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Research Article

Improvement in radiolabelling proteins with the ^{99m}Tc-tricarbonyl-core $[^{99m}Tc(CO)_3]^+$, by thiol-derivatization with iminothiolane: application to γ-globulins and annexin V

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Summary

The aim of this study was to improve the radiolabelling of proteins with the ^{99m}Tc-tricarbonyl-$[Tc(I)(CO)_3]^+$ core by introducing thiol groups to their structure. To achieve this goal, γ-globulins and annexin V were derivatized with mercaptobutyrimidyl groups (MBG) after reaction with 2-iminothiolane. The optimal conditions permitted attachment of an average of 3.3 thiol groups on γ-globulins and 1.0 to annexin V. The radiolabelling assays were carried out by incubating 3.2 nmol of either γ-globulin-SH or unmodified γ-globulin with 60 MBq ^{99m}Tc-tricarbonyl produced from an Isolink® kit (Mallinckrodt) under different reaction conditions. Results clearly showed that the introduction of three MBG could double the radiolabelling yields to more than 90% in a short time (30 mn, 37°C). Such results would never have been reached with unmodified γ-globulins alone. Under the same conditions when using 1–2 nmol derivatized annexin V, the average radiolabelling yield was 55% against 19% with the unmodified protein. The ^{99m}Tc-tricarbonyl-conjugates were challenged with cysteine or histidine and showed good stability. Copyright © 2005 John Wiley & Sons, Ltd.

Key Words: ^{99m}Tc-tricarbonyl; 2-iminothiolane; annexin V; globulins

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Introduction

Due to their high kinetic stability, technetium complexes in low (+1) oxidation state have gained considerable attention in the development of novel site-specific radiopharmaceuticals in recent years. Alberto *et al.*[1] synthesized the Tc (+1)-tricarbonyl complex $[Tc(H_2O)_3(CO)_3]^+$ as a precursor for Tc (+1) radiolabelling of biomolecules. The ^{99m}Tc-tricarbonyl core can easily be obtained from $[^{99m}TcO_4]^-$ in saline, in the presence of a reducing agent and carbon monoxide or from a kit commercialized by Mallinckrodt, in which boranocarbonate is used as a carbon monoxide source.[1,2] The closed shell Tc(I) centre has a d^6 electronic configuration, which is kinetically stable, since the dissociative ligand substitution is quantum mechanically forbidden. Ligand exchange is expected to be based on dissociative or interchange dissociative mechanism of the more labile water ligands, with a bifunctional ligand attachable to biomolecules.[3] The replacement of one water molecule by a monodentate ligand provides good stability but the use of bi- or tridentate ligands improves it.[4] It has been shown that various *N*-containing ligands, such as histidine, histamine, imidazole and Schiff bases are able to react with the organometallic aquaion $[Tc(H_2O)_3(CO)_3]^+$ to give stable complexes.[5,6] Investigations by Egli *et al.*[7] showing that histidine was the most potent ligand among other amino acids, have led to the direct radiolabelling of peptides or proteins.[7–9] Nevertheless, the yields are correlated to the number of histidine residues present on the primary structure, making the ^{99m}Tc-tricarbonyl core not suitable for the radiolabelling of every protein. For example, annexin V, a 33 kDa protein with only three histidine residues[10,11] shows a disappointing yield of 40% when labelled with ^{99m}Tc-tricarbonyl.[12] In order to improve the labelling efficiency, Tait *et al.*[12] developed annexin V mutant with *N*-terminal extensions containing three or six histidine residues, but this method is not suitable for routine clinical practice since mutated annexin is not readily available.

Although less efficient than histidine, Egli *et al.*[7] demonstrated in 1999 that the fixation of ^{99m}Tc-tricarbonyl core was also possible on cysteine. This observation has been confirmed in 2005 by Park *et al.*[13] However, cysteine in proteins is not believed to play an important role in ^{99m}Tc-tricarbonyl fixation, as this amino acid is less common than histidine. Moreover, the thiol function is often oxidized to disulphide bond (cystine), a well known poor chelating group. In the pursuit of these results, we have chosen to modify natural proteins with 2-iminothiolane (2-IT) in order to generate free thiol groups and to observe the consequence on the ^{99m}Tc-tricarbonyl core fixation. 2-IT (Traut's reagent) reacts with primary amines (e.g. *N*-terminal α- or Lys ε-amino groups in peptides or proteins) to produce mercaptobutyrimidyl groups (MBG) as shown in Figure 1.

