On the Design of Affibody Molecules for Radiolabeling and *In Vivo* Molecular Imaging

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Abstract

Affibody molecules have lately shown great potential as tools for in vivo molecular imaging. These small, 3-helical bundles, with their highly stable protein scaffold, are well suited for the often harsh conditions of radiolabeling. Their small size allows for rapid clearance from the blood circulation which permits the collection of images already within hours after injection. This thesis includes four papers aimed at engineering different variants of a HER2-binding Affibody molecule to enable effective and flexible radiolabeling and enhancing the molecular imaging in terms of imaging contrast and resolution.

In paper I an Affibody molecule was engineered to function as a multifunctional platform for site-specific labeling with different nuclides for radionuclide imaging. This was done using only natural amino acids, thereby allowing for both synthetic and recombinant production. By grafting the amino acid sequence -GSECG to the C-terminal of our model-protein, a HER2-binding Affibody molecule, we enabled site specific labeling with both trivalent radiometals and with $^{99m}$Tc. Maleimide-DOTA was conjugated to the cysteine residue for labeling with $^{111}$In, while the peptide sequence was able to chelate $^{99m}$Tc directly. This approach can also be used for site-specific labeling with other probes available for thiol-chemistry, and is applicable also to other protein scaffolds.

In paper II we investigated the impact of size and affinity of radiolabeled Affibody molecules on tumor targeting and image contrast. Two HER2-targeting Affibody molecules, a two-helix (~5 kDa) and a three-helix (~7 kDa) counterpart, were synthetically produced, labeled with $^{111}$In via chelation by DOTA and directly compared in terms of biodistribution and targeting properties. Results showed that the smaller variant can provide higher contrast images, at the cost of lower tumor uptake, in high-expressing HER2-tumors. However, neither the tumor uptake nor the contrast of the two-helix variant is sufficient to compete with the three-helix molecule in tumors with low expression of HER2.

In paper III and IV we were aiming to find methods to improve the labeling of Affibody molecules with $^{18}$F for PET imaging. Current methods are either complex, time-consuming or generate heavily lipophilic conjugates. This results in low yields of radiolabeled tracer, low specific activity left for imaging, undesirable biodistribution or a combination thereof. In paper III we demonstrate a swift and efficient 2-step, 1-pot method for labeling HER2-binding Affibody molecules by the formation of aluminum $^{18}$F-fluoride (Al$^{18}$F) and its chelation by NOTA, all in 30 min. The results show that the $^{18}$F-NOTA-approach is a very promising method of labeling Affibody molecules with $^{18}$F and further investigation of this scheme is highly motivated. In the last paper we pursued the possibility of decreasing the high kidney retention that is common among small radiotracers with residualizing radiometabolites. In this work $^{18}$F-4-fluorobenzaldehyde (FBA) was conjugated to a synthetic HER2-targeting Affibody molecule via oxime ligation. However, to avoid elevated liver retention, as seen in previous studies with this kind of label, a hydrophilic triglutamyl spacer between the aminooxy moiety and the N-terminal was introduced. A comparison of the two constructs (with and without the triglutamyl spacer) showed a clear reduction of retention in both kidney and liver in NMRI mice at 2 h p.i. when the spacer was included. In the light of these promising results, further studies including tumor-bearing mice, are in preparation.

Keywords: Affibody molecule, AC/DC, radionuclide molecular imaging, HER2, SPECT, PET, biodistribution, peptide synthesis, radiolabeling
List of publications

This thesis is based upon the following four papers, which are referred to in the text by their corresponding roman numerals (I-IV). The papers are included in the Appendix.


IV. Rosik D, Thibblin A, Antoni G, Orlova A, Eriksson Karlström A, Tolmachev V. Incorporation of a triglutamyl spacer improves the biodistribution of a synthetic Affibody molecule radiofluorinated at the N-terminus via oxime formation with $^{18}$F-4-fluorobenzaldehyde. Manuscript
“It’s a Long Way to the Top. If you wanna rock ‘n’ roll”

- Angus Young, Malcolm Young and Bon Scott
## INTRODUCTION

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##### 4.3.1 Imaging applications
Abbreviations

%-IA/g  percent of injected activity per gram
C_H  constant domain of the antibody heavy chain
C_L  constant domain of the antibody light chain
CT  computed tomography
DOTA  1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
Fab  fragment antigen-binding
Fc  fragment crystallizable
FISH  fluorescence in situ hybridization
HER2  human epidermal growth factor receptor 2 (ErbB2, neu)
IgG  immunoglobulin G
IHC  immunohistochemistry
K_D  equilibrium dissociation constant
K_a  association rate constant
K_d  dissociation rate constant
mAb  monoclonal antibody
MRI  magnetic resonance imaging
PET  positron emission tomography
p.i.  post injection
scFv  single-chain variable fragment
sdAb  single domain antibody
SPECT  single photon emission computed tomography
t_1/2  half-life
V_H  variable domain of the antibody heavy chain
V_L  variable domain of the antibody light chain

Affibody® molecule is abbreviated "Affibody molecule" throughout this thesis.
Introduction
1 Molecular Imaging

Molecular imaging is an effective and non-invasive way to study biological processes in living subjects, e.g. abnormal growth, enhanced activity/metabolism, inflammation or tissue damage/cell death. This information can in turn be used to diagnose a patient, follow disease progression during treatment or to personalize the treatment of one specific individual [1, 2].

As the name “Molecular Imaging” implies, the aim is to visualize molecular structures. Furthermore, the molecules should preferably be imaged within their native context, i.e. inside the living organism, and are thus inaccessible by conventional imaging techniques such as cameras or microscopes that rely on visible light.

The solution to this problem is to image the molecules indirectly e.g. by attaching some source of radiation that you can detect to the targeted molecule and then acquire an image of the signal distribution. For the radiation source to specifically accumulate at the molecular target of interest and not in the surroundings, a targeting molecule is usually needed. The targeting molecule, also called a tracer, is normally first labeled with the radiation source and then allowed to find and interact with the target. The excess of non-attached tracer is washed away – *in vivo* this is done by the blood circulation and/or lymphatic drainage with subsequent excretion via kidney and/or liver. The signal is collected by a camera, designed to detect the particular emitted radiation. The aim with this kind of imaging is to get as high signal as possible from the targeted area and as low signal as possible from the non-targeted surroundings, i.e. to get as high contrast as possible [3].

An important class of molecular targets for molecular imaging is cancer-associated molecular structures. Since cancer cells are the body’s own cells that are malfunctional in different ways it is not trivial how to distinguish them from normal, healthy cells and thereby administer an effective targeted therapy. If identified, unique molecular structures on the cancer cells can be targeted and imaged, thereby aiding in the diagnosis of the patient. The same molecular structures or related signaling pathways can then be targeted for therapy. By employing targeted therapy the exposure of healthy non-targeted cells to the therapeutic drugs and toxins is minimized, lowering the side effects for the patient and facilitating a more efficient therapy. An illustration on the principles of *in vivo* molecular imaging can be seen in Figure 1.

1.1 HER2 as a Target

The molecular target discussed in this thesis is the HER2/ErbB-2 receptor. HER2, together with the rest of the ErbB-family of tyrosine kinase receptors, was implicated in cancer already in the 1980’s [4, 5]. Today, HER2 is a well-established oncoprotein, related to several types of cancer, e.g. breast, lung, pancreas and colon [6]. The HER2 receptor does not have any known ligand but it is constantly active and can dimerize with any other member of the ErbB-family, including itself. Dimerization is necessary for signaling initiation [7]. Today, the use of trastuzumab is a well-established antibody therapy for patients with advanced relapsed breast cancers that overexpress HER2 [8, 9]. However, choosing patients that would benefit from this therapy is not straightforward [10, 11]. To determine the HER2 expression in patients, immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) are used. Both these methods require biopsies of the tumor. Because of intra- and interlesion heterogeneity of HER2 expression, errors in the detection are not uncommon. Also, the HER2 expression might change during the course of the disease. Furthermore,

![Image](image.png)
the invasiveness of biopsies prevent their multiple and frequent use. Therefore a good molecular imaging agent with the ability to detect and to some extent quantify the HER2 overexpression would be most useful.

In the following chapters, I will give a brief description of how molecular imaging can be performed, the different radiation sources used and how the targeting molecule can be designed, produced and conjugated with a label. Table 1 shows a comparison of some of the different techniques currently used.

