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A new method for the labelling of proteins with radioactive arsenic isotopes

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Abstract

Radioarsenic labelled radiopharmaceuticals could be a valuable asset to positron emission tomography. In particular, the long half-lives of ^{72}As ($T_{1/2} = 26$ h) and ^{74}As ($T_{1/2} = 17.8$ d) allow to investigate slow physiological or metabolical processes, like the enrichment and distribution of monoclonal antibodies (mab) in tumour tissue. In this work, a new method for the labelling of proteins with various radioactive arsenic isotopes was developed. For this purpose, two proteins, namely a chimeric IgG_3 monoclonal antibody, ch3G4, directed against anionic phospholipids, and Rituxan (Rituximab), were labelled as a proof of principle with no-carrier-added radioarsenic isotopes (^{74}As and ^{77}As). The developed labelling chemistry gives high yields (>99.9%), is reliable and could easily be transferred to automated labelling systems in a clinical environment. At least for the mab used in this work, this route of radioarsenic labelling does not affect the immunoreactivity of the product. The arsenic label stays stable for up to 72 h at the molecular mass of the monoclonal antibody, which is in particular relevant to follow the pharmacology and pharmacokinetics of the labelled mab for several days.

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Keywords: Protein labelling; Labelled antibodies; Radioactive arsenic

1. Introduction

Molecular imaging in vivo is a sophisticated approach to answer the question on the molecular basis and mechanisms of binding of specific monoclonal antibodies (mab) to their target. Among the molecular imaging modalities relevant to this aim, quantitative positron emission tomography (PET) might be an excellent choice. Smith-Jones et al. [1] recently demonstrated the use of PET for the molecular imaging of antibody fragments. However, because of the long-term metabolism of the whole antibody, their concept of labelling mab with ^{68}Ga ($T_{1/2} = 68$ min) seems not to be feasible in all cases.

In comparison, arsenic provides several radioisotopes of interest for medical or environmental application, including the positron emitters ^{72}As and ^{74}As, which are excellent candidates for the imaging of longer lasting biological processes using quantitative PET.

The recent increasing interest in the element arsenic in environmental sciences [2], toxicology [3] and carcinogenesis [4,5] and medicine [6–14] stimulates a need to develop convenient and reproducible methods to trace this element and its compounds in subtoxic and subpharmaceutical concentrations.

A number of approaches to develop an easy and practical system to separate these isotopes from cyclotron or reactor irradiated germanium or germanium oxide

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targets have been described [15–19]. We have previously developed strategies towards a versatile radioarsenic labelling chemistry to generate arsenic isotopes in chemical forms suitable for future application in labelling chemistry, radiopharmacy and, ultimately, for molecular imaging using PET.

In the present study, a new method for the labelling of proteins with various radioactive arsenic isotopes was developed. For this purpose, two proteins, namely a chimeric IgG_3 mab, ch3G4, directed against anionic phospholipids, and Rituxan (Rituximab), were labelled as a proof of principle with no-carrier-added (nca) radioarsenic isotopes (^{74}As and ^{77}As).

2. Materials and methods

2.1. Materials

Germanium(IV) oxide (99.9999% grade, PURA TREM) was purchased from Strem Chemicals Inc. Concentrated hydrofluoric acid (48%) and potassium iodide were purchased from Aldrich. BOND ELUT ENV solid phase extraction cartridges with a sorbent mass of 50 mg and a volume of 1 ml were purchased from Varian.

2.2. Proteins/antibodies

Rituximab (MabThera®, CD20) was purchased from Roche. Ch3G4 (Tarvacin®) was provided by Peregrine Inc., USA. HRP goat anti-human IgG was from Jackson Immunoresearch Labs (West Grove, PA).

2.3. Isotopes

In this chemical study, the two nca radioarsenic isotopes ^{74}As and ^{77}As were used.

