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Comparison of tridentate ligands in competition experiments for their ability to form a $[^{99m}Tc(CO)_3]$ complex

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Abstract—In this study we have compared different ligands containing three or more hetero-atoms (N, O and/or S) with respect to their ability to form tridentate complexes with a Tc-tricarbonyl moiety. Comparison of each ligand in a competition reaction with histidine first and then with each other compound allowed to rank the ligands according to their ability of complex formation with the $[^{99m}Tc(CO)_3]^+$ precursor from diethylenetriamine (most efficient of the studied ligands) to nitrilotriacetic acid (weakest complexing properties). The results provide insight in the structural requirements for the formation of stable Tc-tricarbonyl complexes and suggest preferred combinations and arrangements of the hetero-atoms involved in the complex formation. They also give a good indication which type of ligand is most appropriate to modify biomolecules for an efficient and stable labelling with a Tc-tricarbonyl moiety.

Over the last years the radiochemistry of ^{99m}Tc-tricarbonyl complexes has been extended to find useful applications in radiopharmacy and nuclear medicine. This included the labelling of proteins and peptides as well as the search for specific receptor imaging agents.[1,2]

The basis for this kind of radiochemistry is the convenient preparation of the Tc-tricarbonyl precursor $[^{99m}Tc(CO)_3(OH_2)_3]^+$ either in the 'classic' way with CO gas and borohydride reduction or with an IsoLink™ kit.[3,4] In the resulting precursor the three water molecules can easily be replaced by ligands that contain suitable donor atoms to form stable complexes.

In this study we compared the ability of small molecules with different combinations of the hetero-atoms N, O and S, which can act as tridentate ligands, to form complexes with the Tc-tricarbonyl moiety. Earlier results showed that a spacer of two or three atoms between the hetero-atoms might suit best for a complex formation with the Tc-tricarbonyl precursor. In this manner five- or six-membered rings are formed when the ligand attaches to the metal centre. Therefore, we compared different molecules that match these conditions against each other. Histidine (HIS) was used as a reference compound as it is known as one of the most efficient ligands for the complexation of the $[^{99m}Tc(CO)_3]^+$ moiety.[1,5]

Apart from histidine, *N*-β-aminoethylglycine (AEG), diethylenetriamine (DETA), diethylenetriamine pentaacetic acid (DTPA), *N*,*N*′-ethylenediamine diacetic acid (EDDA), ethylenediamine tetraacetic acid (EDTA), iminodiacetic acid (IDA), *N*-(hydroxyethyl)-iminodiacetic acid (he-IDA) and nitrilotriacetic acid (NTA) were included in the study, each containing three hetero-atoms in a linear sequence with two spacers of two carbon atoms in between. As a separate series, also amino acids were studied, namely aspartic acid (ASP), 3-aminopropionic acid (DAP) and homocysteine (h-CYS), in which the hetero-atoms can be seen as substituents on positions 1, 2 and 3 or 4 of a propylene or butylene chain.

In the first place the complex formation of the individual compounds with the $[^{99m}Tc(CO)_3(OH_2)_3]^+$ precursor was investigated. The precursor was prepared by

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addition of 1 mL ^{99m}Tc-pertechnetate solution (370 MBq ^{99m}Tc) to an IsoLink™ labelling kit (Mallinckrodt, Petten, The Netherlands), incubation for 20 min at 100 °C and adjustment to pH 10 with 0.1 M HCl. The reaction mixture was analysed by reversed phase HPLC (X-terra RP18-column 5 mm, 250 mm × 4.6 mm, elution with gradient of 0.1% aqueous trifluoroacetic acid to 0.1% trifluoroacetic acid in acetonitrile over 20 min) at a flow rate of 1 mL/min.

Labelling of the ligands was performed by mixing 50 μL of a 50 mM stock solution (resulting in an amount of 2.5 μmol ligand for each experiment) with 50 μL of the $[^{99m}Tc(CO)_3(OH_2)_3]^+$ precursor solution and heating for 15 min at 70 °C. In all cases excellent labelling yields were obtained. Quality control by HPLC after 20 h indicated also an excellent stability for all examined $[^{99m}Tc(CO)_3]$ complexes. Their assumed structures are shown in Figures 1 and 2.