### Table 1: Properties of the imaging modalities described in this work. (Modified from [12–14].)

<table>
<thead>
<tr>
<th>MODALITY</th>
<th>RADIATION</th>
<th>PENETRATION [mm]</th>
<th>SENSITIVITY [mol/l]</th>
<th>RESOLUTION [mm]</th>
<th>COST</th>
<th>QUANTIFICATION CAPABILITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>Ionizing</td>
<td>No limit</td>
<td>N/A</td>
<td>0.05</td>
<td>$$</td>
<td>N/A</td>
</tr>
<tr>
<td>MRI</td>
<td>Non-ionizing</td>
<td>No limit</td>
<td>$10^4 - 10^5$</td>
<td>$0.01-0.1^*$</td>
<td>$$$</td>
<td>Medium</td>
</tr>
<tr>
<td>PET</td>
<td>Ionizing</td>
<td>No limit</td>
<td>$10^{11} - 10^{12}$</td>
<td>$1-10^*$</td>
<td>$$$</td>
<td>High</td>
</tr>
<tr>
<td>SPECT</td>
<td>Ionizing</td>
<td>No limit</td>
<td>$10^{10} - 10^{11}$</td>
<td>$0.5-15^*$</td>
<td>$$</td>
<td>Medium-high</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>Non-ionizing</td>
<td>1-20</td>
<td>$10^{-4} - 10^{-11}$</td>
<td>1</td>
<td>$</td>
<td>Low-medium</td>
</tr>
<tr>
<td>Bioluminescence</td>
<td>Non-ionizing</td>
<td>1-10</td>
<td>$10^{10} - 10^{16}$</td>
<td>$0.3-10$</td>
<td>$$</td>
<td>Low-medium</td>
</tr>
</tbody>
</table>

* Lower ranges are for small animal imaging.

### 1.2 OPTICAL IMAGING

Optical imaging utilizes emitted photons with wavelengths in or close to the visual light spectrum, i.e. ~400-790 nm in wavelength as illustrated in Figure 2. One advantage of using optical imaging is that there is no exposure to ionizing radiation, as in the case for radionuclide-based imaging. Optical imaging is an excellent technique for imaging of cells, thin tissue slices or small animals, e.g. mice. However, when the object of interest increases in size, the signal gets scattered, absorbed and loses resolution due to the high interaction of the visible light and the tissue. Typically, visible light can penetrate a distance of 1-2 cm of tissue before the signal gets too distorted to generate good image quality [15]. There are mainly two optical methods used in molecular imaging: bioluminescence and fluorescence. The process by which the emitted light is produced is similar for the two methods, but they differ in the process by which the energy needed is delivered.

#### 1.2.1 BIOLUMINESCENCE

Bioluminescence is a subcategory of chemiluminescence, however, in bioluminescence the light-generating reactions occur inside an organism. Here, light is produced by a chemical reaction between a substrate called luciferin and an enzyme called luciferase. Luciferase will oxidize the luciferin and in the process excite an electron on the luciferin molecule. When this electron returns back to its normal state, light is emitted. By coupling the enzyme to a tracer that will bind to a target of interest, then adding the substrate, luciferin, light will be emitted at the target molecule. An advantage with this technique (compared to the other techniques described here) is that a continuous and steady signal can be sustained as long as more substrate is available [16, 17]. When performing imaging *in vivo*, the substrate is normally injected, but in other systems, where the luciferase gene itself is incorporated into the host DNA, e.g. by viral vectors, the substrate can be co-produced in the same cells expressing the luciferase, providing an autonomous bioluminescent expression [18].
1.2.2 Fluorescence

Fluorescence differs from luminescence in the way by which the light-emitting electrons get excited. Instead of a chemical process as in the case of luminescence, in fluorescence imaging the electrons get excited by another light source, often a laser. The light is absorbed by the fluorescent probe and an electron is excited. As in the former case, when the electron relaxes to its ground state, a photon is emitted. Due to energy losses the light absorbed and the light emitted are not of the same wavelength. One drawback with using fluorescence imaging in larger samples is the relatively high background due to autofluorescence from surrounding tissue [2]. There are many different fluorescent probes with various relaxation wavelengths. However, in order to increase the penetration depth of the emitted light, probes emitting infrared or near infrared light are often used [19–21].

1.3 Magnetic Resonance Imaging (MRI)

MRI is an implementation of NMR (Nuclear Magnetic Resonance) for molecular imaging. As for some of the optical imaging techniques, in NMR you first have to apply a source of energy to “disturb the balance of the atoms”. When the balance is restored, photons are emitted and can be detected. Normally, in the clinic, this modality is used to image the subject’s own molecules, i.e. the hydrogen atoms in the water molecules, which are abundant in the body. It can also be used with targeting molecules coupled to contrast-enhancing agents like superparamagnetic iron oxide (SPIO) particles [22, 23].

A powerful external magnetic field is applied in order to align the magnetic nuclear spin of hydrogen atoms in the subject being imaged. A short burst of, right-tuned, radio frequency (RF) pulse can flip the spin. As the spin flips back, an RF-signal is sent back from the nuclei. By repeated RF bursts an image can be accumulated from the echo of the hydrogen atoms. Different concentrations of hydrogen atoms, i.e. varying amounts of water in different tissues, create a contrast in the image. A resolution of 0.1 mm or better is possible with today’s techniques [24].

MRI is best used when imaging soft tissue like brain, muscle, heart, etc., where there is a lot of water and hydrogen atoms that can be imaged. Although this technique is truly exceptional in for instance anatomical imaging it has a large drawback, namely its very low sensitivity. Only about 0.001% of the potential nuclei are in fact detected. Consequently, you need a large amount of hydrogens in your sample or the time that it takes to acquire an image will be very long. In 2003, Paul Lauterbur and Sir Peter Mansfield were awarded the Nobel Prize in Physiology or Medicine for their discoveries concerning magnetic resonance imaging.

1.4 Computed Tomography (CT)

CT, also known as X-ray computed tomography, is a technique where 3D X-ray images can be produced from a large amount of 2D images taken from a camera rotating around the subject. Digital geometry processing is used to reconstruct a 3D model that can then be rotated and viewed from all angles. The two modalities SPECT and PET that are used in radionuclide-based imaging are nowadays often combined with CT to form the fusion systems PET-CT and SPECT-CT, in order to get a high resolution anatomical localization [25].

1.5 Radionuclide Based Imaging

At much shorter wavelengths and higher energies in the electromagnetic spectrum, one finds the gamma (γ) rays at frequencies of around 1021 Hz and wavelengths of about 10-13 m. In radionuclide-based imaging the source of the signal comes from the nucleus of an unstable atom (radionuclide). As the unstable nuclide decays, the composition and energy of its nucleus is changed and it is usually transformed into a new nuclide, which in turn may or may not be unstable. In some cases though, as with metastable isotopes, the decay will not result in a transmutation into a new nuclide, but the nuclei energy state will change. In the different processes of decay, a large amount of energy is released. Depending on the radionuclide, some or all of the energy may be released as a gamma photon that can be detected by a gamma camera. It is important to note that the decay for each atom is completely random and impossible to predict. There is however a probability that the atom will decay within a certain amount of time, i.e. a decay constant, which is specific for each isotope. The time after which half of the atoms have decayed is called the half-life of the radionuclide. This is a very important factor to consider when choosing an appropriate radionuclide for an imaging agent. Since the decay starts as soon as the isotope is created there is only a certain amount of time from the creation of the radionuclide to the acqui-
sition of the signal before the activity falls below the limit needed to generate a high quality image. The half-lives of useful isotopes for imaging span from about 2 minutes (i.e. $^{15}$O) to more than 4 days (i.e. $^{124}$I). Some commonly used radionuclides for molecular imaging are listed in Table 2.

