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Simple and convenient radiolabeling of proteins using a prelabeling-approach with thiol-DOTA

Carmen Wängler [a,*], Ralf Schirrmacher [a], Peter Bartenstein [b], Björn Wängler [b,*]

[a] McConnell Brain Imaging Center, Montreal Neurological Institute, McGill University, University Street 3800, Montreal H3A 2B4, Quebec, Canada
[b] University Hospital Munich, Department of Nuclear Medicine, Ludwig-Maximilians-University, Marchioninistraße 15, Munich, Germany

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ABSTRACT

Commonly applied methods for radiometal-labeling of proteins require complex and protracted derivatization reactions of the protein and the subsequent radiolabeling is time-consuming due to the low reaction temperatures applicable. Therefore, a convenient and efficient prelabeling technique for proteins using the DOTA derivative 2,2′,2″-(10-(2-(2-mercaptoethylamino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (thiol-DOTA) containing a thiol moiety for rapid and selective introduction into maleimide-derivatized proteins was developed. Thiol-DOTA was labeled with ^{68}Ga, ^{90}Y and ^{177}Lu and subsequently introduced into bovine serum albumin and a human IgG with maximum radiochemical yields of 66%. The entire radiolabeling procedure was completed after only 30 min making this a favorable new labeling technique for proteins.

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Although bioactive compounds labeled with radiometals as, for example, ^{68}Ga, ^{90}Y and ^{177}Lu have gained widespread interest in diagnostic imaging as well as for therapeutic purposes, only few examples of proteins labeled with radiometals can be found. Using the commonly applied radiometal chelator DOTA (1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid), which forms highly stable complexes with a broad variety of radiometals, high temperatures for a fast and quantitative complexation reaction are required. These conditions are suitable for the labeling of peptides and oligonucleotides but not for proteins when the chelator is conjugated to the protein prior to the radiolabeling reaction.

Recently, labeling methods for proteins with ^{67}Ga and ^{68}Ga using the chelators NOTA and HBED-CC were described.[1–5] However, these chelators either form stable complexes only with gallium and indium isotopes and are not applicable for the stable labeling with other important radiometals such as ^{86}Y, ^{90}Y and ^{177}Lu or are only accessible by a complex synthesis and have shown to be highly immunogenic.[6–8] As mentioned above, DOTA derivatives are particularly suitable for radiometal-labeling as they form very stable complexes with a broad variety of radiometal ions. Several DOTA derivatives for the modification and subsequent labeling of proteins at mild temperatures were described for the use in post-labeling approaches as, for example, DOTA-L-*p*-isothiocyanato-phenylalanine, DOTA-triglycyl-L-*p*-isothiocyanato-phenylalanine, DOTA *N*-hydroxysulfosuccinimide, DOTA-maleimidoethylacetamide and active esters of DOTA. However, the derivatization reactions of the proteins are complex and time-consuming and the subsequent labeling reactions take between 30 and 60 min due to the low reaction temperature.[9–15] This is relatively long regarding the short half-life of some radionuclides such as ^{68}Ga ($t_{1/2}$: 68 min). In addition, the complexation reactions are mostly not quantitative due to the low reaction temperatures used to avoid protein denaturation.

Using DOTA-triglycyl-L-*p*-isothiocyanato-phenylalanine in a prelabeling approach, the labeling procedure was time-consuming and the reaction of the precomplexed nuclide with the protein required a high excess of the protein to be labeled to ensure acceptable reaction yields of the moderately reactive isothiocyanate with the protein.[16] This resulted in a high amount of non-radioactive protein which can negatively influence the pharmacokinetic and pharmacodynamic properties of the radiolabeled compound.

Thus, a DOTA derivative allowing an efficient and rapid introduction of various radiometals into proteins without intricate and time-consuming derivatization and purification steps would be advantageous. This would also allow the use of short-lived radionuclides such as ^{68}Ga and should result in an uncomplicated

* Corresponding authors.
E-mail addresses: carmen.waengler@med.uni-muenchen.de (C. Wängler), bjoern.waengler@med.uni-muenchen.de (B. Wängler).

