Molecular Imaging of HER2 Expression using Synthetic Affibody Molecules

Design, Synthesis and Biological Evaluation

THUY TRAN
Dissertation presented at Uppsala University to be publicly examined in Rudbecksalen, Rudbecklaboratoriet, Uppsala, Saturday, May 16, 2009 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract

Molecular imaging is an emerging multidisciplinary field that addresses the visualisation of diseases at the cellular and molecular levels. This thesis focuses on the development of a synthetic Affibody molecule-based imaging tracer for the detection of HER2 expression in malignant tumours.

Papers I-IV report the development of the HER2-specific Affibody molecule, Z_{HER2:342} by peptide synthesis and the use of different chelators attached to the N-terminus to allow ^99mTc- labelling. Paper I described the optimisation of labelling of Affibody molecules using cysteine-based chelator sequences, in which the direct labelling method under alkaline conditions was the most suitable one. Papers II-IV report the development and optimisation of the in vivo properties of the HER2-specific Affibody molecule for high-contrast imaging. By using an array of mercaptoacetyl-based chelators, it was found that the substitution of a single amino acid in a 60 amino acid-long Affibody molecule can dramatically change the pharmacokinetics of the tracer. Strategic approaches that utilised hydrophilic amino acids, such as serine, glutamate and lysine, changed the excretion pathway from hepatobiliary to renal excretion. Problems with the high accumulation of radioactivity in the abdomen area and restricted imaging were resolved by the use of mercaptoacetyl-triglutamyl, maEEE or mercaptoacetyl-seryl-lysyl-seryl, maSKS chelators.

Paper V reports the re-engineering of the HER2-specific Affibody molecule to provide a C-terminal SECG sequence using peptide synthesis. Incorporation of this sequence provided a multifunctional platform for labelling (with technetium or trivalent radio metals) and a flexible production route (recombinant or chemical synthesis). Combination of a serine, a glutamic acid and a thiol-bearing group in the chelating sequence reduced the renal accumulation of Affibody molecules.

Altogether, the in vivo efficiency of Affibody molecules to target tumours and their biodistribution properties can be improved by strategic design and suitable chemistry. Hopefully, these observations will be applicable to other small peptide and protein scaffold-based tracers.

Keywords: Molecular imaging, Affibody molecules, HER2, cancer detection, peptide synthesis, radiolabelling, technetium, indium, SPECT, PET, radiometal, chelator engineering

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List of papers


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**Front cover:** Confocal microscopy image of the HER2-specific Affibody molecule ZHER2:342 on living SKOV-3 cells.
Related publications not included in this thesis


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<th>Definition</th>
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<tr>
<td>% IA/g</td>
<td>Percent of injected activity per gram</td>
</tr>
<tr>
<td>ABD</td>
<td>Albumin binding domain</td>
</tr>
<tr>
<td>CEA</td>
<td>Carcinoembryonic antigen</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>CGG</td>
<td>Cysteinyl-di-glycyl</td>
</tr>
<tr>
<td>CGGG</td>
<td>Cysteinyl-tri-glycyl</td>
</tr>
<tr>
<td>CHX-A''-DTPA</td>
<td>([(R)-2\text{-Amino-3-(4-isothiocyanatophenyl)propyl}]\text{-trans-(S,S)-cyclohexane-1,2-diamine-pentaacetic acid})</td>
</tr>
<tr>
<td>CISH</td>
<td>Chromogenic in situ hybridization</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DOTA</td>
<td>1,4,7,10-tetraazacyclododecane-N,N’,N”,N’’’-tetraacetic acid</td>
</tr>
<tr>
<td>ECD</td>
<td>Extracellular domain</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ErbB-1</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ErbB-2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>ErbB-3</td>
<td>Human epidermal growth factor receptor 3</td>
</tr>
<tr>
<td>ErbB-4</td>
<td>Human epidermal growth factor receptor 4</td>
</tr>
<tr>
<td>Fab</td>
<td>Antigen-binding fragment</td>
</tr>
<tr>
<td>FDA</td>
<td>The U.S. Food and Drug Administration</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>Fmoc</td>
<td>9-fluorenlymethyloxycarbonyl</td>
</tr>
<tr>
<td>Fv</td>
<td>Variable fragment</td>
</tr>
<tr>
<td>HER1</td>
<td>Epidermal growth factor receptor, EGFR</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HER3</td>
<td>Human epidermal growth factor receptor 3</td>
</tr>
<tr>
<td>HER4</td>
<td>Human epidermal growth factor receptor 4</td>
</tr>
<tr>
<td>His6</td>
<td>Hexa-histidine tag</td>
</tr>
<tr>
<td>HMW</td>
<td>High molecular weight</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>ITLC</td>
<td>Instant thin layer chromatography</td>
</tr>
<tr>
<td>Kd</td>
<td>Dissociation equilibrium constant</td>
</tr>
<tr>
<td>LMW</td>
<td>Low molecular weight</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAG3/maGGG</td>
<td>Mercaptoacetyl-glycyl-glycyl-glycyl or -triglycyl</td>
</tr>
<tr>
<td>maEEE</td>
<td>Mercaptoacetyl-triglutamyl</td>
</tr>
<tr>
<td>maEEE</td>
<td>Mercaptoacetyl-triglutamyl-glycyl</td>
</tr>
<tr>
<td>maGES</td>
<td>Mercaptoacetyl-glycyl-seryl-glycyl</td>
</tr>
<tr>
<td>maSKS</td>
<td>Mercaptoacetyl-seryl-lysyl-seryl</td>
</tr>
<tr>
<td>maKKK</td>
<td>Mercaptoacetyl-trilysyl</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>neu</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>NMRI</td>
<td>Naval Medical Research Institute.</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>p.i. or pi</td>
<td>Post-injection</td>
</tr>
<tr>
<td>PMSA</td>
<td>Prostate-membrane specific antigen</td>
</tr>
<tr>
<td>scFv</td>
<td>Single chain variable fragment</td>
</tr>
<tr>
<td>SDS PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SECG</td>
<td>Serine-glutamyl-cysteinyl-glycyl</td>
</tr>
<tr>
<td>SPECT</td>
<td>Single-photon emission computed tomography</td>
</tr>
<tr>
<td>SPPS</td>
<td>Solid phase peptide synthesis</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>T/B</td>
<td>Tumour-to-blood</td>
</tr>
<tr>
<td>T/2</td>
<td>Half-life</td>
</tr>
<tr>
<td>tBoc</td>
<td>1-butyloxy carbonyl</td>
</tr>
<tr>
<td>Tm</td>
<td>Melting point</td>
</tr>
<tr>
<td>TGF-α</td>
<td>Transforming growth factor α</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
1. Introduction

1.1 Cancer

Cancer is the generic name of a group of diseases that are characterised by abnormal, uncontrolled cell division, the ability to invade normal tissues and the ability to spread to other parts of the body [1].

In Sweden during 2007, approximately 50 000 cancer cases were newly diagnosed. Despite improvements in the diagnosis and treatment of cancer, its incidence is steadily increasing by around 1.4 % annually. Breast cancer is the most commonly diagnosed cancer in women, constituting approximately 30 % of all female cancers, while prostate cancer is the most common in men and represents 34 % of all male cancer cases [2].

Cancer can be treated primarily with surgery, radiotherapy, systemic therapy (chemotherapy and endocrine therapy), targeted therapy or by some combinations of these [3].

1.2 Cancer detection

The early diagnosis of cancer has a major impact on the potential survival of patients. The prognosis is often better and the treatments are more successful if a cancer is detected at an early stage, i.e. before tumours have spread to other parts of the body [4].

Apart from assessing symptoms that might indicate directly the presence of cancer, there are a number of methods available for the detection of cancers. These include laboratory tests, histological analysis of biopsy samples and the use of x-ray, magnetic resonance imaging (MRI), ultrasound, computed tomography (CT) and radionuclide imaging techniques [4, 5]. The radionuclide imaging techniques of single-photon emission tomography (SPECT) and positron emission tomography (PET) have become important techniques in contemporary cancer detection [6-8] and are further described in section 1.4.2.
1.3 Tumour targeting

The concept of tumour targeting relies on the use of a highly specific targeting agent (e.g. antibody based or non-antibody based molecules) as described in section 1.6, often to selectively destroy cancer cells or to deliver cytotoxic substances to the tumour cells with minimal damage to the surrounding tissues [9]. The target is usually an antigen or a receptor structure that is highly expressed on the tumour cell but is preferably absent or expressed to a lower extent on normal cells (Figure 1).

Radioimmunotherapy uses this targeting concept, in which the antibody mediates the delivery of radioactivity to the target. Many antigens have been identified as potential targets for radioimmunotherapy in different cancer types, such as CEA, the EGFR/HER family, A33, MUC-1, VEGF, the cytokeratins [10] and CD44v6 [11]. The same concept is utilised in cancer diagnosis with the distinction that the radionuclides generally have physical properties that are suitable for imaging in SPECT and PET, which are further discussed in section 1.5.