^{74}As is a positron emitting isotope with a half-life $T_{1/2}$ of 17.8 d. It has a positron emission rate of 29% with an extremely low positron energy of $E_{\beta+\text{mean}} = 128$ keV, providing high local resolution when measured via PET and an electron emission rate of 34.2% and $E_{\beta-\text{mean}} = 137$ keV [20]. It was one of the first isotopes used for very preliminary forms of PET in the 1950s and 1960s [21–26] called *positrocephalography* at those times. Due to its long half-life it will be more appropriate for animal than human use, but as well could provide a useful tool for the study of long lasting metabolic processes, like antibody–antigen interaction or in general long term pharmacokinetics of developmental drugs. ^{74}As can be produced best by ^{74}Ge(p,n)^{74}As or ^{73}Ge(d,n)^{74}As reactions at a small-sized cyclotron. Excitation functions and target yields are described in detail in ref. [27]. ^{74}As was produced by the ^{nat}Ge(p,x)^{74}As reaction ($E_p = 20$ MeV, 3 h irradiation at 15 μA) giving a yield of about 370 MBq at the VUB Cyclotron at the University of Brussels, Belgium.

^{77}As is an 100% electron-emitting isotope with a $T_{1/2}$ of 1.62 d and $E_{\beta-\text{mean}} = 226$ keV. This isotope could be of future use for an endoradiotherapeutic arsenic based radiopharmaceutical. ^{77}As can be produced at nuclear reactors via the ^{nat}Ge(n,γ)^{77}Ge reaction. ^{77}Ge decays to ^{77}As with a half-life of 11.3 h. It was produced in nca state via the ^{76}Ge(n,γ)^{77}Ge, $T_{1/2} = 11.30\,\text{h} \rightarrow \beta^- \rightarrow$ ^{77}As processes at the TRIGA reactors of the Institute of Nuclear Research of the University of Mainz and of the Nuclear Engineering Teaching Laboratory of the University of Texas at Austin.

All nuclear reactions were performed on 100 mg of $^{nat}GeO_2$.

2.4. Radiochemical separations

Irradiated germanium oxide targets were dissolved in 5 ml HF conc. at room temperature for 1 h. Subsequently, potassium iodide was added up to 10 mg/ml HF conc. and stirred for 10 min. The mixture was transferred to an ENV solid phase extraction cartridge. Cartridge holder and fittings to standard size syringes were home made in the machine shop of the Institute of Nuclear Chemistry, University of Mainz. The ENV cartridge was preconditioned with 5 ml of MeOH, 5 ml H_2O and 5 ml HF conc. containing sodium iodide in the concentration of 1 mg/ml. Nca [*As]AsI_3 was fixed to the solid phase, while macroscopic $[GeF_6]^{2-}$ was eluted quantitatively with the mobile phase. After the fixation of [*As]AsI_3, excessive HF conc. was removed with a high pressure nitrogen flow over the cartridge for 5 min. The elution was performed with 500 μl ethanol. The solution was concentrated to 50 μl under a slight N_2 flow at $T = 70$ °C immediately before the subsequent labelling procedure.

2.5. Protein conjugation and testing

The used mabs were SATA modified according to the protocol of Pierce Endogen [28,29]. The deprotection of the sulfhydryl groups of the protein was performed directly before the labelling. SATA-modified antibodies of 100 μg in 3 ml PBS at pH = 7.5 were then combined with the nca [*As]AsI_3 solution at 37 °C for 30 min. Quality control was performed by HPLC, using an Agilent 1100 Series HPLC system, with an LDC/Milton Roy UV-Monitor III at 254 nm and a 'Gabi' NaI-radiation monitor from Raytest. The HPLC column was a Bio-Silect Sec 250-5, 300 × 7.8 mm and PBS + 0.01 M NaN_3 was used as solvent.

2.6. Labelling of SATA-modified proteins

After deprotection of the sulfhydryl by deacetylation of the SATA-modified protein the labelling is directly performed with nca [*As]AsI_3 as labelling synthon. Deacetylation is performed as following: 1.0 ml of SATA-modified (acetylated) protein are combined with 100 μl of the deacetylation solution (0.5 M hydroxylamine, 25 mM EDTA in PBS, pH 7.2–7.5, 1.74 g hydroxylamine. HCl and EDTA (0.475 g of tetrasodium salt or 0.365 g of disodium

salt) were dissolved in 40 ml of reaction buffer; ultrapure water was added to a final volume of 50 ml and pH adjusted to 7.2–7.5 with NaOH. The contents are mixed and incubated for 2 h at room temperature. A desalting column is used to purify the sulfhydryl-modified protein from hydroxylamine in the deacetylation solution. To keep the now free thiols from forming disulphide bridges again, all solutions used contain 1 mM EDTA.