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synthesis of radiolabeled proteins. The DOTA derivative should furthermore allow a facile determination of the number of derivatization sites introduced per protein as strong structural changes exerted by the derivatization can easily result in a significant loss of the biological activity of the protein.[17–19]

A DOTA derivative meeting these requirements is thiol-DOTA (**3**) which was synthesized according to an earlier published procedure.[20] This DOTA derivative, containing a thiol moiety for effective and selective introduction into maleimide-derivatized substances, showed to be oxidation-resistant in solution at pH 7.2 for several hours and also exhibits very favorable coupling properties as the Michael addition to the maleimide generally takes place within minutes. Thiol-DOTA (**3**) was thus investigated regarding its applicability for a prelabeling approach with ^{68}Ga, ^{90}Y and ^{177}Lu and its use as a labeling agent for the maleimide-derivatized proteins bovine serum albumin (BSA) and a human monoclonal IgG. These proteins were derivatized with Sulfo-SMCC (**2**) (sulfosuccinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate sodium salt) to introduce maleimide moieties and subsequently reacted with the prelabeled thiol-DOTA (**5**).[21] The amount of maleimide-derivatization sites per proteins was determined according to Wängler et al. by Ellman's Assay and found to be between 1.5 and 1.8.[19]

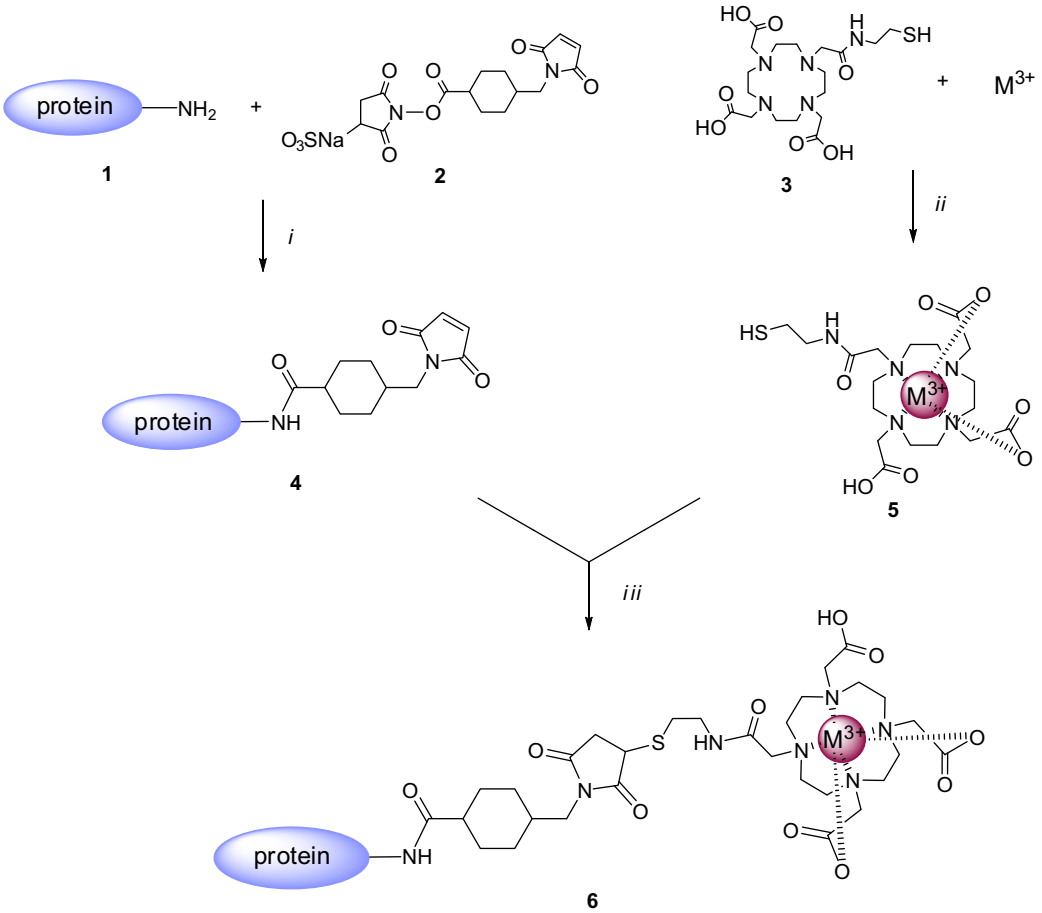
The prelabeling approach using thiol-DOTA (**3**) allowed an efficient complexation of ^{68}Ga, ^{90}Y and ^{177}Lu at high temperatures and subsequently a very rapid introduction into the maleimide-functionalized proteins under mild conditions. The final purification step could be carried out using a size exclusion gel cartridge. Thus, this prelabeling approach enables the very rapid radiolabeling of various proteins.

