Cyclic versus Noncyclic Chelating Scaffold for $^{89}$Zr-Labeled ZEGFR:2377 Affibody Bioconjugates Targeting Epidermal Growth Factor Receptor Overexpression

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ABSTRACT: Zirconium-89 is an emerging radionuclide for positron emission tomography (PET) especially for biomolecules with slow pharmacokinetics as due to its longer half-life, in comparison to fluorine-18 and gallium-68, imaging at late time points is feasible. Desferrioxamine B (DFO), a linear bifunctional chelator (BFC) is mostly used for this radionuclide so far but shows limitations regarding stability. Our group recently reported on fusarinine C (FSC) with similar zirconium-89 complexing properties but potentially higher stability related to its cyclic structure. This study was designed to compare FSC and DFO head-to-head as bifunctional chelators for $^{89}$Zr-radiolabeled EGFR-targeting ZEGFR:2377 affibody bioconjugates. FSC-ZEGFR:2377 and DFO-ZEGFR:2377 were evaluated regarding radiolabeling, in vitro stability, specificity, cell uptake, receptor affinity, biodistribution, and microPET-CT imaging. Both conjugates were efficiently labeled with zirconium-89 at room temperature but radiochemical yields increased substantially at elevated temperature, 85 °C. Both $^{89}$Zr-FSC-ZEGFR:2377 and $^{89}$Zr-DFO-ZEGFR:2377 revealed remarkable specificity, affinity and slow cell-line dependent internalization. Radiolabeling at 85 °C showed comparable results in A431 tumor xenografted mice with minor differences regarding blood clearance, tumor and liver uptake. In comparison $^{89}$Zr-DFO-ZEGFR:2377, radiolabeled at room temperature, showed a significant difference regarding tumor-to-organ ratios. MicroPET-CT imaging studies of $^{89}$Zr-FSC-ZEGFR:2377 as well as $^{89}$Zr-DFO-ZEGFR:2377 confirmed these findings. In summary we were able to show that FSC is a suitable alternative to DFO for radiolabeling of biomolecules with zirconium-89. Furthermore, our findings indicate that $^{89}$Zr-radiolabeling of DFO conjugates at higher temperature reduces off-chelate binding leading to significantly improved tumor-to-organ ratios and therefore enhancing image contrast.

KEYWORDS: FSC, DFO, zirconium-89, EGFR, affibody, PET
peptide-based setting are not entirely translatable for the evaluation of larger biomolecules. Imaging probes based on nonimmunoglobulin engineered scaffold proteins (ESP) represent a higher level of complexity compared to radiolabeled short peptides. Unlike peptides, ESP possess tertiary structure. Although ESP usually refold after denaturing (e.g., under harsh radiolabeling conditions), chelators or other pendant group might interfere with refolding resulting in appreciable loss of binding capacity. An intricate spatial structure of ESP and larger size (molecular weight of 4 to 16 kDa) might lead to formation of “chelator pockets” of electron donating side-chains, which compete for binding of a radiometal with a bifunctional chelator. Although ESP-based probes can often provide good imaging a few hours after injection, in some cases one or several days are required to reach an appropriate contrast. A long-lived positron-emitting label would be attractive for such ESP. Therefore, a head-to-head comparison of FSC and DFO (structures are presented in Figure 1) conjugated to an antibody molecule targeting epidermal growth factor receptor (EGFR) was initiated.

Figure 1. Structures of maleimide-derivatized FSC (A) and DFO (B).

EGFR is a transmembrane tyrosine kinase (TK) and as a promoter for cell proliferation involved in various malignancies like nonsmall-cell lung cancer (NSCLC), head and neck squamous cell carcinoma (HNSCC), colorectal cancer, glioblastoma, pancreatic, and breast cancer. Detection of EGFR overexpression enables to stratify patients with NSCLC and HNSCC for an adequate therapy due to clonal selection makes leading to EGFR overexpression and the discrepancy in primary pathway of EGFR, the susceptibility for different mutations like nonsmall-cell lung cancer (NSCLC), head and neck squamous cell carcinoma (HNSCC), colorectal cancer, glioblastoma, pancreatic, and breast cancer. Detection of EGFR overexpression enables to stratify patients with NSCLC and HNSCC for an adequate therapy due to clonal selection makes leading to EGFR overexpression and the discrepancy in primary pathway of EGFR, the susceptibility for different mutations.

**Experimental Section**

**Analytical RP-HPLC.** Reversed-phase high-performance liquid chromatography analysis was carried out on an UltiMate 3000 RS UHPLC pump, UltiMate 3000 autosampler, Ultimate 3000 column compartment (25 °C oven temperature), UltiMate 3000 Variable Wavelength Detector (Dionex, Germering, Germany) using acetonitrile (ACN)/H2O/0.1% trifluoroacetic acid (TFA) as mobile phase with varied settings.

A. ACE 3 μM C18 100 Å 150 × 3.0 mm (ACE, Aberdeen, Scotland) column; flow rate of 0.6 mL/min; UV detection at λ = 220 nm; gradient: 0.0–1.0 min 10% ACN, 1.0–10.0 min 10–30% ACN, 10.0–11.0 min 30–60% ACN, 11.0–13.0 min 60% ACN, 13.0–17.0 min 60–100% ACN.

