

Research Article

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Radiochemical and biological evaluation of novel $^{153}\text{Sm}/^{166}\text{Ho}$ -amino acid–chitosan complexes

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$^{153}\text{Sm}/^{166}\text{Ho}$ -chitosan complexes have been considered promising agents for internal radiation therapy. By direct administration, complexes solution converts into a gel, at physiological pH, allowing its retention for a long time. Herein, we report on the synthesis of $^{153}\text{Sm}/^{166}\text{Ho}$ complexes with the novel amino acid–chitosan polymers, *N*-(γ -propanoyl-valin)–chitosan (CHICO-val) and *N*-(γ -propanoyl-aspartic acid)–chitosan (CHICO-asp). The main goal of this study was to obtain data on the radiochemical and biological behaviour of these complexes and information regarding their therapeutic potential when compared to $^{153}\text{Sm}/^{166}\text{Ho}$ -chitosan. Radiolabelling yield of $^{153}\text{Sm}/^{166}\text{Ho}$ -amino acid–chitosan complexes was dependent on polymer concentration but less dependent on pH. Radiochemical stability was shown to be higher for amino acid–chitosans than for chitosan, with $^{153}\text{Sm}/^{166}\text{Ho}$ -CHICO-val being stable up to 3 h, while $^{153}\text{Sm}/^{166}\text{Ho}$ -CHICO-asp is stable up to 24 h. In the presence of ascorbic acid radiochemical stability of $^{153}\text{Sm}/^{166}\text{Ho}$ -CHICO-val and $^{153}\text{Sm}/^{166}\text{Ho}$ -CHICO was improved, decreasing for $^{153}\text{Sm}/^{166}\text{Ho}$ -CHICO-asp. *In vivo* behaviour of ^{153}Sm complexes was studied in mice. The radioactive amino acid–chitosans can be directly injected into blood stream without significant retention on injection site, being trapped by liver. Biodistribution studies suggest that the radioactive amino acid–chitosans, due to its water solubility and stability may be considered potential candidates to be further explored for liver targeted nuclear therapy.

Keywords: radiolanthanides; chitosan derivatives; amino acid; polymers; internal radiation therapy

Introduction

Internal radiation therapy (IRT) with unsealed β -emitting radionuclides has emerged for the treatment of cancer as an alternative to external radiotherapy. β -emitters have advantages for the treatment of tumours owing to their physical characteristics, in particular their short soft tissue penetration range, delivering high radiation dose to tumour without adverse radiation effects on the surrounding normal tissues.¹ Among the β -emitters, ^{166}Ho ($t_{1/2}$ 26.8 h, β_{max} 1.85 MeV (51%), γ 0.081 MeV (7.5%) and ^{153}Sm ($t_{1/2}$ 46.8 h, β_{max} 0.81 MeV (21%), γ 0.103 MeV (38%)) appear to be good candidates for IRT.²

Chitosan, a polymer of 2-deoxy-2-amino-D-glucose obtained by *N*-deacetylation of chitin has unique properties for a broad variety of applications.^{3–5} Its chemical structure is characterized by the degree of acetylation (DA), which also determines its ability to chelate metal ions.³ Chemical modifications to enhance chelating properties, selectivity and metal ion capacity of chitosan have been proposed and various chitosan derivatives have been designed to improve these features.⁶ Keeping this in mind, *N*-(2-carboxyethyl)–chitosans have been prepared and their metal complexation properties and potential applications have been studied.^{7–9}

Owing to its capacity to complex β -emitter metal ions through its amino groups, chitosan appears to be a promising therapeutic alternative for local treatment of tumours.¹⁰ The main features of these complexes are their solubility under

acidic conditions and the easy conversion to gel at the local of administration (physiological pH), with the corresponding retention of the radionuclide. Based on this principle, $^{153}\text{Sm}/^{166}\text{Ho}$ -chitosan complexes have been prepared with relatively high radiochemical purity (>90%) and their biological behaviour has been considered promising for local therapy of prostate and skin cancer,^{11–13} brain glioma,¹⁴ renal cysts¹⁵ and rheumatoid arthritis.^{16–17} Following these results, ^{166}Ho -chitosan (DW-166HC) has been approved in Korea as a radiopharmaceutical for the treatment of small hepatocellular carcinoma (HCC).^{18,19} However, DW-166HC is sensitive to degradation induced by radiation and its use immediately after synthesis is highly required.

