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Positioning of ^{99m}Tc-chelators influences radiolabeling, stability and biodistribution of Affibody molecules

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ABSTRACT

Affibody molecules represent a novel class of affinity proteins with a high potential as tracers for radionuclide molecular imaging. In this comparative structure–property study, a series of Affibody molecules with the ^{99m}Tc-chelators maGGG, maSSS, or maESE attached to the ε-amine of the internally positioned K49 was prepared by peptide synthesis, for comparison to molecules with similar chelators positioned at the N-terminus. The conjugates were labeled with ^{99m}Tc and evaluated in vitro and in vivo. It was found that both composition and position of the chelating moiety influence the label stability, biodistribution and targeting properties of HER2-binding Affibody molecules.

Certain novel anti-cancer therapies are directed towards particular oncogene products and successful treatment requires sensitive detection methods for evaluation of the expression levels of such proteins. The use of radionuclide molecular imaging allows for non-invasive detection of overexpressed target proteins both in primary tumors and metastases. Affibody molecules constitute a new class of targeting proteins, which has demonstrated favorable properties as tracers for radionuclide molecular imaging.[1] Affibody molecules are three-helix bundle proteins originating from the 58 amino acid scaffold of the staphylococcal protein A-derived Z domain. The scaffold has been subjected to randomization of 13 surface-exposed residues in helices 1 and 2 to generate a library from which high-affinity binders have been selected by phage display.[2] The small size (about 7 kDa) of Affibody molecules enables rapid extravasation and diffusion in the extracellular space of tumors, and also allows for rapid clearance from blood and non-specific compartments.[1] The selection of the Affibody molecule $Z_{HER2:342}$, which binds to the proto-oncogene product human epidermal growth factor receptor type 2 (HER2) with picomolar affinity, has recently been reported.[3] In vivo assessment of HER2 expression is important for selection of breast cancer patients, who would benefit from therapy using the humanized monoclonal anti-HER2 antibody trastuzumab. Radiolabeled Affibody molecules have previously demonstrated specific targeting and high-contrast imaging of HER2-expressing xenografts.[4] Furthermore, data from a clinical pilot study has confirmed the value of $Z_{HER2:342}$ for imaging of HER2-expressing breast cancer in patients.[5] The ability to produce Affibody molecules by peptide synthesis[6] allows for site-specific incorporation of chelators for radiometal labeling and generation of well-defined conjugates with uniform chemical and biodistribution properties.[7–12]

The radionuclide ^{99m}Tc ($T_{1/2}$ = 6.0 h) is the most frequently used label for nuclear medicine tracers due to its low cost, good logistics and low dose burden to patients. Earlier studies have demonstrated that mercaptoacetyl-containing peptide-based chelators

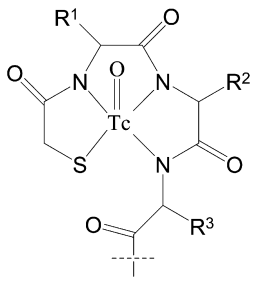

Figure 1. General structure of mercaptoacetyl-containing peptide-based ^{99m}Tc-chelators. R^1 = H, CH_2OH or $(CH_2)_2COOH$, R^2 = H or CH_2OH, and R^3 = H, CH_2OH or $(CH_2)_2COOH$.

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incorporated at the N-terminus of Affibody molecules allow for stable attachment of ^{99m}Tc(V) (Fig. 1).[13] Moreover, the use of polar amino acids in the chelator suppresses hepatobiliary excretion of the radioactivity and shifts the clearance to the more favorable renal excretion pathway.[9–12] It has also been found that the use of glutamic acid, serine and lysine instead of glycine in the chelator improves the catabolic stability of the chelates.[9–12] Furthermore, it has been observed that a combination of serine and glutamic acid in the chelator reduces renal accumulation of the radioactivity while providing a predominant renal excretion.[11] Since the amino acids located close to the chelator can influence the stability of a ^{99m}Tc chelate,[14] we wanted to investigate if our previously observed results are valid also when mercaptoacetyl-containing chelators are positioned elsewhere on the Affibody molecules. To study this, a series of $Z_{HER2:342}$ derivatives, containing mercaptoacetyl-glycyl-glycyl-glycyl ($N^{\varepsilon 49}$-maGGG-$Z_{HER2:342}$), mercaptoacetyl-seryl-seryl-seryl ($N^{\varepsilon 49}$-maSSS-$Z_{HER2:342}$) and mercaptoacetyl-glutamyl-seryl-glutamyl ($N^{\varepsilon 49}$-maESE-$Z_{HER2:342}$) chelators were synthesized and evaluated. The chelators were incorporated as a peptide branch at the side chain of K49, located in the middle of helix 3 (Fig. 2). This position is distant from the binding surface and modification at this site was hypothesized to cause minimal interference with the HER2-binding.