B. Jupiter 4 μM Proteo 90 Å 250 × 4.6 mm (Phenomenex Ltd. Aschaffenburg, Germany) column; flow rate of 1 mL/min; UV detection at λ = 220 nm; gradient: 0.0–3.0 min 0% ACN, 3.0–23.0 min 0–80% ACN, 23.0–25.0 min 80% ACN, 25.0–30.0 min 0% ACN.

C. Jupiter 5 μM C18 300 Å 150 × 4.6 mm (Phenomenex Ltd. Aschaffenburg, Germany) column; flow rate of 2 mL/min; UV detection at λ = 280 nm; gradient: 0.0–1.0 min 30% ACN, 1.0–15.0 min 30–60% ACN, 15.0–17.0 min 60% ACN, 17.0–20.0 min 60–30% ACN.

**Preparative RP-HPLC.** Sample purification via RP-HPLC was performed on a Gilson 322 Pump with a Gilson UV/simultaneous detector and the single fractions were collected by a PrepFC automatic fraction collector (Gilson, Middleton, WI, USA). UV detection at λ = 220 nm and a flow rate of 2 mL/min on a Eurosil Bioselect Vertex Plus 30 × 8 mm 5 μM C12A 300 Å precolumn and Eurosil Bioselect Vertex Plus 300 × 8 mm 5 μM C18A 300 Å column (Knauer, Berlin, Germany) were used.
applied using following ACN/H₂O/0.1% TFA multistep gradients.

**Gradient A.** 0.0–1.0 min 0% ACN, 1.0–20.0 min 0–50% ACN, 20.0–23.0 min 50% ACN, 23.0–25.0 min 0% ACN

**Gradient B.** 0.0–1.0 min 20% ACN, 1.0–25.0 min 20–60% ACN, 25.0–30.0 min 60% ACN, 30.0–35.0 min 20 % ACN

**Gradient C.** 0.0–1.0 min 20% ACN, 1.0–45.0 min 20–80% ACN, 45.0–50.0 min 100% ACN, 50.0–55.0 min 20 % ACN

**MALDI-TOF MS.** Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was performed on a Bruker microflex benchtop MALDI-TOF MS (Bruker Daltonics, Bremen, Germany). Samples were prepared on a micro scout target (MSP96 target ground steel BC, Bruker Daltonics, Bremen, Germany) equipped with a Nanospray Flex ionization source. The samples were separated on a homemade fritless Bremen, Germany) equipped with a Nanospray Flex ionization source. All spectra were recorded by summarizing 800 laser shots per spot and Flex Analysis 2.4 software was used for data processing.

**ESI-MS.** Samples were purified by ZipTip C18 pipet tips (Millipore) according to the manufacturer’s instructions prior to nanoLC-ESI-MS analysis. Purified samples were analyzed using an UltiMate 3000 nano-HPLC system coupled to a Q Exactive HF mass spectrometer (both Thermo Scientific, Bremen, Germany) equipped with a Nanospray Flex ionization source. The samples were separated on a homemade fritless fused-silica microcapillary column (75 μm i.d. × 280 μm o.d. × 10 cm length) packed with 3 μm reversed-phase C18 material (Reprosil). Solvents for HPLC were 0.1% formic acid (solvent A) and 0.1% formic acid in 85% acetonitrile (solvent B). The gradient profile was as follows: 0–2 min, 4% B; 2–40 min, 4–50% B; 40–45 min, 50–100% B, and 45–50 min, 100% B. The flow rate was 250 nL/min. The Q Exactive HF mass spectrometer was operating in the data dependent mode selecting the top 20 most abundant isotope patterns with charge >1 from the survey scan with an isolation window of 1.6 mass-to-charge ratio (m/z). Survey full scan MS spectra were acquired from 300 to 1750 m/z at a resolution of 60,000 with a maximum injection time (IT) of 120 ms, and automatic gain control (AGC) target 1e6. The selected isotope patterns were fragmented by higher-energy collisional dissociation (HCD) with normalized collision energy of 28 at a resolution of 30,000 with a maximum IT of 120 ms, and AGC target 5e5.

**Precursor Preparation.** [Fe]Fusarinine C ([Fe][FSC]). Fusarinine C (FSC) could be obtained from fungal culture in good yield as previously described.45 Brieﬂy, the iron saturated culture media was passed through a C18 cartridge and the [Fe][FSC] was eluted with methanol to give a red brown colored solid after evaporating the organic solvent. A sufﬁcient purity (>90%) permitted its use for further derivatization without additional puriﬁcation. Analytical data: RP-HPLC (setting A) t_R = 6.3 min; MALDI TOF-MS: m/z [M+H]+ = 780.86 [C_{30}H_{44}FeN_{12}O_{14}] exact mass: 779.63 (calculated). N,N'-Diacctyl-Fe[Fusarinine C ([dAc[Fe][FSC]])]. An aliquot of 500 μL [Fe][FSC] (32 mM) was dissolved in MetOH/DMAF (9:1) and reacted with 5 μL of acetic anhydride (7.5 mg, 0.07 mmol) for 5 min at room temperature (RT) under vigorously stirring. After analytical RP-HPLC analysis (setting A), the resulting mixture of mono- (t_R = 7.7 min), di- (t_R = 9.3 min), and triacetylfusarinine C (t_R = 10.7 min) in a ratio of 1:2.1:2 was immediately puriﬁed via preparative RP-HPLC using gradient A (t_R = 19.4 min) to obtain [dAc[Fe][FSC] in high purity (>95%). MALDI TOF-MS: m/z [M+H]+ = 864.68 [C_{37}H_{55}FeN_{6}O_{14}] exact mass: 863.709 (calculated).