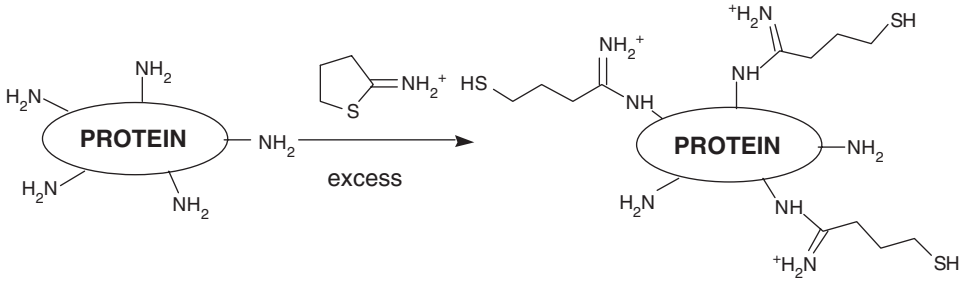

Figure 1. Reaction between 2-iminothiolane (2-IT) and amino groups on proteins

The use of 2-IT offers several advantages: the compound is commercially available, reaction with proteins or peptide can be performed in non denaturing conditions,[14] the number of introduced thiol groups can be determined by spectrometry using Ellman's reagent.[15] Iminothiolane has already been used to cross link proteins by reversible disulphide linkage, as a bifunctional agent to conjugate other molecules bearing electrophilic groups or as bifunctional chelating agent (BFCA) to radiolabel peptides or proteins with ^{99m}Tc. As a BFCA, mercaptobutyrimidyl groups (MBG) were used to chelate ^{99m}Tc-glucoheptonate[16–18], but showed relatively low radiochemical purity and stability. Annexin V derivatized with MBG was commercialized by Mallinckrodt but is not yet available. The thiol groups generated were also used to chelate ^{99m}Tc in a '3 + 1' mixed ligand approach with a $[Tc(V)O]^{3+}$ core.[19] Nevertheless, the harsh conditions of temperature required to label following this strategy, do not allow this technique to be applied without denaturation of proteins. If thiol groups generated by reducing disulphide bond (e.g. with dithiothreitol, DTT) allow the direct labelling of proteins, it led to the denaturation of proteins.

In our study, we had chosen to derivatize and radiolabel γ-globulins first because immunoglobulin G, a major class of γ-globulins, is known to present free lysine residues able to react with BFCA.[14,20] The results obtained were adapted for radiolabelling of annexin V which normally binds ^{99m}Tc-tricarbonyl with low efficiency. Radiolabelled annexin V is well characterized for *in vivo* apoptosis imaging. This protein has high affinity for phosphatidylserine, a phospholipid normally retained on the intracellular surface of the plasma membrane but externalized particularly during early stages of apoptosis, or after cell disruption resulting from a necrosis phenomenon. Several radiolabelling methods of this protein with ^{99m}Tc have been described.[21] Among them annexin V labelled using HYNIC methodology is the most promising annexin conjugate because it offers simple and fast labelling, while giving satisfactory radiochemical yields.[22,23] Nevertheless, this approach suffers from two drawbacks: the relatively low stability of the label

in solution[24] and the requirement of chemical synthesis, because HYNIC precursor is not commercially available. Our objective was also to find a simple and efficient method of radiolabelling annexin V with ^{99m}Tc-tricarbonyl core.