Table 2: Example of commonly used radionuclides and their nuclear properties. (Modified from [13])

<table>
<thead>
<tr>
<th>Nuclide</th>
<th>Half-life</th>
<th>Imaging modality</th>
<th>Position $E_{\gamma}$ [MeV]</th>
<th>Most abundant gamma [keV (abundance)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{99m}$Tc</td>
<td>6.01 h</td>
<td>SPECT</td>
<td>-</td>
<td>140.5 (89%)</td>
</tr>
<tr>
<td>$^{123}$I</td>
<td>13.27 h</td>
<td>SPECT</td>
<td>-</td>
<td>159.0 (83%)</td>
</tr>
<tr>
<td>$^{111}$In</td>
<td>2.8 days</td>
<td>SPECT</td>
<td>-</td>
<td>171.3 (90%), 245.4 (94%)</td>
</tr>
<tr>
<td>$^{11}$C</td>
<td>20.33 min</td>
<td>PET</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td>$^{67}$Ga</td>
<td>67.6 min</td>
<td>PET</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>$^{18}$F</td>
<td>109.8 min</td>
<td>PET</td>
<td>0.635</td>
<td></td>
</tr>
<tr>
<td>$^{64}$Cu</td>
<td>3.33 h</td>
<td>PET</td>
<td>1.22</td>
<td></td>
</tr>
<tr>
<td>$^{68}$Ga</td>
<td>9.49 h</td>
<td>PET</td>
<td>4.15</td>
<td></td>
</tr>
<tr>
<td>$^{65}$Cu</td>
<td>12.7 h</td>
<td>PET</td>
<td>0.657</td>
<td></td>
</tr>
<tr>
<td>$^{86}$Y</td>
<td>14.7 h</td>
<td>PET</td>
<td>4.16</td>
<td></td>
</tr>
<tr>
<td>$^{82}$Br</td>
<td>16.2 h</td>
<td>PET</td>
<td>3.98</td>
<td></td>
</tr>
<tr>
<td>$^{57}$Co</td>
<td>17.5 h</td>
<td>PET</td>
<td>2.52</td>
<td></td>
</tr>
<tr>
<td>$^{89}$Zr</td>
<td>78.4 h</td>
<td>PET</td>
<td>0.897</td>
<td></td>
</tr>
<tr>
<td>$^{124}$I</td>
<td>4.17 days</td>
<td>PET</td>
<td>2.1</td>
<td></td>
</tr>
</tbody>
</table>

1.5.1 Single Photon Emission Computed Tomography (SPECT)

The most commonly used modality for radionuclide-based imaging is SPECT. In Sweden, all of the largest hospitals have clinical SPECT facilities, whereas only a few of the bigger cities have a clinical PET facility (the PET modality is discussed later in the text). Today, SPECT is used in many different applications in the clinic (e.g. imaging of cancers, atherosclerosis, thrombosis, neurological disorders and infections) [25]. As the name suggests, in SPECT, a single gamma photon from one decay event is registered. When SPECT radionuclides decay, all or part of the energy released in the event is emitted as a gamma photon. Depending on the radionuclide, emission of different types and energies are produced (Table 2). Typically in SPECT, nuclides emitting gamma quanta with energies of 90-300 keV are used [13]. For example, the most common isotope used in SPECT imaging is technetium-99m ($^{99m}$Tc). About 88% of the emission from the decay of $^{99m}$Tc is in the form of gamma radiation ($^{99m}$Tc $\rightarrow$ $^{99}$Tc + $\gamma$) where the photons can have one of two possible energies, 140.5 keV (98.6%) or 142.6 keV (1.4%). The remaining 12% of the emission is released as high energy electrons due to “internal photoeffect” ($^{99m}$Tc $\rightarrow$ $^{99}$Tc + e). However, for imaging purposes, only the gamma photons, which we can detect with the camera, are of interest. The electrons, in this case, will only travel a small distance and deposit energy into the surroundings and contribute to the total dose for patient. The high energy gamma photons on the other hand will pass through the patient and can be detected outside of the body by the camera. The gamma camera or scintillation camera is mainly built up by three components (not counting all the essential electronics): collimator, scintillation crystals and the photomultiplier tubes.

1.5.1.1 The Gamma Camera

One of the challenges with the technique of imaging by detecting single gamma photons is that it is not possible to determine from which direction a photon hitting the detector came from. There is always background radiation that can interfere with the real source of interest. Photons can interact with the surrounding atoms and be scattered in a process called the Compton Effect. In order to be able to determine the point of origin of the photons detected by the camera, a perforated, thick (about 2-8 cm) lead shield (the collimator) is placed in front of the detector as seen in Figure 3. Typically, holes in the collimator are directed towards the target so that only photons on a straight path from the target can pass through. Unfortunately, this will allow less than 1% of the emitted photons to find their way to the detector, resulting in an overall sensitivity of about 0.01% [26]. Also, some photons hitting the collimator do find their way to the detector but might be deflected on the way or coming in from a direction other than the focus point of the camera (i.e. pass through the collimator walls), contributing to most of the degradation of the SPECT image.

As the photon enters the camera it hits the scintillation crystal, most commonly consisting of sodium iodide, doped with thallium [NaI(Tl)]. The photon will transfer some of its energy to the iodide, which will emit a valence electron. Upon de-excitation a flash of visible light is emitted – approximately one light photon per 30 eV is produced. The very weak light pulse is transformed into
an electric signal and amplified in the photomultiplier tube. The signal is then passed along to yet another amplifier and further processing. Finally an image can be put together by a computer and signal processing software.

To produce 3D images, a SPECT camera can be rotated around the subject and/or consist of multiple camera heads in order to collect signals from different angles.

**Figure 3:** Schematic representation of a gamma camera.

### 1.5.2 Positron Emission Tomography (PET)

An alternative to SPECT, the PET technology, is now on the rise. Developed in the 1950’s this modality is in many ways superior to SPECT (e.g. has higher sensitivity and enhanced quantification possibilities) but due to its dependence on short-lived isotopes and expensive hardware it has yet a long way to go in terms of availability in hospitals around the world compared to SPECT [26]. The PET modality allows for three-dimensional visualization of the distribution of radioactivity utilizing positron-emitting radionuclides.

With PET we are moving further into the mysteries of quantum physics. As noted in the name, the emission is not a photon, but a positron (β⁺). A positron is the antiparticle of an electron, i.e. antimatter. Positron emitters for PET have to be produced using a particle accelerator, i.e. a cyclotron [27]. As the unstable nuclei decompose, positrons are created and released. The positron travels a short distance until it collides with its antiparticle, the electron, and annihilate with the release of energy in the form of two gamma photons.

Depending on the radionuclide, the expelled positron will have different maximum kinetic energy which in turn determines the maximum distance it will travel in the tissue before finally annihilating upon the encounter with an electron. As an example, the most frequently used PET-isotope, fluorine-18 (¹⁸F) decays by emitting a positron with a maximum kinetic energy (E_{max}) of 0.635 MeV which will travel a maximum of 2.3 mm in the tissue before annihilating. See Table 2 for some of the currently used positron emitters.

At the annihilation a total energy of 1.022 MeV is released in the form of two 511 keV gamma photons, regardless of the nuclide. The two photons travel in opposite directions, nearly at 180 degrees from each other, and can be detected with similar cameras as used with SPECT. However, there is one significant difference in the detector design. With PET, a collimator is not needed. Instead the PET camera can detect the source of annihilation by simultaneously detecting the two photons at opposite sides of the subject and calculating the most probable path and thereby the point of origin, a method known as coincidence detection [28].

**Figure 4:** Schematic representation of the setup and function of the PET camera.
needed, allowing for a higher sensitivity as compared to SPECT. The spatial resolution of the image will now mostly depend on the size of the detector and the distance the positron traveled before annihilation, i.e. the energy of the positron, which in turn is determined by the radionuclide used. However, random coincidences do occur and add to the background noise in PET images.

1.5.3 PET vs. SPECT

It seems that the PET camera in most aspects is superior to the SPECT modality. It has about 100 fold higher sensitivity and about 2-3 fold better spatial resolution in clinical settings – also the quantification ability is more enhanced. However, sensitivity and resolution are not the only factors that matter when planning an imaging study. The availability of PET is considerably lower as compared to SPECT. Modern PET is a relatively new technology and a much more expensive set of equipment is needed, whereas SPECT cameras are present in almost every bigger hospital in the Western world. Also, the resolution and sensitivity of SPECT are sufficient for many applications.

Aside from the equipment, you need to think about the production of the tracer itself. SPECT nuclides are often more long-lived than the PET counterpart, i.e. allowing for more time to carry out the chemistry to produce the tracer and to acquire the image. Also the chemistry of radiolabeling with $^{18}$F, the most common and attractive PET nuclide, is all but straightforward. Nevertheless, PET has a high potential for the future, which motivates the continued work on developing and improving methods for labeling of PET tracers.

2 Radiolabeling

When doing molecular imaging with a radiotracer it is important to remember that the images produced are not of the targeting agent per se, but rather of the radionuclide attached to it. Therefore it is of extreme importance that the radiolabeling produces a stable conjugate where the radionuclide is stably attached to the targeting molecule. Also, equally important, that the produced imaging agent does not contain any free radionuclide that could spread into the surroundings and accumulate in non-target areas, or simply raise the background signal in general, leading to a low-contrast image.

Another aspect to consider when developing protein-based imaging agents is at what position on the tracer the labeling is performed. If the radionuclide is attached randomly on the tracer there is a risk that the labeling might occur in, or near, the binding site, thereby compromising the binding to the target molecule. This will in turn lead to weaker signal in the targeted area and likewise produce an image with suboptimal contrast. Therefore it is always better to have full control over the labeling and direct it to a specific site on the protein [13].