**Preparation of EGFR-Targeting Bioconjugates.** Single C-terminal cysteine bearing afibody molecule (anti-EGFR ZEGFR:2377) was produced as previously described and was conjugated to three-hydroxamate bifunctional chelators via maleimide-sulphonyl cross-link reaction to facilitate radiolabeling with zirconium-89. Conjugation of acyclic deferoxamine (DFO) to provide DFO-ZEGFR:2377 was conducted according to Garousi and co-workers.42 Coupling of cyclic fusarinine C derivative ([dAc-FSC]-mal) to provide DFO-ZEGFR:2377 was carried out as follows. The disульﬁde bond stabilized anti-EGFR ZEGFR:2377-dimer (ESI-MS 14780.25 Da) was dissolved in phosphate buffered saline (PBS, pH 7.4) to a concentration of 1 mg/mL and after a 10-fold molar excess of trif(2-carboxyl-bisphosphino)hydrochloride (TCEP, Sigma-Aldrich, Handels GmbH, Vienna, Austria) freshly prepared in 100 μL PBS, the mixture was incubated at 37 °C to reduce intermolecular disulﬁde bonds. After 1 h incubation time, reduction completion was conﬁrmed by ESI-MS (7390.68 Da) and reduced afibody molecules were puriﬁed via size exclusion chromatography by using disposable, PBS/BSA (0.1%) pre-equilibrated PD-10 column (GE Healthcare, Vienna, Austria) according to manufacturer’s protocol. Hereafter 20-fold molar excess of [dAc-FSC]-mal was dissolved in PBS and added to anti-EGFR ZEGFR:2377 containing fraction. After 2 h reaction time at RT, when ESI-MS showed complete consumption of unconjugated afibody molecule the bioconjugate (further designated as FSC-ZEGFR:2377) was isolated by preparative RP-HPLC (gradient C; t_R = 27.2 min) and was obtained as colorless solid after lyophilization (0.18 μmol; 66.4% of theoretical yield). Purity of the bioconjugate (>96%) was conﬁrmed by analytical RP-HPLC (setting B; t_R = 17.01 min and setting C: t_R = 7.99 min) and ESI-MS analysis to conﬁrm the identity was in good agreement with the expected value (ESI-MS 8352.12 Da measured; 8352 Da calculated).

**Radiolabeling with Zirconium-89.** 89Zr-oxalic acid solution (1M) was purchased from PerkinElmer (Waltham, US). ZEGFR:2377 targeting bioconjugates were labeled with zirconium-89 according to following protocol, corresponding to a modiﬁed variant of Vosjan and co-workers.46 89Zr solution (8.7 μL, 10–12 MBq) was neutralized by adding 8.3 μL of Na₂CO₃ (1M) and incubated for 3 min at RT. Hereafter, 66.7 μL of HEPES buffer (0.5M; pH 6.98) was added to the radionuclide containing solution. After subsequent addition of 20 μL of bioconjugate solution (1 μg/μL in H₂O/ETOH 90/10 v/v), the resulting mixture was incubated up to 120 min at 28 and 85 °C, respectively. Radiolabeling was monitored by radioactivity.
instant thin layer chromatography (radio-ITLC) using silica gel-impregnated glass microfiber sheets (ITLC-SG strips, Varian, Lake Forest, CA) as stationary and 0.2 M citric acid (pH 5) as mobile phase. Distribution of the radioactivity among the strips was measured on Cyclone Phosphor Storage Screen using OptiQuant software for data processing (both Packard Instrument Company, Meriden, CT, US) as well as Fujifilm Bioimaging Analyzers (BAS) 1800II using MultiGauge V3.0 analysis software (both Fujifilm, Tokyo, Japan). Radiolabeled bioconjugates were purified for further studies using phosphate buffer saline pre-equilibrated NAP-5 size exclusion columns (GE Healthcare, Uppsala, Sweden) according to manufacturer's protocol.

EDTA-Challenging Assessment. Aliquots of 50 μL of zirconium-89 labeled NAP-5 purified tracers (1.8 nmol) were incubated in duplicates with 1000-fold excess of ethylenediaminetetraacetic acid (EDTA) and in PBS as a control. Transchelation ability was evaluated at 1, 2, and 24 h via radio-ITLC measurement as described above, where radiolabeled conjugate remains at the start while 89Zr-EDTA migrates with the solvent front.