All reaction mixtures were analysed also by electrophoresis (0.05 M ammonium acetate buffer, pH 7, 300 V, 20 min) to check the charge of the radiolabelled compounds. The ^{99m}Tc complexes with DETA, HIS, IDA and he-IDA showed the expected charge, while the complex with AEG appeared to be positively charged instead of neutral. Electrophoresis at pH 10, however, showed a neutral ^{99m}Tc complex as expected for a tridentate binding of AEG to the $[Tc(CO)_3]^+$ moiety. The $[Tc(CO)_3]$ complexes with the polycarbonic acids DTPA, EDDA, EDTA and NTA revealed a negative charge as anticipated, but this is also due to the presence of one or more deprotonated carboxylic acid groups at pH 7, which are not involved in the complex formation.

For EDDA, the Tc-tricarbonyl core uses two nitrogen atoms and one of the acid groups for the complex formation to form the more stable five-membered rings. For $[Tc(CO)_3]$ complexes with DTPA and EDTA,

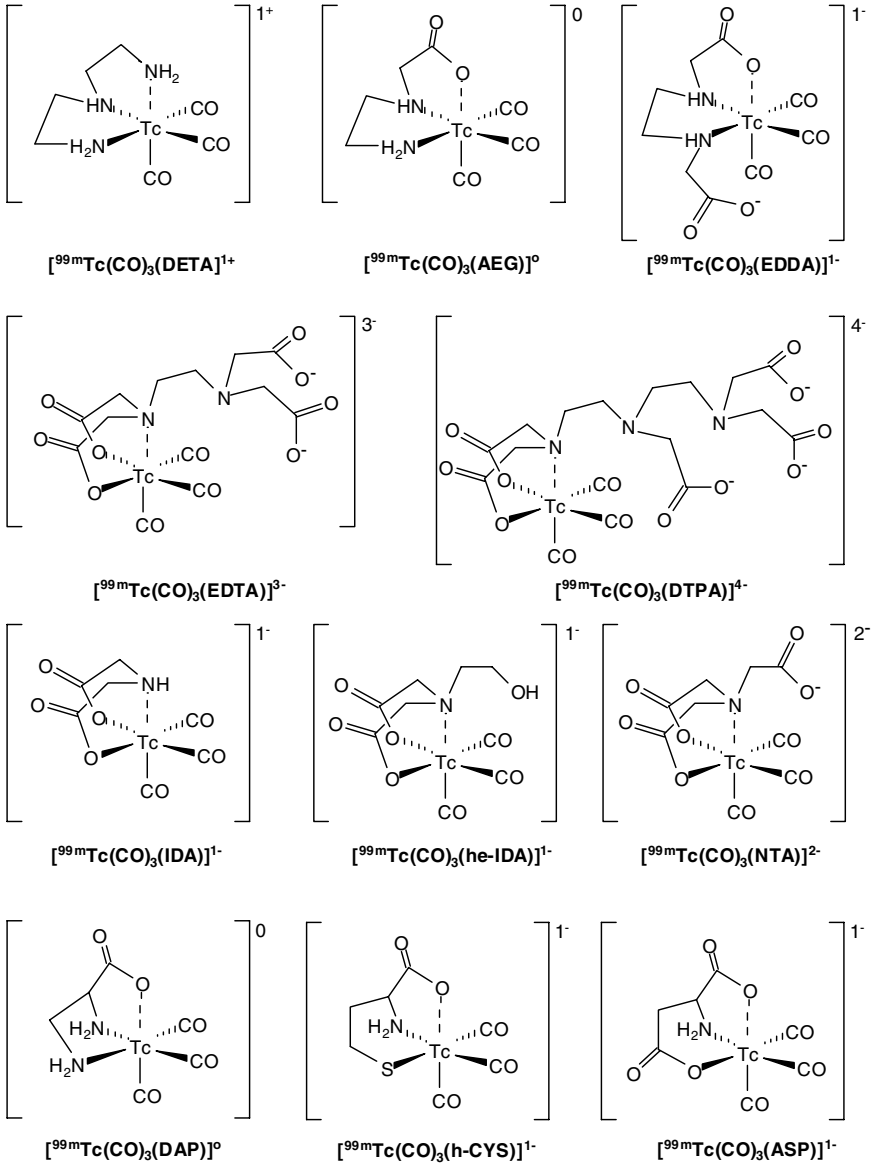

Figure 1. Suggested structure for the radiolabelled $[^{99m}Tc(CO)_3]$ complexes (at pH 10).