We have been working on the chemical modification of chitosan amino groups in order to improve both the polymer water solubility over a broader pH range and its chelating

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properties, for potential biomedical applications.^{7–9} Recently, we have prepared two novel amino acid–chitosan polymers, *N*-(γ -propanoyl-valin)–chitosan and *N*-(γ -propanoyl-aspartic acid)–chitosan, from low molecular weight chitosan and *N*^z-(3-bromopropanoyl)–valine or *N*^z-(3-bromopropanoyl)–aspartic acid, respectively.²⁰ Structures of chitosan (CHICO, **1**), *N*-(γ -propanoyl-valin)–chitosan (CHICO-val, **2a**) and *N*-(γ -propanoyl-aspartic acid)–chitosan (CHICO-asp, **2b**) are presented in Figure 1. In view of the interesting properties of our novel amino acid–chitosan derivatives,²¹ we decided to explore the coordination capability of these modified chitosan towards ¹⁵³Sm/¹⁶⁶Ho, two therapeutically relevant radionuclides. Herein, we describe the synthesis of ¹⁵³Sm/¹⁶⁶Ho-*N*-(γ -propanoyl-valin)–chitosan and ¹⁵³Sm/¹⁶⁶Ho-*N*-(γ -propanoyl-aspartic acid)–chitosan as well as our data on their radiochemical and

biological behaviour as compared with the respective chitosan analogous.

Results and discussion

Synthesis and stability of the radiolabelled complexes

Irradiation of ¹⁵²Sm/Ho-nitrates yielded 140 MBq/mg (¹⁵³Sm) and 190 MBq/mg (¹⁶⁶Ho) with high radionuclidic purity as confirmed by the typical γ -ray spectrum.²² The complexation reaction conditions were optimized in order to obtain ¹⁵³Sm/¹⁶⁶Ho-complexes with high radiochemical purity. The labelling yield of ¹⁵³Sm/¹⁶⁶Ho-CHICO–amino acid complexes was not highly dependent on the pH, but depends on the polymer concentration. Complexes were obtained with yields higher than 98% at pH 3–7 with 1% (w/v) polymer solution, while the complexation yield drastically decreases at lower concentration (Table 1). In contrast, the labelling yield of ¹⁵³Sm/¹⁶⁶Ho-CHICO complexes was highly dependent on both parameters, reaching only 98% at pH \approx 3 and using a 1% CHICO solution.^{11,16}

The stability of the CHICO–amino acid complexes was followed by ITLC, using the chromatographic systems indicated in the experimental section. Both complexes have *in vitro* radiochemical behaviour different than that of their CHICO analogous. While the latter complex remains in the origin, in accordance with its colloidal nature, the CHICO-asp and CHICO-val complexes migrate with *R_f* values of approximately 0.4 and 0.6, respectively (Table 2). Nonetheless, in both cases the presence of free radiolanthanide was never detected.

After formation, ¹⁵³Sm/¹⁶⁶Ho-CHICO-asp/CHICO-val complexes showed relatively high radiochemical purity within a sufficient time period for eventual clinical applications (¹⁵³Sm/¹⁶⁶Ho-CHICO-val is stable up to 3 h and ¹⁵³Sm/¹⁶⁶Ho-CHICO-asp is stable up to 24 h), while ¹⁵³Sm/¹⁶⁶Ho-CHICO appeared to be more sensitive and degrading with storing.²³ As an example, we show in Figure 2 how fast ¹⁵³Sm-CHICO degrades leading to new radiochemical species with time. As shown in Figure 2, ¹⁵³Sm-CHICO leads to a new species with *R_f* \approx 0.22, 30 min after formation, and with time new species are formed progressively with higher *R_f* values. The amino acid derivatives are stable for a longer period of time and the decomposition corresponds to the formation of a new radiochemical species along with the original ones. The different behaviour found for our complexes may be due to the presence of one or two carboxylate groups in the chitosan backbone, which may contribute for structural stabilization of the radioactive complexes.