SDS-PAGE. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out on XCell SureLock system at 200 V constant for 30 min using NuPAGE 12% Bis-Tris Gel, LDS sample and MES running buffer (Invitrogen AB Foster City, CA, USA).

In Vitro Studies. High level EGFR-expressing A431 human epidermoid carcinoma and MDA468 human breast cancer as well as low level EGFR-expressing DU-145 human prostate cancer cell lines were purchased from American Type Culture Collection (ATCC, via LGC Promochem, Borås, Sweden).

Cells were grown in a humidified atmosphere of 95% air/5% carbon dioxide using RPMI-1640 media supplemented with 10% v/v fetal bovine serum (FBS) and 1% v/v Penicillin-Streptomycin-Glutamin (PSG) solution (= complete media).

For binding specificity studies, cells were seeded in 6-well plates (4 × 10⁵ cells/dish for A431 and MDA-468; 1 × 10⁶ cells/dish for DU-145) 3 days before the experiment. On the day of the experiment media was aspirated and all dishes were washed with 2 mL of RPMI-1640 media without additives (= incomplete media). Three sets of dishes were blocked by adding 1 mL complete media containing 0.5 μM blocking agent (cetuximab, His6-ZEGFR:2377 a body molecule or bevacizumab in 100-fold molar excess over radioligand) while three dishes remained unblocked containing 1 mL complete media. Finally cells were washed with 1 mL incomplete media and 1 mL complete media was added. Finally cells were incubated with 1 mL radioligand solution (5 nM in complete media) at 37 °C for 1, 2, 4, 8, and 24 h in triplicates. At the appointed time, the incubation media was aspirated from the dishes, cells were washed with 2 mL ice-cold incomplete media and the supernatant was discarded. To determine the membrane-bound amount of radiolabeled conjugate cells were treated at RT as follows. First cells were incubated for 15 min with 1 mL acid wash buffer (0.1 M glycine buffer, pH 2.5, containing 4 M urea solution) and after collecting the supernatant this step was repeated with 1 min incubation and acid wash fractions were pooled. After this, cells were rinsed twice with 1.5 mL PBS buffer combining the fractions. Subsequently after PBS buffer collection 1.5 mL of phosphate buffer (pH 8.0) was added to each dish and cells were incubated for 15 min. Phosphate buffer was collected followed by a second basic wash step and phosphate buffer fractions were pooled. To determine the internalized amount of radioligand cells were lysed by adding 1 mL of sodium hydroxide solution (1 M NaOH) and dishes were incubated at 37 °C for at least 45 min. NaOH fractions were collected and the step was repeated without incubation but using a cell scraper. Alkaline fractions were pooled and all fractions were taken for gamma-counter measurement.

Real-time receptor interaction measurements were carried out on LigandTracer Yellow instrument (Ridgeview Instruments AB, Vänge, Sweden) according to previous published protocol. Briefly, living A431 cells were incubated with increasing concentrations (0.33, 1, and 3 nM) of zirconium-89 labeled EGFR-targeting bioconjugates to determine binding kinetics. After replacement of incubation media by fresh cell culture media the dissociation rate was monitored and the obtained data were analyzed using InteractionMap software (Ridgeview Diagnostics AB, Uppsala, Sweden) to calculate receptor affinity.

In Vivo Studies. Biodistribution and imaging studies in tumor xenograft bearing female outbred BALB/c nu/nu mice (Taconic M&B a/S, Ry, Denmark) were conducted under xylazine/ketamine anesthesia to minimize animal suffering and number of animals was reduced as much as possible, this being in accordance with Swedish national regulation on laboratory animals’ protection and approval by the Ethics Committee for Animal Research in Uppsala (permission C 4/2016). For tumor xenograft preparation, 1 × 10⁷ A431 cells were injected subcutaneously to the right hind leg and the tumors were grown for 2 weeks. For biodistribution experiments mice were randomized into five groups with four mice. Only one group was pretreated by subcutaneous injection of 10 mg cetuximab per mouse 24 h before the experiment to determine in vivo specificity of 89Zr-FSC-ZEGFR:2377 as specificity of 89Zr-DFO-ZEGFR:2737 has been shown before. Zirconium-89 labeled bioconjugates were injected to mice via tail vain (40 kbq in 100 μL PBS per mouse) and the tracer dose was adjusted to 38 μg per mouse with nonlabeled bioconjugate. Distribution of radioactivity among blood and organs was measured 3 and 24 h post injection (p.i.).

Whole body positron emission tomography (PET)/computed tomography (CT) imaging was performed using Triumph trimodality system (TriFoil Imaging, Inc., Northridge, CA, USA) at 3 and 24 h p.i. The animals (n = 2 per time point) were injected with 4 MBq of zirconium-89 labeled bioconjugate (dose adjustment with nonlabeled tracer to 38 μg per mouse) and sacrificed by CO₂ asphyxiation immediately before being
placed in the camera. The CT scans were performed at the following parameters: 80 mm field of view (FOV); 1:48 magnification; one frame and 512 projections for 2.13 min followed by PET scans conducted for 1 h. CT raw files were reconstructed by filter back projection (FBP). PET data were reconstructed using OSEM-3D (20 iterations). CT data were used for scatter and attenuation correction. PET and CT files were fused and analyzed using PMOD v3.510 (PMOD Technologies Ltd., Zurich, Switzerland). Coronal and sagittal PET-CT images are presented as maximum intensity projections (MIP) in RGB color scale.