Finally, the label itself could potentially alter the characteristics of the tracer, especially for small targeting proteins, e.g. Affibody molecules. The label can alter the charge and lipophilicity of the whole or parts of the molecule and thereby alter its biodistribution and clearance pathway. If the tracer becomes too lipophilic the excretion pathway could be altered from urinary, where it exits the circulation primary via the kidneys, to hepatic where the pathway is mainly altered to pass through the liver and exit the circulation into the intestine and stool. This will produce images with a high signal from the liver and intestine area which is quite undefined and irregular and also heavily limiting the possibility to image, not uncommon, liver metastases. The kidneys on the other hand are very well-defined, an uncommon place for metastases and distinct from many areas of interest to image [29–31].

2.1 Radionuclides for Imaging

Suitable radionuclides are found primarily among the metals and the halogen elements in the periodic table, but there are some exceptions like $^{11}$C and $^{13}$N [32, 33], etc. However, their half-lives are quite short, 20 and 10 min respectively, and therefore there is very little time to synthesize the tracer. $^{11}$C has a limited use in
targeted imaging whereas $^{15}$N is mainly used as a gas or in the form of ammonia in lung perfusion or blood flow studies, respectively [3].

2.1.1 Radiometals

Transition radiometals are attractive candidates for radiolabeling due to the relatively facile chemistry of chelation. These metals, e.g. indium-111 ($^{111}$In) and cobalt-57 ($^{57}$Co) for SPECT and gallium-68 ($^{68}$Ga) and cobalt-55 ($^{55}$Co) for PET, can be chelated by bifunctional organic ligands where one part chelates the metal and another functional group can be conjugated to the targeting molecule. The process of chelation creates multiple non-covalent bonds that hold the metal in place with high thermodynamic stability and/or kinetic inertness. The chelator typically contains several electron donors like oxygen, nitrogen or sulfur that contribute to the bonding of the central metal atom. However, the chelating agents do not distinguish between the radiometals that we want to load them with and free metal impurities, e.g. commonly appearing iron ($\text{Fe}^{3+}$). Therefore caution has to be taken not to have metal contamination in the buffers when working with chelators. Although $^{111}$In sits very firmly in DOTA (1,4,7,10-tetraazacyclododecan-1-yl)acetic acid), a well-established chelator for indium [34, 35], the contaminant can be present in several thousand times of excess which will make chelation of indium highly unlikely. With a contaminant-free medium the labeling is often quite straightforward. The radiometal is added to the chelator-conjugated peptide and heat is applied (mainly for macrocyclic chelators) – after an incubation period the metal should be firmly attached to the peptide. However, the heat treatment, usually >50º C, is not suitable for all proteins, where improper refolding after denaturation is an issue. In Figure 5, the different chelators, used in the work presented is this thesis, are shown.

Figure 5: Structures of three chelators used in this work. From left to right: Maleimide-DOTA, Maleimide-NOTA and the peptide-based chelator composed of the amino acid sequence SECG, in complex with technetium.

2.1.2 Fluorine-18

If $^{99m}$Tc is the king of SPECT, then $^{18}$F is the dominator of PET. It has a very high probability of positron decay, almost 97%. The remaining 3% of the decay is in the form of electron capture. It has one of the lowest $E_{\text{max}}$ (0.635 MeV) amongst the PET nuclides and thereby a very short positron range in tissue (max 2.3 mm). Among the positron emitters it has a quite long half-life of almost 110 min which allows for transport for a couple of hours from the PET production centers. Today it is mostly produced as fluoride ($^{18}$F) through the process of proton bombardment of oxygen-18 ($^{18}$O) in water causing a (p, n)- or "knock-out" reaction. The most common use of $^{18}$F is to couple it to a glucose to form $[^{18}$F]fluorodeoxyglucose ($[^{18}$F]FDG) [44]. ($[^{18}$F]FDG)-PET is used to visualize areas of high metabolism in vivo as the sugar is taken up by highly active cells. The sugar is trapped intracellularly since it cannot be further metabolized due to the substitution of the hydroxyl group in position 2 by fluorine. An increased

Technetium-99m

$^{99m}$Tc is by far the most commonly used isotope for SPECT imaging. It is considered to have ideal physical characteristics such as almost pure gamma radiation with an appropriate energy for gamma cameras (~140 keV) and a convenient half-life of 6 h. Technetium, with the atomic number 43, is the lightest element in the periodic table without any stable isotopes. $^{99m}$Tc is the decay product of molybdenum-99 ($^{99}$Mo) and can be very cheaply and easily produced using the $^{99}$Mo/$^{99m}$Tc generator, which also makes the nuclide very convenient to transport from the production facilities to the SPECT centers where it will be used [36]. When eluted from the generator, $^{99m}$Tc is in the form of pertechnetate $^{99m}$TcO$_4^-$, with the oxidation state +7. For labeling, the pertechnetate first has to be reduced – this is predominantly done with stannous chloride ($\text{SnCl}_2$), although other reducing agents have been used. Technetium can be chelated by many different chelators with varying stability. A particularly stable class of chelators for the technetium (+5) is composed of three nitrogen atoms from a peptide backbone and a sulfur moiety, from e.g. a cysteine residue or a mercaptaoacetyl moiety. This type of chelator can be designed as N$_2$S or SN$_{13}$. Today, $^{99m}$Tc is used in many different kinds of imaging, e.g. lung perfusion, heart perfusion, kidney perfusion or tumor imaging [40–43].
amount of the FDG accumulates and can then be imaged by the PET camera, often combined with a CT, as mentioned earlier. As tumors, active parts of the brain, and inflammations normally have an increased metabolism, they can be visualized by a higher signal than the surrounding tissue.

Although $^{18}$F is widely used, the radiochemistry is all but simple. The conditions needed for labeling are often not compatible with proteins and peptides, which often are the objects for labeling when considering targeted radiolabeling. Since ($^{18}$F)FDG is so widely used, dedicated synthesizers have been developed to facilitate fast, efficient and reproducible synthesis of this compound [45]. However, when labeling proteins, the fluorine is often first coupled to a small organic group, i.e. a prosthetic group, and then this labeled prosthetic group is conjugated to the protein. This approach results in many synthesis steps, with decreasing yield for each step and prolonged synthesis time. Today much research is focusing on new and improved prosthetic groups and their synthesis to facilitate higher yields in a shorter amount of time [27, 46, 47].

In summary, when deciding on a strategy for targeted molecular imaging, using proteins or peptides as tracers, the radiolabeling is a central determining factor. If your radionuclide of choice has a short half-life, the labeling chemistry must be very swift and efficient in order to maintain enough activity for the imaging experiment. Fast and efficient chemistry often involves harsh labeling conditions in terms of temperature and pH which in turn sets high demands on the protein being labeled. For a homogeneous and well-defined final product, it is preferable to perform the labeling site-specifically. To prepare the protein for site-specific labeling, unique amino acids with selective chemical reactivity can be incorporated recombinantly [35, 48, 49]. Alternatively, by employing synthetic production of the peptide, chelators or other functionalized groups can be introduced at any position during the synthesis [50, 51]. This may not only allow site-specific labeling, but also enable new labeling chemistries that are not possible with only natural amino acids.

3 **Solid Phase Peptide Synthesis – SPPS**

Peptide or protein synthesis is the step-wise process of coupling the carboxyl group of one amino acid to the amine of another, thereby building a peptide chain one amino acid at a time. This is a convenient way to produce peptides and proteins since at each step you can make a decision to stop, repeat the last step, change an amino acid, use an unnatural amino acid or simply choose to incorporate another building block in the peptide chain. However, a small error in each step will rapidly accumulate and dramatically decrease the yield of the final product. Therefore, a reaction yield of 99% or more in each step is typically needed to efficiently produce proteins longer than 40-50 amino acids. In chemistry one often employs an excess of one reactant in order to drive a reaction to completion. In 1963 Merrifield suggested a new way to achieve this, namely to anchor the C-terminal of the growing peptide chain on a solid but porous support [52]. This method allowed using an excess of reagents to be added followed by an easy removal of the reagents, preparing the peptide chain for the next reaction. In addition, this strategy greatly reduced the problems during synthesis associated with poor solubility of the protected peptide chain. For this achievement he was later awarded the Nobel Prize in Chemistry.

