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Glucose-holmium for radiotherapy: Characterization and in vitro assays☆

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HIGHLIGHTS

- Breast cancer cellular uptake of holmium glucose complexes.
- Glucose and holmium interaction in solution, helping cellular uptake.
- Continuous-time cell uptake of holmium in glucose´s solution.
- Saccharide-holmium complexes provides characteristic peaks in DESI-MS.
- Glucose helps holmium to be internalized on cancerous cells *in vitro*.

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ABSTRACT

Background: The existence of saccharide-holmium complexes, containing mono or polysaccharide molecules, is an attractive hypothesis toward a radiation therapy (RT) with beta-emitters targeting high glucose metabolic human sites. To exam such hypothesis, the aim of this study was to investigate the possible chemical interactions of Ho and glucose molecules and if glucose may be a facilitator to holmium cell internalization based on *in vitro* uptake assays and mass spectrometry analyses.

Methods: The ionic-solution preparations were based on glucose-anhydrous and holmium-nitrate hydrated in aqueous solution, in non-radioactive condition. The uptakes in MDAMB231 cell lineage were evaluated, at 0 and 50 μg mL^{-1} holmium solution, in incubation times of 10, 30 and 50 min. The measurements of the holmium mass into the dried cell were evaluated by Neutron Activation Analysis – NAA method. Also, the ionic solution was tested in Electrospray Ionization Mass Spectrometry (ESI-MS) in order to identify Ho and glucose interactions.

Results: There were intracellular holmium-uptake in MDAMB-231 of 3.6 ± 0.1, 6.8 ± 0.2 and 9.7 ± 0.3 μg increasing linearly with incubation time. The *m/z* ions at 523, 586, 649, 991 and 1054 were attributed to the positively loaded species containing Ho^{+3}, glucose (GLU) and NO_3^-, making up the possible molecular compound formulae, involving Ho, GLU, and anions.

Conclusions: The findings of the *in vitro* assay and the ESI-MS suggested a suitable holmium cell uptake, increased in function of incubation time, due to the presence of glucose and holmium chemical interactions in solution.

1. Introduction

Primary or metastatic tumors promote higher metabolism of glucose; therefore, human tumor sites have been targeted by glucose-analog compounds considering their possible high uptake. (Labak et al., 2016) The use of sugar complexes or derivatives thereof, containing mono or polymolecules, type polysaccharides, attached to metal centers as particle-emitting radionuclides, is attractive to radiation therapy, among other clinical protocols. (Liu et al., 2014; Geng et al., 2011; Shan et al., 2016) There is a strong interest in the therapy of high-metabolic sites through radiopharmaceuticals carrying saccharide or other analog molecules, monitored by the simultaneous emissions of gamma and X-

☆ Study developed in Radiobiology and Radiochemistry laboratory of Nuclear Engineering Department, Institute of Chemistry at Federal University of Minas Gerais (UFMG), Belo Horizonte, MG, Brazil.

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rays of the excited states. Such high metabolic sites may represent a tumor or metastatases, but also aseptic or septic infections induced by different pathogenic. (Bleeker-Rovers et al., 2011; Payen et al., 2016)

The feasibility of a systemic radiotherapy of high metabolism sites, as primary tumors and metastases, can be questioned by the inevitable high heart and brain saccharide uptakes. However, the possibility of peri- or intra-tumoral internalization may bring a new perspective to minimize such effects on healthy organs that are in the natural pathways of the radiopharmaceutical time kinetics. (McDougall, 2000) However, such concerns can be brought to light only after *in vivo* imaging studies on an animal model, when uptake ratios are estimated based on the activities in the target site to blood or healthy organs.

Another possibility of the use of a high metabolic-seeker radiopharmaceutical is in radioisotopic synovectomy. (Lima and Campos, 2005) The radiosynovectomy consists of applying the radiopharmaceutical into the articular capsule in order to reduce inflammation in the synovial membrane. Indications are for rheumatoid arthritis, psoriatic arthritis, osteoarthritis with inflammation, hemophilic arthropathy; with more than six months of systemic and local treatment with corticosteroids. (Das, 2007) Such a technique can represent an alternative to surgical synovectomy or synovitis with osmic acid that achieves synovial destruction. The radiopharmaceutical can eliminate specific macrophage cells which act in the destruction of the synovial membrane. Such suitable target cells hold high glucose uptake and have a larger radio sensibility than the epithelial and fibroblast cells present in the synovial membrane (Lima and Campos, 2002).