For radioarsenic labelling of the proteins, the 0.05 ml nca [*As]AsI_3 containing ethanolic solution is added to 1 ml of PBS. The pH is then adjusted to 7.0 with NaOH. This solution is combined with the solution of the SATA modified mab [containing 0.1 mg mab] and incubated for 30 min at $T = 35\,^{\circ}C$. The [*As]AsI_3 couples to one SH under elimination of the iodide, which is catched by cationic ions in the buffer solution.

The coupling of nca [*As]AsI_3 to the introduced free SH is summarized in Fig. 1.

2.7. Determination of radioarsenic protein labelling yields

After a labelling time of 30 min, an aliquot of 20 μl of the ^{74}As[SATA]ch3G4 solution is given over a size-exclusion column for radio-HPLC (Agilent 1100 Series, with LDC/Milton Roy UV-Monitor III at 254 nm and a 'Gabi' NaI-radiation Monitor from Raytest, Column: Bio-Silect Sec 250-5, 300×7.8 mm 20 μl injection volume, solvent: PBS + 0.01 m NaN_3, runtime 30 min). No free ^{74}As was detectable, remaining unreacted in solution. The radioarsenic protein labelling yield was > 99.9%.

2.8. In vitro stability

In vitro stability of the radioarsenic labelled protein ch3G4 was tested in terms of competition via incubation in fetal bovine serum (FBS). Aliquots of 50 μl were taken at $t = 30$ min, 24, 48, and 72 h, diluted with 200 μl water.