![Figure 1](image)

**Figure 1.** The tumour targeting concept utilises a targeting agent that carries a radiolabel or other toxic substance and binds specifically to its targeted structure. The structure is overexpressed on the tumour cells but is expressed to a very small amount or preferably absent on normal cells. The targeting concept can be utilised both for cancer therapy and diagnosis.

1.3.1 The HER/ErbB family

The human epidermal growth factor receptor (EGFR or HER) family belongs to one of the most extensively studied growth receptor families. This family has four members: EGFR/ErbB-1, HER2/neu/ErbB-2, HER3/ErbB-3
and HER4/ErbB-4. All members except HER2 have several known natural ligands including transforming growth factor α (TGF-α), betacellulin, heparin-binding growth factor, epiregulin and neuregulins [12, 13]. The binding of ligands to the extracellular domain of ErbB receptors induces the formation of receptor homo- or heterodimers. Subsequent activation of intrinsic tyrosine kinase regulates their cellular effects (proliferation, apoptosis, migration and differentiation) via different pathways [12, 14]. All possible homo- and heterodimeric receptor complexes between the members of the ErbB family have been identified in different systems [12-14].

The overexpression of EGFR and HER2 has often been associated with malignant transformation. Tumour targeting against these targets have been clinically useful in many cancer types. Cancer biology of the HER3 and HER4 receptors is not as well characterised as the other two. Currently available data suggest that HER3 expression is found in many malignancies [15] while the role of HER4 in cancer is still unclear [16].

1.3.2 The target HER2

A major focus of this research involved targeting of the HER2 receptor which is therefore described here in greater detail. HER2 (also known as ErbB-2 or neu) has no natural ligands and serves as a co-receptor for hetero- and homodimerisation with other receptors [17].

Studies have demonstrated that HER2 possesses many features of an appropriate therapeutic target: It is associated with the characteristics of a cancerous cell (uncontrolled proliferation, resistance to apoptosis and increased mobility). Its overexpression is specific to tumour cells, making it suitable for targeted radionuclide therapy. As a surface-associated protein, it is easily accessible to drugs and as a kinase it is amenable to targeted inhibition by small molecules [18].

The overexpression of HER2 receptors has been found in a variety of cancers (http://www.proteinatlas.org), including 14-20 % in breast [19-21], 2-23 % in lung [22, 23], 2-76 % in ovary [24], 12-64 % in prostate [25-27], 8-79 % in urinary bladder [28, 29] and 2-82 % in colorectal cancers [30, 31]. Further, the overexpression of HER2 is a prognostic biomarker for breast [29, 32], ovarian [33, 34], bladder [35, 36] and lung [37] cancers. In breast cancer, HER2 overexpression/amplification is also a predictive biomarker [38, 39], and is associated with more aggressive disease and a poorer prognosis [38, 40].

1.3.3 HER2-targeted treatment

Currently, nine monoclonal antibodies are approved for cancer therapy. For anti-HER2 treatment only trastuzumab (Herceptin®, Roche) is currently commercially available but there are emerging targeting agents under pre-
clinical and clinical investigation, for example the monoclonal antibody per-
tuzumab (Omnitarg®, Genentech). Other potential HER2-targeted treatments
include the heat shock protein (Hsp90) inhibitor 17-AAG [41], the tyrosine
kinase inhibitor lapatinib [42], and others [43].

The humanized monoclonal antibody trastuzumab (Herceptin®, Roche)
was approved by the U.S. Food and Drug Administration (FDA) in 1998 for
the treatment of HER2-positive metastatic breast cancers. The anticancer
effects of trastuzumab are mediated through several mechanisms, such as the
reduction of receptor signalling, the induction of apoptosis, the inhibition of
angiogenesis and the inhibition of HER2-ECD cleavage [44, 45].

The addition of trastuzumab in breast cancer treatment reduces the recur-
rence by approximately 50 % and improves the overall survival rate by 30 %
[46].

1.3.4 Evaluation of HER2 status

In order to select patients who will benefit from trastuzumab treatment, it is
currently recommended that the HER2 status of every primary tumour will be
determined at the time of diagnosis or recurrence of breast cancer [39, 47, 48].

The standard methods for HER2 determination are immunohistochemistry
(IHC) or fluorescence in situ hybridisation (FISH) of biopsy samples. IHC
staining is a relatively simple and cost-effective test but wide variations in
sensitivity and specificity have been observed. FISH is a more sensitive
technique which quantifies the number of gene copies in the cancer cell and
objectively reflects the HER2 status, whereas IHC is a more subjective test.
Current literature suggests that FISH provides a more accurate and consist-
ent scoring system for the measurement of HER2 amplification than IHC
using the HercepTest [49].

The FDA recently approved another test, chromogenic in situ hybridisa-
tion (CISH) for HER2 testing [50]. CISH uses the in situ hybridisation tech-
nology of FISH, but the chromogenic signal can be detected using ordinary
light microscopy which makes the cost much lower [51].

However, these techniques can give rise to false-negative and false-positive
results. All such tests require the assessment of biopsy samples, which are
most often taken from the primary tumours. It has been shown that heteroge-
neity can occur in tumour expression [32], and that there is discordance in
expression between primary tumours and metastases [52, 53], although most
data show that HER2 overexpression is retained in metastases [54, 55]. A
HER2 testing algorithm using both IHC and FISH methods is generally
adopted as the standard approach to take. The American Society of Clinical
Oncology and the College of American Pathologist expert panel reported re-
cently that that approximately 20 % of current HER2 test results may be inac-
curate [48]. There is so far no overall agreement on the best method for HER2
testing [48, 56] and when the difficulties in taking a biopsy sample from cancer patients is also considered, robust alternative methods are needed.

Therefore, we propose Affibody molecule-based molecular imaging for the assessment of HER2 status. Molecular imaging of HER2 has the potential advantages of 1) being non-invasive with the ability to monitor the total expression of biomarkers, 2) offering the opportunity of real-time monitoring of the HER2 status both in the primary tumour and metastases in a single scan, 3) having a strong ability for quantification and 4) potentially achieving suitably low false-negative and false-positive rates.

\[\text{Figure 2. HER2 assessment using IHC (left) and FISH (right). Images modified from Hicks JD and Tubbs RR [57]. With permission from Elsevier.}\]

1.4 Molecular imaging

Molecular imaging is defined as “the visualisation, characterisation, and measurement of biological processes at the molecular and cellular levels in humans and other living systems” (www.snm.org).

Today, molecular imaging is increasingly used, not only in clinical oncology but also in neurology, cardiology and in a number of other areas of medicine to study biological processes, e.g. apoptosis, angiogenesis, metastasis, gene expression and inflammation [58-62]. Applications of molecular imaging provide sensitive and non-invasive means for early diagnosis, patient stratification, patient prognosis, response prediction, real-time monitoring of therapy, optimisation of dosage and validation of drug targets. Molecular imaging also contributes to the acceleration of drug discovery, drug development and cost reduction [58, 63-65].

1.4.1 Implications for personalised therapy

Cancer is a highly complex disease that can have different phenotypes. There are types and subtypes of cancers that differ significantly and subsequently respond differently to treatment. The need to tailor individual therapy for
each tumour at a molecular level, so called personalised therapy, has become increasingly important in contemporary patient management [65, 66].

In oncology, molecular imaging has the potential to become a powerful tool with which to detect biomarkers *in vivo*, enable the visualisation of phenotypic expression in cancer lesions and thus helps clinicians to rapidly access an accurate diagnosis in order to identify those patients that will benefit from specific treatment [65, 67]. Different imaging modalities have also been used for staging, guiding treatment, evaluating the progress of treatment and monitoring for cancer recurrence [68-70].

1.4.2 Radionuclide imaging techniques

The principles of radionuclide imaging can be divided into single photon imaging (planar gamma-camera imaging or single-photon emission computed tomography, SPECT) and positron emission tomography (PET). Several factors determine the suitability of each modality. These include the nature of the nuclide used (physical properties, cost and availability), the registration efficiency, resolution, quantification capacity and the availability of imaging devices [71, 72]. A brief comparison of the two modalities is provided in Table 1. More detailed descriptions and additional technical details are presented elsewhere [71, 73].

SPECT imaging requires the use of radionuclides that emit gamma ray photons (\(\gamma\)) or high-energy X-ray photons, preferably with the energy range of 100-300 keV. Only a single photon is detected per event and the photon is emitted directly from the radioactive atom. In SPECT the most commonly used radionuclides are \(^{99m}\text{Tc}\), \(^{123}\text{I}\), and \(^{111}\text{In}\). The gamma-camera used in SPECT generally contains one or more photon detectors, which are typically large scintillation detector containing a NaI(Tl) crystal.