The Ho-166 nuclide decays by beta, gamma and X-rays emissions, which enable simultaneous: (i) monitoring of the biodistribution through scintigraphy in simple photon emission tomography (SPECT) or Gamma Camera through the 80 keV gamma-ray or the X-ray emissions that are characteristic photons from the excited state of Er-166; (ii) induction of systemic therapy through the deposition of beta-particle emissions since maximum energies are of 1.854 MeV (48%) and 1.773 MeV (50.5%) (Nuclear Data, 2018).

The holmium is of the Lanthanide group, 6th period, with Ho-165 having 100% of isotopic abundance (CRC, 2009; Nelson and Cox, 2002). The Ho-166 nuclide is generated by radioactive capture reactions, $Ho^{165}(n,\gamma)Ho^{166}$, with thermal and epithermal neutron cross sections of 26 and 200 barns, respectively (Chart of Nuclides, 2017). Holmium does not play biological interactions in human beings, and the same in plants with holmium content found lower than a hundred parts per trillion (Hammond, 2000; Emsley, 2011).

In this stage of research development, the main goal was the investigation of the *in vitro* holmium uptake in a breast cancer cell line at specific incubation times in a solution of glucose. The hypothesis is the cell uptake occurs due to metal complexation of holmium and glucose. The Electrospray Mass Spectrometry was used to characterize the holmium-glucose complexes in aqueous solution.

2. Materials and methods

2.1. Materials

In tissue culture development, the materials T-75 and T-25 flasks, 15.0 mL Falcon tubes and 1.0 mL polyethylene bottles were used for tissue culture. Controlled speed centrifuges, a CO_2 incubator for cell growth, an inverted microscope, a distiller, a deionizer and a cell defrost system were employed, with its respective instrumentations and disposable materials from Sigma-Aldrich.

Complete RPMI-1640 (RPMI) with L-glutamine, glucose-free culture medium (Sigma-Aldrich), fetal bovine serum (FBS) medium and phosphate buffered saline medium (PBS) were used. PBS is a buffer isotonic and saline containing sodium chloride, sodium phosphate, potassium chloride and potassium phosphate, for keeping the neutral pH of the solution, prepared in distilled water and deionized water.

2.2. Methods

2.2.1. Cell culture

The cell lineage was established and maintained *in vitro* following the protocols of literature. (Falcão et al., 2015) Cell cultures were maintained in RPMI-1640 supplemented with 10% fetal bovine serum medium and antibiotic Gentamicin ($50\,\mu g\,\mu L^{-1}$) and Streptomycin ($500\,mg\,mL^{-1}$), in the T-75 culture flasks in a humid atmosphere containing 5% CO_2 at 37 °C. Human breast carcinoma MDA-MB231 cells were donated by Dr. Mirian Paz from the Pharmacology Department of Sciences Institute of Biology - ICB/UFMG. The lineage source of breast adenocarcinoma has epithelial morphology, derived from a metastatic site.

The T-75 flasks with cells in confluence were trypsinized and the cells were placed in suspension. The RPMI complete medium was removed, centrifugated for cell pellet formation, and the suspension removed. The cell pellet was resuspended in PBS buffer medium and centrifuged again. The procedure was repeated for removing the residues of the complete medium. Then, the cellular pellet was resuspended in PBS buffer medium. An aliquot was removed to be counted in a hemocytometer. Three aliquots of 2.5 mL with 2×10^6 cell each were transferred to three T-25 bottles and kept in the incubator for 2 h.

2.2.2. Ho-Glu solution preparation

Two primary solutions were prepared based on the following reagents: $Ho(NO_3)_3{\cdot}5H_2O$ (Sigma-Aldrich) with 99.8% purity and anhydrous glucose and deionized water. Both aqueous solutions were prepared with the concentrations of 1×10^{-2} mol L^{-1} of glucose (GLU) and holmium nitrate. An ionic solution was prepared in the proportion of 1:1 of both primary solutions, namely Ho-Glu solutions.

