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Labeling and preliminary *in vivo* assessment of niobium-labeled radioactive species: A proof-of-concept study

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ABSTRACT

The application of radionuclide-labeled biomolecules such as monoclonal antibodies or antibody fragments for imaging purposes is called *immunoscintigraphy*. More specifically, when the nuclides used are positron emitters, such as zirconium-89, the technique is referred to as *immuno-PET*. Currently, there is an urgent need for radionuclides with a half-life which correlates well with the biological kinetics of the biomolecules under question and which can be attached to the proteins by robust labeling chemistry. ^{90}Nb is a promising candidate for *in vivo immuno-PET*, due its half-life of 14.6 h and low β^+ energy of $E_{mean} = 0.35$ MeV per decay. ^{95}Nb on the other hand, is a convenient alternative for longer-term *ex vivo* biodistribution studies, due to its longer half-life of ($t½ = 35$ days) and its convenient, lower-cost production (reactor-based production).
In this proof-of-principle work, the monoclonal antibody bevacizumab (Avastin®) was labeled with $^{95/90}$Nb and *in vitro* and *in vivo* stability was evaluated in normal Swiss mice and in tumor-bearing SCID mice.
Initial *ex vivo* experiments with ^{95}Nb-bevacizumab showed adequate tumor uptake, however at the same time high uptake in the liver, spleen and kidneys was observed. In order to investigate whether this behavior is due to instability of *Nb-bevacizumab or to the creation of other *Nb species *in vivo*, we performed biodistribution studies of ^{95}Nb-oxalate, ^{95}Nb-chloride and ^{95}Nb-Df. These potential metabolite species did not show any specific uptake, apart from bone accumulation for ^{95}Nb-oxalate and ^{95}Nb-chloride, which, interestingly, may serve as an "indicator" for the release of ^{90}Nb from labeled biomolecules. Concerning the initial uptake of ^{95}Nb-bevacizumab in non-tumor tissue, biodistribution of a higher specific activity radiolabeled antibody sample did show only negligible uptake in the liver, spleen, kidneys or bones. *In-vivo* imaging of a tumor-bearing SCID mouse after injection with ^{90}Nb-bevacizumab was acquired on an experimental small-animal PET camera, and indeed showed localization of the radiotracer in the tumor area. It is the first time that such results are described in the literature, and indicates promise of application of ^{90}Nb-labeled antibodies for the purposes of *immuno*-PET.
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1. Introduction

Targeted imaging of cancer is crucial to modern-day cancer management, and radionuclides attached to biomolecules are an exciting strategy for tumor diagnosis and therapy [1]. An attractive feature, but also the key challenge of this strategy is to select radionuclides and targeting vehicles with characteristics which are best suited for a particular application.

Angiogenesis is the development of new blood vessels from pre-existing ones. Angiogenesis is regulated by chemical signals of the organism, which function as an "angiogenesis switch", regulating the formation of new vasculature. Bevacizumab is a humanized monoclonal antibody (mAb) which binds all VEGF-A isoforms [2]. Currently many studies focus on labeling of bevacizumab with radionuclides for *in vivo* evaluation [3–7]. An important criterion for selecting of radionuclides for mAb is the half-life of the radionuclide which should favorably correlate to the biological kinetic of large-size biomolecules. Due to the long circulation of intact antibodies, optimal tumor-to-non tumor ratios

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can be reached from approximately 2 to 5 days post-injection. Therefore, radionuclides with an appropriate half-life should be chosen. Positron-emitting radionuclides with long and medium-long half-lives of interest for PET-imaging with antibodies and antibody fragments are, for example, ^{89}Zr ($t_{1/2}$ = 78.4 h) [8,9], ^{64}Cu ($t_{1/2}$ = 12.7 h) [10,11], ^{86}Y ($t_{1/2}$ = 14.7 h) [12], ^{76}Br ($t_{1/2}$ = 16.0 h) [13].