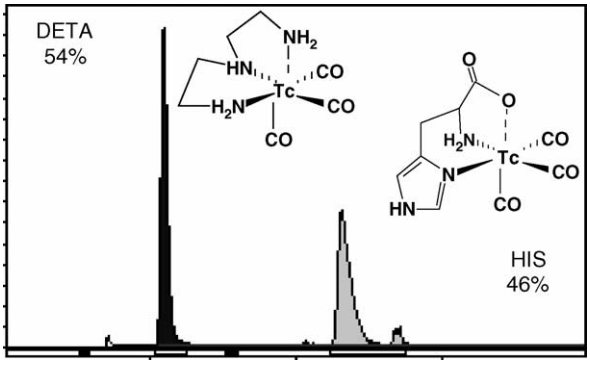

Figure 2. HPLC chromatogram of the reaction mixture after a competition experiment in which equimolar amounts of DETA and histidine were labelled with a $[^{99m}Tc(CO)_3]$ core.

however, two possible isomers can be formed: one in which two carboxylic acid groups and a nitrogen atom are bound to the metal centre (resulting in a negatively charged complex) and the other with only one carboxylic acid group and two nitrogen atoms bound to the Tc atom (resulting in a neutral complex). Further investigations by X-ray structure analysis are necessary to determine which of the isomers is preferred.

As a sulfur containing triligand we first chose *N*-(2-(*S*-benzyl-mercaptoethyl))glycine (Bz–$SCH_2CH_2NHCH_2$COOH). The *S*-benzyl protective group was removed with sodium/liq. ammonia prior to the labelling experiment, but the labelling with the Tc-tricarbonyl precursor did not result in a single, well-defined product. On the other hand, the *S*-benzyl protected ligand itself did form two $[Tc(CO)_3]$ complexes, both characterised by a late retention time in the HPLC chromatogram, which suggests that the *S*-benzyl group was still present in the complex. The *S*-benzyl protected ligand, however, could not even compete with the weakest of the other ligands and for that reason, it finally was not included in the study.

In a typical procedure for the competition experiments ($n = 3$–4 for each ligand) equimolar amounts of two of the ligands (50 μL of a 50 mM aqueous stock solution, i.e., 2.5 μmol of each ligand) were mixed with 50 μL of the $[^{99m}Tc(CO)_3]^+$ solution (pH 10), the mixture heated for 15 min at 70 °C and analysed by HPLC to determine the relative amount of each of the formed $[Tc(CO)_3]$ complexes. From these results the studied ligands with a linear sequence of the hetero-atoms can be ranked as follows regarding their ability to form Tc-tricarbonyl complexes:

DETA (98.8%) > AEG (96.3%) > EDDA (92.6%) > EDTA (91.9%) > IDA (84.9%) > DTPA (72%) > he-IDA > NTA.

The percentages in brackets represent the relative amounts of $[Tc(CO)_3]$ complex formed with that ligand in competition with NTA, the weakest ligand shown here. NTA and he-IDA could not be compared directly because their $[Tc(CO)_3]$ complexes had almost the same retention time in the HPLC analysis, but the position in the ranking was confirmed indirectly by experiments against the other compounds. All ligands were cross-checked against each other and the results confirmed the order shown above.

For the examined amino acids the labelling with the Tc-tricarbonyl moiety results in the formation of a five-membered and a six-membered ring (HIS, h-CYS, ASP) or two five-membered rings (DAP) in which only one hetero-atom is different (Figs. 1 and 2). In competition experiments with equimolar amounts of histidine, $[Tc(CO)_3]$-DAP was formed for 37% and $[Tc(CO)_3]$-ASP for 0.5%. $[Tc(CO)_3]$-h-CYS could not be compared directly to histidine as the retention time of the complex was too similar to that of the corresponding $[Tc(CO)_3]$-HIS. However, cross-checking against the other amino acids resulted in the following ranking of the ligands: HIS > DAP > h-CYS > ASP.

To enter it into the study, additional competition experiments were performed against the other compounds shown above. For the ability to form a complex with the $[Tc(CO)_3]^+$ moiety the following order of the complete set of studied ligands was obtained:

DETA > HIS > AEG > DAP > h-CYS > EDDA > EDTA > IDA > DTPA > he-IDA > ASP > NTA

Although the differences between the ligands in the competition experiments against NTA seem small, there are actually huge varieties in the ability to form a complex with the $[Tc(CO)_3]$ core. These can be demonstrated best by listing the results of the competition experiments of consecutive ligands as shown in Table 1.

Under the chosen conditions DETA with two primary and one secondary amine was slightly superior (54%) to histidine (46%). To determine a possible influence of the pH this reaction was also performed at pH 7, resulting in almost identical percentages of complex formation. The original chromatogram is shown in Figure 2.