A typical labeling procedure comprised the following steps (Scheme 1):

1. the prelabeling of thiol-DOTA (**3**, 4.35 μg, 10 nmol) with the radiometal (230–250 MBq ^{68}Ga in sodium acetate buffer (1.1 mL, 0.1 M, pH 3.8) or 0.5–1.1 GBq ^{90}Y in sodium acetate buffer (0.5 mL, 0.4 M, pH 4.8) or 550–850 MBq ^{177}Lu in sodium acetate buffer (0.5 mL, 0.4 M, pH 4.8)) for 10 min at 99 °C,
2. the introduction of the prelabeled complex (**5**) into the maleimide-derivatized protein (**4**, 10–35 nmol)[21] by reacting 10 min at room temperature in phosphate buffer (1 mL, 0.5 M, pH 7.2) and
3. the purification of the radiolabeled protein (**6**) using a NAP-10-column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

The prelabeling of thiol-DOTA (**3**) with ^{68}Ga (Fig. 1A), ^{90}Y and ^{177}Lu (Fig. 1D) proceeded with high yields, giving the radiolabeled thiol-DOTA complex (**5**) in radiochemical yields of 93–98% and high radiochemical purities within 10 min at 99 °C. As the thiol-DOTA (**3**) was used in low quantities of only 10 nmol, the achieved specific activities were between 20–25 GBq/μmol for ^{68}Ga, 45–100 GBq/μmol for ^{90}Y and 50–80 GBq/μmol for ^{177}Lu.

The ^{68}Ga–thiol-DOTA-complex (^{68}Ga-**5**) (Fig. 1A) and the ^{90}Y- and ^{177}Lu–thiol-DOTA-complexes (^{177}Lu-**5**) (Fig. 1D) show differ-



Scheme 1. Labeling of proteins (bovine serum albumin or human IgG) with radiometals M^{3+} ($^{68}Ga^{3+}$, $^{90}Y^{3+}$ or $^{177}Lu^{3+}$). Reagents and conditions: (i) 100 nmol protein (**1**), 7 equiv Sulfo-SMCC (**2**), 1 h, room temperature, phosphate buffer (0.5 mL, 0.1 M, pH 7.2), 95–98%[21]; (ii) 10 nmol thiol-DOTA (**3**), 230–250 MBq ^{68}Ga in sodium acetate buffer (1.1 mL, 0.1 M, pH 3.8) or 0.5–1.1 GBq ^{90}Y in sodium acetate buffer (0.5 mL, 0.4 M, pH 4.8) or 550–850 MBq ^{177}Lu in sodium acetate buffer (0.5 mL, 0.4 M, pH 4.8), 10 min, 99 °C; (iii) 10–35 nmol maleimide-derivatized protein (**4**), prelabeled thiol-DOTA from ii (**5**), 10 min, room temperature, phosphate buffer (1 mL, 0.5 M, pH 7.2).