The basic principle for solid phase peptide synthesis (SPPS) is illustrated in Box 1. In short, the carboxyl group of the first (C-terminal) amino acid is coupled to the solid support (also called a resin) via a linker. The amino group of each amino acid is protected in order to avoid the free amino acids to react with each other. The amino protecting group is cleaved off under basic conditions. The next amino acid is now ready to be coupled to the previous one. Coupling is done by activating the carboxylic group with an electron-withdrawing group in order to starve the carbon atom of electrons, which will make the carbon of the carboxylic group more attractive for a nucleophilic attack by the amino group in the previously coupled amino acid. Now, the amino protection of the second amino acid can be removed. This process is repeated for each amino acid in the synthesis until the polypeptide chain is complete. Some amino acids (i.e. Arg, Asn, Asp, Cys, Gln, Glu, His, Lys, Ser, Thr, Trp, Tyr) also have reactive side chains that need protection. These are protected for the entire duration of the synthesis and are only cleaved off at the end of the synthesis, together with the release of the peptide from the resin. Different types of side chains (i.e. alcohols, amines, phenol, guanidine, thiol, carboxyls and the imidazole) require different types of protecting groups. In the Merrifield method, or Boc chemistry, the cleavage
of the different protecting groups and the release from the resin is achieved by gradients of acidity, with the final cleavage from the solid support only possible by extremely strong and toxic acids such as hydrofluoric acid (HF). To get milder reaction conditions, Carpino and Han suggested the use of another temporary protecting group for the amines, the 9-fluorenylmethoxycarbonyl (Fmoc) group, in 1972 [53].

**Box 1:** Schematic illustration showing the basic steps in SPPS. Alternatively, the linker can contain an amino group (other functionalities are also available) to anchor the first amino acid. This makes the attachment of the first amino acid much easier and will produce a polypeptide chain containing a C-terminal amide.
3.1 FMOC CHEMISTRY

The Fmoc chemistry is the most commonly used method for SPPS today. By using the base-labile Fmoc group as protection for the amino groups, more acid-labile (compared to Boc-chemistry) protecting groups can be used for the amino acid side chains, and also the linker anchoring the peptide chain to the solid support can be made more acid labile. This orthogonal strategy allows for weaker acids to be used in the final cleavage. With this approach the Fmoc protected α-amino group is usually deprotected using a 20% piperidine solution. The side chains that now can be protected with e.g. tert-butyl-derivatives (tBu or tBoc) or trityl-based groups are along with the resin linkers easily cleaved off by trifluoroacetic acid (TFA). Another useful property of the Fmoc group is the fact that it absorbs UV light. This enables monitoring of the amount of Fmoc that is cleaved off in each deprotection step. If adequate deprotection is not achieved in the first attempt the process can be repeated with fresh reagents before moving on to the coupling reaction. The orthogonality of the Fmoc strategy can be further expanded to include the side chain protecting groups. Specific amino acids can be protected by unique protecting groups which can be removed selectively, thereby exposing a single functional site for further modification [54, 55]. As an example, the methyltrityl group (Mtt) can be used to protect the side chain amino group of lysine. The Mtt group can later be removed with as little as 1% TFA in DCM, leaving the rest of the “normal” protecting groups intact. For orthogonal protection of carboxylic acids, hydroxyls, or amino groups, an allyl-based protecting group can also be used. The allyl-based protection is then removed by palladium catalysis.

3.2 MICROWAVE ASSISTANCE

As with many chemical reactions the synthesis process can be accelerated by raising the temperature of the reaction. However, heating the reaction vessel on a plate or using an oil bath is not a viable option. This simply takes too long and creates temperature gradients in the reaction mixture which would lead to unwanted side reactions. A better option is to use microwave energy to heat the reaction [56]. The microwaves interact with ions and molecules with inherent dipolar momentum. The constantly changing electric field produced by the electromagnetic wave agitates the molecules, as they try to align themselves with the field. However, when the molecules align themselves in one direction, the field has already changed, resulting in a constant and rapid movement of the molecules, which in turn results in heat. This process, in contrast to conventional heating, is almost instantaneous throughout the whole sample and the heating is very rapid. The microwaves can be applied during deprotection, coupling and final cleavage. Although this helps in reducing the time for many reactions, as with conventional heating, this also leads to an increase in side reactions. However, by optimizing the applied power and temperature and by using the right reagents, most of the side reactions can be kept to a minimum [57].

SPPS clearly is an attractive method for generating high quality peptides and proteins. It allows for fast production of many different analogs for evaluation, including the use of unnatural amino acids, alternative building blocks and on-resin conjugation. It also produces a product free from host cell proteins, toxins and viruses that often need to be removed when using a recombinant production method for in vivo studies. However the method is not without problems – the biggest problems of course being the side reactions and the relatively low yields. Furthermore, the peptide has to be purified from a complex crude mixture, which typically requires reversed phase high performance liquid chromatography (RP-HPLC). In RP-HPLC, highly flammable and toxic buffers are used, which can be problematic when scaling up the process. The large amount of organic solvents used in the synthesis and the following purification is also neither easily handled, cost efficient nor environmentally friendly.
Protein-based affinity reagents

In order to achieve high contrast images in targeted molecular imaging, the targeting agent should have sufficiently high affinity and high specificity for the target molecule [58]. To further enhance the contrast of the image the tracer should exhibit rapid clearance from the blood and the rest of the non-targeted tissue. Rapid clearance is achieved with small tracers (with a molecular weight below the glomerular filtration cutoff, approximately 60 kDa) as they often are efficiently filtered in the glomeruli and exit the circulation via the kidney and into the urine. Also, the small size allows for a more rapid extravasation and diffusion into the extracellular space. However, with rapid clearance from the circulation the tracer has little time to find and bind to its target. Therefore, a small targeting agent needs to have a very high affinity to its target in order to accumulate in sufficient amount to provide a good signal. As a targeting agent different types of ligands can be used – the natural ligand of the target, antibodies, or a novel affinity protein, preferably derived from one of the existing protein-based scaffolds. Some common, non-immunoglobulin, alternative scaffolds are listed in Table 3.

In recent years, a large number of different affinity reagents have been developed. The most obvious choice has been the use of monoclonal immunoglobulins (Ig), i.e. antibodies, and fragments thereof [59, 60]. However, lately the development of non-immunoglobulin, protein-based scaffolds has been used with great success [61]. These so-called “scaffold” proteins originate from various structural classes with mainly one feature in common; a highly stable core structure which allows for grafting or otherwise incorporating affinity functions without compromising the integrity of the “framework”. The “framework” refers to the 3-dimensional structure of the protein which is highly dependent on the amino acid sequence and the surrounding environment of the protein. Although antibodies and their derivatives usually are highly tolerant towards sequence variations in their binding sites they are relatively sensitive to harsh environments in terms of extremes in buffers, pH and temperature. Also, in the world of molecular imaging, as mentioned earlier, size matters. In reference to this, alternative non-immunoglobulin based protein scaffolds have the potential to far surpass antibodies as affinity reagents in various applications.

Table 3: Examples of common, non-immunoglobulin, protein scaffolds.

<table>
<thead>
<tr>
<th>NAME</th>
<th>LENGTH [aa]</th>
<th>MW [kDa]</th>
<th>DISULFIDE STABILIZED</th>
<th>RANDOMIZED STRUCTURE</th>
<th>STRUCTURE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Affibody</td>
<td>58</td>
<td>7</td>
<td>No</td>
<td>Helices</td>
<td>3-helix bundle</td>
</tr>
<tr>
<td>Anticalin</td>
<td>170</td>
<td>20</td>
<td>Yes</td>
<td>Loops</td>
<td>ß-barrel</td>
</tr>
<tr>
<td>Knottins</td>
<td>30</td>
<td>3</td>
<td>Yes</td>
<td>Loops</td>
<td>Cysteine knot</td>
</tr>
<tr>
<td>DARPin</td>
<td>67 + n x 33</td>
<td>14 + n x 3</td>
<td>No</td>
<td>Helices &amp; loops</td>
<td>Ankyrin repeat</td>
</tr>
<tr>
<td>Fn3 (Adnectins)</td>
<td>94</td>
<td>10</td>
<td>No</td>
<td>Loops</td>
<td>ß-sandwich</td>
</tr>
</tbody>
</table>

4.1 Size Matters

How does the size of the tracer molecule impact the tumor targeting and thereby image quality? Tumor uptake is dependent on many parameters including plasma clearance rate, permeability across the tumor capillary wall, diffusion within the tumor interstitium and available volume fraction in the tumor [62–64]. However, how these parameters interact and produce a final result is not elementary. Schmidt and Wittrup have done a fantastic work in trying to simulate and predict tumor uptake with respect to these parameters [58]. Although computer simulations of this kind do not in any way replace in vivo experiments, Schmidt and Wittrup’s data show a good overall conformity when compared with existing in vivo data. Their model shows that small (<10 kDa) and large (>100 kDa) tracers exhibit the highest tumor uptake, whereas intermediate size results in an uptake minimum, as shown in Figure 6. They also show that small tracers need to have a high affinity toward the target in order to get a high retention in the tumor, whereas large enough molecules (~50 nm range) like liposomes and nanoparticles exhibit little or no increase in tumor uptake with targeting, but are taken up and accumulate in the tumor nonspecifically.

