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Transglutaminase-mediated conjugation and nitride-technetium-99m labelling of a bis(thiosemicarbazone) bifunctional chelator

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ABSTRACT

An assessment study involving the use of the transglutaminase (TGase) conjugation method and the nitride-technetium-99m labelling on a bis(thiosemicarbazone) (BTS) bifunctional chelating agent is presented. The previously described chelator diacetyl-2-(N^4-methyl-3-thiosemicarbazone)-3-(N^4-amino-3-thiosemicarbazone), H_2ATSM/A, has been functionalized with 6-aminohexanoic acid (ε-Ahx) to generate the bifunctional chelating agent diacetyl-2-(N^4-methyl-3-thiosemicarbazone)-3-[N^4-(amino)-(6-aminohexanoic acid)-3-thiosemicarbazone], H_2ATSM/A-ε-Ahx (**1**), suitable for conjugation to glutamine (Gln) residues of bioactive molecules *via* TGase. The feasibility of the TGase reaction in the synthesis of a bioconjugate derivative was investigated using Substance P (SP) as model peptide. Compounds **1** and H_2ATSM/A-ε-Ahx-SP (**2**) were labelled with nitride-technetium-99m, obtaining the complexes [^{99m}Tc][Tc(N)(ATSM/A-ε-Ahx)] (**^{99m}Tc1**) and [^{99m}Tc][Tc(N)(ATSM/A-ε-Ahx-SP)] (**^{99m}Tc2**). The chemical identity of **^{99m}Tc1** and **^{99m}Tc2** was confirmed by radio/UV-RP-HPLC combined with ESI-MS analysis on the respective carrier-added products **$^{99g/99m}$Tc1** and **$^{99g/99m}$Tc2**. The stability of the radiolabelled complexes after incubation in various environments was investigated. All the results were compared with those obtained for the corresponding ^{64}Cu-analogues, **^{64}Cu1** and **^{64}Cu2**. The TGase reaction allows the conjugation of **1** with the peptide, but it is not highly efficient due to instability of the chelator in the required conditions. The SP-conjugated complexes are unstable in mouse and human sera. However, indeed the BTS system can be exploited as nitride-technetium-99m chelator for highly efficient technetium labelling, thus making compound **1** worthy of further investigations for new targeted technetium and copper radiopharmaceuticals encompassing Single Photon Emission Computed Tomography and Positron Emission Tomography imaging.

1. Introduction

In the last two decades, bis(thiosemicarbazones) (BTS) have been extensively utilized as chelators for copper radionuclides in the development of copper-based radiopharmaceuticals [1]. Since BTS are avid and very selective chelators for Cu^{2+} (log K > 17), radiolabelling occurs virtually instantaneously at room temperature and in mild reaction conditions, offering advantages in the development of a convenient one-pot kit formulation. [^{64}Cu]Cu(II)-diacetyl-bis(N^4-methylsemithiocarbazone), [^{64}Cu][Cu(ATSM)], has been approved for multicentric human trials for the Positron Emission Tomography (PET) imaging of hypoxic tissues [2,3]. Besides, BTS were exploited with other radionuclides [3,4] such as technetium-99m for Single Photon Emission Computed Tomography (SPECT) applications (*vide infra*) and, more recently, with indium-111 (for SPECT) and gallium-68 (for PET) [5,6]. In this connection, literature describes several ^{99m}Tc-labelled BTS complexes generated by simple tin chloride reduction of the pertechnetate ion in presence of the ligand. Compounds were evaluated *in vitro* and *in vivo* as metal-essential radiopharmaceuticals, and some of them have shown interesting features for nuclear medicine applications [7–15]. However, in all these studies the exact chemical structure of the technetium complexes was not determined; only putative neutral structures based on the cores [^{99m}Tc][Tc^{IV}(O)]$^{2+}$ or [^{99m}Tc][Tc^{V}(O)(X)]$^{2+}$ (X = Cl^- or OH or H_2O) were proposed. In addition, to the best of our knowledge, none of the studies involving technetium and BTS have considered other potentially suitable metal cores such as the nitride-technetium-99m. In a recent communication, Nguyen et al. described the synthesis and the chemical structure of two nitride

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complexes with tetradentate thiosemicarbazone/thiocarbamoylbenzamidine hybrid ligand (L) of general formula [M(N)L] (M = ^{99g}Tc and Re); however, the preparation of the corresponding compounds at tracer level has not been reported [16]. Indeed, nitride-technetium core is more advantageous than the oxo-technetium, as nitride-technetium complexes are generally more stable in a wide range of conditions and more inert toward redox reactions, thus being interesting candidates for nuclear medicine applications [17,18].