Considering that ascorbic acid (AA), an antioxidant radical scavenger, is often used as a food additive or as a stabilizer in radiopharmaceutical compositions,^{24,25} we have decided to evaluate the effect of this compound on the radiochemical stability of ¹⁵³Sm/¹⁶⁶Ho-chitosan derivatives and at the same time on ¹⁵³Sm/¹⁶⁶Ho-chitosan. As can be seen in Figure 2,

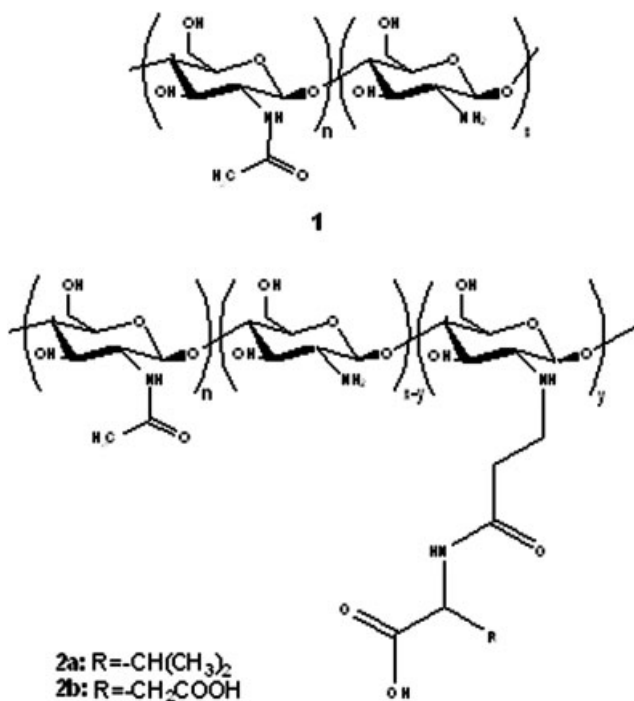


Figure 1. Molecular structures of chitosan (**1**), *N*-(γ -propanoyl-valin)–chitosan (**2a**) and *N*-(γ -propanoyl-aspartic acid)–chitosan (**2b**).

Table 1. Labelling yield vs polymer concentration at the pH range 3–7

Polymer	Conc. (% w/v)	Labelling yield (%)
CHICO-val	0.5	\sim 0
	1	\sim 100
CHICO-asp	0.5	\sim 0
	1	\sim 100

Table 2. Radiochemical behaviour of ¹⁵³Sm/¹⁶⁶Ho-amino acid–CHICO complexes

<i>R_f</i>			
¹⁵³ Sm/ ¹⁶⁶ Ho-CHICO	¹⁵³ Sm/ ¹⁶⁶ Ho-CHICO-val	¹⁵³ Sm/ ¹⁶⁶ Ho-CHICO-asp	¹⁵³ Sm/ ¹⁶⁶ Ho(NO ₃) ₃
\sim 0	\sim 0.6	\sim 0.4	\sim 0.9–1.0

the radical scavenger has slightly improved the stability of ^{153}Sm -CHICO, in agreement with the results previously described by Zoldners *et al.*²⁴ The stability of this compound seems to be relatively dependent on the nature of the solution components. In acetic acid solutions, for example, Zoldners *et al.* described that chitosan degrades with the formation of more water-soluble fragments along the time, as a result of oxidative and hydrolytic splitting of the polymer chains. Moreover, it has also been referred that ^{166}Ho -chitosan is radiation sensitive, heading for decreasing in the complex viscosity.²³ The increase stability of ^{153}Sm -CHICO in the presence of AA may be due to the effect of AA as a scavenger of possible radical species and/or to the additional coordination capability of AA.^{23,24} Regarding the $^{153}\text{Sm}/^{166}\text{Ho}$ -CHICO-amino acid complexes, the addition of AA (2% AA solution) has significantly affected its radiochemical behaviour. For compound $^{153}\text{Sm}/^{166}\text{Ho}$ -CHICO-val its stability enhanced significantly up to 24 h. In contrast, the addition of AA to $^{153}\text{Sm}/^{166}\text{Ho}$ -CHICO-asp resulted in a high degree of degradation of the initial complexes, with formation of several species. Based on the thermo-stability and structural studies previously performed for CHICO and for CHICO-amino acid polymers, by differential scanning calorimetry and scanning electron microscopy, the CHICO-asp has a looser

macromolecular structure than its CHICO-val counterpart and a highest thermo-sensitivity and water-holding capacity, which implies that its supramolecular structure is weaker than that of its counterpart (Figure 3).^{20,21} Thus, while CHICO-val has a compact and continuous film-like structure, CHICO-asp has a highly porous morphology, probably due to electrostatic repulsions between its higher number of carboxylate groups. The porous morphology of CHICO-asp may allow AA, a well-known chelating agent,^{26,27} to efficiently penetrate into the polymeric matrix and compete for metal complexation with the polymer chelating groups. In CHICO-val the structure is more compact and stable so another oxygen donor co-ligand may lead to a mixed-ligand complex, which will be anchored on the donor groups of the amino acids as well as on the AA, leading to the stabilization of the radiometal by a synergic effect.^{24,26}