Results and discussion

Derivatization of γ-globulins and annexin V with iminothiolane

As shown in Figure 1, the protein linkage was accomplished using the Traut's reagent 2-iminothiolane (2-IT), which reacts with the free amino-residue of lysine on γ-globulins, to produce the mercaptobutyrimidyl group (MBG), under similar conditions depicted by McCall *et al.*[14]

In our case, 2-IT (45-fold excess) was incubated with γ-globulins at different pH (8, 9 or 10), duration (1, 2, 3 or 4 h) and temperature (room temperature or 37°C). The number of sulfhydryl groups fixed on proteins was determined with Ellman's reagent (5, 5′-dithiobis-2-nitrobenzoic acid or DTNB) using cysteine as standard, following the spectrophotometric assay of thiol groups.[15] We have checked that no thiol group was detected on underivatized γ-globulins with this technique. The best fixation yield was obtained after 2 h incubation at room temperature in phosphate buffer, pH 8. In this condition an average of 3.3 moles ($\sigma_{n-1} = 0.53$, $n = 10$) of thiol groups per mole of γ-globulin was found. In accordance with literature, elevating the pH to 9 or 10 did not improve the efficiency.[14] Reactions at lower pH were not tested because the amino group of lysine has a pK_a of 9.5. Lower pH would have led to an increase of protonated amine form, unreactive towards 2-IT. At pH 8, one hour incubation was not sufficient to obtain maximal fixation. No change was observed with either longer incubation period (3–4 h) or temperature at 37°C. The number of MBG fixed on γ-globulins is very similar to the number of HYNIC groups fixed on the same lysine amino residues of immunoglobulins G, according to Abrams *et al.*[20] Excess of 2-IT was easily removed by centrifugation and the resulting derivatized γ-globulins were stored at −70°C.

Following the same conditions, human 33 kDa annexin V was derivatized with MBG. Despite the fact that this protein is composed of 22 lysine residues[10,11], an average of only 1.0 thiol group is bound ($\sigma_{n-1} = 0.06$, $n = 4$). Blanckenberg *et al.* found a very similar result in the course of annexin V's lysine amino-residues derivatization with HYNIC.[22] In these conditions only one histidine seems to be accessible to derivatization with iminothiolane or HYNIC.

Stability of thiol groups fixed on protein

Singh *et al.*[25] demonstrated that the thiol adduct initially formed by the reaction of an amine with 2-iminothiolane could be unstable and decayed in a

Figure 2. Formation of *N*-substituted 2-iminothiolane (non thiol product)

Table 1. Number of thiol groups fixed on γ-globulins after 0, 1, 3, 7 and 31 days storage at −70°C

Time after fixation of 2-IT on γ-globulins (days)	Average number of SH groups per γ-globulin (3 repeats per time point)
0	3.20
1	3.13
3	3.14
7	3.34
31	3.21

first-order process to a cyclic non-thiol product which is inappropriate for bonding to a metal (Figure 2).

They also demonstrated that thiol adducts derived from amines of high pK_a values (e.g. lysine residues) were less labile and cyclized more slowly than those derived from amines of low pK_a values (e.g. α-amino groups). For example, at pH 8 the half-lives of thiol groups fixed on lysine residues ranged from 3 h at room temperature to 44 h at 0°C, whereas such α-amino groups varied from 0.3 h at room temperature to 1 h at 0°C. We checked the presence of the SH groups generated on γ-globulins after storage at −70°C during 1, 2, 3, 7 and 31 days. The results are shown in Table 1. Our results suggested that derivatized protein did not show loss of thiol function during the storage at −70°C.

Radiolabelling assay

Thiol derivatized γ-globulins were radiolabelled with ^{99m}Tc-tricarbonyl obtained from an Isolink® kit (Mallinckrodt, Petten, Netherlands) (Figure 3).

In order to define the optimal radiolabelling conditions, several assays were carried out, using about 3.2 nmol of γ-globulins-SH (about 9 nmol thiol groups; about 3 SH groups per molecule) prepared 24 h before the experiments and stored at −70°C. A fixed activity of 60 MBq ^{99m}Tc-tricarbonyl (less than 0.1 nmol ^{99m}Tc) was used but the duration and the temperature of reaction varied. In the same conditions, the radiolabelling of unmodified γ-globulins was performed for comparison. Results are shown in Figure 4. Each experimental condition was performed two times (2 repeats with less than 3% difference in ^{99m}Tc incorporation yield).