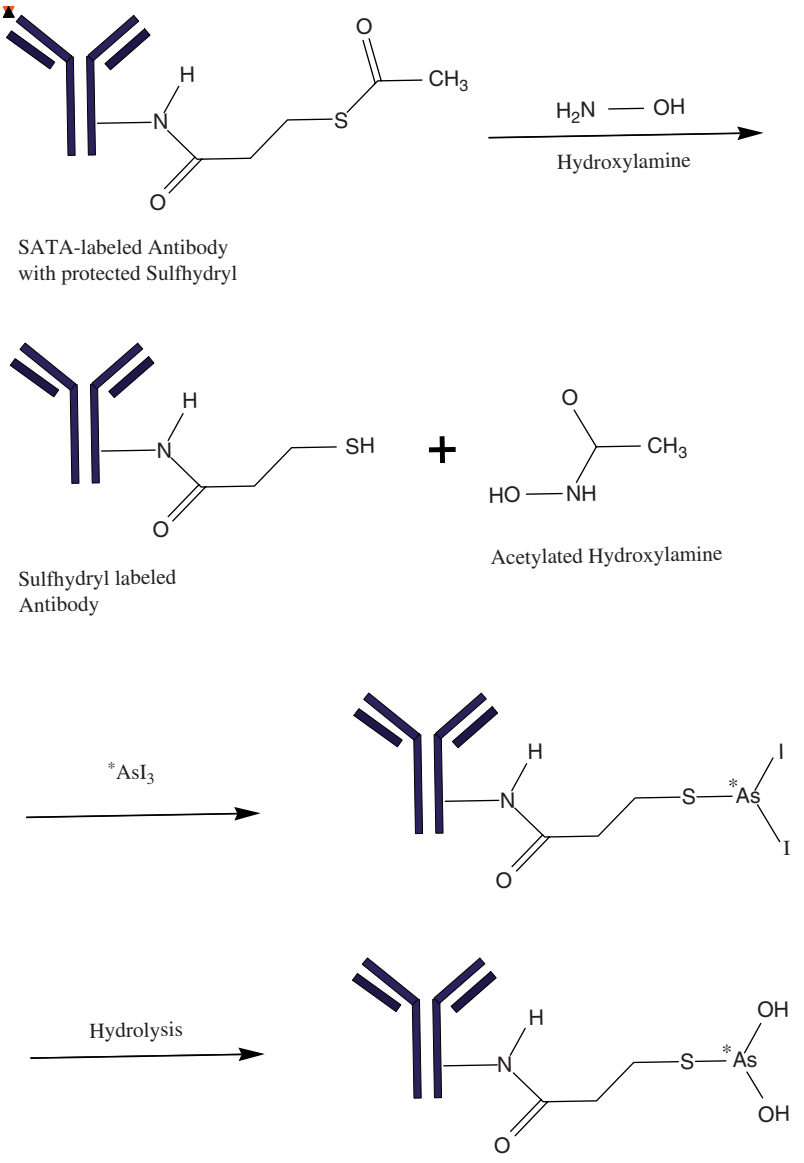

Fig. 1. Reaction scheme for the labelling of SATA-modified antibodies with radioactive arsenic isotopes.

Radioarsenic labelled ch3G4 of 10 μg in 50 μl PBS was combined with 500 μl FBS and incubated at $T = 37\,^{\circ}C$. Aliquots of 20 μl volume were taken for analysis. HPLC measurements were performed as described above at various time points up to 72 h (Fig. 3).

2.9. Reactivity of ch3G4 antibody with plastic-immobilized phospholipids (ELISA)

The immunoreactivity of ^{77}As[SATA]ch3G4 was tested with ELISA and compared with unlabelled and unmodified ch3G4 as positive control and ^{77}As[SATA]Rituxan as negative control.

Consequently, phospholipids were dissolved in *n*-hexane to a concentration of 50 μg/ml, 100 μl of this solution were added to wells of 96-well microtiter plates. After evaporation of the solvent in air, the plates were blocked for 2 h with 1% BSA diluted in PBS (binding buffer). The ch3G4 antibody was diluted in the binding buffer at an initial concentration of 33 nM. Serial two-fold dilutions were prepared in the plates (100 μl per well). The plates were then incubated for 1 h at room temperature. After washing with PBS, HRP goat anti-human IgG (diluted 1:2000) was used to detect ch3G4. Secondary reagents were detected by using chromogenic substrate OPD followed by reading plates at 490 nm using a microplate reader (Molecular Devices, Palo Alto, CA).

3. Results

As described previously [30,31], nca [*As]AsI_3 (* = 71, 72, 73, 74, 76, 77) could be ideal as a versatile labelling synthon for the coupling of radioactive arsenic isotopes to biomolecules. In this work, a new method for the labelling of mab, i.e. proteins, with radioactive arsenic isotopes via this labelling synthon was developed. Arsenic has a high affinity to sulphur and [*As]AsI_3 is able to bind covalently to sulfhydryl groups which could be shown on reference substances like cysteine and glutathione [32,33]. In antibodies, sulphur is mainly bonded to dithiol bridges. To increase the number of free thiols, the antibodies were modified with SATA (*N*-succinimidyl *S*-acetylthioacetate). The amount of free thiol groups per antibody molecule was calculated to be 3.5 using Ellmann's reagent and cysteine-based standards according to the protocol by Pierce Endogen [28].

Quality control of the labelling using radio-HPLC implementing a size-exclusion column indicated an almost complete transfer of *As to the SATA-modified proteins. As no free *As was detectable, the labelling yields after 30 min reaction time in general were above 99.9%, Fig. 2.

After a labelling time of 30 min, an aliquot of 20 μl of the ^{74}As[SATA]ch3G4-solution was given over a size-exclusion column for radio HPLC. The upper graph shows the UV spectrum, the lower the corresponding radioactivity progression. No free ^{74}As was detectable. Labelling yield was > 99.9%.

The in vitro stability of the radioarsenic label was evaluated by incubating the radioarsenic labelled protein in serum, containing an excess of other sulphide-containing proteins, followed by HPLC characterization. No release of radioarsenic from the labelled protein and no formation of antibody fragments were observed for incubation times up to 72 h, Fig. 3. This clearly indicates the chemical stability of the radioarsenic binding to the SATA-modified proteins.