** Autoradiography. After the gamma-counter measurement was completed, tumors were frozen at ∼80 °C and embedded in a cryomedium. Frozen tumors were cut in serial sections (20 μm thick) using a cryomicrotome and thaw-mounted on glass slides. For the digital autoradiography, the slides with sections were put in an X-ray cassette and exposed to phosphor screens overnight. The screens were scanned on a Cyclone Storage Phosphor System at a resolution of 600 dpi and analyzed using the OptiQuant software (PerkinElmer, USA).

After that, the same tumor sections were stained using hematoxylin and eosin following a standard protocol. Digital scanning of hematoxylin and eosin stained sections at 5× magnification was performed at the SciLifeLab Tissue Profiling Facility, Uppsala University. Digital images were analyzed using Aperio ImageScope software (Leica Biosystems, USA).

** Statistical Analysis. ** In order to determine statistical differences, two-tailed t test was performed using GraphPad Prism (version 4.00 for Windows GraphPad Software).

### RESULTS

** Precursor Synthesis. ** Preparation of EGFR targeting bioconjugates was straightforward utilizing maleimide-sulfhydryl cross-link reaction for chelator-affibody conjugation. DFO-ZEGFR:2377 and FSC-ZEGFR:2377, respectively, were obtained in good yield and high chemical purity (>95%) and ESI-MS analysis was in excellent agreement with theoretical calculations (see Supporting Information).

** 89Zr Radiolabeling and Stability. ** Radiolabeling of FSC-ZEGFR:2377 as well as DFO-ZEGFR:2377 with zirconium-89 in HEPES buffer at neutral pH was performed according to the protocol of Vojsan and co-workers with slight variations (Table 1). Both bioconjugates showed slow complex formation over a period of 2 h at 28 °C while the labeling rate could be increased significantly when the reaction was carried out at 85 °C. The radiochemical yield (RCY) was already >96% after 30 min when elevated temperature was used whereas the corresponding RCY was around 60% for 89Zr-FSC-ZEGFR:2377 and around 50% for 89Zr-DFO-ZEGFR:2377 at 28 °C. After quantitative radiolabeling (RCY > 90%), 89Zr-FSC-ZEGFR:2377 and 89Zr-DFO-ZEGFR:2377 were purified via disposable NAP-5 columns with a recovery rate ranging from 70 to 85% and a radiochemical purity of >99%. Radio-SDS-PAGE analysis confirmed the purity the isolated bioconjugates. Stability assessment of 89Zr-labeled conjugates is summarized in Table 2. 89Zr-FSC-ZEGFR:2377 labeled at 28 °C and after radiolabeling at elevated temperature increased the complex stability in both media (>97%).

### Table 1. Radiolabeling of FSC-ZEGFR:2377 and DFO-ZEGFR:2377 with Zirconium-89 (2.4 nmol, pH 7 at 28 °C and 85 °C)

<table>
<thead>
<tr>
<th>Time [min]</th>
<th>FSC-ZEGFR:2377</th>
<th>DFO-ZEGFR:2377</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>28 °C</td>
<td>85 °C</td>
</tr>
<tr>
<td>10</td>
<td>33.7 ± 10.9</td>
<td>83.6 ± 7.9**</td>
</tr>
<tr>
<td>30</td>
<td>63.2 ± 11.6</td>
<td>96.8 ± 1.8**</td>
</tr>
<tr>
<td>45</td>
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<td>n.d</td>
</tr>
<tr>
<td>60</td>
<td>82.7 ± 10.3</td>
<td>97.7 ± 0.1</td>
</tr>
<tr>
<td>90</td>
<td>88.1 ± 7.4</td>
<td>98.6 ± 1.0</td>
</tr>
<tr>
<td>120</td>
<td>91.9 ± 5.8</td>
<td>n.d</td>
</tr>
</tbody>
</table>

* Data were calculated from ITLC measurement of the nonpuriﬁed bioconjugates and are presented as mean ± standard deviation (n = 4), statistical analysis was performed using the Student’s t test with P values indicating very signiﬁcant (** P < 0.01) and signiﬁcant (*) P < 0.05) difference between various temperature.