2.2.3. Uptake assay

An amount of Ho-Glu solution had been added to the buffer medium in each T-25 bottle, such as the MDAMB231 cell culture was exposed to 150 μg of Ho in 3.0 mL medium. The control group was prepared with MDA-MB231 cells in the same culture condition; however, in medium without the holmium-glucose solution (control, n = 3). The bottles had been returned to the incubator and kept in the humid atmosphere containing 5% CO_2 at 37 °C whose cells were incubated with Ho-Glu. At the time of 10, 30 and 50 min, the bottles were removed from the incubator (for a specific time, n = 3). Cells of the T-25 bottles were resuspended by means of some refluxes, homogenized, and a whole content was withdrawn and transferred to a Falcon tube. The Falcon tubes were centrifuged, extracted the Ho-Glu solution, washed again with pure PBS buffer medium, and later the pellet was resuspended in 0.5 mL. From each Falcon tube, the 0.5 mL volume of the suspension was transferred to the 1.0 mL polyethylene vial used to NAA.

Later, the samples were dried in oven 60 °C for 1 h. The residues of the PBS buffer together with the contained cells were dried, generating a film in the vial bottom. It produced a residual mass of cells that had been incubated with Ho-Glu solution, possible internalized during the specific incubation time with the established $50\,\mu g\,mL^{-1}$ concentration. The same was made for the control samples, where cell aliquots in pure PBS buffer only were removed and dried, without washing.

2.2.4. NAA measurements

The metal mass were evaluated by Neutron Activation Analysis - NAA method performed in the TRIGA MARK I IPR-R1 research reactor, CDTN/CNEN, Belo Horizonte, on the k0-method. The requirements of elemental standards were used in the analysis for NAA (De Corte, 1986). The thermal neutron flux was about 6.35×10^{11} $ncm^{-2}s^{-1}$ at 100 kW, the time of exposition was 8 h, and the major k0-method parameters were f was 22.32 and α −0.0022 (De Corte, 1986), on the PI-7 irradiation position (Menezes and Jacimovic, 2006). The sample had been conditioned in the polyethylene vial. After neutron

irradiations, the sample activities and spectra had been analyzed in the Gamma Spectrometry System associated to a gamma detector HPGe and Genie 2000 software for spectra acquisition and electronic association from CANBERRA. The spectra had been analyzed in HyperLab® (HyperLab, 2013; Simonits et al., 2003) and the elemental concentrations had been calculated in the Kayzero code for Windows® (Kayzero for Windows, 2011).

2.2.5. Mass Spectrometry protocol

The ESI-MS of Ho-Glu solution was conducted in an LCQFleet mass spectrometer (Thermo Electron). The electrospray source conditions were as follows: positive mode; spray voltage 5 kV, capillary voltage 36 V, tube lens voltage 65 V sheath gas flow rate (N_2) 8 arb, and capillary temperature 275 °C. The *m/z* range analyzed was 50–2000. The samples were introduced directly in the electrospray source at 20-μL min^{-1} using a syringe pump.

3. Results

3.1. Ho cell uptake

The holmium mass recovered in the dried samples of MDAMB231 cells incubated with 150 μg of Ho in 3.0 mL of medium, in incubation times of 10, 30 and 50 min, were 3.6 ± 0.1, 6.8 ± 0.2, and 9.7 ± 0.3 in μg, with difference statically significant, respectively. At 0 μg mL^{-1}, the Ho concentration was lower than a detectable value. Thus, the Ho element was not found in the control samples. At 50 μg mL^{-1}, the holmium the uptake increases linearly in function of 10, 30 and 50 min of incubation time.

3.2. Mass spectroscopy analysis

The ESI-MS spectrum of the Ho-Glu solutions is shown in Fig. 1, whose complexion was prepared with cold nuclide. The *m/z* ion of major intensity 649 was attributed to species containing Ho^{+3}, two molecules of glucose and two ions NO_3^-. The other *m/z* ions at 523, 586, 991 and 1054 had also been attributed to the positive species containing Ho^{+3} and deprotonated glucose molecules. The deprotonation of the glucose-lead complex under ESI-MS condition was previously reported by Salpin and Tortajada (2003). Following the same analysis, our findings can point to the formation of Ho-Glu complexes in solution.