Figure 6: Graph of tumor uptake vs. molecular weight. The gray area represents computer simulation of tumor uptake as a function of effective molecular weight and experimental data from various HER2-targeting experiments from the literature is plotted as black dots. The colored line marks the molecular weight of monomeric Affibody molecules. Simulated and experimental data are in very good agreement. The graph is reconstructed and modified from Schmidt and Wittrup [58].
4.2 Antibodies

Antibodies are the natural affinity ligands of the human body. The terms antibody and immunoglobulin (Ig) are often used interchangeably and are considered synonyms in this text. They are large Y-shaped proteins produced by the body’s B-cells. Secreted antibodies circulate in the blood and are part of the humoral immune system. Their role in the immune system is to identify and bind to all possible antigens and thereby either neutralize a toxin, mark a cell or foreign object for elimination, or help to dispose of debris and other antigens.

The most abundant isotype of immunoglobulins in humans is the IgG molecule. This fairly large protein (~150 kDa) is composed of four subdomains, two identical light (L) chains and two identical heavy (H) chains. One light chain and one heavy chain are held together by a disulfide bridge and several non-covalent interactions. This heterodimer comes together with its identical counterpart by disulfide bridges and non-covalent interactions between the H-chains to form the homodimer known as an IgG molecule, as seen in Figure 7. Both H- and L-chains consist of a constant (C\text{H} and C\text{L}) and a variable (V\text{H} and V\text{L}) domain. The constant part of an antibody, especially the Fc-region, makes up the effector function and communication with the rest of the immune system. The Fc-region is identical in different molecules within a class but differs between the classes and subclasses, e.g. IgA, IgM, IgG1, IgG2. There is a small number of variants of the constant regions that constitute the different classes. The variable domains of the IgG however are unique to each molecule and its clones. At the tip of each chain there are three loops called complementarity determining regions (CDRs). These are highly variable regions, both in the sequence and in the length of the loops. The CDRs of both the V\text{H} and V\text{L} make up the binding area toward the antigen. Since the two H-chains and L-chains are identical, one IgG has two identical binding sites and the potential to bind two antigen molecules at the same time. [65]

To generate an antibody towards a specific target the most straightforward method is to immunize an animal with the target in question and then isolate a pool of polyclonal antibodies from its serum. However, this method produces a heterogeneous product that will potentially differ for each batch produced [66], which might be problematic from a clinical viewpoint. Monoclonal antibodies (mAbs), that are well defined and renewable, are typically produced by hybridoma technology or recombinant production [67, 68]. It is not always desirable to use the antibody in its natural form, as the effector function of the Fc-region or the large size of the protein might be undesirable for the particular application. With the development of recombinant or enzymatic methods it is possible to produce antibody fragments of different sizes and properties [69, 70], as seen in Figure 7.

4.2.1 Imaging Applications

Antibodies and their derivatives are widely and successfully used in most areas where affinity reagents are needed. For molecular imaging, however, full-sized antibodies are generally considered too big. The size of the protein and the effects of the Fc-region lead to a long circulation time in the blood [70]. Still, some mAbs are used in the clinic as tracers for tumor imaging, e.g. [\(^{99m}\text{Tc}\)]Arcitumomab (CEA-Scan\textsuperscript{®}) and [\(^{111}\text{In}\)]Capromab pendetide (ProstaScint\textsuperscript{®}) [71, 72]. In an effort to minimize the size of the antibody, the single domain antibody (sdAbs) fragment (12-15 kDa) has been developed [73]. Normally a single domain is unstable and has very poor solubility but during recent years naturally occurring antibodies consisting of only the heavy chains have been found in some animals belonging to the families of camels and sharks [74]. These findings have helped in the successful development of the sdAbs. Although the size has been reduced tenfold there is still the task of labeling the tracer. Many labeling methods require quite harsh conditions which normally are not suited for most proteins, including immunoglobulins and derivatives thereof. The high temperatures and extreme pH often needed for the reaction simply denature the proteins and irreversibly destroy their 3D structure and binding capacity.
4.3 **AFFIBODY MOLECULES**

One of the previously mentioned alternative scaffolds to antibodies is the Affibody technology. Affibody molecules originate from the staphylococcal protein A where the 58 amino acid B-domain was selectively mutated in order to achieve higher chemical stability [75]. The resulting protein was called the Z-domain. By further engineering of the Z-domain combinatorial protein libraries were produced, from which binders to virtually any protein target could be selected [76, 77].

The Affibody molecule is a small three helical bundle protein. Of the three helices normally only two (helix 1 and helix 2) participate in the actual binding to the target – the third helix though is needed for stability.

Synthetic combinatorial libraries are produced by randomizing 13 surface-exposed amino acid positions in helices 1 and 2. To screen for binders in these libraries, *in vitro* selection systems like phage display or bacterial display are usually employed, which also enable the selection of high affinity binders through the process of affinity maturation. The selected Affibody molecule can then be produced in *Escherichia coli* (*E. coli*) in high yields [78]. Furthermore, because of their small size and rapid folding [79], Affibody molecules can be produced by chemical peptide synthesis in high yields [80]. Although the size of an Affibody molecule is much smaller than an antibody, as seen in Figure 8, the surface area available for binding is approximately the same [81, 82]. However, since the binding surface of the Affibody molecule is more flat compared to the CDR-loops of the antibody the preferred binding epitopes might differ.

Affibody molecules are naturally cysteine-free but because of the high robustness of the Z-domain, modifications to the amino acid sequence and/or further conjugations are highly acceptable. Recently an extensive redesign of the Affibody scaffold was undertaken in order to improve stability, peptide synthesis and increase hydrophilicity. The result “The new Affibody” has about 40% of its amino acids substituted compared to the original Z-domain [83]. Over the years a large number of applications and different binders have been developed that utilize the Affibody technology [84]. The most studied of the Affibody binders is the ZHER2:342 Affibody molecule and its derivatives. In fact, this binder is a set of binders, developed and improved over the years. To better understand the sequence evolution and relationship of the different binders mentioned in this thesis please see Box 2 – showing an alignment of the amino acid sequences and the differences between the constructs.

![Figure 8: Size comparison between an Affibody molecule (PDB ID: 2KZJ) [81] and an antibody (PDB ID: 1IGT) [86].](image-url)
4.3.1 Imaging Applications

For molecular imaging applications, at present, Affibody molecules might be considered the perfect candidate.

From a production point of view, the expression in E. coli allows for cost efficient recombinant production at high yields. The small size enables synthetic production which gives the opportunity to try different mutant iterations within a short amount of time.

From a radiolabeling point of view, the high structural and chemical stability and rapid refolding allows for effective site-specific conjugation of chelators and prosthetic groups. Also, the conjugations and labeling reactions can be performed at chemically harsh conditions and elevated temperatures, often required for a rapid and efficient results [85].

From an imaging point of view, the small size and the ability to generate high affinity binders allow for high contrast images due to the rapid clearance from blood and non-targeted tissue and relatively high uptake at the target site – also the robustness of the scaffold allows for a high degree of manipulation of the amino acid sequence in order to modulate the excretion path from the body.
Present Investigation
5 Aim of the Thesis

The overall aim with this thesis was to find different ways of optimizing the Affibody molecule as an imaging agent for radionuclide imaging. The Affibody technology has already been shown to be very well suited for this task, mainly due to the molecule’s small size, robust structure and the ability to generate high-affinity binders to many relevant targets [84, 87]. However, in order to be truly exceptional and make it all the way to the clinic for routine use, further optimization is needed. Besides specific and efficient targeting, most importantly the labeling has to be swift and straightforward. Also, preferably the same molecule should be able to be labeled with different radionuclides.

In the four papers presented in this thesis, the first two papers focus on improvements to the framework of the molecule in order to facilitate production capabilities and create better images, but also to make the molecule more versatile, allowing for a wider range of radionuclides to be attached. The last two papers focus on developing new and improved ways of labeling the Affibody molecule with $^{18}$F. This is done in order to position the scaffold in the frontlines of tracers suitable for the fast growing area of PET imaging. In all papers Affibody molecules binding HER2 have been evaluated in vivo using murine models. Mice with HER2-expressing xenografts were used as a tumor model.