### Table 2. Stability of 89Zr-FSC-ZEGFR:2377 and 89Zr-DFO-ZEGFR:2377 in PBS and EDTA

<table>
<thead>
<tr>
<th>Time [h]</th>
<th>89Zr-FSC-ZEGFR:2377</th>
<th>89Zr-DFO-ZEGFR:2377</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>28 °C</td>
<td>85 °C</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>EDTA</td>
</tr>
<tr>
<td>1</td>
<td>96.8 ± 0.1</td>
<td>94.6 ± 0.3</td>
</tr>
<tr>
<td>2</td>
<td>96.5 ± 0.5</td>
<td>93.5 ± 0.5</td>
</tr>
<tr>
<td>24</td>
<td>90.2 ± 0.1</td>
<td>85.7 ± 0.4</td>
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<tr>
<td></td>
<td>99.3 ± 0.2</td>
<td>95.9 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>90.7 ± 4.5</td>
<td>81.7 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>88.3 ± 1.3</td>
<td>67.7 ± 3.6</td>
</tr>
</tbody>
</table>

* Data were calculated from ITLC measurement and are presented as average ± maximum error (n = 2).
Receptor specificity for both conjugates is summarized in Figure 3. 89Zr-FSC-ZEGFR:2377 showed 18.7 ± 0.9% (A431) and 24.7 ± 0.4% (MDA468) of total added activity bound to the cells after 1 h-incubation. Corresponding studies with 89Zr-DFO-ZEGFR:2377 resulted in 17.7 ± 0.3% (A431) and 23.8 ± 0.2% (MDA468) cell-associated radioactivity. Pretreatment of A431 and MDA468 cells with EGFR specific antibody cetuximab led to a highly significant (<0.3% of total activity; P < 1 × 10⁻⁶) reduction of binding of 89Zr-labeled antibody bioconjugates indicating highly specific targeting properties (Figure 3A). Furthermore, extended specificity studies (Figure 3B) with 89Zr-FSC-ZEGFR:2377 showed level of EGFR-expression dependent cell binding. Receptor saturation with cetuximab and ZEGFR:2377 antibody molecule (His6-ZEGFR:2377) resulted again in highly significant reduction (P < 2 × 10⁻⁶) of 89Zr-FSC-ZEGFR:2377 binding to the cells while blocking studies with bevacizumab showed no significant reduction, as this antibody is vascular endothelial growth factor (VEGF) and not EGFR specific, substantiating the highly specific EGFR mediated cell binding of FSC-ZEGFR:2377 biomolecule. Cellular processing profile of 89Zr-FSC-ZEGFR:2377 and 89Zr-DFO-ZEGFR:2377 by MDA468 and A431 cells is presented in Figure 4. Both bioconjugates showed very similar internalization, which was cell-line dependent and relatively slow as the uptake of cell-bound radioactivity was ~6% in A431 and ~9% in MDA468 cells after 24 h-incubation.

**In Vivo Characterization.** Specificity of 89Zr-FSC-ZEGFR:2377 in tumor xenografted mice is presented in Figure 5 while in vivo specificity of 89Zr-DFO-ZEGFR:2377 has already been reported by Garousi and co-workers. Pretreatment of the mice with cetuximab as blocking agent resulted in a nonblocked-to-blocked tumor ratio of ~12 revealing high specificity of 89Zr-FSC-ZEGFR:2377 binding to EGFR expressing malignant tissue. Ex vivo biodistribution of 89Zr-FSC-ZEGFR:2377 and 89Zr-DFO-ZEGFR:2377 labeled at elevated temperature as well as previous published 89Zr-DFO-ZEGFR:2377 labeled at ambient temperature in A431 tumor xenografted nude mice 3 and 24 h after injection is shown in the...
Table 3. Biodistribution of $^{89}$Zr-FSC-ZEGFR:2377 and $^{89}$Zr-DFO-ZEGFR:2377 in EGFR-Expressing A431 Tumor Xenografted Female BALB/C nu/nu Mice Expressed as Percentage of Injected Dose per Gram Tissue (% ID/g; Mean ± SD; n = 4)\(^a\)

<table>
<thead>
<tr>
<th>organs</th>
<th>$^{89}$Zr-FSC-ZEGFR:2377 (radiolabeling at $85^\circ$C)</th>
<th>$^{89}$Zr-DFO-ZEGFR:2377 (radiolabeling at $85^\circ$C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 h</td>
<td>24 h</td>
</tr>
<tr>
<td>blood</td>
<td>1.02 ± 0.29(^b)</td>
<td>0.31 ± 0.08(^c)</td>
</tr>
<tr>
<td>salivary gland</td>
<td>1.18 ± 0.15</td>
<td>1.08 ± 0.19</td>
</tr>
<tr>
<td>lung</td>
<td>1.43 ± 0.06</td>
<td>0.88 ± 0.17</td>
</tr>
<tr>
<td>liver</td>
<td>6.71 ± 1.38</td>
<td>5.71 ± 0.94</td>
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<tr>
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<td>1.38 ± 0.14</td>
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<td>1.19 ± 0.2</td>
</tr>
<tr>
<td>kidney</td>
<td>291.04 ± 19.8</td>
<td>251.54 ± 23.87</td>
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<tr>
<td>tumor</td>
<td>8.42 ± 1.53(^b)</td>
<td>5.25 ± 0.83(^a)</td>
</tr>
<tr>
<td>muscle</td>
<td>0.30 ± 0.04</td>
<td>0.24 ± 0.03(^*)</td>
</tr>
<tr>
<td>bone</td>
<td>1.00 ± 0.09</td>
<td>0.99 ± 0.26</td>
</tr>
</tbody>
</table>

$^a$Statistical analysis was performed using the Student’s t test with P values indicating significant (P < 0.05) difference (+) between 3 and 24 h, (*) between $^{89}$Zr-FSC-ZEGFR:2377 (85 °C) and $^{89}$Zr-DFO-ZEGFR:2377 (85 °C), (§) between $^{89}$Zr-FSC-ZEGFR:2377 (85 °C) and $^{89}$Zr-DFO-ZEGFR:2377 (RT) and (#) between $^{89}$Zr-DFO-ZEGFR:2377 (RT) at corresponding time points.