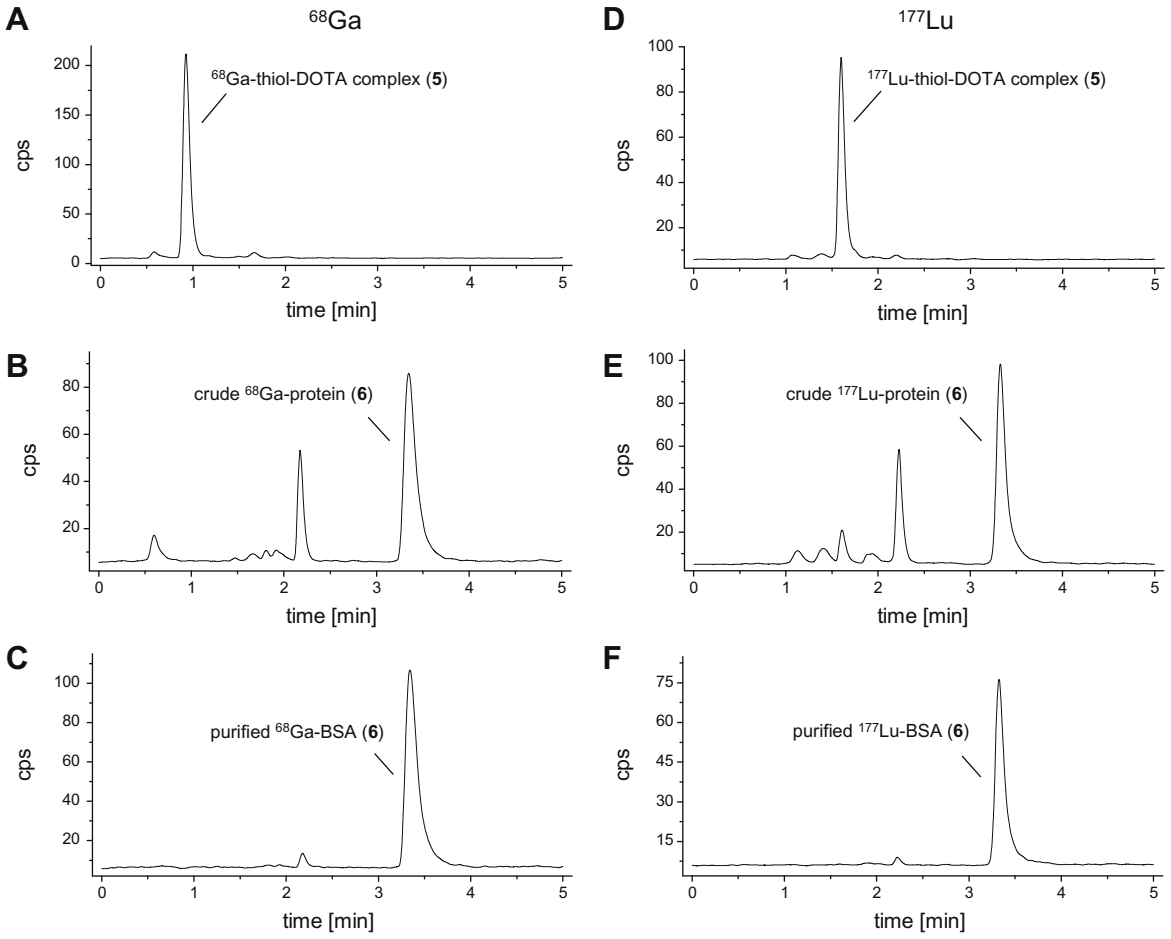


Figure 1. Analytical radio-HPLC chromatograms at different stages of the labeling reaction: A and D show the finished precomplexation reactions of thiol-DOTA with ^{68}Ga and ^{177}Lu, B and E the reactions of maleimide-derivatized BSA (**4**) with the labeled ^{68}Ga- and ^{177}Lu–thiol-DOTA complexes (**5**) and C and F the purified ^{68}Ga- and ^{177}Lu-labeled BSA (**6**). ^{90}Y labeling reactions result in equivalent chromatograms to those of ^{177}Lu.

ent retention times under the same HPLC conditions of 0.95 min and 1.6 min, respectively, which might be attributed to the different ion radius and therefore to the different complexation behavior of the radiometals. The free thiol function does not seem to contribute to the complex formation as it subsequently reacts with the maleimide-derivatized protein. Moreover, the contribution of the thiol moiety in the complexation is not likely regarding the known complex geometry of DOTA complexes.[22]

In contrast to the prelabeling step yielding only the desired complexation product (**5**), its reaction with the maleimide-derivatized proteins (**4**) shows the formation of some unidentified side products (Fig. 1B and E) which are likely to consist of small labeled molecules as can be deduced from analyzing the same reaction mixtures with size exclusion FPLC.[23] However, these side products can be easily removed by the final gel filtration purification (Fig. 1C and F), yielding the pure radiolabeled proteins (**6**).

The overall radiochemical yields of the labeling reactions were between 18% and 66% and the specific activities achieved were between 1.1–3.2 GBq/μmol for ^{68}Ga, 8.1–27 GBq/μmol for ^{177}Lu and 4.5–32 GBq/μmol for ^{90}Y which is in the range of published values.[9,11,12,16]

By applying Ellman's assay,[24] the extent of the maleimide-derivatization of the proteins can be verified. Using this assay, we introduced a well defined and low number of 1.5–1.8 maleimide-derivatization sites into the proteins which results in only minor structural alteration and by this in a highly preserved biological activity of the protein to be labeled. As the maleimide-derivatized proteins can be stored in solution at 4 °C for several hours without loss of maleimide reactivity, the derivatization can be accomplished before labeling, further shortening the labeling procedure.[25]

The radiolabeling of the maleimide-derivatized proteins (**4**) can be accomplished within 30 min as the prelabeling of thiol-DOTA (**3**) takes only 10 min as well as the reaction of the prelabeled complex with the protein and the purification of the final product via a gel cartridge can be carried out within 5–10 min.

In summary, it has been demonstrated that thiol-DOTA can be used as a valuable tool for the convenient and rapid radiolabeling of maleimide-derivatized proteins with ^{68}Ga, ^{90}Y and ^{177}Lu. Particularly the very simple, mild and defined derivatization of the protein and the efficient radiolabeling procedure which is completed after a short time span of only 30 min make this labeling technique a valuable alternative to applied methods.

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