Biodistribution of the ^{153}Sm -labelled complexes in mice

Preliminary biodistribution studies were only performed for ^{153}Sm -complexes. For comparison, ^{153}Sm -CHICO was also studied in the same animal model. As expected, the ^{153}Sm -CHICO was almost completely retained at the site of administration and no radioactivity excretion was found up to 24 h. On the contrary, the CHICO-amino acid complexes could be injected directly into the blood stream by the tail vein. For both complexes a high liver uptake was found, despite i.v. injection and the excretion was very slow or negligible. However, the two complexes have shown slightly different biodistribution patterns (Figure 4). While ^{153}Sm -CHICO-val was associated to high hepatic radioactivity retention that enhanced over time, the ^{153}Sm -CHICO-asp was slowly cleared from the liver into the intestine, which is a disadvantage if one wants to treat liver cancer. Additionally, the uptake by the liver should be speed up in order to reduce the radiation dose to the blood cells. The significant retention of activity in the liver found for ^{153}Sm -CHICO-val can probably be due to the *in vivo* formation of radiochemical species of colloidal/polymeric nature.

Experimental

Chemicals: Enriched samarium oxide (Sm_2O_3 , 98.4% as ^{152}Sm) was purchased from Campro Scientific and natural Ho_2O_3 (99.9%) from Strem. Chitosan (abbreviated CHICO) (MW ~ 150 000; DA = 0.10) was supplied by Sigma-Aldrich.

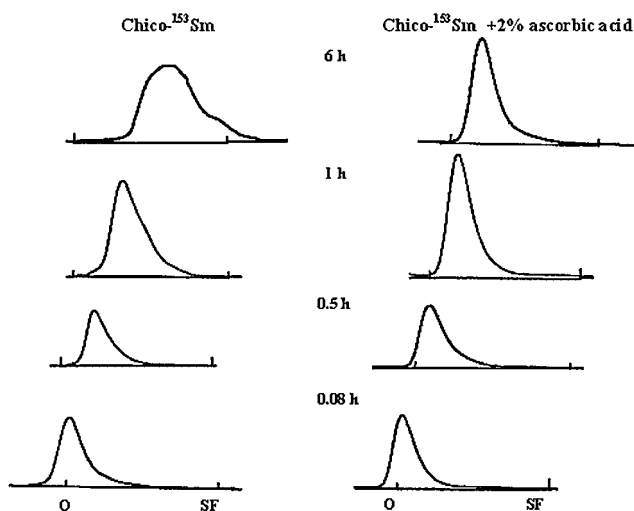


Figure 2. Radioactive distribution on the ITLC-SA strips of ^{153}Sm -CHICO complex obtained in the absence and presence of ascorbic acid over time.

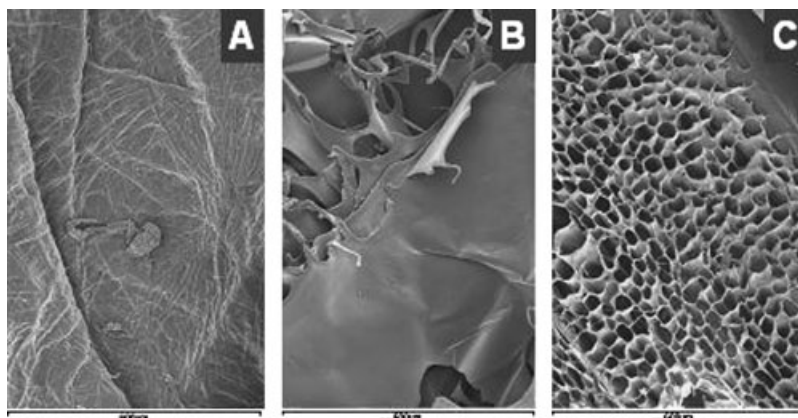


Figure 3. SEM micrographs (100 × magnification) of CHICO (A), CHICO-val (B) and CHICO-asp (C).