Table 4. Tumor-to-Organ Ratios of $^{89}$Zr-FSC-ZEGFR:2377 and $^{89}$Zr-DFO-ZEGFR:2377 in BALB/C Nude Mice Bearing EGFR-Expressing A431 Tumor Xenograft (Mean ± SD; n = 4)\(^a\)

<table>
<thead>
<tr>
<th>organs</th>
<th>$^{89}$Zr-FSC-ZEGFR:2377 (radiolabeling at $85^\circ$C)</th>
<th>$^{89}$Zr-DFO-ZEGFR:2377 (radiolabeling at $85^\circ$C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 h</td>
<td>24 h</td>
</tr>
<tr>
<td>blood</td>
<td>9.0 ± 3.3(^e)</td>
<td>17.5 ± 5.6(^e)</td>
</tr>
<tr>
<td>salivary gland</td>
<td>7.3 ± 2.1(^f)</td>
<td>5.0 ± 1.6</td>
</tr>
<tr>
<td>lung</td>
<td>5.9 ± 1.3(^a)</td>
<td>6.0 ± 1.2(^a)</td>
</tr>
<tr>
<td>liver</td>
<td>1.3 ± 0.4</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>spleen</td>
<td>5.6 ± 1.2</td>
<td>3.8 ± 0.4</td>
</tr>
<tr>
<td>colon</td>
<td>6.4 ± 1.3(^a)</td>
<td>4.5 ± 1.2</td>
</tr>
<tr>
<td>kidney</td>
<td>0.03 ± 0.006</td>
<td>0.03 ± 0.00</td>
</tr>
<tr>
<td>muscle</td>
<td>27.9 ± 4.9(^a)</td>
<td>21.9 ± 2.2(^a)</td>
</tr>
<tr>
<td>bone</td>
<td>8.5 ± 1.8(^e)</td>
<td>5.6 ± 1.8(^e)</td>
</tr>
</tbody>
</table>

$^a$Statistical analysis was performed using the Student’s t test with P values indicating significant (P < 0.05) difference (+) between $^{89}$Zr-FSC-ZEGFR:2377 (85 °C) and $^{89}$Zr-DFO-ZEGFR:2377 (85 °C), (§) between $^{89}$Zr-FSC-ZEGFR:2377 (85 °C) and $^{89}$Zr-DFO-ZEGFR:2377 (RT) and (#) between $^{89}$Zr-DFO-ZEGFR:2377 (RT) at corresponding time points.

Both conjugates labeled at 85 °C showed rapid blood clearance over time, being consistent with the report of Garousi et al.

$^{89}$Zr-FSC-ZEGFR:2377 showed increased tumor-to-organ ratios for all organs except from liver and spleen 3 h p.i. and except from liver, salivary gland and colon 24 h p.i. compared to $^{89}$Zr-DFO-ZEGFR:2377 labeled at room temperature. Somewhat lower uptake in salivary gland, liver, spleen and muscle compared to $^{89}$Zr-FSC-ZEGFR:2377 and lung and muscle compared to $^{89}$Zr-DFO-ZEGFR:2377 labeled at room temperature. Corresponding tumor-to-organ ratios as a predictor for imaging contrast are presented in Table 4. Comparing $^{89}$Zr-FSC-ZEGFR:2377 and $^{89}$Zr-DFO-ZEGFR:2377 the resulting ratios were not significantly different at both time points except from tumor-to-liver ratio which was 1.4 fold higher for $^{89}$Zr-DFO-ZEGFR:2377.
do not offer additional binding pockets for radionuclides. In this study radiolabeling at room temperature was sufficient for stable coordination of zirconium-89 as animal experiments showed generally low bone uptake (∼1%) without increasing over time (e.g., 1, 2, and 4 h).16

FSC-conjugation did not alter receptor binding or specificity as compared to DFO. Both bioconjugates showed high EGFR affinity (Figure 1) in the picomolar range, being in good agreement with earlier findings.41,42 Highly specific receptor binding correlating with the level of EGFR-expression for 89Zr-FSC-ZEGFR:2377 is shown in Figure 2. Internalization on A431 cells (Figure 3) was slow and somewhat lower compared to previous reports on 89Zr-DFO-ZEGFR:2377,42 99mTc-ZEGFR:2377,41 and 111In-DOTA-ZEGFR:2377.43 In vivo specificity results of 89Zr-FSC-ZEGFR:2377 were similar to the data from Garousi and co-workers42 except from liver uptake. 89Zr-FSC-ZEGFR:2377 liver uptake was 2-fold higher compared to 89Zr-DFO-ZEGFR:2377 and showed no significant reduction after EGFR presaturation with cetuximab, indicating that this uptake is compound not receptor related. The extraordinary high radioactivity accumulation in kidney tissue is a common feature of radiometal labeled affibody molecules due to prevalent renal elimination followed by tubular reabsorption. The exact molecular mechanism is still not fully understood, but it is clear that the uptake is not megalin-dependent and cannot be prevented by pre- or co-injection of cationic amino acids or Gelofusine.38 This might be limiting toward affibody-mediated radionuclide therapy but is tolerable in case of diagnostic imaging, an equally high renal uptake of anti-HER2 affibody molecules did not prevent clinical imaging of metastases in lumbar area.49,50