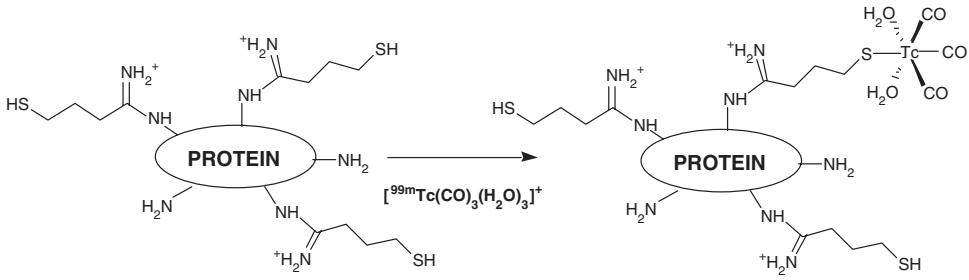

Figure 3. Proposed structure of conjugate [(thiol derivatized protein)-^{99m}Tc $(CO)_3$]

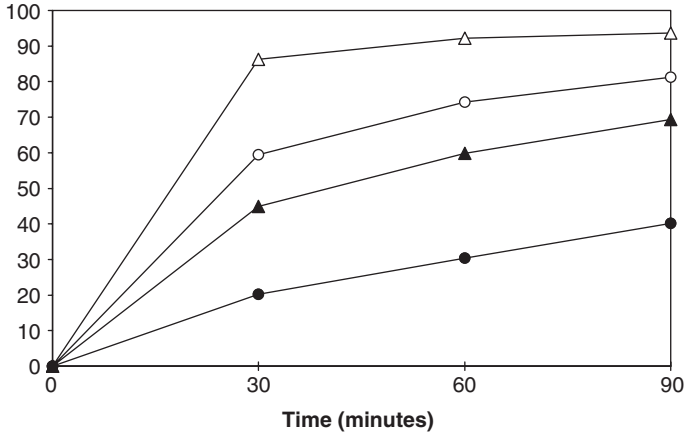

Figure 4. Time and temperature course of radiolabelling; 3.2 nmol derivatized (unfilled symbols) and unmodified γ-globulins (filled symbols) were incubated with ^{99m}Tc-Tricarbonyl (60 MBq) at room temperature (-○- and -●-) and at 37°C (-△- and -▲-)

The curves clearly indicate that MBG-derivatized γ-globulins are superior to the underivatized one since they result in higher radiolabelling yields in a shorter time. If an increase in duration and temperature are beneficial to the radiochemical yields of unmodified γ-globulins, it never reaches those of γ-globulins-SH in the timescale studied. The optimal time and temperature of radiolabelling for modified protein were 30 min at 37°C. In such conditions the specific activity was about 16 MBq nmol^{-1} for γ-globulins-SH and only 7 MBq nmol^{-1} for the unmodified. Increasing the ^{99m}Tc-tricarbonyl activity to 250–300 MBq allowed higher radiolabelling yield (95%) with γ-globulins-SH and thus better specific activity (80–155 MBq nmol^{-1}), depending on the amount of protein-SH employed. The radiolabelling yield never reached 50%

with unmodified γ-globulins under the same conditions. After purification, the radiochemical purity, as determined by TLC, was greater than 95%.

As a confirmation of the stability of the mercaptobutyrimidyl link during storage at $-70^{\circ}C$, radiolabelling of γ-globulins-SH prepared for 7, 3 and 1 day gave exactly the same labelling efficiency (about 93%). Similar results were found with annexin V. Results are shown in Table 2.

The labelling efficiency of annexin was always poorer than those obtained with the γ-globulins. This can be explained by the very low quantity of annexin V used (1–2 nmol) and by the fact that there is only one thiol group on the protein (against 3 thiols on γ-globulins). Increasing the amount of annexin V effectively increases the radiolabelling yield as clearly shown in Figure 5. These results and the ones obtained with γ-globulins, suggest that the presence of 3 nmol thiol groups would be sufficient to reach radiolabelling yields higher than 90%. In practice this would mean the use of 1 nmol γ-globulins-SH or 3 nmol annexin V-SH.