PET requires radionuclides that emit positrons (\(\beta^+\)), having an energy of 511 keV. In contrast to SPECT, two 511keV photons (resulted by annihilation of \(\beta^+\)) travelling in opposite directions are simultaneously detected in a PET system. The most commonly used \(\beta^+\)-emitters are \(^{18}\text{F}\), \(^{15}\text{O}\), \(^{13}\text{N}\) and \(^{11}\text{C}\). The PET camera is surrounded by a complete cylindrical shell comprising contiguous rings of many photon detectors.

In comparison to PET, SPECT has the advantages of wide clinical availability of imaging devices and the relatively low cost of radionuclides used. However, PET possesses superior resolution, higher registration efficiency and better quantification capability.
### Table 1. Properties of SPECT and PET for in vivo imaging

<table>
<thead>
<tr>
<th></th>
<th>SPECT</th>
<th>PET</th>
</tr>
</thead>
<tbody>
<tr>
<td>Form of energy used</td>
<td>γ rays</td>
<td>Annihilation photons</td>
</tr>
<tr>
<td>Spatial resolution (mm)</td>
<td>7-15</td>
<td>2-4</td>
</tr>
<tr>
<td>Acquisition time per frame (s)</td>
<td>60-2000</td>
<td>1-300</td>
</tr>
<tr>
<td>Availability of radionuclides</td>
<td>High</td>
<td>Medium-high</td>
</tr>
<tr>
<td>Availability of equipment</td>
<td>High</td>
<td>Medium-high</td>
</tr>
<tr>
<td>Cost</td>
<td>Medium-high</td>
<td>High</td>
</tr>
</tbody>
</table>

## 1.5 Radiolabelling

When developing a radiopharmaceutical several criteria must be considered. The stability of the radiohalogen-carbon bond or the metal-chelator complex is one of the most important aspects. The choice of chelator for radiolabelling with radiometals is vital. A chelator should form a stable bond and withstand the physiological conditions in vivo. It should not interfere with the binding site as this could reduce the binding capability towards its targets. The radiopharmaceutical should have physico-chemical properties that are advantageous for the in vivo pharmacokinetics (e.g. rapid blood clearance and renal excretion).

### 1.5.1 Radiohalogenation

Proteins or peptides can be radiolabelled with halogens, such as iodine, bromine or fluorine via covalent bond formation. The radiohalogenation of proteins using $^{125}$I has been the most widely used method for the initial characterisation of proteins. Direct labelling with protein functional groups such as tyrosine residues, with an electrophilic radioiodine after oxidisation by Chloramine-T or Iodogen is the most straightforward procedures [74].

Indirect radiohalogenation using prosthetic groups (for example N-succimidyl-para-iodobenzoate, PIB) is employed if directly labelled conjugates loose their binding capability to the target or if they suffer from metabolic instability. Indirectly iodinated targeting agents also show better excretion of radiocatabolites [75].

### 1.5.2 Radiolabelling with $^{99m}$Tc

For gamma camera based imaging there is a wide range of radionuclides (Table 2) to choose from, among them the most commonly used is technetium-99m. More than 80% of all radiopharmaceuticals used in clinics are $^{99m}$Tc-based because of its low cost, good availability and its ideal physical properties ($γ$-energy of 140 keV, $T_{1/2}$ ~ 6 h). The half-life of $^{99m}$Tc is long
enough to carry out a labelling preparation and perform imaging without unnecessary radiation dose to the patients. The gamma-energy is suited to the low-energy, high-resolution collimators used in gamma-cameras. Also, $^{99m}$Tc has high single energy gamma abundance (~ 89 %), which helps to achieve high imaging resolution.

Table 2. Radionuclides commonly used for diagnostic SPECT imaging.

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Half-life</th>
<th>$\gamma$-energy (keV)</th>
<th>Abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{99m}$Tc</td>
<td>6.0 h</td>
<td>140</td>
<td>89</td>
</tr>
<tr>
<td>$^{111}$In</td>
<td>2.80 d</td>
<td>247</td>
<td>94</td>
</tr>
<tr>
<td>$^{123}$I</td>
<td>13.2 h</td>
<td>159</td>
<td>83</td>
</tr>
<tr>
<td>$^{201}$Tl</td>
<td>3.04 d</td>
<td>167</td>
<td>11</td>
</tr>
<tr>
<td>$^{67}$Ga</td>
<td>3.25 d</td>
<td>300</td>
<td>17</td>
</tr>
</tbody>
</table>

The coordination chemistry of $^{99m}$Tc contains many oxidation states (from -1 to +7). Pertechnetate ($^{99m}$TcO$_4^-$), with the oxidation state +7, eluted from the $^{99}$Mo/$^{99m}$Tc generator in saline can be used directly for thyroid imaging. $^{99m}$Tc radiopharmaceuticals are used in several clinical diagnostic procedures, such as the use of $^{99m}$Tc-MAG3 for examining renal functions and the use of $^{99m}$Tc-HYNIC-octreotide for the imaging of neuroendocrine tumours.

A number of technetium cores have been investigated for technetium-labelling, as reviewed by Liu et al [76-78], where the most important cores are of [Tc=O]$^{3+}$, Tc[HYNIC] and [Tc(CO$_3$)]$^+$. In order to label complexes or chelates with $^{99m}$Tc, pertechnetate has to be reduced to a lower oxidation state. This can be achieved using reducing agents such as stannous chloride (SnCl$_2$). Labelling with $^{99m}$Tc can be done by direct or indirect labelling method. The direct labelling method involves the reduction of a disulphide bridge in a protein and complexation of the resulting free thiol groups with $^{99m}$Tc. The indirect labelling method involves the use of a chelator, to which $^{99m}$Tc can be bound. Chelating agents contain hetero atoms for the binding of technetium, such as nitrogen (in amines, amides, imines, oximes and N in aromatic rings), sulphur (in thiols and thioethers), phosphorus (in phosphines), and oxygen (in carboxylates, phenols, alcohols, enols and phosphonates) [79].

1.5.3 Radiolabelling with trivalent radiometals

Radiolabelling with trivalent radiometals (indium, gallium and yttrium) and their isotopes are of increasing interest in nuclear medicine because they are possible labels for both SPECT and PET applications.

DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) is an important chelator for the labelling of proteins with these radionuclides. DOTA is a macrocyclic chelator that provides extraordinary thermodynamic
stability and kinetic inertness with these radiometals. For example, DOTA is used for indium-labelling of the clinical somatostatin analogue octreotide [80, 81]. The utility of other chelators, including DTPA, DTPA derivatives and CHX-A\(^{-}\)-DTPA, has also been investigated for trivalent radiolabelling (Figure 3).

![Chelator structures of DOTA and CHX-A\(^{-}\)-DTPA](image)

*Figure 3. Chelator structures of DOTA and CHX-A\(^{-}\)-DTPA, often used for radiolabelling with trivalent radionuclides (indium, gallium and yttrium) and their isotopes for both SPECT and PET applications.*

### 1.6 Tumour targeting agents

Several molecular imaging agents have been approved for clinical use in nuclear medicine. Many others are under preclinical evaluation with high potential for clinical applications. Certain key properties of an imaging probe must be verified and optimised in preclinical models. These include specificity, size, affinity and \textit{in vivo} biodistribution properties [67, 82, 83].

#### 1.6.1 Antibodies

Monoclonal antibodies (mAbs) have been extensively studied for targeted therapy and imaging. The schematic structure of an intact IgG molecule is presented in Figure 4. For therapy, molecules with long circulation time in the blood might be suitable but for imaging, it is highly desirable that targeting agents should be rapidly excreted from the body. It is also essential that the targeting agent binds rapidly to its target, reducing the time between injection and imaging [84].

The use of mAbs has presented challenges in radionuclide imaging. Because of their large size (molecular weight of ~150 kDa), mAbs penetrate slowly and have long residence times in the blood circulation (days-weeks), leading to limited tumour-to-normal organ ratios in biodistribution and low contrast images for the detection of biomarkers/tumours [85].
1.6.2 Antibody derivatives

Advances in protein engineering have led to a number of alternative constructs for imaging. These alternatives are characterised by smaller size, derivatives of antibody fragments such as F(ab’)2 (110 kDa), Fab (~55 kDa) single chain fragment, scFv (~25 kDa), diabodies (80kDa) and minibodies (55 kDa) [85]. Examples are illustrated in Figure 4. When compared to intact IgGs, these antibody fragments have the advantages of faster biodistribution and improved tumour-to-normal organ ratios, more suitable for imaging but are still too large for optimal imaging purposes. At the same time, they present issues associated with solubility and stability [84].

