Chapter

Strategies for Site-Specific Radiolabeling of Peptides and Proteins

Ingrid Dijkgraaf, Stijn M. Agten, Matthias Bauwens and Tilman M. Hackeng

Abstract

Although anatomical imaging modalities (X-ray, computed tomography (CT), magnetic resonance imaging (MRI)) still have a higher spatial resolution (0.1–1 mm) than molecular imaging modalities (single-photon emission computed tomography (SPECT), positron emission tomography (PET), optical imaging (OI)), the advantage of molecular imaging is that it can detect molecular and cellular changes at the onset of a disease before it leads to morphological tissue changes, which can be detected by anatomical imaging. During the last decades, noninvasive diagnostic imaging has encountered a rapid growth due to the development of dedicated imaging equipment for preclinical animal studies. In addition, the introduction of multimodality imaging (PET/CT, SPECT/CT, PET/MRI) which combines high-resolution conventional anatomical imaging with high sensitivity of tracer-based molecular imaging techniques has led to successful accomplishments in this exciting field. In this book chapter, we will focus on chemical synthesis techniques for site-specific incorporation of radionuclide chelators. Subsequently, radiolabeling based on complexation of a radionuclide with a chelator will be discussed, with focus on: diethylenetriaminepentaacetic acid (DTPA), 1,4,7,10-tetraazacyclododecane-tetraacetic acid (DOTA), 1,4,7-triazacyclononane-triacetic acid (NOTA), hexa-histidine (His-tag), and 6-hydrazinonicotinic acid (HYNIC) that allow the production of peptides labeled with ^{18}F, ^{68}Ga, ^{99m}Tc, and ^{111}In – the currently most widely used isotopes.

Keywords: radiolabeled peptides, chelator, PET, SPECT, radionuclide, peptide synthesis, protein synthesis

1. Introduction

1.1 Application of peptides and proteins as molecular imaging agents

The concept of using radiolabeled receptor-binding peptides and proteins to target receptor-(over)expressing tissues *in vivo* has stimulated a large body of research in nuclear medicine. Peptides and small proteins for receptor imaging and targeted radiotherapy have particular advantages over antibodies and antibody fragments. Peptides are small molecules and show rapid diffusion in target tissue. They rapidly clear from the blood and non-target tissues, resulting

in high target-to-background ratios. Furthermore, peptides have a low toxicity and are generally not immunogenic. However, ubiquitously occurring amino- and carboxypeptidases in the circulation will rapidly degrade most peptides preventing intact imaging agents to reach the target tissue in sufficient quantities. Thus, to prevent rapid enzymatic degradation of peptide-based imaging agents, most peptides have to be modified [1, 2]. Several methods to prevent enzymatic peptide proteolysis have been developed, including substitution of L- by D-amino acids, replacement of amino moieties by imino groups, substitution of peptide bonds, insertion of unusual amino acids or side chains, amidation, cyclization, *C*-terminal amidation or reduction, *N*-terminal acylation or methylation, and use of peptidomimetics. Cyclization of peptides results not only in resistance to enzymatic degradation, it can also lead to conformationally more constrained compounds with enhanced receptor affinity and biological activity [1, 3].

Besides stability toward enzymes, lipophilicity is very important. The preferred route of clearance of a peptide-based radiopharmaceutical is via the kidneys. For targeting of tumors, cardiovascular diseases, and infections or inflammation, the lipophilicity of the compound should not be too high (log P < 1) as lipophilic compounds result in non-specific binding and slower blood clearance via mainly the hepatobiliary route. In contrast, molecular imaging tracers that target brain diseases such as Alzheimer require a higher lipophilicity (log P > 1) in order to cross the blood–brain barrier (BBB). Lipophilicity of molecular imaging tracers can be reduced by linking them to polyethylene glycol (PEG) chains, a technique called PEGylation. An alternative method to reduce lipophilicity of a tracer is attachment of carbohydrates, as this enhances the hydrophilicity, resulting in reduced hepatobiliary uptake, enhanced urinary excretion, and reduced nonspecific binding [4].