The molecular weights evaluated to the theoretical assignment of the chemical formulae were presented in Table 1. The *m/z* experimental signals were found equivalent to the *m/z* theoretical signals of the assignment chemical formulae, based on Isotope Patterns Calculator, taken into consideration the uncertain order of the measurements in ESI-MS experiments.

Table 1
Attributions of the *m/z* signals in the spectrum of the Ho-Glu solution, the theoretical assignment of the chemical formula, molecular weight and possible ion species in the formations.

m/z	Theoretical Assignment	MW (a.m.u.)	Proposed species
523	$C_{12}H_{22}O_{12}Ho$	523,229	$[Ho(Glu)_2 - 2H]^+$
586	$C_{12}H_{23}O_{15}HoN$	586,242	$[Ho(Glu)_2NO_3 - H]^+$
649	$C_{12}H_{24}O_{18}HoN_2$	649,255	$[Ho(Glu)_2(NO_3)_2]^+$
991	$C_{18}H_{33}O_{24}Ho_2N_2$	991,318	$[Ho_2(Glu)_3(NO_3)_2 - 3H]^+$
1054	$C_{18}H_{34}O_{27}Ho_2N_3$	1054,331	$[Ho_2(Glu)_3(NO_3)_3 - 2H]^+$

4. Discussion

The natural high uptake in heart and brain predicts a clinical failure of a possible radiation therapy with ^{166}Ho-glucose. Contra measurements can be taken to overcome such clinical situation. Radiopharmaceutical can have different vascular assessments, as intratumoral, peritumoral or directly in a specific artery of the target organ. A suitable infusion time-rate shall be chosen to obey the diffusion and absorption parameters of the tumor. A short-time control of the natural glucose tissue-uptake based on the management of the physiological hormonal dependence may be also a contra measurement, in cases of the cancer cells does not respond similarly.

Free Ho^{3+} ions are found in $Ho(NO_3)_3{\cdot}5H_2O$ solution, as similar to Ho^{3+} from $HoCl_3$ aqueous solution. Thus, uptake of Ho^{3+} ions from $HoCl_3$ provides similar data to holmium nitrate in solution. Indeed, Ho-free ions uptake data can be recalled to address this issue. Zolghadri et al. (2013), investigated the uptake of free Ho^{3+} ions in solution, among other radiopharmaceuticals. The free Ho-ion uptakes in stomach, skin, xyphoid, lung, intestine of rats were lower than 0.25% of the Injectable Dose (ID) per gram of the extracted organ, *i.e.* %ID.g^{-1}. The large uptake of 2.5% at 2 h was in the excretion system (kidney) that fallen down to 1% up 24 h. The brain did not uptake free Ho ions. The uptake in heart, blood, and lung dropped from 0.5% (2 h) to lower than 0.2 up to 4 h. Zolghadri et al. (2013), addressed the Ho^{3+} cation excretion *in vivo*, with earth accumulation in liver and excreted through hepatobiliary excretion route, leading to the reduction in liver