The peptide $Z_{HER2:342}$ was assembled using automated Fmoc/*t*Bu solid phase peptide synthesis according to protocols described earlier (see Supplementary data).[6] Reagents and amino acids were as previously reported except for K49 which was incorporated as Fmoc-Lys(Mtt)-OH.[13] After completed synthesis the N-terminal amino group was protected by reaction with di-*tert*-butyl pyrocarbonate. The orthogonal protection group Mtt was then liberated from the resin-bound peptide.[15] The ^{99m}Tc-chelating moieties maGGG, maSSS, or maESE were incorporated at the ε-amino group of K49 by step-wise manual synthesis.[16] The synthetic products were analyzed by RP-HPLC and by MALDI MS.[13,17] The purity of all peptides was more than 95%.

The melting temperatures of $N^{\varepsilon 49}$-maGGG-$Z_{HER2:342}$, $N^{\varepsilon 49}$-maSSS-$Z_{HER2:342}$ and $N^{\varepsilon 49}$-maESE-$Z_{HER2:342}$ were determined by variable temperature measurements.[13] All three variants were found to have a lower melting temperature (6–7 °C) compared with analogues carrying the chelator at the N-terminus (Table 1). Circular dichroism wavelength scans from 250 to 195 nm collected before and after melting indicated that the proteins refolded after heating to 90 °C.

The binding affinities of $N^{\varepsilon 49}$-maGGG-$Z_{HER2:342}$, $N^{\varepsilon 49}$-maSSS-$Z_{HER2:342}$ and $N^{\varepsilon 49}$-maESE-$Z_{HER2:342}$ were evaluated by real-time biospecific interaction analysis on a Biacore 2000 using Recombinant Human ErbB2/Fc Chimera (R&D Systems) as immobilized ligand.[13] $N^{\varepsilon 49}$-maSSS-$Z_{HER2:342}$ and $N^{\varepsilon 49}$-maESE-$Z_{HER2:342}$ were found to have K_D values similar to their N-terminally modified analogues, whereas $N^{\varepsilon 49}$-maGGG-$Z_{HER2:342}$ displayed a lower affinity (Table 1).

^{99m}Tc-labeling was performed using either a direct labeling approach[9] or indirect transchelation from tartrate.[13,18] Analysis of the labeled Affibody molecules was performed by instant thin layer chromatography.[13,19] The label stability was evaluated by challenge with 300-fold excess of cysteine during 2 h at 37 °C (Table 1).[13] Indirect labeling resulted in poor stability of all three conjugates during cysteine challenge (17.9–44.4%) and they were therefore excluded from further evaluation. The cysteine challenge stability of ^{99m}Tc-$N^{\varepsilon 49}$-maGGG-$Z_{HER2:342}$ was poor also following the direct labeling approach (58.1 ± 4.7% of Affibody-bound activity), while the N-terminally modified ^{99m}Tc-maGGG-$Z_{HER2:342}$ was much more stable (87.5 ± 1.8%).[13] These data indicate that the chelator position can influence the properties of ^{99m}Tc-labeled conjugates. Due to the poor stability, ^{99m}Tc-$N^{\varepsilon 49}$-maGGG-$Z_{HER2:342}$ was

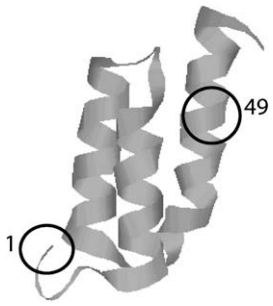

Figure 2. Schematic figure of an Affibody molecule showing the two positions (N-terminus and K49 in helix 3) used for attachment of the mercaptoacetyl-containing peptide-based chelators. The figure is based on the NMR solution structure of the Affibody scaffold molecule Z (pdb ID 2spz).