5.1 Design, Synthesis and Biological Evaluation of a Multifunctional HER2-specific Affibody Molecule for Molecular Imaging. (Paper I)

In this paper we aimed to develop a multifunctional platform for site-specific labeling of Affibody molecules with different nuclides for radionuclide imaging. The goal was to design and produce an Affibody molecule that would be suitable both for labeling with trivalent radiometals and with $^{99m}$Tc. Another requirement was to allow for both recombinant and synthetic production. This excluded the use of unnatural amino acids. As a model, a HER2-binding Affibody molecule was used.

Previous studies on labeling of Affibody molecules with $^{99m}$Tc employed mercaptoacetyl-containing peptide based chelators at the N-terminus [29–31, 38, 88]. These studies provided an important insight into how the amino acid composition of the chelator influences the biodistribution profile and the excretion pathway of $^{99m}$Tc-labeled Affibody molecules. It was shown that the use of maESE [31] and maSKS [88] provided low retention of $^{99m}$Tc in kidneys. However, a serious limitation to this approach was that it could only be applied to synthetic Affibody molecules, which limited the production alternatives. It was further shown that the use of cysteine-containing peptide-based chelators at the N-terminus [89] produced $^{99m}$Tc-labeled Affibody molecules capable of specific targeting in vivo. However, such conjugates demonstrated elevated uptake in salivary glands and stomach, presumably due to insufficient stability of the chelator because of suboptimal complex geometry. On the other hand, the use of the cysteine-containing peptide based chelator –VDC on the C-terminus provided stable labeling with $^{99m}$Tc [39]. However, the use of –VDC resulted in high retention of radioactivity in the kidneys.

In the light of the previous studies, an N,S peptide chelator with the sequence Ser-Glu-Cys (SEC) was placed at the C-terminus, flanked by Gly at both sides. The –SEC chelator is a C-terminal “mirror” of the N-terminal maESE- chelator, and we hypothesized that this would reduce renal retention of $^{99m}$Tc in a similar way. This construct allowed for efficient and stable chelation of $^{99m}$Tc at the same time as providing a cysteine residue that can be used for maleimide conjugation of a DOTA to accommodate for the chelation of trivalent metals. Also, by only using natural amino acids, recombinant or synthetic production is possible.

In this study, the Affibody construct PEP05352 was produced by microwave-
assisted SPPS and successfully labeled with \(^{99}\)Tc. The DOTA conjugated variant of the same peptide was named PEP05541 and labeled with \(^{111}\)In. The binding kinetics were analyzed using a Biacore instrument and the \(K_D\) shown to be 74 pM and 84 pM for PEP05352 and PEP05541, respectively. The difference in affinity is within the error of the method. The biodistribution in normal mice showed very low uptake in non-targeted tissue, suggesting a stable chelation in both cases. In addition, there was an impressive reduction of renal uptake from 191 to 48 %IA/g compared to previous data with \(^{99}\)Tc-labeled Affibody molecules [39]. \(^{111}\)In-PEP05541 showed a renal uptake of 209 %IA/g which is not surprising considering the residualizing nature of the \(^{111}\)In-DOTA label. Both variants showed HER2-specific binding in nude mice bearing LS174T xenografts. \(^{99}\)Tc-PEP05352 showed a tumor uptake of 6.3 %IA/g whereas \(^{111}\)In-PEP05541 had an uptake of 8.2 %IA/g. This small difference did however not affect the tumor-to-blood ratio, which was approximately 30 for both candidates at 4 h p.i.

In this paper we show that by adding the -GSECG sequence to the C-terminal of an Affibody molecule, we create a highly versatile platform for radionuclide molecular imaging. The tracer can be labeled with \(^{99}\)Tc, the most common and widely used SPECT radionuclide but also, through the cysteine residue and conjugation to other chelators, trivalent radiometals (e.g. indium, gallium, cobalt, and yttrium) are available for labeling. In addition the system can be used for site-specific labeling with other probes available for thiol-chemistry, e.g. fluorophores and cell toxins. The platform is compatible with both synthetic and recombinant production of the Affibody molecules, providing flexibility in the selection of manufacturing method. Also, this approach is not necessarily restricted only to Affibody molecules. This approach might be useful for other scaffolds as well, as demonstrated for octreotide- or RGD-based imaging [90, 91].

5.2 Direct comparison of \(^{111}\)In-labelled two-helix and three-helix Affibody molecules for in vivo molecular imaging. (Paper II)

In this work we further pursued the potential optimization of the Affibody framework – this time, an investigation into the role of tracer size and affinity on tumor targeting and image contrast.

Previously, the use of radiolabeled \(Z_{HER2:342}\) derivatives for radionuclide molecular imaging of HER2-expressing tumors had demonstrated excellent results in both preclinical [92] and clinical [93] settings. In efforts to increase the sensitivity of the Affibody-based imaging, further size reduction of the Affibody molecule by removing the third \(\alpha\)-helix was recently accomplished by Webster et al. [94], based on earlier work by Braisted and Wells [95]. The concept was that the smaller size would increase imaging contrast by better tumor penetration and a more rapid clearance from blood circulation and non-targeted areas. However, removing the third helix causes severe instability in the Affibody scaffold and multiple mutations are needed in order to counteract this. Also, in this version two homocysteines were introduced to form an intramolecular S-S-bridge to further increase stability. Still the affinity was reduced to a \(K_D\) of 4.8 nM as compared to 78 pM for the original three-helix variant. Comparing the published data with similar studies on a full-length, three-helix Affibody molecule [96] suggested that the truncated variant had significantly lower tumor uptake 2 h after injection, 12.4±3.8 %ID/g and 4.12±0.83 %ID/g, respectively. However, comparing biodistribution data, published by other groups, is not reliable, as the data might be affected by differences in mice strains, cell-lines transformed during multiple passages and experimental techniques at different research sites. A direct comparison of the two variants in the same study, using the same batch of tumor-bearing mice, would provide much more reliable comparative information.

In this study the goal was to perform a direct comparison of the two synthetic variants; the two-helix variant, here denoted PEP09239, and the full-length ABY-002 (DOTA-\(Z_{HER2:342}\)) in terms of biodistribution and targeting properties. This was done in a murine model with two different xenografts, with both high (SKOV-3) and low (LS174T) HER2 expression. Radiolabeling was done with \(^{111}\)In. As described earlier, PEP09239 was shown to have a greatly reduced affinity, with a \(K_D\) of 2 nM according to our measurements. According to the circular dichroism (CD) measurements, the helicity was also somewhat...
reduced. The melting point could not be determined, as the melting curve did not show any distinct transition. $^{111}$In-PEP09239 showed specific HER2 binding in blocking studies. The animal studies showed a more rapid clearance from the blood for $^{111}$In-PEP09239. In xenografts with high HER2 expression, $^{111}$In-ABY-002 showed a significantly higher tumor uptake than $^{111}$In-PEP09239. The tumor-to-blood ratio was higher for $^{111}$In-PEP09239 at 4 h after injection, while there was no significant difference in other tumor-to-organ ratios. However, the tumor uptake of $^{111}$In-ABY-002 was eightfold higher than that of $^{111}$In-PEP09239 in xenografts with low expression. The tumor-to-blood ratios were equal while the other tumor-to-organ ratios were higher for the three-helix variant.

To conclude, two-helix Affibody molecules can provide a higher imaging contrast, at the cost of lower uptake in tumors with high expression of HER2. However, for tumors with low expression, both tumor uptake and tumor-to-organ ratios are substantially lower for the two-helix variant, compared to the original three-helix Affibody molecule.

5.3 Imaging of human epidermal growth factor receptor type 2 expression with $^{18}$F-labeled Affibody molecule Z$_{HER2:2395}$ in a mouse model for ovarian cancer. (Paper III)

In the following studies, the focus was changed from scaffold improvements to conjugation and radiolabeling development. Because of the higher sensitivity and better quantification accuracy of PET compared with SPECT, imaging of HER2 expression may be improved. Using $^{18}$F as the positron-emitter would also improve image quality due to its low positron energies. However, radiolabeling with $^{18}$F is not a straightforward feat. Kramer-Marek et al. have conjugated $^{18}$F-HER2:342 with [N-2-(4-$^{18}$F-fluorobenzamido)ethyl] maleimide ($^{18}$F-FBEM) [97] and successfully visualized HER2-expressing tumors. However these kind of labeling reactions, including the preparation of the prosthetic group, are not straightforward and/or require a lot of time with concerns regarding the short half-life of the radionuclide.