![Figure 4](image1.png)

*Figure 4. Schematic overview of antibody-based targeting structures. Intact IgG molecules contain two antigen binding domains, Fab (fragment antigen-binding), two Fc (fragment crystallisable) modulating immune cell activity. Each Fab arm has a variable light (V_L) chain and a variable heavy (V_H) chain. Each chain, in turn, contains three complementary determining regions (CDRs).*

1.6.3 Affibody molecules

Affibody molecules are 58 amino-acid, three-helix bundle affinity proteins and are derived from the B-domain of the five-domain Ig-binding region protein A from *Staphylococcus aureus*. They represent highly specific binders, selected by phage display from a library generated by randomization of 13 amino acids in helix 1 and 2, which are responsible for the Fc-binding site. Phage display technology can create and maintain a link between phenotype and genotype during a selection process and allows the construction of large libraries from which clones with a desired target can be selected [86] (Figure 5). Using the affibody library (2 x 10¹⁰ variants) a variety of binders have been identified with affinities ranging from micro- to femtomolar, for example insulin [87], IgA [88], Alzheimer amyloid beta peptides [89], HER2 protein [90], HIV-1 [91], EGFR [92] and other targets [86]. Enhanced affinity (K_D) can be obtained by affinity maturation, performed through helix shuffling [93] or sequence alignment and directed combinatorial mutagenesis [94, 95].
Affibody molecules have been applied to numerous biotechnological applications based on molecular recognition, including affinity purification (e.g. affinity chromatography; ELISA and Western blotting), specific molecular detection, inhibition of receptor interactions, protein arrays and molecular recognition-based therapy (e.g. adenoviral targeting for gene therapy), as reviewed by Nygren [86]. Recently, Affibody molecules have been investigated for tumour targeting purposes both for imaging and targeted therapy [96-98]. The first Affibody molecule generated and used for radionuclide imaging was the $Z_{\text{HER2:4}}$, with a binding affinity of 50 nM to HER2 protein [90].

Figure 5. The three-helix bundle Affibody molecule. Helix 1 and 2 are responsible for binding (blue) and helix 3 is stabilizing (red). The 13 randomized positions (yellow) are located on helix 1 and 2. With kind permission from Affibody AB.

1.6.4 Peptides

Another class of targeting agents is the natural peptide receptor ligands and their analogues, which consist of a few (5-20) amino acids and have great potential as imaging tracers [99]. A good example is the clinical OctreScan ($^{111}$In-labelled somatostatin analogue), which is approved for the imaging of neuroendocrine tumours. Others include neurotensin (a 13 amino acid neuropeptide), which is expressed in both the central and the periphery nervous system. The use of neurotensin is limited in cancer diagnosis because of its metabolic instability [100]. RGD-peptides [101] and bombesin peptides have also been investigated as targeting agents for the $\alpha_v\beta_3$ integrin receptor and the bombesin receptors, respectively [102].
The major characteristics of peptides that favour their use as imaging agents are their small size, which allows rapid binding to their target, and rapid clearance from blood and non-target tissues, which leads to high contrast images. In addition, they can tolerate harsh chemical conditions and are easy to purify and modify. However, they often possess agonistic effects and some targets (for example the HER2) unfortunately lack natural ligands.

1.7 Chemical Synthesis

Peptide synthesis of biomolecules is an established method which has generated a number of synthetic peptides of different sizes for clinical use [103]. Chemical synthesis provides an efficient method to site-specifically incorporate “non-natural” sequences, pseudo-peptides and polymers and thus provides an opportunity to modulate the structure and function of proteins [104, 105]. The peptides are chemically defined and stable and no infectious material is involved in their manufacture [106].

Chemical synthesis employs mainly either solid phase (SPPS) or liquid phase [107, 108]. SPPS is generally the method of choice as it has advantages over the liquid phase such as rapid coupling reactions and simple washing procedures.

1.7.1 Principle of solid phase peptide synthesis

The fundamental principle of SPPS is based on the stepwise assembly of a peptide by the consecutive coupling of amino acids. A schematic overview of the process is outlined in Figure 6. Briefly, the C-terminal amino acid residue of the target peptide is attached to an insoluble support, resin, via its carboxyl group. The α-amino group of the first amino acid residue is deprotected in order to attach the next amino acid. This is done in such conditions so that the permanent side-chain protecting group is not cleaved. The next Nα protected amino acid residue is coupled to the resin-bound amino acid residue. This coupling/deprotection process is repeated in cycles until the length of the target peptide is achieved. After completed synthesis, the side-chain protecting groups are cleaved and the peptide is released from the resin [109, 110].

SPPS employs mainly two formats: an acid labile protecting group tBoc (t-butyloxy carbonyl) or a base labile alpha-amino protecting group, Fmoc (9-fluorenylmethyloxycarbonyl). Each method utilizes different linkers, amino acid side-chain protection and consequent deprotection strategy. The advantages of the Fmoc chemistry are higher quality and better yields while the tBoc chemistry is most essential in synthesis of base-sensitive peptides [109].

The Affibody molecules reported here have been synthesised by Fmoc SPPS. There is a great diversity of conditions under which Fmoc SPPS may employ. More details of the synthesis method, the particular linkers and de-protection strategies are provided elsewhere [110].
1.7.2 Advantages and limitations with chemical synthesis

Developments in peptide synthesis have brought the field of SPPS to a new level and have opened the door to the rapid synthesis of many biologically active peptides and small proteins. Commercially available automation systems have been optimised to produce longer peptides and the final products are obtained in high yields [109].

Although tremendous achievements have been made since Fmoc chemistry appeared as a practical approach, SPPS still has limitations. During the course of SPPS, the growing peptide chain can fold over onto itself, or aggregate with a neighbouring chain. The result is incomplete couplings which may create a truncated fragment of the desired peptide. This compromises purification and product yields. A number of side-chain reactions during synthesis are difficult to control. By-products arising from incomplete reactions, side reactions and impurities can accumulate on the resin during chain assembly and contaminate the final product. Synthetic efficiency is thus
highly dependent on the amino acid chain length and sequence, and the synthesis of peptides over 100 amino acids is limited because of the low yield. Peptides with sequences containing, for example, hydrophobic and ß-branched residues are difficult to synthesize [111].
2. Aims

The aims of this thesis were to develop a $^{99m}$Tc-labelled Affibody molecule-based tracer for the imaging of HER2 expression in tumours and to design a novel, multifunctional Affibody molecule that would provide a flexible platform for radiolabelling and production.

The specific aims were to:

1. utilise Fmoc peptide synthesis for the generation of Affibody molecules;
2. optimise the $^{99m}$Tc-labelling method for Affibody molecules;
3. evaluate the stability, \textit{in vitro} and \textit{in vivo} targeting properties of the radiolabelled conjugates;
4. investigate the influence of chelator sequences on the biodistribution properties;
5. study the influence of chelator (N- or C-terminus) position on the biodistribution properties;
6. evaluate the influence of different labels on the biodistribution properties.
3. The present study

3.1 Effects of N-terminal cysteine-based chelators (I)

3.1.1 Development of a $^{99m}$Tc-labelling method

Technetium chemistry was first applied to Affibody molecules using a $^{99m}$Tc-tricarbonyl core [$^{99m}$Tc(CO)$_3]^+$ but the results were unsatisfactory [112]. Although improvements were achieved by using the [Tc=O] core with the mercaptoacetyl-tri-glycyl (MAG3) chelator, high hepatobiliary excretion obscured the detection of tumours and metastases in the abdominal area [113].

In an attempt to improve the biodistribution properties, cysteine-based chelators containing cysteine-diglycyl (CGG) and cysteine-triglycyl (CGGG) were investigated as alternatives for $^{99m}$Tc-labelling of Affibody molecules, as described in paper I.

A major contribution of radiochemistry has been the development of kit formulations which facilitate simple preparation. A radiopharmaceutical should be simple to use in a clinical application and require minimal preparation by the hospital radiopharmacist. The formulation should be stable under ordinary conditions of use and storage and a heating step or separation step should be avoided. The labelling yield of a $^{99m}$Tc-based radiopharmaceutical must be over 90 % to avoid the need for purification and the radiochemical purity should preferably be over 95 %.

In order to optimise labelling, direct and indirect $^{99m}$Tc-labelling methods were evaluated. Na/K tartrate was used in indirect labelling as a weak chelating agent to form an intermediate $^{99m}$Tc-complex, to stabilize Tc in its intermediate oxidation state, followed by addition of this intermediate complex to the final ligand/protein. The direct labelling method involved a single-step reaction in which $^{99m}$TcO$_4^-$ was added to both the coordinating chelator/protein and the reducing agent under alkaline conditions for deprotonation of the sulphur and donor atoms [114].