Tait *et al.*[12] used the ^{99m}Tc-tricarbonyl core (prepared with $NaBH_4$, $^{99m}TcO_4^-$ and CO gas) to radiolabel annexin V-mutants with *N*-terminal

Table 2. Comparison of radiochemical yield and specific activities obtained with derivatized or unmodified annexin V. Labellings were performed with 1–2 nmol of annexin, 250–300 MBq of ^{99m}Tc-tricarbonyl for 30 min at 37°C

Labelled annexin	Radiochemical yield	Specific activity (MBq nmol^{-1})
Annexin V-SH	$55.2 \pm 10.9\%$ ($n = 4$)	61–124
Unmodified annexin V	$19.0 \pm 4.4\%$ ($n = 4$)	23–70

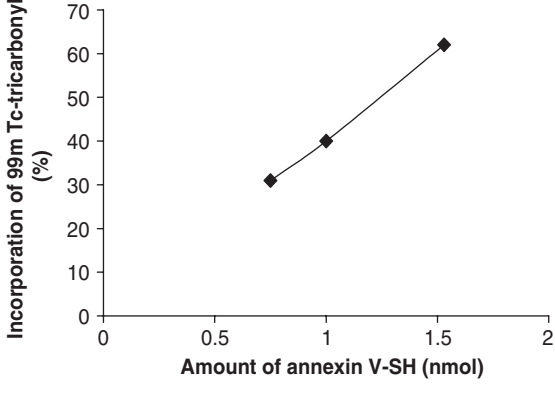

Figure 5. Radiolabelling efficiency as a function of the amount of [annexin V-SH] employed. Derivatized annexin V was incubated with 300 MBq of ^{99m}Tc-tricarbonyl for 30 min at 37°C

extensions containing three or six histidine residues. In these experiments, wild-type annexin V was radiolabelled with a radiochemical yield of 40%. Our lower results (19% on average) are probably due to the lower amount of proteins used (1–2 nmol against 3 nmol), the shorter reaction time (30 min against 1 h) and the use of ^{99m}Tc-tricarbonyl obtained from an Isolink® kit. Effectively, Satpati *et al.*[26] demonstrated that the kinetics of radiolabelling reaction was significantly lower when ^{99m}Tc-tricarbonyl was produced from Mallinckrodt's kit than from a reducing reaction in the presence of CO gas. Tait *et al.*[12] showed that three residues of histidine had to be incorporated to annexin V to increase the radiochemical yield from 40 to 60%, and six residues were needed to reach 80%. Our experiments clearly indicated that only one sulphhydryl group had to be added to the protein to, at least, double the radiolabelling yield (19–55%).

In vitro stability

Stability of the radiolabelled compounds was tested after 4 h incubation in PBS at room temperature (RT) or 37°C. Analysis was performed by TLC. The results, presented in Table 3, indicate that the radiolabelling stability was similar using derivatized or unmodified proteins.

Radiolabelled proteins were also challenged with cysteine or histidine in order to determine their stability against exchange and/or decomposition. Cysteine or histidine was added to reach a 10-fold excess over the number of thiol groups present in solution. The samples were analyzed by TLC. Results obtained with γ-globulins are summarized in Table 4. After 4 h at 37°C, the

Table 3. Stability of derivatized or native radiolabelled proteins after incubation in PBS

	γ-globulins-SH (%)	γ-globulins (%)	Annexin V-SH (%)	Annexin V (%)
T_0	97	96	96	95
T+4 h (RT)	93	92	92	91
T+4 h (37°C)	92	91	90	89

Table 4. Radiochemical purity of [γ-globulins-S–^{99m}Tc-tricarbonyl] and unmodified [γ-globulins–^{99m}Tc-tricarbonyl] incubated for 4 h at 37°C either in PBS or in the presence of cysteine or histidine

Conditions of incubation	γ-globulins-SH–^{99m}Tc-tricarbonyl (%)	γ-globulins–^{99m}Tc-tricarbonyl (%)
Alone	92	91
+ Cysteine	88	85
+ Histidine	92	90

