3 h. The final pH of each solution ranged from 5.5 to 5.8, with a final ^{153}Sm concentration of 1.7 nM.

Samples were analysed on ITLC-SG Gelman plates and eluted with 0.1 M sodium acetate (pH 5.8)/methanol (1:1) for phosphonic chelating agents (EDTMP and CDTMP) and 0.1 M sodium acetate (pH 5.8)/methanol (1:3) for carboxylic chelating agents (EDTA and CDTA). The chelation yield and the chromatographic profiles were determined using a Phosphorimager 445SI.

5.1.2. EDTA, CDTA, EDTMP, and CDTMP competition study

The competition study investigated the influence of a carboxylic or phosphonic group on radionuclide chelation properties. Chelating agents were identified in two different groups, one labelled as C (EDTA and CDTA) for carboxylic-carrying chelating agents and the other P (EDTMP and CDTMP) for phosphonic-carrying chelating agents. This study was performed according to the following procedure: 4 equiv. amounts of each chelating agent and 2 μl of stock solution radionuclide (10.4 MBq) were mixed in an Eppendorf tube and incubated at 37 °C for 2 h to obtain complete chelation efficiency. Four samples of each chelating agent were prepared in this manner. One to four equivalents (competitor/chelating agent ratio = respectively, 0.25, 0.5, 0.75 and 1) of competitor ligand were then added to each ^{153}Sm complex solution, and the final volume was adjusted to 100 μl by addition of 0.1 M sodium acetate, pH 5.8. The samples were incubated at 37 °C for a last 2 h period, and the dissociation from the ^{153}Sm-(C or P) chelate complex towards the formation of ^{153}Sm-(P or C) competitor complexes was quantified using the Phosphorimager procedure. TLC analysis of a 2 μl aliquot on ITLC-SG Gelman plates was performed by elution with 0.1 M sodium acetate (pH 5.8)/methanol (1:3). The ^{153}Sm-C chelate complex moved to

the solvent front, while the ^{153}Sm-P chelate complex remained at the point of origin.

5.2. ^{53}Sm chelation properties of the four chelating agents (**1–4**)

5.2.1. Effect of chelator concentration on radiolabelling efficiency

Four chelating agents (**1–4**) were evaluated as chelate complexes of ^{153}Sm. Stock solutions of each chelate in 0.1 M sodium acetate, pH 5.8 (5, 0.5, and 0.05 mg ml^{-1}), were used to examine the effect of chelator concentration on radiolabelling efficiency. A range of each chelator from 0, 0.5, 1, 2, 10 to 50 equiv. was added to a constant quantity of 2 μl (10.4 MBq) of ^{153}Sm stock solution before adjustment of the final volume to 100 μl. The final ^{153}Sm concentration was 1.7 nM, pH 5.6.

After incubation at 37 °C for 3 h, quantification was performed, as described above. Cellulose plates, by elution with 0.1 M sodium acetate (pH 5.8)/methanol (3:5), were used.

5.2.2. Competition with EDTMP

A 50 equiv. excess of each chelating agent (compounds **1–4**) was used to ensure complete ^{153}Sm chelation. Two microlitres (10.4 MBq) of ^{153}Sm stock solution were added separately to each chelating agent (85 nmol), and the solutions were incubated at 37 °C for 3 h. The ^{153}Sm-EDTMP complex was prepared in the same conditions for stability control. An equal amount (85 nmol) of EDTMP (stock solution of 5 mg ml^{-1} in 0.1 M sodium acetate, pH 5.8; chelating agent/competitor = 1) was then added separately to the chelating agent solutions, and the volume was adjusted to 100 μl by addition of 0.1 M sodium acetate, pH 5.8. The stability of ^{153}Sm-chelating agent complexes was measured at 3, 6, 24, 48, and 72 h. Separate cellulose plates were used for each chelating agent and the controls, and elution was performed with 0.1 M sodium acetate (pH 5.8)/methanol (3:5). Radioactivity was quantified using a Phosphorimager apparatus. Two microlitres of each solution of the ^{153}Sm-chelating agent, ^{153}Sm-EDTMP, and the reaction mixture containing ^{153}Sm-chelating agent and EDTMP were spotted onto ITLC plates. The ^{153}Sm-EDTMP solution was used as a control since the two different species (^{153}Sm-chelating agent and ^{153}Sm-EDTMP) could be distinguished on the same ITLC plate.

5.3. Human serum stability

Human serum (pH 7.4) in sterile tubes was provided by the Nantes University Hospital for stability studies. A 100 μl solution of ^{153}Sm-**3** complex was prepared in duplicate according to the procedure previously described and added to 0.5 ml of human serum for a final radionuclide concentration of 1.7 nM. Another sample containing 2 μl of stock ^{153}Sm solution (10.4 MBq), 98 μl of 0.1 M sodium acetate, pH 5.8, and 0.5 ml of human serum was also prepared. After both samples were incubated at 37 °C, a 4 μl aliquot of each was spotted onto ITLC plates to quantify the percentage of radioactivity remaining in the chelating agent. Elution was performed with 0.1 M sodium acetate (pH 5.8)/methanol (3:5). The ^{153}Sm associated with the serum remained at the point of origin, while the ^{153}Sm-**3** complex moved up to R_f 0.7. This evaluation was conducted daily over a 10-day-period.

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