In this study we focus on a new method recently described for labeling NOTA-conjugated peptides with $^{18}$F [98, 99]. This method utilizes the formation of aluminum $^{18}$F-fluoride (Al$^{18}$F) and its chelation by NOTA. With this method, the number of steps and the time for radiolabeling can be reduced, resulting in more activity left for imaging. The aim of the present study was to determine whether the $^{18}$F-labeled Affibody molecule Z$_{HER2:2395}$ is a suitable imaging agent for HER2 expression in mice with SKOV-3 xenografts. NOTA-conjugated Z$_{HER2:2395}$ was radiolabeled with Al$^{18}$F and compared with $^{111}$In- and $^{68}$Ga-labeled Z$_{HER2:2395}$.

The radiolabeling yield for $^{18}$F, $^{68}$Ga, and $^{111}$In was 21.0%, 84.6%, and 94.0%, respectively. No release of free Al$^{18}$F could be detected after incubation in human or mouse serum at 37º C for 4 h, indicating excellent stability. Biodistribution studies showed a tumor uptake, 1 h after injection, of 4.4 %ID/g for $^{18}$F-NOTA-Z$_{HER2:2395}$, compared to 5.6 %ID/g and 7.1 %ID/g for $^{68}$Ga- and $^{111}$In-NOTA-Z$_{HER2:2395}$ respectively. All constructs showed rapid clearance from the blood circulation and non-targeted organs, resulting in tumor-to-blood ratios of 7.4, 8.0 and 4.8 for $^{18}$F-, $^{68}$Ga- and $^{111}$In-NOTA-Z$_{HER2:2395}$ respectively. The images produced by both PET/CT and SPECT/CT clearly visualized HER2-expressing xenografts, with high contrast to normal tissues. Also, high stability of the Al$^{18}$F-NOTA complex in vivo was indicated by the fact that PET/CT did not reveal any bone uptake.
In conclusion, all three radiolabeled Affibody molecules were able to target and visualize HER2-expressing tumors. However, the use of positron-emitting radionuclides, e.g. $^{18}$F and $^{68}$Ga, is preferred over $^{111}$In due to the higher sensitivity and better quantification accuracy of PET as compared with SPECT. As a PET radionuclide, $^{18}$F is more desirable than $^{68}$Ga because of its longer half-life (110 vs. 68 min) and shorter positron range (2.3 mm vs. 8.9 mm in water), which will improve image quality. Although other methods of labeling proteins and peptides with $^{18}$F exist, they often require synthesis times of several hours, whereas we were able to radiolabel Affibody molecules with $^{18}$F within 30 min using a 2-step, 1-pot reaction.

This study showed that $^{18}$F-NOTA-Z$_{HER2}$:2395 is a promising new imaging agent for HER2 expression in tumors, although further research is needed to determine whether this technique can be used for patient selection for HER2-targeted therapy.

5.4 Incorporation of a triglutamyl spacer improves the biodistribution of a synthetic Affibody molecule radiofluorinated at the N-terminus via oxime formation with $^{18}$F-4-fluorobenzaldehyde. (Paper IV)

In the previous paper we demonstrated a swift and elegant way of labeling Affibody molecules with $^{18}$F. However, as is common with small tracers and residualizing radiometabolites, we observed high kidney uptake. Since the PET modality, and especially the favorable properties of the positron emitter $^{18}$F, is a good way of increasing the sensitivity of radionuclide imaging, we continued our work with optimizing the Affibody molecule for $^{18}$F-labeling.

In this paper we wanted to label the Affibody molecule using $^{18}$F-4-fluorobenzaldehyde (FBA) via an oxime formation. The use of FBA to label Affibody molecules has successfully been shown before [100–102]. However, in all previous cases there was a problem with elevated liver uptake which might prevent imaging of liver metastases in clinic. Considering that liver metastases are quite common for many cancers, this could pose a serious problem [103]. The elevated liver uptake is however not a property of the Affibody molecule per se but a consequence of the labeling method and the following increase in lipophilicity of the entire construct. The same Affibody molecules labeled with other methods have shown considerably lower liver uptake [96, 97, 104]. Previous attempts of suppressing a high liver uptake by incorporation of glutamyl residues and thereby lowering the lipophilicity have proven successful [30, 105]. Could this approach also be used in conjunction with $^{18}$F-FBA?

In this work we hypothesized that incorporation of a hydrophilic triglutamyl spacer between the aminooxy moiety and the N-terminus of a synthetic Affibody molecule would decrease the hepatic uptake of $^{18}$F-FBO-labeled Affibody molecules.

Two variants of the Affibody molecule Z$_{HER2}$:342 were synthetically produced: OA-PEP4313, where aminooxyacetic acid was conjugated directly to the N-terminal alanine, and OA-E$_3$-PEP4313, where a triglutamyl spacer was introduced between the aminooxy moiety and the N-terminus. The two molecules were synthesized using microwave-assisted solid phase peptide synthesis (SPPS). A 0.1 mmol batch was synthesized up to the first amino acid of PEP4313 (A1). Thereafter, the batch was split into two equal parts for the completion of the two different molecules. At this stage, the E$_3$-linker and the final, protected, ami-
nooxyacetic acid (Bis-Boc-aminoxyacetic acid) (BB-AOA) were attached using manual peptide synthesis. Both variants and their N-(4-fluorobenzylidine)-oxime derivatives were characterized by biophysical methods. The biodistribution of the radiofluorinated variants was evaluated in normal mice to evaluate the influence of the triglutamyl spacer on hepatic uptake.

Both FB-PEP4313 and FB-E3-PEP4313 retained capacity to bind to HER2 with very high affinity, 50 pM and 180 pM, respectively. The presence of the E3-linker did not interfere with the refolding of FB-E3-PEP4313, as shown by circular dichroism (CD) measurements before and after heating to 90 °C. The biodistribution experiment confirmed our hypothesis concerning the liver uptake. The uptake of 18F-FB-E3-PEP4313 in liver (1.9±0.1 %ID/g) was nearly threefold (p <0.05) lower than that of 18F-FB-PEP4313, 2 h p.i. Interestingly however, there was no significant difference in the radioactivity in the gastrointestinal tract, which means that the presence of the spacer did not influence hepatobiliary excretion of radioactivity. The spacer did however influence the renal uptake. Although renal uptake was considerably lower for both molecules (≤7 %ID/g) compared with residualizing radiometal labels (e.g. 68Ga-DOTA or 111In-DOTA, usually >200 %ID/g), 18F-FB-E3-PEP4313 showed a kidney uptake of 2.8±0.3 %ID/g, which is more than a twofold reduction compared to 18F-FB-PEP4313. Hypothetically, the triglutamyl spacer might interfere with the processing of the protein in the proximal tubuli or aid in enzymatic cleavage of hydrophobic 18F-bearing catabolites from the Affibody molecule [106].

In conclusion, the introduction of a triglutamyl linker between the N-(4-fluorobenzylidine)-oxime and the N-terminus of the Affibody molecule provided a conjugate with high (low picomolar) affinity. In mice, both hepatic and renal uptake of 18F-FB-E3-PEP4313 was reduced in comparison with 18F-FB-PEP4313. With the encouraging biodistribution properties of 18F-FB-E3-PEP4313, further studies in tumor-bearing mice, are justified.

6 Conclusions

I believe that, in these four papers, we have demonstrated the great robustness and high versatility of the Affibody molecule. It has previously been shown that it is possible to produce binders towards a large set of targets, e.g. HER2, EGFR, insulin, amyloid β-peptide and TNF-α, [77, 107–109]. With our Affibody variants, targeting HER2, we have added multifunctional features to the binder that enable a wide variety of radionuclides to be used for molecular imaging. We also enabled the possibility to produce the targeting proteins using both peptide synthesis and recombinant production (Paper I). In Paper II we show the remarkable strength of the scaffold by being able to retain binding and specificity towards the target despite removing as much as one third of the amino acids in the protein. However, the study also shows that the smaller size and lower affinity greatly compromise the ability to image low-expressing tumors. Finally, in the last two papers, we show two efficient methods for 18F-labeling of Affibody molecules allowing for PET imaging. The first method (Paper III), employing AlF-chemistry, could be especially useful for fast internalizing surface proteins as targets, due to the residualizing radiometabolites. The second method (Paper IV), giving non-residualizing radiometabolites, resulted in a remarkable decrease in kidney uptake while keeping the hepatic uptake low levels, with the added triglutamyl linker. This work should provide important data and experience in the pursuit of a highly potent radioimaging agent, which is not limited to imaging of HER2, but could prove to be useful in the development of tracers to other targets and even using other protein scaffolds.
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Daniel Rosik

On the Design of Affibody Molecules for Radiolabeling and In Vivo Molecular Imaging

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...jo det var en sak till...


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