As can be seen in Table 3, a greater labelling efficiency of $^{99m}$Tc-CGGG-ZHER2:342 was achieved using the direct labelling method (90 ± 4 %) than the indirect method (54 ± 10 %). Simple purification on a disposable NAP-5 column gave a high radiochemical purity of over 95 %.
Table 3. Labelling and characterisation of $^{99m}\text{Tc}$-labelled cysteine-based conjugates

<table>
<thead>
<tr>
<th></th>
<th>$^{99m}\text{Tc}$-CGGG-ZHER2:342</th>
<th>$^{99m}\text{Tc}$-CGG-ZHER2:342</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Indirect</td>
<td>Direct</td>
</tr>
<tr>
<td>Labelling yield, %</td>
<td>54 ± 10</td>
<td>90 ± 4</td>
</tr>
<tr>
<td>Isolated yield, %</td>
<td>49 ± 9</td>
<td>72 ± 6</td>
</tr>
<tr>
<td>Radiochemical purity, %</td>
<td>98 ± 2</td>
<td>98 ± 2</td>
</tr>
<tr>
<td>Antigen binding capacity, %</td>
<td>75 ± 12</td>
<td>63 ± 6</td>
</tr>
<tr>
<td>Stability in cysteine, %</td>
<td>67 ± 15</td>
<td>70 ± 6</td>
</tr>
<tr>
<td>Stability in PBS, %</td>
<td>85 ± 17</td>
<td>83 ± 5</td>
</tr>
</tbody>
</table>

3.1.2 Stability studies

**Cysteine challenge**

Transchelation to cysteine in blood proteins can cause significant losses of technetium labels in vivo. The stability of a $^{99m}\text{Tc}$-label can be assessed using a cysteine challenge test in which the labelled conjugate is incubated with excess of cysteine. The amount of radioactivity still attached to the conjugate relative to the amount transchelated to free cysteine is a measure of the binding strength of the $^{99m}\text{Tc}$ to the protein. The stability of the labelled Affibody conjugates in a 300-fold molar excess of cysteine, incubated for 2 h at 37 °C, can be expressed as the percentage of radioactivity still attached to the protein. The stability of the cysteine-based conjugates was 70 % which was comparatively high.

**Plasma stability**

Plasma contains several proteins and enzymes that can cause protein binding or enzymatic degradation of radiopharmaceuticals. High protein binding leads to a prolonged retention of radioactivity in the blood circulation, while instability towards enzymatic degradation can result in several product fragments of a protein. Both cases generate high backgrounds for imaging, decreased tumour uptake and possibly unnecessary radiation burden. To investigate their stability in plasma, the labelled conjugates were incubated in mouse blood and analysed using SDS PAGE gels.

The results (Figure 7) demonstrated that the conjugates were stable as no degradation to low molecular weight product was observed. However, the peak of $^{99m}\text{Tc}$-CGG-ZHER2:342 had a small amount of radioactivity associated with larger proteins which was probably due to minor transchelation to blood plasma proteins.
Figure 7. Serum stability of A. $^{99m}$Tc-CGGG-ZHER2:342 and B. $^{99m}$Tc-CGG-ZHER2:342. The first peak in each figure indicates the labelled compound while the arrows indicate the reference sample of free pertechnetate, run on the same SDS PAGE gel.

3.1.3 In vivo targeting properties

![Graph showing biodistribution of $^{99m}$Tc-maGGG-ZHER2:342 and $^{99m}$Tc-labelled cysteine-based ZHER2:342 in NMRI mice, 4 h p.i.]

Figure 8. Biodistribution of $^{99m}$Tc-maGGG-ZHER2:342 and $^{99m}$Tc-labelled cysteine-based ZHER2:342 in NMRI mice, 4 h p.i.

The $^{99m}$Tc-labelled Affibody molecules containing cysteine-based chelators were compared with a previously studied conjugate, $^{99m}$Tc-maGGG-ZHER2:342 [113]. A biodistribution study was performed in tumour-free mice with all conjugates (see Figure 8). It was found that the labelling method (direct or indirect) did not have a significant effect on biodistribution properties as the radioactivity concentrations were similar in all organs. While the $^{99m}$Tc-labeled cysteine-based conjugates behaved similarly, the maGGG chelator exhibited a 3-fold higher accumulation of radioactivity in the intestines. This effect was explained by the use of cysteine instead of mercaptoacetyl as a thiol group donor, which increases the overall hydrophilicity of the chelate due to the presence of a charged amino group and changes the excretion pathway from hepatobiliary to renal (Figure 8).
In tumour-bearing mice, $^{99m}$Tc-CGG-Z$_{HER2:342}$ bound specifically to HER2 receptors and a tumour-to-blood (T/B) ratio of 9.2 was obtained at 6 h post-injections (p.i.).

3.2 Effects of mercaptoacetyl-based chelators (II-IV)

Papers II-IV report the optimization of the tumour targeting properties of the Affibody molecule Z$_{HER2:342}$ for HER2 imaging. An array of different mercaptoacetyl-based chelators (Figure 9) coupled to the N-terminus of Z$_{HER2:342}$ was generated using peptide synthesis. The chelator mercaptoacetyl-triglycyl, maGGG, originally studied by Engfeldt et al [113], was included for comparison. The mercaptoacetyl-triseryl, maSSS (paper II), mercaptoacetyl-triglutamyl, maEEE (paper III), mercaptoacetyl-seryl-lysyl-seryl, maSKS (paper IV) chelators and other derivatives were labelled and characterised in vitro and in vivo.

Figure 9. Chelator structures used for $^{99m}$Tc-labelled Z$_{HER2:342}$ in paper II-IV. The dashed lines indicate border to the Affibody molecule. All chelators are anchored on the N-terminus of Z$_{HER2:342}$.

Biophysical characterisations, including circular dichroism (CD) spectra, melting point measurement and Biacore analysis, were performed to confirm that the synthetic proteins retained their folding and binding capacity after peptide synthesis. The CD spectra are exemplified in Figure 13 (paper IV). The peptide synthesis yield, melting point ($T_m$) and the $K_D$ (equilibrium dissociation constant = affinity) of the proteins are summarised in Table 4.
<table>
<thead>
<tr>
<th>Affibody molecule</th>
<th>Synthesis yield (%)</th>
<th>Calculated/Experimental Mw (Da)</th>
<th>Tm (°C)</th>
<th>Kd (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z_{HER2:342}</td>
<td>21</td>
<td>6718/6718</td>
<td>64</td>
<td>76</td>
</tr>
<tr>
<td>maGGG-Z_{HER2:342}</td>
<td>17</td>
<td>6494/6493</td>
<td>65</td>
<td>270</td>
</tr>
<tr>
<td>maGSG-Z_{HER2:342}</td>
<td>15</td>
<td>6994/6993</td>
<td>68</td>
<td>280</td>
</tr>
<tr>
<td>maSSS-Z_{HER2:342}</td>
<td>14</td>
<td>7054/7053</td>
<td>65</td>
<td>430</td>
</tr>
<tr>
<td>maGEG-Z_{HER2:342}</td>
<td>17</td>
<td>7036/7035</td>
<td>ND</td>
<td>360</td>
</tr>
<tr>
<td>maEEE-Z_{HER2:342}</td>
<td>13</td>
<td>7180/7179</td>
<td>68</td>
<td>410</td>
</tr>
<tr>
<td>maSKS-Z_{HER2:342}</td>
<td>14</td>
<td>7095/7094</td>
<td>65</td>
<td>310</td>
</tr>
<tr>
<td>maKKK-Z_{HER2:342}</td>
<td>12</td>
<td>7177/7177</td>
<td>68</td>
<td>270</td>
</tr>
</tbody>
</table>

ND: not determined

3.2.1 Improved targeting with maSSS chelator (paper II)

A previously studied Affibody molecule, $^{99m}$Tc-maGGG-Z_{HER2:342} binds specifically to HER2 tumours but undergoes significant hepatobiliary excretion, causing a high accumulation of radioactivity in the abdomen [113]. This would restrict the imaging of tumours and metastases in the lumbar area at the day of injection. Potential improvement of the biodistribution of Z_{HER2:342} was explored by substituting glycyl residues in the chelating sequence with more hydrophilic seryl residues.

The biodistribution properties in normal NMRI mice 4 h p.i. of $^{99m}$Tc-maGGG-, $^{99m}$Tc-maGSG- and $^{99m}$Tc-maSSS-Z_{HER2:342} are illustrated in Figure 10. The substitution of one glycine with serine (maGGG to maGSG) resulted in a significant 2-fold reduction (p < 0.001) of radioactivity in the gastrointestinal tract (GI) compared to mice injected with $^{99m}$Tc-maGGG-Z_{HER2:342}. The substitution of all three glycines in the chelator sequence with three serines (maGGG to maSSS) reduced the GI radioactivity almost 3-fold, indicating that the reduction of radioactivity correlated with the number of substituted serines. Accordingly, the radioactivity accumulation in the kidneys increased with the number of substituted glycine residues, indicating a change to predominantly renal excretion. The renal accumulation of $^{99m}$Tc-maSSS-Z_{HER2:342} was significantly higher than that of either $^{99m}$Tc-maGGG-Z_{HER2:342} or $^{99m}$Tc-maGSG-Z_{HER2:342} (p < 0.01) (see Figure 10).