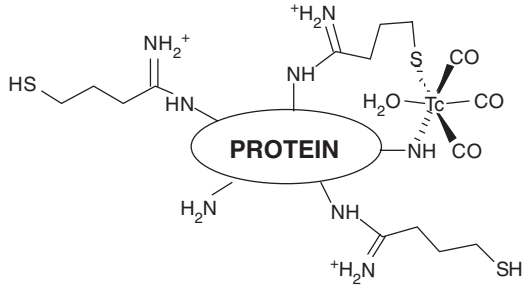

Figure 6. Stabilization of the ^{99m}Tc-tricarbonyl complex by a neighbouring ligand

displacement of ^{99m}Tc-tricarbonyl was not significantly accelerated in the presence of cysteine or histidine, indicating the very high stability of ^{99m}Tc-tricarbonyl on both MBG and histidine residues. The percentage dissociation using derivatized or unmodified annexin V was not significantly modified in the presence of histidine or cysteine. These results are encouraging as the cysteine molar ratio concentration above 5/1 may not be relevant *in vivo*.[27]

To date, derivatization of proteins with thiol groups in order to radiolabel them with ^{99m}Tc-tricarbonyl has not been done. Our results clearly showed that the introduction of mercaptobutyrimidyl groups allowed the acceleration of ^{99m}Tc-tricarbonyl fixation and provide radiolabelling yields that could never have been attained with unmodified proteins. The exact mode of complexation of the ^{99m}Tc-tricarbonyl core on protein-SH is not known. One water molecule of the aquaion is very likely substituted by the thiol function; nevertheless, other groups, i.e. a nitrogen atom close to the complex could also participate in its stabilization (Figure 6).

However, even if the ^{99m}Tc core is not complexed by bi- or tri-dentate ligands, results clearly indicate the stability of the fixation.

Experimental

All chemicals were obtained from Sigma-Aldrich unless otherwise specified. Annexin V was available in aliquots of 3.2 nmol in 100 µL of Tris-buffer. γ-globulins were composed of 90% IgG, 6% IgM and 4% IgA. Technetium-99m was obtained from a Shering-Cisbio generator (Saclay, France) and pertechnetate $[^{99m}TcO_4]^-$ was eluted in saline solution on a daily basis. The [^{99m}Tc-Tricarbonyl] core was produced from an Isolink® kit (Mallinckrodt, Peten, Netherlands). Purifications were performed by centrifugation through a Microcon® filter with 10 or 30 kDa cut off (Millipore) or with a Sephadex G 25 column (Amersham Bioscience) previously equlibrated with 5 ml of albumin (3% in PBS) and 25 ml of PBS (0.1 M; pH = 7.4). Protein concentrations were determined using biscinchoninic assay kits, Micro BC

Assay® and BC Assay® (Pierce), limit of quantification 5 and 20 $\mu g\,ml^{-1}$, respectively. Radiochemical purity analysis was performed by Instant Thin Layer Chromatography (ITLC) using silica gel coated fiberglass sheet (Gelman Science Inc.), Whatman DE 81 paper (Whatman International Ltd). Solvents for the mobile phase were aqueous NaCl (0.9%) and acetone of HPLC grade.

Fixation of mercaptobutyrimidyl groups (MBG) on γ-globulins or annexin V

The reaction between 2-iminothiolane and amino-residues present on proteins was first performed using bovine γ-globulins. An aliquot of γ-globulins (150 µl, 21.3 nM) in phosphate buffer (NaH_2PO_4 0.1 M at pH = 8, 9 or 10) in saline [NaCl] = 0.15 M) was incubated for 1, 2, 3 or 4 h at room temperature or at 37°C with 10 µl of a 2-IT solution (8.5 $g\,l^{-1}$ in phosphate buffer 0.1 M, pH = 8, [NaCl] = 0.15 M, DMSO 10% v/v). The solutions were purified by two centrifugations through a 30 kDa filter. The concentration of thiol groups was determined with Ellman's reagent (DTNB) as described: 90 µl of sample were incubated with 60 µl of DTNB at 4 $g\,l^{-1}$ in phosphate buffer (pH = 8) during 30 min at room temperature in a 96 well microplate. A standard curve was created using 90 µL of cysteine solutions ranging from 0 to 60 µM in phosphate buffer pH = 8, 9 or 10. The absorbances were read at 405 nm. The number of thiol groups fixed on unmodified γ-globulins were estimated with the same technique. The γ-globulin-SH derivative obtained was then stored at −70°C.