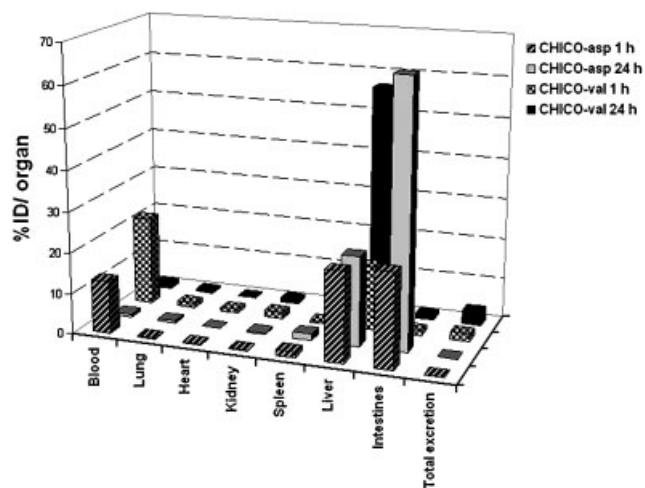


Figure 4. Whole body excretion and biodistribution in the most relevant organs of ^{153}Sm -CHICO complexes at 1 and 24 h after administration in mice.

Instant thin layer chromatography (ITLC) strips were supplied by Polygram, Macherey-Nagel. *N*-(γ -propanoyl-valin)-chitosan (abbreviated CHICO-val) and *N*-(γ -propanoyl-aspartic acid)-chitosan (abbreviated CHICO-asp) were synthesized and purified according to methods previously reported.²¹ All materials were reagent grade unless otherwise specified.

Synthesis of the peptide chitosan polymers: The synthesis of the two novel CHICO-based polymers was reported in detail elsewhere.²¹ Briefly, *N*^z-(3-bromopropanoyl)-valine and *N*^z-(3-bromopropanoyl)-aspartic acid were prepared by reacting the relevant L-amino acid *tert*-butyl esters with 3-bromopropanoic acid in the presence of dicyclohexylcarbodiimide (DCCI) as coupling reagent. After being isolated by column chromatography on silica, the *tert*-butyl esters were successfully identified by ^1H and ^{13}C NMR. These esters were then cleaved by acidolysis with neat TFA, with quantitative formation of the corresponding free carboxylic acids *N*^z-(3-bromopropanoyl)-valine and *N*^z-(3-bromopropanoyl)-aspartic acid, whose structures were also confirmed by NMR. The carboxylic acids obtained as above described were covalently attached to chitosan as follows: chitosan was dissolved in water containing 4 M equivalents of *N*^z-(3-bromopropanoyl)-valine or *N*^z-(3-bromopropanoyl)-aspartic acid. The reactions were allowed to proceed at 60°C under magnetic stirring and with daily additions of NaHCO_3 to keep pH within the 6–8 range. The peptide-chitosans were purified by dialysis against deionized water for 5–7 days, and then freeze-dried.

Production of ^{153}Sm and ^{166}Ho : ^{153}Sm and ^{166}Ho were produced by $^{152}\text{Sm}(n,\gamma)^{153}\text{Sm}$ and $^{165}\text{Ho}(n,\gamma)^{166}\text{Ho}$ reaction, respectively, in the ITN Nuclear Research Portuguese Reactor (1 MW). Irradiation was performed using nitrate targets (Sm/Ho, 10 mg) prepared from the correspondent isotopically enriched $^{152}\text{Sm}_2\text{O}_3$ or natural Ho_2O_3 under a thermal neutron flux of $1.5 \times 10^{13} \text{ n/cm}^2\text{ s}$ and epithermal neutron flux of $3.1 \times 10^{11} \text{ n/cm}^2\text{ s}$ for 1–2 h. Following irradiation, the targets were reconstituted in H_2O to yield 1% (w/v) $^{153}\text{Sm}/^{166}\text{Ho}$ -nitrate solution for complex preparation. The radionuclide purity was assessed by γ spectrometry with a Ge(Li)-detector (Canberra) and activities were measured by a radioisotope calibrator (Aloka, Curimeter IGC-3, Tokyo, Japan).

Radiolabelling procedure: The preparation of $^{153}\text{Sm}/^{166}\text{Ho}$ -complexes with a high labelling yield (>98%) was optimized.