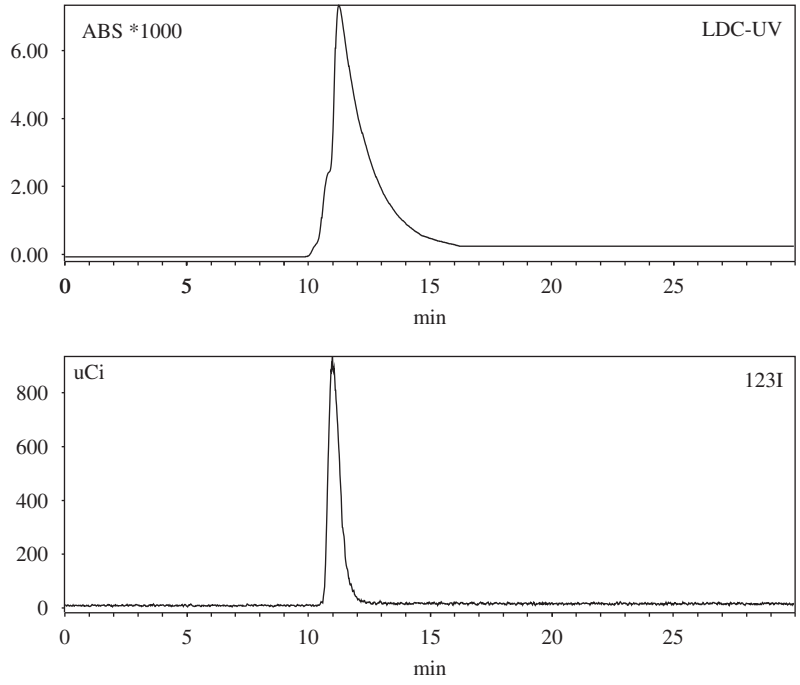

Fig. 2. Quality control of the labelling of ch3G4 with radioactive arsenic isotopes.