Serine substitution also improved catabolic stability in vivo, as verified by low uptake in the stomach and salivary glands (Figure 10).
The introduction of serine into the chelating sequence created a chiral centre. It has been reported that the introduction of D-alanine into the GAGG peptide chelator results in a highly stable label [115]. Different forms (L- or D-form) might give different stability and different in vivo behaviour. To elucidate whether this was also the case for serine-containing chelators, the biodistribution data of $^{99m}$Tc-maGSG-ZHER2:342 and $^{99m}$Tc-maG(D-S)G-ZHER2:342 at 4 h p.i. were compared. There was no significant difference between the conjugates, indicating that chirality did not influence the distribution of the conjugate in mice, at least not at the selected time point.

The $^{99m}$Tc-maSSS-ZHER2:342 conjugate was further investigated in human ovarian carcinoma (SKOV-3) xenografts because it had the lowest degree of hepatobiliary excretion, the best in vivo stability, the longest cellular retention and the highest antigen binding capacity. The tumour targeting was efficient and resulted in 11.5 ± 0.5 %IA/g in tumours at 4 h p.i., while accumulation in other organs was low and especially in the blood (less than 1 % at 4 h p.i.). The tumour-to-blood (T/B) ratio was 76 at 4 h p.i., which was much higher than $^{99m}$Tc-maGGG-ZHER2:342, which had a T/B ratio of 24 at the same time point [113].

In conclusion, the use of the mercaptoacetyl-triseryl (maSSS) sequence resulted in a stable conjugate with specific binding to HER2 receptors, good retention of radioactivity and good in vivo targeting properties. However, the radioactivity accumulation in the GI tract (11 ± 1.5 % IA) was still high and further improvements were therefore needed.
3.2.2 Improved stability using a maEEE chelator (paper III)

The hydrophilicity could be further increased by employing amino acids with charged side chains, such as glutamic acids. Glutamic acid (E) is a polar, negatively charged and very hydrophilic amino acid. Glutamic acid is more suitable for peptide synthesis than aspartic acid because the formation of aspartimide commonly occurs during synthesis as a side reaction by cyclization of aspartyl residues [116].

In paper III, chelators containing mercaptoacetyl-glycyl-glutamyl-glycyl (maGEG) and mercaptoacetyl-triglytamyl (maEEE) were investigated as alternatives to maGGG in order to improve the imaging contrast in the abdomen area.

Both maGEG-Z\textsubscript{HER2:342} and maEEE-Z\textsubscript{HER2:342} were successfully $^{99m}$Tc-labelled with a labelling yield of over 90 % and a radiochemical purity of more than 95 %. Both $^{99m}$Tc-maGEG- and $^{99m}$Tc-maEEE-Z\textsubscript{HER2:342} were stable during incubation in 300-fold molar excess of cysteine for 2 h at 37 \degree C. Furthermore, no evidence of release of radioactivity from $^{99m}$Tc-maEEE-Z\textsubscript{HER2:342} or any technetium transchelation to blood plasma proteins was detected after incubation in mouse serum for 1 h, signifying its suitability for \textit{in vivo} use. The labelled conjugates also retained their specific binding to HER2 receptors in SKOV-3 cells \textit{in vitro}.

In \textit{vivo}, the substitution of a single glycine with glutamic acid (maGGG to maGEG) exerted a clear effect on the excretion pattern in normal mice, with a significant 3-fold reduction of radioactivity accumulated in the intestines (hepatobiliary excretion). The hepatobiliary clearance was further reduced when all three glycines in the chelator were replaced by glutamic acids (maGGG to maEEE), resulting in a 10-fold decrease in intestinal accumulation.

The \textit{in vivo} tumour-targeting properties of $^{99m}$Tc-maEEE-Z\textsubscript{HER2:342} were investigated in BALB/c \textit{nu/nu} mice bearing HER2-expressing SKOV-3 xenografts (Figure 11). The radioactivity was rapidly cleared from the blood circulation, reaching $0.15 \pm 0.05 \% \text{ IA/g}$ at 6 h p.i. The clearance of radioactivity from other organs followed the same pattern. The tumour uptake was $9.1 \pm 1.4 \% \text{ IA/g}$ at 1 h p.i., $7.9 \pm 1.0 \% \text{ IA/g}$ at 4 h p.i. and $6.1 \pm 0.4 \% \text{ IA/g}$ at 6 h p.i., indicating a decrease over time.
Figure 11. Biodistribution of $^{99m}$Tc-maEEE-Z$_{HER2:342}$ at 1, 4 and 6 h p.i. in SKOV-3 tumour bearing mice. Data are presented as % IA/g from 4 animals ± SEM. Data for intestines is expressed as % IA per whole organ.

In order to test the binding specificity of the conjugate, one group of mice was pre-injected with a 1,000-fold molar excess of non-labelled Z$_{HER2:342}$ to saturate the receptors before administration of $^{99m}$Tc-maEEE-Z$_{HER2:342}$. The tumour uptake (Figure 12A) in the blocked group was 0.3 ± 0.07 % IA/g but was 7.9 ± 1.0 % IA/g in the non-blocked group (p < 0.001), demonstrating that the tumour uptake was HER2-specific. The gamma-camera imaging also confirmed that binding was HER2-specific (Figure 12B).

Figure 12. A. Dot plot of tumour uptake (% IA/g) in BALB/c $nu/nu$ mice with SKOV-3 xenografts 4 h p.i. Blocked mice received an excess of non-labelled Z$_{HER2:342}$ prior to injection of $^{99m}$Tc-maEEE-Z$_{HER2:342}$. Student’s t-test gave p < 0.001. B. Gamma-camera imaging of tumours using $^{99m}$Tc-maEEE-Z$_{HER2:342}$. The image was taken at 5 h p.i. and the tumours were blocked with a pre-injection of excess of non-labelled Z$_{HER2:342}$. 
In conclusion, increased hydrophilicity, combined with improved stability of the mercaptoacetyl-triglutamyl chelator resulted in minimal abdominal accumulation which enabled the rapid detection of HER2-positive tumours and their metastases in the abdominal area. Thus, $^{99m}$Tc-maEEE-ZHER2:342 appears to be a promising tracer for the clinical imaging of HER2 expression in tumours and metastases.

3.2.3 Increased liver accumulation with maKKK (paper IV)

In the previously reported study it was found that the use of negatively charged side chains in the chelator, maEEE led to the hepatobiliary excretion [117]. The combination of two serines and one glutamate in the chelator, as in mercaptoacetyl-glutamyl-seryl-glutamyl (maESE), produced favourable properties for imaging [118].

One purpose of the study reported in paper IV was to investigate the influence of the positively charged hydrophilic amino acid lysine on the biodistribution properties. For this purpose, two novel Affibody molecules, mercaptoacetyl-seryl-lysyl-seryl, maSKS-ZHER2:342 and mercaptoacetyl-trilysyl, maKKK-ZHER2:342, were synthesised and labelled with $^{99m}$Tc.

Biophysical characterisation of the synthetic molecules was carried out. As well as Biacore analysis, the maKKK-ZHER2:342 and maSKS-ZHER2:342 were also subjected to CD analysis. As illustrated in Figure 13, the CD spectra of the proteins showed that the Affibody molecules were similar, retained their $\alpha$-helical structures and refolded after heating to 90 °C. The melting points of the conjugates were between 64 and 68 °C, which is close to that previously reported for ZHER2:342.

![Figure 13. Circular dichroism (CD) spectra of A. maKKK-ZHER2:342 and B. maSKS-ZHER2:342 obtaining before heating and after heating to 90 °C.](image)

The in vivo results indicated that the substitution of a single serine with one lysine in the chelator (maSSS to maSKS) decreased the hepatobiliary excretion 2-fold and that kidney uptake was increased. This shift might be explained by the increased hydrophilicity of the lysine in the chelator. However, substitution of all three serines with lysines (maSSS to maKKK) did not decrease the hepatobiliary excretion further (intestinal uptake 4 ± 0.3 %)
IA/sample for labelled maKKK-Z_{HER2:342} compared to 4.3 ± 0.5 % IA/sample for maSKS-Z_{HER2:342}, p = 0.34), while renal accumulation was significantly higher. The radioactivity of $^{99m}$Tc-maKKK-Z_{HER2:342} was also found to be higher in other organs including the liver, spleen, stomach and salivary glands, compared with the levels observed when using labelled maSSS-Z_{HER2:342} or maSKS-Z_{HER2:342}.

An unexpected result was the elevation of radioactivity in the liver for $^{99m}$Tc-maKKK-Z_{HER2:342}. In a previous study, the use of multiple copies of the amino acid histidine (in His-tagged Affibody molecules) also resulted in the increased accumulation of radioactivity in the liver [119]. There is a clear indication that the use of multiple positively charged amino acids in Affibody molecules can lead to increased accumulation in the liver. However, the mechanism behind this phenomenon is unclear.

3.2.4 Comparative data of mercaptoacetyl-based chelators

In the papers (II-IV) presented above, we have investigated the use of a number of amino acid sequences as chelators for $^{99m}$Tc-labelling of Affibody molecule in an attempt to improve the imaging contrast in the abdominal area for detection of tumours at the day of injection.