Aliquots of annexin V, available in Tris-buffer, were first purified by filtration through a 10 kDa filter and placed in a phosphate buffer (0.1 M at pH = 8). The reaction conditions which allowed the higher fixation yield of thiol groups on γ-globulins were maintained for annexin V: one aliquot (3.2 nmol) was incubated with a 45-fold excess of 2-IT (10 µl of a 2 $g\,l^{-1}$ solution in phosphate buffer 0.1 M, pH = 8, [NaCl] = 0.15 M, DMSO 10% v/v).

Stability of mercaptobutyrimidyl group fixed on protein

Five samples of 150 µl γ-globulins (21.3 nM in phosphate buffer 0.1 M, pH = 8, [NaCl] = 0.15 M) were each incubated with 10 µl of 2-IT (2 $g\,l^{-1}$ in DMSO) for 2 h at room temperature. The solutions were then centrifuged through a 30 kDa membrane, mixed, divided into 5 equal aliquots and stored at −70°C. Concentrations of protein and thiol groups were determined as described previously on the day of fixation and then after 1, 3, 7, and 31 days.

Radiolabelling assays of derivatized and native proteins via ^{99m}Tc-Tricarbonyl

^{99m}Tc-tricarbonyl was obtained from an Isolink® kit (Mallinckrodt) using the following procedure: 1800–3700 MBq (in 1 ml of NaCl 0.9%) of daily eluted

sodium [^{99m}Tc]pertechnetate were introduced into an Isolink® vial and mixed gently. The vial was maintained for 20 min in boiling water then cooled for 10 min in cold water (15°C) and finally the pH was brought to pH = 8 with HCl (0.1 M).

The radiolabelling assays were performed using one aliquot (about 3.2 nmol) of γ-globulins-SH prepared 24 h before and stored at −70°C.

γ-globulins-SH were incubated with 60–70 MBq of the previously prepared ^{99m}Tc-tricarbonyl at room temperature or 37°C over during 30, 60 or 90 min. The solutions were purified through a Sephadex G25 column. Fractions of 1 ml were collected. Native γ-globulins were radiolabelled under exactly the same conditions. Concentrations of proteins were measured with a micro BCAssay, using bovine serum albumin as standard. Radiochemical purity was determined by ITLC, either with silica gel plates in NaCl 0.9% in order to define the percentage of free ^{99m}Tc-pertechnetate and ^{99m}Tc-tricarbonyl, or with Whatman DE 81 paper (anion exchanger) in acetone, in order to determine the percentage of free ^{99m}Tc-pertechnetate alone.

Annexin V was derivatized with thiol groups the day before the radiolabelling and stored at −70°C. The protein-SH obtained was incubated for 30 min at 37°C with 230–300 MBq of ^{99m}Tc-tricarbonyl. The reaction mixture was then treated as previously described.

In vitro stability assays

In vitro stability assays were carried out by incubating radiolabelled proteins either in PBS, either with excess of cysteine or histidine for 4 h at room temperature or at 37°C. After purification, the protein concentrations were estimated. Thirty microliters of each radiolabelled mixture were then incubated with 10 µl of solutions, containing ten fold excess cysteine or histidine per MBG. After incubation, each solution was analyzed simultaneously with ITLC SG in NaCl 0.9% and Whatman DE 81 paper in acetone. In acetone, only pertechnetate migrates while in saline, pertechnetate, ^{99m}Tc-tricarbonyl and ^{99m}Tc-cysteine migrate.[12,19,28]

Conclusion

In this study, we have shown that thiol-derivatization with 2-iminothiolane of proteins is an effective method to radiolabel them with the ^{99m}Tc-tricarbonyl core. The good availability of 2-iminothiolane, the simplicity of the derivatization and radiolabelling and the good stability of the final products make the technique very attractive. The introduction of mercaptobutyrimidyl groups to γ-globulins or annexin V allowed at least doubling of the radiolabelling yields of ^{99m}Tc-tricarbonyl core against unmodified proteins in short incubation times. Results showed that the complexation of the ^{99m}Tc-tricarbonyl core *via* MBG was as stable as *via* histidine residues.

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