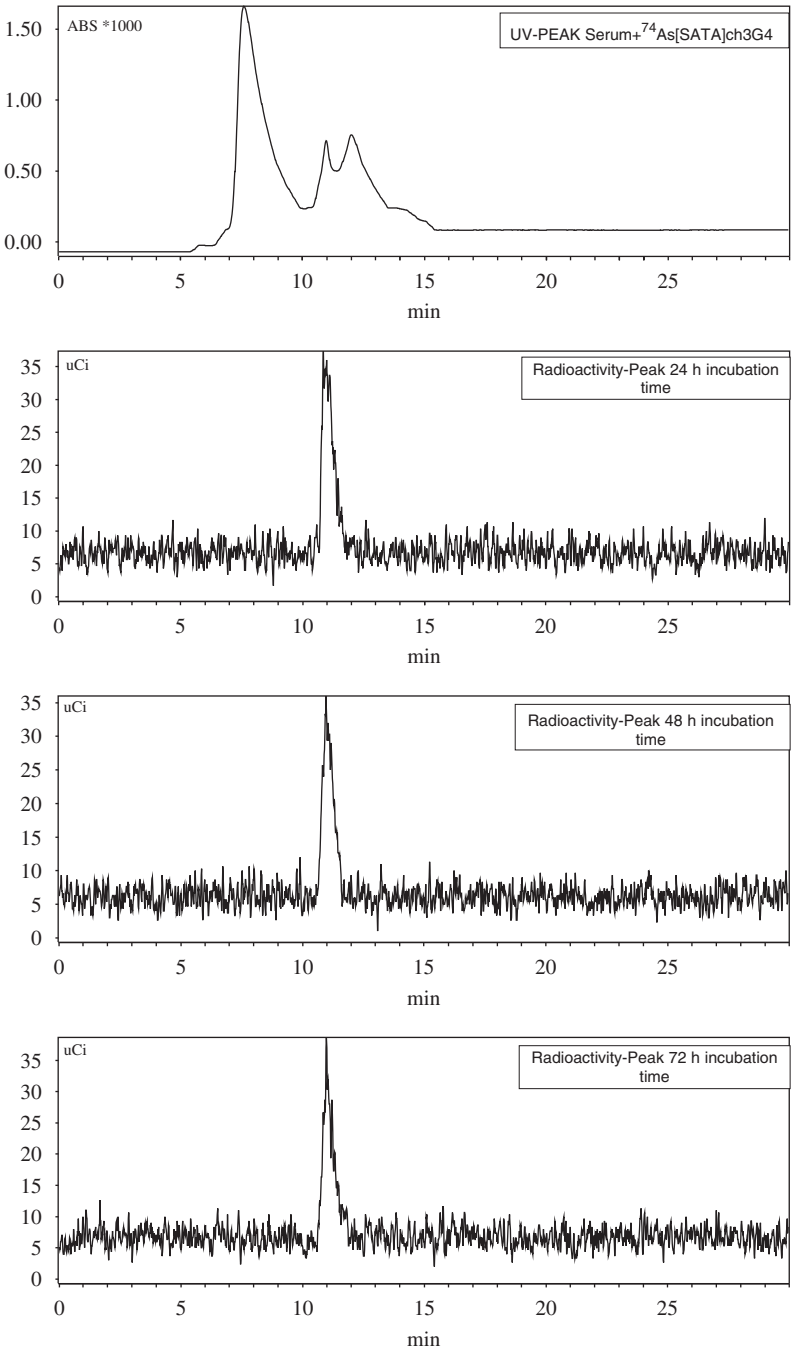

Fig. 3. In vitro stability of ^{74}As[SATA]ch3G4. The upper graph shows the UV spectrum. Because of the low concentration of the mab versus serum proteins, a typical serum profile is observed. The lower graph shows the corresponding radioactivity progression. The radioactivity peak remains unchanged in position and peak area.

The immunoreactivity of the labelled ch3G4 could be demonstrated using ELISA. No inhibition of immunoreactivity following SATA modification and subsequent labelling with nca [*As]AsI_3 could be observed (Fig. 4).

4. Conclusion

The new techniques introduced in this study are noteworthy for several reasons:

The developed labelling chemistry gives high yields (>99.9%), is reliable and could easily be transferred to automated labelling systems in a clinical environment. At least for the mab used in this work, this route of radioarsenic labelling does not affect the immunoreactivity of the product. The arsenic label stays stable for up to 72 h at the molecular mass of the mab, which is in particular relevant because of the molecular imaging strategy, namely to follow the pharmacology and pharmacokinetics of the labelled mab for several days.

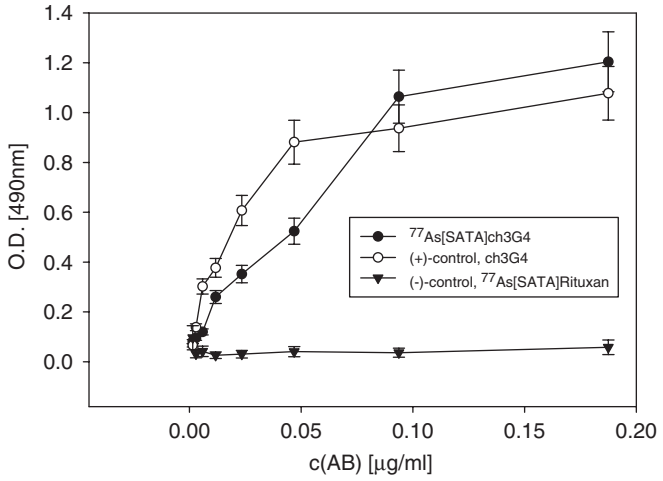

Fig. 4. Immunoreactivity of ^{77}As[SATA]ch3G4 as tested with an ELISA unlabelled and unmodified ch3G4 used as positive control and ^{77}As[SATA]Rituxan as negative control were used. No reduction of immunoreactivity through the applied SATA modification with subsequent radioarsenic labelling was detectable.

As demonstrated here for the two functionalised globulines, the mabs ch3G4 and Rituxan (Rituximab) as a proof of principle for the radioarsenic labelling chemistry of proteins, the described technique may be relevant to all sorts of proteins/antibodies with slow pharmacokinetics.

In addition to proteins/antibodies, the chemical approach might be transferred to other problems where the localization, follow-up and imaging of drugs over more then day-long periods is relevant, like e.g. stem-cell trafficking.

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