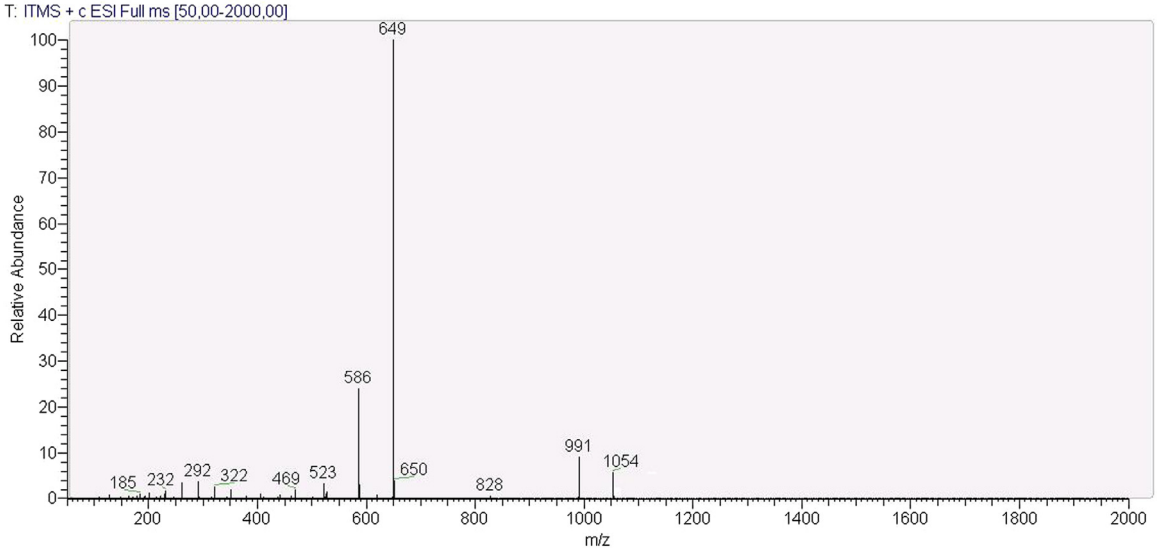

Fig. 1. The ESI-MS spectrum of Ho-Glu solution.

accumulation to about 0.7% of the activity after 24 h. A late uptake in the bone was identified 4–24 h at 0.7% ID g^{-1}. Soft-tissues incorporated non-significant later uptake larger than 0.05% ID g^{-1} showing the ion incapacity of internalization in tissue cells.

The formation of metal complexes of mono-, oligo-, and polysaccharides has been attracting interest (Alekseev et al., 1998). Angyal et al. (1974) had already observed interactions of lanthanide ions and polyols in aqueous solution.

Previous Ho-Glu characterization studies with ^{166}Ho-radioactive were carried out; however, those did not prosper in separating an R_f for the Ho-Glu complexes, maybe due to the presence of a diversity of complexes. The ITLC system presented a low resolution for such studies. Thus, the mass spectroscopy was considered to analyze the complexes.

The Ho-Glu complexes were characterized by mass spectroscopy. The ESI-MS has been mainly used in the analysis of biomolecules in solution. The ESI-MS is an accurate technique for specifying the masses of the complexes (Eberlin et al., 2011). Electrospray ionization provides ion transfers directly from the solution to the gaseous phase and thus allows their analysis of masses, becoming a suitable technique for the ionic complex studies (Ho et al., 2003).

A Ho-Glu solution in a 50 μg mL^{-1} concentration was chosen in our experiment, which means that 1 g of PBS-buffer solution holds 50 μg of holmium or $6.08 \times 10^{+19}$ atoms of holmium per gram of the solution. This amount was chosen in the analogy of the measurements of the boron concentration in blood after fructose-boron-phenylalanine, a non-toxic compound, following injection in clinical trials in patients treated with BNCT – Boron Neutron Capture Therapy, which found a boron concentration of 45–50 μg mL^{-1} in blood (Brandão and Campos, 2015).

Blood glucose achieves a usual measurement of 108 mg dL^{-1} or 6 mmolL^{-1} after a meal. Since the usual plasma concentration holds 55% of the blood, with 1.053 gcm^{-3} of mass density, the glucose concentration of 1.08 mg mL^{-1} in blood represents 1.96 mgmL^{-1} or 1.86 mg g^{-1} (%w) of plasma. Let us consider this value as a limit of the number of glucose molecules on the plasma. Taking the glucose molar mass of 180 g mol^{-1}, 1.86 mg g^{-1} of glucose represents $6{,}22 \times 10^{+18}$ glucose molecules per gram of plasma. Our experiment predicted 1 glucose-molecules per holmium cation in the complexes of greater intensity; therefore, 150 μg in 3.0 mL of Ho means $3.47 \times 10^{+17}$ molecules per gram of the Ho-Glu complex. It produces an *in vivo* ratio of Glu *in vitro* per Glu in plasma Ho-Glu/Glu of ~1:36, which means 1 coordinate compound per 36 glucose molecules in the blood. Thus, our *in vitro* concentration is small when we compare and translate this data into an *in vivo* ratio of 1:36. Thus, a large concentration of Ho-Glu complexes can be expected to be injected *in vivo*, which means a possible larger uptake in cancerous cells *in vivo*.