![Figure 14. Retention of the investigated $^{99m}$T-labelled Z_{HER2:342} containing different mercaptoacetyl-based chelators on SKOV-3 cells over time.](image)

In summary, the in vitro data showed that all labelled conjugates bound specifically to HER2 receptors on cells. Cellular retention of the investigated Affibody molecules was high at about 70 % cell-associated radioactivity 2 h after interrupted incubation and the retention did not appear to decline much (Figure 14).

The tumour-to-blood ratio (T/B) is often used as an indicator to evaluate the imaging contrast. Of the four conjugates that were investigated, $^{99m}$Tc-maSSS-Z_{HER2:342} had the highest T/B ratio. As can be seen, the contrast
achieved using $^{99m}$Tc-maSSS-Z$_{HER2:342}$ (Figure 15B) clearly visualised the tumour but there was a high intestinal accumulation. The inclusion of multiple negatively charged glutamic acids in $^{99m}$Tc-maEEE-Z$_{HER2:342}$ resulted in a change from hepatobiliary to renal excretion (Figure 15C). The substitution of a single serine with lysine seems to be the most effective way to improve the contrast by achieving high tumour uptake, low intestinal accumulation and moderate kidney accumulation (Table 5).

Table 5. Biodistribution data of $^{99m}$Tc-labelled conjugates. Tumour uptake and kidney accumulation are presented as % IA/g while intestinal accumulation is presented as % IA per whole organ. Data were accessed from Balb nu/nu mice bearing SKOV-3 xenografts at 4 h p.i. *Data for maGGG-Z$_{HER2:342}$ was accessed from LS174T xenografts.

<table>
<thead>
<tr>
<th>Affibody molecule</th>
<th>Tumour uptake</th>
<th>Intestinal accumulation</th>
<th>Kidney accumulation</th>
<th>T/B ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>*maGGG-Z$_{HER2:342}$</td>
<td>6.1 ± 2.0</td>
<td>ND</td>
<td>5.5 ± 0.15</td>
<td>24</td>
</tr>
<tr>
<td>maSSS-Z$_{HER2:342}$</td>
<td>11.5 ± 0.5</td>
<td>11 ± 1.5</td>
<td>34 ± 1.5</td>
<td>76 ± 8</td>
</tr>
<tr>
<td>maEEE-Z$_{HER2:342}$</td>
<td>7.9 ± 1</td>
<td>3.7 ± 0.9</td>
<td>104 ± 14</td>
<td>38 ± 7</td>
</tr>
<tr>
<td>maSKS-Z$_{HER2:342}$</td>
<td>17 ± 8</td>
<td>4.3 ± 0.9</td>
<td>67 ± 14</td>
<td>48 ± 23</td>
</tr>
</tbody>
</table>

ND: not determined

In conclusion, the use of more hydrophilic, polar or charged amino acids in the chelating part of the Affibody molecule is an efficient way to modify the in vivo targeting properties for imaging. The issue of hepatobiliary excretion of $^{99m}$Tc-labelled synthetic Affibody molecules was resolved by the incorporation of negatively charged side chains, such as glutamic acids (as in maEEE), or positively charged lysine-containing amino acids (as in maSKS) (Figure 15). The overall in vivo stability was also improved by the use of these conjugates.

Figure 15. Illustrative SPECT images of tumours, using A. $^{99m}$Tc-maGGG-Z$_{HER2:342}$ at 6 h p.i. B. $^{99m}$Tc-maSSS-Z$_{HER2:342}$ 6 h. i. C. $^{99m}$Tc-maEEE-Z$_{HER2:342}$ at 5 h p.i. and D. $^{99m}$Tc-maSKS-Z$_{HER2:342}$ at 4 h p.i. Imaging was performed on LS174T xenograft in A. and on SKOV-3 xenografts in all others.
3.3 Design of a multifunctional Affibody molecules (V)

Several aspects of the development of a radiopharmaceutical drug require careful consideration. Ideally, an imaging tracer should be flexible to allow labelling with a number of different radionuclides for both SPECT and PET applications. The chelating amino acid sequence should be suitable not only for recombinant production but also for high-yield chemical peptide synthesis.

Such flexible design is exemplified by the novel HER2-specific Affibody molecule PEP05352, which was discussed in paper V. The incorporation of a C-terminal sequence of SECG (serine-glutamic acid-cysteine-glycine) in the Affibody molecule has created a general multifunctional platform. The SECG sequence provides a site-specific chelator for $^{99m}$Tc-labelling, while the same cysteine in the sequence also offers the possibility to couple numerous chelator derivatives (DOTA, DTPA, CHX-A”-DTPA) for indium-labelling and for labelling with other radiometals (gallium, cobalt or yttrium). The DOTA-conjugated PEP05352 used in this study was designated PEP05541.

3.3.1 C-terminal modifications preserve the binding affinity

The binding strength/affinity (the dissociation equilibrium constant, $K_D$) was determined using surface plasmon resonance (SPR) technology on a Biacore instrument. Figure 16 illustrates typical sensorgrams showing the binding kinetics of PEP05352 before ($K_D = 74 \text{ pM}$) and after ($K_D = 84 \text{ pM}$) DOTA conjugation. These values agree with earlier results ($K_D = 64 \text{ pM}$) for the synthetic ZHER2:342, which has an N-terminal DOTA.

One observation made from this study concerned the effects of modifications on the binding affinity. As summarised in Table 6, DOTA conjugation either on the N-terminal or C-terminal of the Affibody molecule does not seem to affect the binding strength significantly. However, the position of the technetium chelator has a significant impact on the binding strength. For example, the $K_D$ values were 280 and 250 pM for CGG-ZHER2:342 and CGGG-ZHER2:342, respectively [120] and the $K_D$ of another homologous conjugate, maESE-ZHER2:342 was 500 pM [118]. These binding affinities are weaker than the original binding affinity of ZHER2:342, which has a $K_D$ of 80 pM [113]. Although the affinities were not determined in the same experiment, the data indicate that it is more favourable to attach the chelating sequence at the C-terminus to preserve the binding affinity.
Figure 16. Binding affinity (K_D) and binding kinetics of PEP05352 determined by surface plasmon resonance (SPR) using a Biacore 2000 instrument. Sensorgrams of PEP05352 determined before DOTA conjugation (upper) and after DOTA conjugation (lower) using 6 concentrations ranging 0.3 – 10 nM.

Table 6. Binding affinity (K_D) of Affibody molecules

<table>
<thead>
<tr>
<th>Molecule</th>
<th>K_D (pM)</th>
<th>Chelator/Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z_HER2:342</td>
<td>80</td>
<td>Synthetic</td>
</tr>
<tr>
<td>CGG-Z_HER2:342</td>
<td>280</td>
<td>CGG on N-terminal</td>
</tr>
<tr>
<td>CGGG-Z_HER2:342</td>
<td>250</td>
<td>CGGG on N-terminal</td>
</tr>
<tr>
<td>maESE-Z_HER2:342</td>
<td>500</td>
<td>maESE on N-terminal</td>
</tr>
<tr>
<td>DOTA-Z_HER2:342</td>
<td>64</td>
<td>DOTA on N-terminal</td>
</tr>
<tr>
<td>PEP05352</td>
<td>74</td>
<td>SECG on C-terminal</td>
</tr>
<tr>
<td>PEP05541</td>
<td>84</td>
<td>DOTA on C-terminal</td>
</tr>
</tbody>
</table>

3.3.2 Combination of serine, glutamic acid and a thiol-bearing moiety reduces renal uptake

Earlier studies indicated that the combination of a serine and a glutamic acid next to a thiol-bearing moiety of the chelator (as in maESE) reduces the retention of 99mTc in the kidney [118]. The uptake of 99mTc-PEP05352 did not differ significantly from the uptake of 99mTc-Z\_HER2:2395-C, which was a re-
combinantly produced anti-HER2 Affibody molecule with the C-terminal sequence –KVDC [121]. The tumour uptake of $^{99m}$Tc-PEP05352 in LS174T xenografts at 4 h p.i. (6.30 ± 0.67 % IA/g) did not differ significantly from the uptake of $^{99m}$Tc-ZHER2:2395-C (6.9 ± 2.5 % IA/g) in this model. The most striking difference was the reduced renal accumulation. The renal uptake of $^{99m}$Tc-ZHER2:2395-C was 191 ± 15 % IA/g at 4 h p.i. in nude mice [121], while the uptake of $^{99m}$Tc-PEP05352 was only 48 ± 6.1 % IA/g at 4 h p.i. in the same mouse strain, i.e. nearly 4-fold lower. This confirmed the hypothesis that the C-terminal placement of a combination of serine and glutamic acid with a thiol-bearing moiety (cysteine in this case) in the chelator would reduce renal uptake.