89Zr-FSC-ZEGFR:2377 showed slower blood clearance in comparison to 89Zr-DFO-ZEGFR:2377 resulting in slightly increased uptake in normal tissue but also significantly higher tumor uptake especially 3 h p.i. indicating higher bioavailability51 of 89Zr-FSC-ZEGFR:2377. Corresponding tumor-organ ratios (Table 4) were not significantly different except from liver indicating that 89Zr-FSC-ZEGFR:2377 is equivalent to 89Zr-DFO-ZEGFR:2377 for imaging of EGFR-expression in vivo. This was confirmed by small animal PET-CT in A431 tumor xenografted mice (Figure 5). Lower tumor-to-liver ratio of 89Zr-FSC-ZEGFR:2377 might be disadvantageous toward sensitivity against EGFR-expressing liver metastases but might be compensated by the higher tumor uptake. The most striking difference was observed between 89Zr-ZEGFR:2377 conjugates labeled at elevated temperature and 89Zr-DFO-ZEGFR:2377 labeled at room temperature.52 Tumor-to-organ ratios were generally significantly higher allowing to detect EGFR-expression with higher contrast and therefore improved sensitivity. This should be taken into account for radiolabeling of biomolecules with zirconium-89. In case of heat sensitive molecules, pre-radiolabeling approaches, where radiolabeling is carried out prior to conjugation to the biomolecule, may lead to improved targeting. In comparison to other EGFR-affibody tracers evaluated in the same mouse model (e.g., 111In-DOTA-ZEGFR:190744, 18F-CBT-ZEGFR:190744, and 18F-FBEM-Cys-ZEGFR:190745) both 89Zr-DFO- and 89Zr-FSC-ZEGFR:2377 showed ~5-fold higher tumor-to-blood ratio. Additionally 89Zr-panitumumab53 and 89Zr-cetuximab53 in A431 xenografted mice showed ~5-fold lower tumor-to-blood ratios after 120 and 96 h, respectively. This indicates that both 89Zr-FSC-ZEGFR:2377 as well as 89Zr-DFO-ZEGFR:2377 are very good radiotracers for imaging of EGFR-related diseases already
3 h after administration, but imaging at 24 h would increase imaging contrast further.

It has to be noted that efficient and more stable radiolabeling at elevated temperature might be a limitation for the use of FSC for radiolabeling of heat sensitive biomolecules, e.g. monoclonal antibodies. Such targeting vectors would require alternative approaches for stable radiolabeling with zirconium-89. A number of novel chelators are under development for this purpose, as it is described in the recent review by Heskamp and co-workers.\textsuperscript{54} Particularly, such chelators as DFO\textsuperscript{6}, DFO-Sq, and HOPO enable radiolabeling at lower temperature and seem to be a good alternative to DFO and FSC for this purpose. However, so far it has not been shown that they are also superior to DFO when labeled at higher temperatures.

\section{CONCLUSION}

The results of this study showed that FSC is a suitable alternative to DFO as chelating scaffold for EGFR-targeting affibody molecules radiolabeled with zirconium-89. Furthermore, our findings indicate that radiolabeling at elevated temperature increases complex stability and tumor-to-organ ratios resulting in considerably improved imaging properties of \textsuperscript{89}Zr-DFO-bioconjugates.

\section{ASSOCIATED CONTENT}

\subsection{Supporting Information}

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.7b00787.

Representative ESI-MS chromatogram of FSC-ZEGFR:2377 and representative autoradiography and hematoxylin and eosin staining of A431 tumor section obtained at 24 h after injection of \textsuperscript{89}Zr-FSC-ZEGFR:2377 (PDF)

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\subsection{Notes}

The authors declare the following competing financial interest(s): Vladimir Tolmachev, John Loebblom, and Anna Orlova are members of the scientific advisory board of Affibody AB. Other authors declare no potentially competing interests.

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\section{ABBREVIATIONS USED}

FSC, fusarinine C; DFO, desferrioxamine B; EGF(R), epidermal growth factor (receptor); PET, positron emission tomography; mAbs, monoclonal antibodies; ZEGFR:2377, EGFR targeting affibody molecule; RP-HPLC, reversed-phase high-performance liquid chromatography; ITLC, instant thin layer chromatography; RT, room temperature; PBS, phosphate buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazinethesulfonylic acid; EDTA, thylenediaminetetraacetic acid

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