Several crucial factors and characteristics apply to radionuclide candidates for *immuno*-PET. The most important ones are: i) a physical half-life paralleling the biological half-life of the mAb or antibody fragment; ii) a high positron branching with no or weak accompanying other radiation (β^-, γ) to offer high-sensitivity PET imaging while reducing the radiation burden of the patient; iii) a preferably low β^+-energy to allow for high-resolution PET imaging; and iv) the availability of the radionuclide, i.e., an efficient production and radiochemical separation route.

In previous works we have reported a convenient way for production of ^{90}Nb–labeled biomolecules and proposed ^{90}Nb as a promising candidate for application in *immuno*-PET [14–17]. Its intermediate half-life of 14.6 h and a high positron branching of 53% may make ^{90}Nb an ideal candidate for application with antibody fragments, monoclonal antibodies, drug delivery systems and nanoparticles. Moreover, the chelate desferrioxamine has been identified as an excellent moiety to label *Nb to proteins [17,18].

In this work we report on the *in vitro* stability and *in vivo* behavior of $^{95/90}$Nb radiolabeled bevacizumab. Biodistribution studies of ^{95}Nb-oxalate, ^{95}Nb-chloride and ^{95}Nb-Df were performed in healthy mice, to provide more information on the fate of *Nb-labeled species *in vivo*.

2. Materials and methods

2.1. Materials

Reagents were purchased from Sigma-Aldrich (Germany) and used without further purification, unless otherwise stated. Deionized water (18 MΩ cm^{-1}) and ultra-pure HCl solution were used. No further special measures were taken regarding working under strict metal-free conditions. The mAb Bevacizumab (Avastin®, Roche) directed against the VEGF-A family of isoforms was bought from Roche Ellas S. A. (Greece). For the purification of conjugated and labeled antibodies, PD-10 columns (GE Healthcare Life Science) were applied, for ion exchange separation Aminex A27, 15 ± 2 μm and AG1x8, 200–400 mesh anionic exchange resins and DOWEX50x8, 200–400 mesh (BioRad) were used. For solid phase extraction, UTEVA® resin (Triskem Int., France) was applied.

The production yield, radionuclidic purity, radiochemical purity and separation yield of $^{95/90}$Nb were determined by γ-ray spectroscopy using an Ortec HPGe detector system and Canberra Genie 2000 software. The dead time of the detector was always kept below 10%. The detector was calibrated for efficiency at all positions with the certified standard solution QCY48, R6/50/38 (Amersham, UK).

VEGF165-transfected MDA MB 213 cells (M165) were kindly provided by Cancer Research UK. MDA MB 231 breast cancer cells were infected with virus expressing VEGF 165. The virus was made in Pheonix cells using the plasmid pLXRSpBMN-IRES-GFP. The VEGF clone is human. The cells are cultured at safety level I in minimum essential medium (Eagle) with 2 mM L-glutamine in the presence of 10% fetal bovine serum, at 37 °C in a humidified 5% CO_2 incubator.

Labeling efficiency and stability of the $^{95/90}$Nb labeled mAb was monitored by instant thin layer chromatography (iTLC) and high performance liquid chromatography (HPLC). ITLC was performed on chromatography strips (Biodex, NY). As mobile phase, 0.02 M citrate buffer (pH 5.0) was used. HPLC monitoring was performed on a Waters HPLC system using a TSKgel G3000SWXL size exclusion column (TOSOH Bioscience, Germany). As eluent, a mixture of 0.05 M sodium phosphate and 0.15 M sodium chloride (pH 6.8) solution was used at a flow rate of 0.8 mL/min.

Formation of ^{95}Nb-Df was measured *via* ITLC at conditions described above.

All numerical data were expressed as the mean of the values ± the standard error of the mean. Statistical analysis was performed using the t-test. A p value less than 0.05 was considered statistically significant.