Table 2
Summary of properties of Affibody molecules with ^{99m}Tc-chelators at K49 compared to N-terminally modified analogues

	MaGGG	MaSSS	MaESE
Melting temperature	Lower	Lower	Lower
Refolding	Equal	Equal	Equal
HER2-binding affinity	Lower	Equal	Equal
^{99m}Tc-labeling yield	Lower	Equal	Equal
Label stability	Lower	Equal	Equal
Cell-binding specificity	n.d.	Equal	Equal
Cell-binding capacity	n.d.	Lower	Lower
In vivo stability	n.d.	Lower	Lower
Tumor uptake	n.d.	Lower	Lower
Intestine uptake	n.d.	Lower	Equal
Kidney uptake	n.d.	Higher	Lower

Table 1
Characterization, labeling and stability of $Z_{HER2:342}$ Affibody molecules with mercaptoacetyl-containing chelators in different positions

Peptide	Mw theoretical/ experimental (Da)	T_m (°C)	K_D (pM)	Radiochemical yield (%)	Reduced hydrolyzed Technetium (%)	Stability under cysteine challenge (%)
$Z_{HER2:342}$	6718/6718	64	80	n.a.	n.a.	n.a.
maGGG-$Z_{HER2:342}$[a]	7021/7021	64	200	82 ± 12	<2	87.5 ± 1.8
maSSS-$Z_{HER2:342}$[b]	7054/7054	65	400	82.3 ± 4.9	<1	97.1 ± 0.5
maESE-$Z_{HER2:342}$[c]	7138/7138	62	500	87 ± 8	<1	89.5 ± 0.5
$N^{\varepsilon 49}$-maGGG-$Z_{HER2:342}$	6964/6964	57	530	88.5 ± 4.6	0.2 ± 0.2	58.1 ± 4.7
$N^{\varepsilon 49}$-maSSS-$Z_{HER2:342}$	7054/7054	59	450	98.5 ± 2.3	0.2 ± 0.1	92.1 ± 1.3
$N^{\varepsilon 49}$-maESE-$Z_{HER2:342}$	7138/7138	55	500	94.2 ± 3.5	0.1 ± 0.1	91.3 ± 0.12

Radiochemical yield was determined as percent of affibody-bound ^{99m}Tc at the end of the labeling. Reduced hydrolyzed technetium was determined as percent of ^{99m}Tc in colloid form at the end of the labeling. Labeling and stability data are presented as an average from 3 to 6 labelings ± SD.

[a] Data from 13, molecular weight includes an acetamide group (+57 Da) for thiol capping.
[b] Data from 9.
[c] Data from 11.

excluded from further evaluation. Both labeling yields and in vitro stability of ^{99m}Tc-$N^{\varepsilon 49}$-maSSS-$Z_{HER2:342}$ and ^{99m}Tc-$N^{\varepsilon 49}$-maESE-$Z_{HER2:342}$ were quite similar to the analogues with chelators attached to the N-terminus (Table 2).

Cell-binding specificity and antigen binding capacity of ^{99m}Tc-$N^{\varepsilon 49}$-maSSS-$Z_{HER2:342}$ and ^{99m}Tc-$N^{\varepsilon 49}$-maESE-$Z_{HER2:342}$ were measured as previously described.[13] Both conjugates demonstrated specific binding to HER2-expressing cells. However, the antigen binding capacity (21.1 ± 0.7% for ^{99m}Tc-$N^{\varepsilon 49}$-maSSS-$Z_{HER2:342}$ and 13.7 ± 1.8% for ^{99m}Tc-$N^{\varepsilon 49}$-maESE-$Z_{HER2:342}$) was appreciably reduced in comparison to conjugates with N-terminal chelators (69.3 ± 2.5% and 64.3 ± 2.0% respectively). Since the binding affinities were only slightly reduced by the introduction of the chelator functions at K49, and the circular dichroism wavelength scans indicated that all Affibody conjugates refold properly after heating, the incorporation of the Tc atom and the harsh labeling conditions are most likely the causes of the loss of function. It is possible that the Tc-chelator complex interferes with refolding, while a free chelator does not. This interference could be much more pronounced when the chelator is positioned at K49 than at the N-terminal. Furthermore, a direct interaction of Tc with the nearest amino acid outside the chelator cannot be excluded.