Histidine is stronger in the competition than AEG, so an aromatic amine seems to be superior to a secondary

Table 1. Results of the competition experiments of consecutive ligands in the ranking

DETA versus HIS	HIS versus AEG	AEG versus DAP	DAP versus h-CYS	h-CYS versus EDDA	EDDA versus EDTA	EDTA versus IDA	IDA versus DTPA	DTPA versus he-IDA	he-IDA versus ASP	ASP versus NTA
54:46	70:30	53:47	69:31	58:42	83:17	99:1	74:26	63:37	52:48	77:23

The values indicate the relative amount of the $[Tc(CO)_3]$ complex formed (in %).

amine (both molecules have a primary amine and a carboxylic acid group). AEG and DAP both use two aliphatic amines and a carboxylic acid group for complex formation, but DAP has two primary amines. As AEG (with its secondary amine) is superior to DAP in its ability to form a $[Tc(CO)_3]$ complex (Table 1), the linear sequence of AEG seems slightly more favourable than the amino acid configuration of DAP.

EDDA uses two secondary amines and a carboxylic acid group while IDA uses two carboxylic acid groups and one secondary amine. This shows a preference for a secondary amine in competition with a carboxylic acid group, which is also supported by the HSAB principle ('hard and soft acids and bases').[6] The metal centre in the $[Tc(CO)_3]$ complex is considered as 'soft' and therefore favours another 'soft' hetero-atom (N) instead of the 'hard' oxygen (O) in the acid group.

EDTA, DTPA, he-IDA and NTA are different due to the fact that they contain tertiary amines and moreover sterical hindrance may influence the reaction. It yet remains unclear why EDTA has a significantly superior ability to bind the $[Tc(CO)_3]$ core as compared to DTPA, which in most conditions is the stronger complexing agent of the two compounds. It has to be further studied, whether DTPA and EDTA are superior to he-IDA and NTA because of the involvement of two amines in the complex formation or because each molecule contains two iminodiacetic acid groups as additionally available binding sites (compared to one in he-IDA and NTA). NTA and he-IDA surely use a tertiary amine in combination with two carboxylic acid groups for complexation and they are clearly at the end of the queue of all investigated ligands. The ranking of he-IDA confirms that in compounds with identical Tc-binding hetero-atoms, a secondary amine is clearly superior as compared to a tertiary amine (see IDA vs he-IDA and IDA vs NTA). In addition, the comparison of he-IDA and NTA (both with tertiary amines) seems to indicate that an additional negative charge in the ligand has an adverse effect on complex formation. This might also explain why EDTA was superior to DTPA in this study.

Although the kinetics of the competition experiments were not a major issue of this study, we performed a set of experiments to evaluate the progression of the labelling reaction in the first 15 min (according to our standard conditions). The percentage of complex formation was determined after 3, 5, 10 and 15 min (separated labelling experiment for each time point). We selected two pairs of ligands for these experiments, namely HIS versus AEG as a pair of the stronger ligands and ASP versus NTA, a pair of the weaker ligands. The selection was made on the basis that they follow each other in the ranking and that both form a significant amount of the Tc-tricarbonyl complex in the competition against each other (see Table 1). The results indicate that at these time points no changes in the relative amount of formed complexes was observed (overall difference less than 2%). The labelling reaction was for each pair fast and already completed after 3 min. Only for a very early time point of 1 min slight differences were obtained: For HIS versus AEG less than 3% free Tc-tricarbonyl was detected, for ASP versus NTA 9%, another indication for the different ability to form a complex with the $[Tc(CO)_3]$ precursor.

While it is not yet fully explored whether a five- or a six-membered ring is preferred when a $[Tc(CO)_3]$ complex is formed, both appear to be superior to any other possible ring size, as mentioned before. The arrangement of the hetero-atoms (linear sequence vs nonlinear sequence in the case of the studied amino acids) seems to have some, although minor influence on complex formation, while the type of hetero-atom is a decisive factor. Certainly in all comparable cases primary or aromatic amines were superior to carboxylic acid groups, which corresponds fairly with the HSAB principle. Although not amply explored, the tested thiol-sulfur atoms showed a higher capacity in the competition experiments than carboxylic acid groups (as demonstrated with h-CYS vs ASP).

These results give a good indication which type of ligand to choose to modify biomolecules for an efficient and stable labelling with the Tc-tricarbonyl core.

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