Table 7. Biodistribution of $^{99m}$Tc-PEP05352 and $^{111}$In-PEP05352 in BALB/c nu/nu mice, 4 h p.i. The data are presented as % IA/g (mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>$^{99m}$Tc-PEP05352</th>
<th>$^{111}$In-PEP05352</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>0.21 ± 0.02</td>
<td>0.27 ± 0.05</td>
</tr>
<tr>
<td>Lung</td>
<td>0.35 ± 0.04</td>
<td>0.56 ± 0.23</td>
</tr>
<tr>
<td>Liver</td>
<td>1.5 ± 0.2</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.20 ± 0.03</td>
<td>0.41 ± 0.03***</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.34 ± 0.05</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Kidney</strong></td>
<td><strong>48 ± 6.1</strong></td>
<td><strong>256 ± 17</strong>*</td>
</tr>
<tr>
<td>Salivary glands</td>
<td>0.28 ± 0.03</td>
<td>ND</td>
</tr>
<tr>
<td>Thyroid*</td>
<td>0.007 ± 0.002</td>
<td>ND</td>
</tr>
<tr>
<td>Tumour</td>
<td>6.30 ± 0.67</td>
<td>8.20 ± 1.2**</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.12 ± 0.08</td>
<td>0.081 ± 0.009</td>
</tr>
<tr>
<td>Bone</td>
<td>0.35 ± 0.13</td>
<td>0.25 ± 0.024</td>
</tr>
<tr>
<td>Intestine*</td>
<td>1.9 ± 0.10</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Intestine uptake are measured as % IA per whole content, not per gram
** Student’s t-test give p = 0.03 and *** p < 0.0001

3.3.3 Tumour imaging using a multifunctional Affibody molecule

A multifunctional Affibody molecule has been created and its flexible labelling platform has been demonstrated using $^{99m}$Tc and $^{111}$In (a model label for other trivalent radiometals). Imaging of HER2 expression in LS174T xenografts using PEP05352 is shown in Figure 17. The tumours were detected as early as 1 h after injection of $^{111}$In-labelled Affibody molecule. The tumours were also clearly visualised using $^{99m}$Tc-PEP05352 at 4 h p.i. (Figure 17A).
In conclusion, the incorporation of a C-terminal SECG sequence in Affibody molecules provides a general multifunctional platform for labelling with a number of radionuclides (technetium/indium/gallium/cobalt/yttrium) and for production using recombinant or chemical synthesis which could be extended to other scaffold protein-based tracers.
4. Conclusion remarks

The works presented in this thesis is part of the preclinical development of an Affibody molecule-based tracer for cancer imaging. Much effort has been dedicated to the design and development of a synthetic Affibody molecule for HER2 imaging. The primary goal was to develop a $^{99m}$Tc-based radiotracer for SPECT detection but this was adapted to include the development of a multifunctional tracer suitable for both SPECT and PET applications. Experimental work included the design, synthesis and biological evaluation of a number of variants of the tracers. A major part has focused on the chelator engineering for $^{99m}$Tc-labelling, because it is known that the chelating construction has essential strong impact on the in vivo targeting properties of the tracer. The results will be applicable not only to the development of other Affibody molecules (for instance EGFR-, CD44v6-, PMSA-specific tracers) but hopefully also to the design of other small-sized peptide or scaffold-protein based probes.

The major conclusions from this research are:

- Solid phase peptide synthesis can be utilized for site-specific chemical modifications of Affibody molecules, to tailor imaging properties with desired characteristics
- Chemical synthesis is a powerful method which enables the incorporate of both natural and unnatural sequences without compromising the synthetic yield
- For $^{99m}$Tc-labelling, suitable chelators are highly importance to obtain a stable tracer in vitro and in vivo. Synthetic Affibody molecules can be radiolabelled with $^{99m}$Tc with high yield and high radiochemical purity
- The synthetic Affibody molecule $Z_{\text{HER2:342}}$ can be labelled with $^{99m}$Tc both directly and indirectly without adverse effects in vitro or any significant differences in biodistribution properties in vivo
- Chirality (L- or D-form) has no significant effect on biodistribution properties
- Modification of the chelating moiety of the Affibody molecule is an efficient way to change the in vivo biodistribution characteristics. The substitution of a single amino acid in a 60-amino acid long protein has a pronounced effect on biodistribution and especially on the excretion pathway
• Strategic approaches have been applied to decrease the lipophilicity or increase the hydrophilicity, i.e., by incorporating more hydrophilic amino acids into the chelating sequence. Substitution with amino acids containing hydroxyl, carboxylate or amine groups, such as serine, glutamic acid and lysine efficiently changes the excretion pathway from hepatobiliary to renal excretion.

• The problems caused by the hepatobiliary excretion pathway that impede the detection of tumours and metastases in the abdomen area are solved by the use of maEEE and maSKS chelator.

• Multiple copies of positively charged amino acids (as in maKKK or His-tag) result in increased uptake in the liver which is inappropriate because the liver is a common metastatic site in breast cancer.

• The incorporation of chelators on the N-terminus of an Affibody molecule seems to affect the binding affinity compared to their incorporation at the C-terminus. Consequently, attachment of the chelating sequence at the C-terminus preserves the binding affinity.

• A combination of serine, glutamic acid and a thiol-bearing group reduces renal uptake.

• The introduction of a unique cysteine in Affibody molecules enables site-specific labelling with $^{99m}$Tc and other radionuclides.

• Coupling of the DOTA chelator permits labelling with a wide variety of trivalent radionuclides.

• Renal retention of $^{111}$In-label is much higher than for $^{99m}$Tc-label.

• The incorporation of a SECG sequence provides a general multifunctional platform for labelling and flexibility of production of Affibody molecules.

In conclusion, some of the obvious problems that occurred during development of an Affibody molecule-based imaging probe have been resolved during the study. Significant efforts were directed first to improve the quality of imaging provided by the tracer and second to decrease the renal uptake, with a final goal of making clinical HER2 imaging a reality. To date, molecular imaging based on Affibody molecules is beginning clinical trials (www.affibody.com) that are intended to improve the diagnosis of HER2-positive breast cancer without the need for biopsies. This will enable clinicians to rapidly identify, localize and assess HER2 status in order to select a suitable treatment for each individual cancer patient.
5. Ongoing and future studies

Affibody molecules are an attractive class of targeting agents for molecular imaging. The most important characteristics of an efficient diagnostic agent are sensitivity and specificity [67, 82, 83]. These features depend, in turn, on the key properties of an imaging agent, including binding specificity, molecular size, binding affinity and biodistribution properties [98].

It is evident that Affibody molecules can be selected with high affinity (picomolar affinity) and high binding specificity to the target. Affibody molecules are small and are cleared rapidly from the blood circulation, which gives good imaging contrast. This thesis has demonstrated in detail that the in vivo biodistribution properties depend on the chosen conjugation chemistry and radiochemistry (labelling method, type of radionuclide, chelator sequence, location of the chelator position etc.).

An important finding has been that within a sequence a modification of only a few amino acids can significantly reduce the renal accumulation of Affibody molecules in vivo, while the incorporation of different amino acids can lead to high accumulation in the liver. The underlying mechanisms of these phenomena are currently unknown. A logical continuation of this research would be to investigate these mechanisms in greater detail.

Discussion of binding affinity is controversial in targeted radionuclide tumour imaging and therapy. It is assumed that molecules with higher binding affinities target tumours more effectively [122]. Consequently significant effort in protein engineering is committed to increasing the affinity of mAbs to their targets. This approach is also applied for Affibody molecule-based binders [95]. However, this assumption has been questionable by Weinstein et al [123] who proposed the theory of a “binding site barrier’. Such a barrier is hypothesised to retard the penetration of antibodies into tumour masses because of high-affinity interactions with the antigen, which retain the antibody at the binding site and prevent its movement deeper into the tumour mass [123]. Various mathematical simulations [124] and a limited number of in vivo studies [124-127] have been performed to elucidate this hypothesis. For example, Adams et al reported that the optimal affinity for tumour targeting in vivo using HER2-binding scFv is in the nanomolar range and higher affinity scFv counterparts impeded further tumour penetration [126].

In the case of Affibody molecules, there is an indication that high-affinity binding molecules (in the picomolar range) are better for tumour targeting.
For example, the first generation HER2-binder $Z_{\text{HER2:4}} \ [95, 128]$ with a $K_D$ of 50 nM exhibited a tumour uptake of 2.6 % IA/g while the second generation, maturated HER2 molecule $Z_{\text{HER2:342}}$ had a tumour uptake of 9.5 % IA/g in the same xenograft model at the same time point [95].

Tremendous efforts are invested in the development of more strongly binding Affibody molecules. It is therefore important to understand the influence of binding affinity on penetration into tumours and thus to identify an optimal binding affinity for effective tumour targeting. In the first instance, it is planned to examine the effects of binding affinity by applying a set of Affibody molecules with different affinities to tumour spheroids. Further studies will be conducted \textit{in vivo}. The results will hopefully be used to translate to a further generation of Affibody molecules for tumour targeting and should also provide greater insight into the binding site barrier theory.
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Royal Institute of Technology, KTH, Stockholm:

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Uppsala, 02-04-2009
Thuy


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