The experimental condition imposes an MDAMB231-cell starving condition during a period of 2 h in PBS buffer, and a long-term glucose-starvation condition in the RPMI medium, since the buffer and the medium were glucose-free. Such condition was followed by a short-time exposition to Ho-GLU solution for 10–50 min. Thus, such experimental condition may be induced by a large Ho-GLU cell uptake in a short time period. Measurements of Ho uptake in cells increased since the mass recovery were 3.3 up to 9.7 μg from 10 m to 50 min exposition in accord to the experimental conditions. The metal uptake increased with time when cells were incubated to Ho-GLU solution, achieving a holmium mass concentration in cancerous cells of ~1:23 taken the wet mass of cells.

The average percentage of Ho mass was 0.11% in relation to a total wet cell mass, considering a recovering cell rate of 40% in the *in vitro* assay that means 2.10^6 cells with a total mass of 8.796 mg. Taken 9.7 ± 0.3 μg Ho mass recovery in the cells at 50 min, the concentration ratio of Ho in the cells in relation to the solution can be estimated. A value of 150 μg of Ho was present in 3.0 mL of solution, while 9.7 μg of Ho in 8.796 mg of cell mass. Therefore, a concentration ratio of 1:23 of Ho in the cell mass was obtained. If a ratio of 1:1 were considered on cells in relation to Ho concentration in the solution, no detectable data should be found by NAA. A concentration ratio of solution-tissue of 1:23 is suitable for radiation therapy.

A possible clinical Ho-Glu/RT protocol must be defined having a maximum exposition of the target tissue and lower toxicity in the Organs at Risk (OARs), which means that clinical contra measurements are adopted to reduce radiotoxicity in OARs. The activity in a possible RT will be defined by the maximum tolerated dose (MTD) in OARs, as heart and brain, taken into consideration the percentage of the total injected activity uptake in OARs. Such local OARs activities will be translated to a possible tolerant blood activity. Based on *in vitro* assay data, uptake ratio, as found 1:23, are applied to estimate the target activity (tumor or metastasis) and the target-dose, as consequence.

Despite the amount of counting cells of 2×10^6 cell mL^{-1} was seeded in the T-25 bottles and held up to 02:50 h (including 50 min exposed to Ho-GLU solution), there was no possibility to have counted the final number of cells enclosed in polyethylene bottle after dried. Although the number of cells was not estimated at the experimental end, the cell dilution in the T-75 medium, followed by the same volume transferred to each T-25 flask, held the equivalent cell number in each T-25 flasks.

The ESI-MS experimental spectra provided a set of peak mass-charge ratios *versus* intensities, related to the ion presences. Theoretical spectra were evaluated by the Isotope Patterns Calculator, provided by supposing chemical formulae related to the elemental chemical constituents of the reagents. (ChemPutter, 2018) The analysis was performed matching the theoretical and experimental *m/z* intensity data. The supposed chemical formulae in Table 1 were proposed in order to meet the experimental data. Thus, exact masses were evaluated, using the proposed chemical formula.

The findings of the *in vitro* assay and the mass spectrometry at ESI-MS environment suggested a suitable holmium cell uptake, increased in function of incubation time, due to the presence of glucose and holmium interactions in solution. Therefore, glucose has been acted in our experiments emerging as a facilitator to Ho internalization, possible due to the formation of Ho-Glu complexes in aqueous solution, stabilized by inorganic anions. Further experiments shall be addressed to test our findings *in vivo*, especially the stability of such complexes in serum.

5. Conclusion

Holmium uptakes *in vitro* were demonstrated in breast cancer cells, with concentration increased in function of the incubation time in a glucose and holmium solution. Characteristic *m/z* ions reported the interaction of holmium and glucose, which were demonstrated by ESI-MS. The findings in the *in vitro* assay and on the ESI-MS environment suggested that the holmium cell uptake emerged with the presence of glucose, as a facilitator to Ho internalization, through the Ho^{165}-glucose interactions.

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Conflicts of interest

The authors declare no conflict of interest.

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