2.2. Production of ^{90}Nb and ^{95}Nb

^{90}Nb was produced *via* the ^{90}Zr(p,n)^{90}Nb reaction at the cyclotron MC32NI of the German Cancer Research Center, Heidelberg. For irradiation, a stack of three disks of natural zirconium (natural abundance: 51.45% ^{90}Zr) foils of 10 mm diameter and a thickness of 0.25 mm each was used. Irradiation was performed at 20 MeV proton energy and a current of 5 μA for 1 h. This initial proton energy decreased, by using an aluminum holder cover of 0.5 mm thickness, to 17.5 MeV while entering the first foil of Zr. Twenty-four hours after end of irradiation (EOB), production yield and impurities were measured by gamma ray spectroscopy. The absolute activity of ^{90}Nb was calculated as average of its two gamma-lines at 141.2 keV (66.8% abundance) and 1129.2 keV (92.7%).

^{95}Nb ($t_{1/2}$ = 35 days) was employed for the *ex vitro* biodistribution experiments. ^{95}Nb was produced *via* the ^{94}Zr(n, γ) → ^{95}Zr(β^-, $t_{1/2}$ = 64 days) → ^{95}Nb reaction from natural zirconium granules (1–3 mm, 99.8% ChemPur, Germany). Neutron irradiations were performed at the BR2 reactor at the Belgian Nuclear Research Centre, Belgium and at BERII reactor at Helmholtz Centre in Berlin, Germany.

The production of the radionuclides ^{95}Zr/^{95}Nb and ^{90}Nb was monitored by gamma ray spectroscopy, *via* emissions at 724.2 keV (44.2%) and 756.7 keV (54.0%) for ^{95}Zr, and at 765.8 keV (100%) for ^{95}Nb and 1129 KeV (92%) for ^{90}Nb.

2.3. Separation and purification of n.c.a. $^{95/90}$Nb

2.3.1. First separation strategy

The first separation strategy was applied for biodistribution studies in tumor-bearing mice. The separation procedure was modified following the procedure described by Busse et al. [14]. In short, the zirconium metal target (260 ± 3 mg) was transferred into a 50 mL vial and 2 mL of water was added. Under ice-cooling, 48% HF (0.63 mL) was added in small portions. After complete dissolution, 10 M HCl (6 mL) and saturated boric acid (3.4 mL) were added. The $^{95/90}$Nb fraction was extracted with 0.02 M *N*-benzoyl-*N*-phenylhydroxylamine (BPHA) in $CHCl_3$ (5 mL) by vigorous stirring of the two phases in a 50 mL vial for 20 min. The aqueous phase was additionally washed with $CHCl_3$ (3 mL). The organic phases were combined and washed with a mixture of 9 M HCl/0.001 M HF (2 mL) and with 9 M HCl (2 mL) and finally extracted with aqua regia (5 mL).

For a final purification of $^{95/90}$Nb from remaining trace amounts of zirconium, an anionic exchange method was employed. After the aforementioned back extraction, the aqueous phase was evaporated to dryness. The residue was dissolved in a mixture of 0.25 M HCl/0.1 M oxalic acid (0.5 mL) and adsorbed onto a small Aminex A27 (15 ± 2 μm) anionic exchange column (20 × 1.5 mm). Elution was performed under slight overpressure of 0.3 bars. After loading, the column was washed with 10 M HCl (100 μL). Residues of Zr were removed by washing with a mixture of 9 M HCl/0.001 M HF (200 μL). $^{95/90}$Nb was eluted by a mixture of 6 M HCl/0.01 M oxalic acid (200 μL).

2.3.2. Alternative separation strategy

A second separation strategy was applied to provide a sample of ^{95}Nb with a higher radioactive concentration, for use in consequent biodistribution studies. Crude separation from the Zr target was applied following a published protocol [16]. In short, 2 mL of 21 M hydrofluoric acid containing the irradiated zirconium target was passed through the cation exchange resin (DOWEX 50 × 8, 100 mg, 200–400 mesh, 10 × 5