BTS have also been investigated as bifunctional chelating systems to develop new target specific PET imaging agents [2,3]. In particular, in the last decade, a series of unsymmetrical BTS-based pro-ligands containing a pendant amino group (indicated as H_2ATSR/A) has been proposed as promising bifunctional chelators, paving the way to the possibility to conjugate a stable ^{64}Cu-complex to a molecular vector [19–23]. Such ligands were used also in the preparation of the corresponding ^{99m}Tc-complexes [20,21], even though radiochemical yields (RCY) of the resulting compounds was assessed in the range 30–40%, and purification *via* Sep-pak C18 was required to obtain compounds suitable for a biological evaluation [21]. Anyway, preliminary serum stability studies clearly show that the ^{99m}Tc-species are stable, suggesting that these ligands can be exploited, in a radiopharmaceutical context, for both technetium-99m and copper-64 encompassing SPECT and PET imaging.

The described methods to conjugate BTS bifunctional chelators to biomolecules generally involve chemical reactions [2,3,19–23] whereas, to the best of our knowledge, other interesting approaches such as the use of the transglutaminase (TGase) enzymatic reaction have never been implemented. TGase catalyses the reaction between the γ-carboxamide group of a protein-bound glutamine (Gln) residue (–$CONH_2$, the acceptor) and an amino group (–NH_2, the donor) of an alkyl-amine, such as the ε-amino group of the lysine (Lys), leading to the formation of an isopeptide bond [24]. The main advantages of protein conjugation by TGase are that the modification is performed under physiological conditions and it is site-specific for one or few Gln or Lys residues of a protein substrate [25,26]. Currently, TGase is exploited to conjugate proteins and peptides to a variety of molecular entities, including drugs and metal chelators, for a large number of applications [27–32]. Therefore, it is a potentially useful and versatile tool to synthesize bioconjugates for target-specific radiopharmaceuticals, including those BTS-based.

According to all these considerations, we present here an assessment study involving the use of the TGase conjugation method and the nitride-technetium-99m labelling on a BTS bifunctional chelating agent. Considering the recognized high reactivity of BTS toward copper ions, ^{64}Cu-labellings were also performed for comparative purposes in terms of radiochemical yields, labelling efficiency and stability of the final complexes, in view of the development of target-specific systems based on both technetium-99m and copper-64, thus comprising SPECT and PET imaging. The molecules used in this work are sketched in Fig. 1.