Typically, 40 μL of 1% $^{153}\text{Sm}(\text{NO}_3)_3$ solution or 20 μL of 1% $^{166}\text{Ho}(\text{NO}_3)_3$ solution was added to 700 μL of CHICO or CHICO-based polymer solution in 1% (v/v) acetic acid, followed by thorough stirring for 20 min at room temperature. The radiolabelling pH of the resulting solutions was 3.0 for $^{153}\text{Sm}/^{166}\text{Ho}$ -CHICO complex and 7.0 (by adding an appropriate volume of 1 N NaOH solution) for $^{153}\text{Sm}/^{166}\text{Ho}$ -peptide-chitosan based complexes. The labelling efficiency was accomplished by ITLC, using ITLC-SA strips developed with $\text{MeOH}:\text{H}_2\text{O}:\text{acetic acid}$ (50:50:0.5) as the mobile phase. In this system, the radiochemical species migrate with the R_f presented in Table 2. Radioactive distribution on the ITLC-SA strips was detected by using a Berthold LB 505 detector coupled to a radiochromatogram scanner. The stability of the $^{153}\text{Sm}/^{166}\text{Ho}$ -CHICO or $^{153}\text{Sm}/^{166}\text{Ho}$ -CHICO-based complexes was assessed by measuring the radiochemical purity by ITLC over time, up to 24 h. The effect of the radical scavenger AA (2% AA solution) on the stability of the complexes was also studied.

In vivo studies: Biodistribution studies were carried out using normal CD-1 mice from Charles River, Spain, according to previously reported procedures.²² Animals were intravenously injected, *via* the tail vein, or intraperitoneally injected with 100 μL ($\sim 3.7 \text{ MBq}$) of the radiolanthanide complexes. After 1 and 24 h animals were killed by cervical dislocation, the main organs were dissected and the radioactivity counted in a γ counter (Berthold LB 2111, Germany). Biodistribution results were expressed as percent of injected dose per total organ (% I.D./organ).

Concluding remarks

Concerning the best strategy to deal with inoperable HCC, there is a general lack of consensus. Classical approaches include trans-arterial chemo-embolization (TACE) or IRT through administration of radionuclide-lipiodol complexes that have the advantage of being highly retained by hepatic tumours.^{28–35} However, TACE is not suitable for advanced disease or patients with an obstructed (thrombotic) portal vein, whereas radiolabelled lipiodol (e.g. ^{131}I -lipiodol) is associated with pain on injection and occasional induction of severe pneumopathies.^{30,31} Radioimmunotherapy can be a way to circumvent the above problems. But treatments with the monoclonal ^{131}I -Hepama-1, though associated with low toxicity, usually requires hepatic artery ligation, whereas polyclonal ^{131}I rabbit antiferritin IgG has been related to thrombocytopenia.³¹ Alternatively, radiolabelled glass or resin microspheres have been explored as potentially safer IRT agents, but their clinical efficacy has not been proved so far. Successful treatment of liver tumours in an animal model was achieved with ^{166}Ho -labelled poly-(L-lactic acid) microspheres, which opens new possibilities towards the use of microspheres built from biocompatible materials.³¹ In this context, radiolabelled chitosan-based polymers appear as a most attractive and cost-effective choice, as chitosan is cheap, biodegradable, bioadhesive and biocompatible, and has low toxicity and low immunogenicity. Indeed, the approved ^{166}Ho -chitosan complex (DW-166HC), was found to be a highly effective and safe new radiopharmaceutical agent for IRT against liver cancer.¹⁹ The CHICO-amino acid polymers, described herein, were found to be suitable ligands for the preparation of stable $^{153}\text{Sm}/^{166}\text{Ho}$ -complexes with high labelling yields (>98%) and high radiochemical purity over the time. As compared with unmodified chitosan, the amino

acid derivatives present several advantages for eventual clinical applications: water solubility over a wide pH range and quantitative formation of non-colloidal radiolanthanide complexes. Despite their higher fluidity, the radiolabelled CHICO-amino acid complexes have higher stability than their CHICO counterparts and may be potentially interesting to be explored for the treatment of HCC and liver metastasis, when treating the whole liver makes it more suitable as compared with resection or liver transplantation.³⁶ In addition, in a clustered metastasis or primary tumour, the cross-fire effect of β^- energy particles might also be beneficial.^{36,37} Preliminary biological results have shown that ^{153}Sm -CHICO-amino acid complexes present high liver uptake specially CHICO-val. This targeting of the liver may also be exploited for liver-specific targeting and with the appropriate radionuclide, for imaging.³⁸ Whether the biodistribution profile of this radioactive amino acid-chitosan could be improved in order to increase the blood clearance and then minimize the radiation dose to non-target organs the chance to administer it *via* i.v. injection may be a significant advantage relatively to the compounds advocated for the same purpose, which require local administration. The improved radiochemical and biological characteristics of $^{153}\text{Sm}/^{166}\text{Ho}$ -CHICO-amino acid complexes highlight the interest to further explore the chemical modification of chitosan in order to introduce functional groups to modulate its pharmacokinetics, coordination capability towards different radionuclides and specificity to design specific radiotracers.³⁹

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