To evaluate the influence of chelator position on biodistribution and targeting properties of ^{99m}Tc-labeled Affibody molecules, an animal study with Balb/*c nu/nu* mice, bearing HER2-expressing SKOV-3 xenografts was performed using the protocol reported earlier.[9,20] The general pattern of the biodistribution was similar for the conjugates (high accumulation in tumors and rapid clearance from blood and healthy tissues), however, certain differences were observed (see Supplementary data). Both conjugates with chelators attached to K49 demonstrated significantly higher radioactivity uptake in thyroid, stomach and salivary glands compared to the N-terminally modified analogues, which indicates a lower in vivo stability of the labels. The radioactivity concentration in tumors was higher than in any organ or tissue, except for kidney, for ^{99m}Tc-$N^{\varepsilon 49}$-maSSS-$Z_{HER2:342}$ and ^{99m}Tc-$N^{\varepsilon 49}$-maESE-$Z_{HER2:342}$. However, the tumor uptake was significantly lower than for conjugates with chelators at the N-terminus. One possible explanation is the decreased antigen binding capacity. The accumulation of ^{99m}Tc-$N^{\varepsilon 49}$-maSSS-$Z_{HER2:342}$ in the intestines content was reduced compared to ^{99m}Tc-maSSS-$Z_{HER2:342}$. This suggests that the excretion pathway is dependent on the positioning of the chelator in the Affibody molecule, and not only on its composition. Charge and lipophilicity of the chelating moiety have previously been found to be crucial parameters that govern the biodistribution of Affibody molecules carrying the chelator at the N-terminus. This effect was less pronounced when the chelator was located at K49. Another interesting observation was the difference in the renal retention. When the maESE-chelator was positioned at K49, the renal retention was reduced compared to the N-terminally modified analogue. However, the reversed effect was observed for the maSSS chelator. Taken together, these results show that the effect on global molecular properties from chelating moieties of a given composition may differ depending on whether they are placed internally or at the N-terminus. The biodistribution results in normal organs were confirmed in another mouse strain NMRI, not carrying xenografts (data not shown).

In conclusion, both chelator composition and position of the chelating moiety influence the label stability, biodistribution and targeting properties of Affibody molecules. This is of general relevance to consider for targeting agents where alternative positions for site-specific chelator introduction are available.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.03.083.

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15. Mtt was removed by 8 × 2 min incubation in DCM with 1% TFA and 5% TIS.
16. Coupling reactions were performed using 5 equiv of amino acid or *S*-trityl-mercaptoacetic acid, 5 equiv HBTU/HOBt and 10 eq DIEA in NMP.
17. RP-HPLC analyses was run using a 4.5 × 150 mm polystyrene/divinylbenzene matrix column with a particle size of 5 μm (Amersham Biosciences) at a flow of 1 ml/min and solvent A: 0.1% TFA–H_2O, B: 0.1% TFA–CH_3CN. A 20 min gradient of 25–45% B was used for purification of full-length proteins from the crude synthetic product. Purified proteins were lyophilized and analyzed in a 20 min elution gradient from 20% to 60% B.
18. Direct labeling was performed by mixing 30 μl of 1 mg/ml Affibody-chelator conjugate in deionized, degassed water with 20 μl 0.15 M NaOH to obtain pH 11. 10 μl $SnCl_2{\cdot}2H_2O$ in 0.01 M HCl (1 mg/ml) was added, followed by 100 μl of fresh pertechnetate solution. The mixture was slightly vortexed and incubated at room temperature for 60 min. Indirect labeling was performed by mixing 30 μl of 1 mg/ml Affibody-chelator conjugate in deionized, degassed water with 80 μl PBS, 20 μl of a solution containing Na/K tartrate (50 g/l) in 0.5 M $NaHCO_3$ and 0.25 M NH_4OAc, and adding10 μl $SnCl_2{\cdot}2H_2O$ in 0.01 M HCl (1 mg/ml). To this mixture, 100 μl of fresh Tc-generator eluate was added and the mixture was incubated at room temperature for 60 min.
19. Instant thin layer chromatography was performed using glass fiber sheets impregnated with silica gel (ITLC SG, Gelman Sciences Inc.). The ITLC SG strips were eluted with PBS for yield and stability determinations, and with pyridine:acetic acid:water (5:3:1.5) to determine presence of reduced hydrolyzed technetium (RHT).
20. The animal study was performed in accordance with Swedish law and was approved by the Uppsala Committee of Animal Research Ethics.