2. Results and discussion

In this study we assessed the application of the TGase-mediated conjugation and the labelling with nitride-technetium-99m on a BTS bifunctional chelating agent. We modified the previously reported diacetyl-2-(N^4-methyl-3-thiosemicarbazone)-3-(N^4-amino-3-thiosemicarbazone), H_2ATSM/A [19], with 6-aminohexanoic acid (ε-Ahx) to generate the bifunctional chelating agent diacetyl-2-(N^4-methyl-3-thiosemicarbazone)-3-[N^4-(amino)-(6-aminohexanoic acid)-3-thiosemicarbazone], H_2ATSM/A-ε-Ahx (**1**, Fig. 1), having a pendant primary amino group suitable for the conjugation to Gln residues of bioactive molecules *via* TGase. A spacer length of five carbons was indeed previously shown to give a good reactivity of the primary amine toward TGase [33,34]. The synthesis of **1** is reported and discussed in the Supplementary Information. We investigated the feasibility of the TGase reaction on the undecapeptide Substance P (SP), as an example of peptide vector for molecular targeting, thus achieving the compound H_2ATSM/A-ε-Ahx-SP (**2**, Fig. 1). SP is the most important member of the tachykinin peptide neurotransmitters family: it represents the major endogenous ligand for the neurokinin type-1 receptor (NK1R) [35,36]. This receptor, besides being overexpressed in several malignancies (including breast, ovarian, and prostate cancers as well as glioblastoma and melanoma), is also present on tumor cells infiltrating the intratumoral and peritumoral vasculature. The SP/neurokinin-1 receptor system plays an important role in the mitogenesis, cell migration, angiogenesis, and metastasis of the above-mentioned tumors. Binding of SP to NK-1 results in internalization process providing a potential strategy for developing new theranostic agents [37–47]. SP contains two Gln residues (Gln^5 and Gln^6) and one Lys residue with very low reactivity. Previous studies indicated that the Gln^5 is preferably modified by TGase, leading to the production of a mono-derivative [48–50] which is still biologically active [51]. We assessed the labelling efficiency of **1** and **2** toward nitride-technetium-99m, obtaining the carrier-free radiocomplexes [^{99m}Tc][Tc(N)(ATSM/A-ε-Ahx)] (**^{99m}Tc1**) and [^{99m}Tc][Tc(N)(ATSM/A-ε-Ahx-SP)] (**^{99m}Tc2**); the labelling procedure was thoroughly optimized. The chemical identity of **^{99m}Tc1** and **^{99m}Tc2** was achieved by radio/UV-RP-HPLC combined with ESI-MS analysis on the respective carrier-added products **$^{99g/99m}$Tc1** and **$^{99g/99m}$Tc2**. Finally, the behaviour of the radiolabelled complexes after incubation in various challenging and biological environments was investigated, to assess the stability of both the metal-coordination sphere and the bioconjugate construct. All the results were compared with those obtained for the copper-64 analogues [^{64}Cu][Cu(ATSM/A-ε-Ahx)] (**^{64}Cu1**) and [^{64}Cu][Cu(ATSM/A-ε-Ahx-SP)] (**^{64}Cu2**). Data are presented and discussed below.

2.1. Preliminary stability studies on compound 1

The derivatization of proteins with TGase is generally conducted in phosphate buffer pH 7.0–8.0, at 37 °C in at least 4 h, usually resulting in good conjugation yields [27–29,52,53]. Thus, the stability of **1** was investigated under such conditions, *i.e.* 4 h in phosphate buffer 0.10 M, pH 7.0. Variations were followed by RP-HPLC (Method 1a) and ESI (+)-MS of the collected peaks and reported in Fig. 2A–B. After 4 h incubation, the formation of other products was detected. ESI(+)-MS spectra of the peaks eluted at 4.27 min and 3.34 min display the ions at *m/z* 270, 288 and 106, respectively. These species might result from in-solution hydrolysis and subsequent cyclization of **1** as illustrated in Fig. 2C; these events have been already observed for BTS, although under different chemical conditions [54,55].

Spectra of both the peaks eluted at 10.65 and 7.04 min display the ion at *m/z* 375, corresponding to the protonated molecular ion of compound **1**. MS^2 analyses of the ions at m/z 375 revealed different patterns of fragmentation at the same collision energy voltage; nonetheless, both the spectra display the ions at *m/z* 270 and 146 (Fig. 3).

All of the observed fragments are in accordance with the MS^2 analysis of the pure compound **1** (Fig. S4). According to this, it is reasonable to assume that compounds eluted at 10.65 and 7.04 min are isomeric species, likely the *thione-thiol* tautomers of compound **1**. We assume that the cyclic species giving the ion at *m/z* 270 (*vide supra*) is structurally different from the ion at m/z 270 generated by collisionally induced fragmentation of the ion at m/z 375 (see Fig. S4 and 2C for a comparison). Finally, the species eluted at 7.04 min should be less chemically stable in solution. In fact, the degradation products corresponding to the m/z 270, 288 and 106 ions were found also in its ESI-MS spectrum, but not in the spectrum of the compound eluted at 10.65 min, to indicate that they should be formed *after* collection from the RP-HPLC and *before* the MS analysis.

Thus, it seems that **1** isomerizes from a tautomer to the other one, which in turn is prone to degradation *via* hydrolysis and cyclization. Basing on this hypothesis of degradation, both the products characterized by the ions at *m/z* 288 and 270, (MW 287.14 and 269.13 Da