Furthermore, conjugation of chelators like DOTA (1,4,7,10-tetraazacyclododecane-tetraacetic acid), NOTA (1,4,7-triazacyclononane-triacetic acid), or DTPA (diethylenetriaminepentaacetic acid) also reduce the lipophilicity of an imaging agent. However, modification of a tracer by for example PEGylation, glycosylation or conjugation to a chelator, can also affect the affinity of a peptide for the receptor and thus the effectiveness of the radiotracer.

1.2 Introduction of bifunctional chelating agents (BFCAs) into the peptide or protein

A chelating agent will not only influence the hydrophilicity of a peptide or protein, but it will also increase the overall size of the radiotracer and thus the pharmacokinetics. To preserve biological activity and receptor-binding affinity, conjugation of a chelator must be performed at a site remote from the active and receptor-binding region of the tracer [5]. Total chemical protein synthesis enables single site-specific protein modification, which cannot be achieved through regular labeling methods of biologically obtained proteins. To prevent interference of the chelator with the active and receptor-binding region of the peptide, introduction of a linker may be necessary. These linkers (PEG chains, amino acids, aliphatic hydrocarbon chains, etc.) can be used as pharmacokinetic modifiers (PKMs) to adjust the pharmacokinetics of the probe.

Several acyclic and cyclic bifunctional chelators have been developed for both diagnostic and therapeutic applications (**Figure 1**). A bifunctional chelator is a molecule which can be covalently coupled to the targeting compound and has the ability to chelate a (radio)metal. The most widely used chelators are DTPA, DOTA, and NOTA or derivatives thereof. A chelator should effectively sequester the radionuclide in high-yields (quantitative) and with high stability. Unstable complexation of the radionuclide by the chelator can lead to trans-chelation of the radionuclide
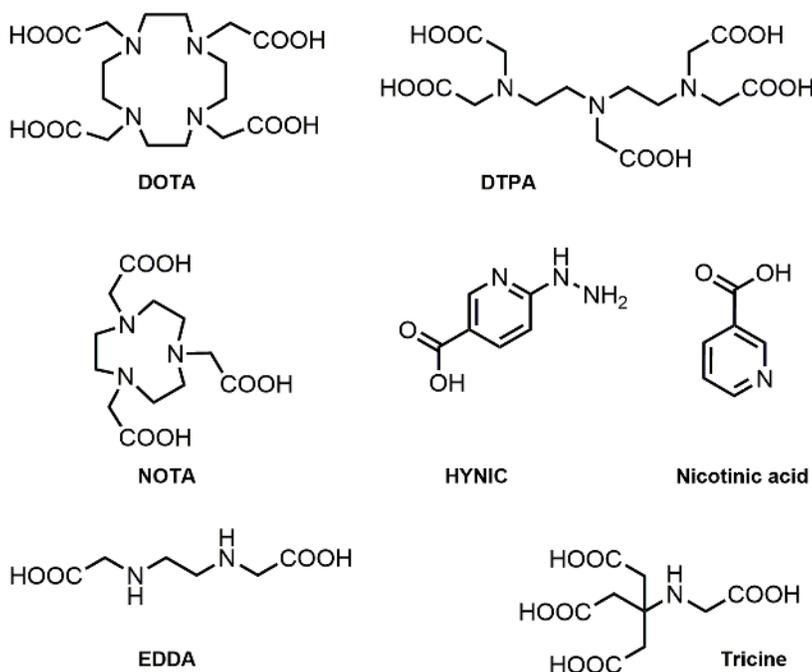
Figure 1.
Structural formula of different chelators and co-ligands for radiolabeling peptides and proteins.

to blood proteins and enzymes (e.g. transferrin, ceruloplasmin, superoxide dismutase). For a detailed review of chelating agents and the optimal match between chelator and radionuclide see Price *et al*. 2014 [6] and references therein.

The introduction of BFCA in proteins or peptides can be achieved using bioconjugation methods based on reactive functional groups, such as amide coupling (carboxylic acids and their activated *N*-hydroxysuccinimide (NHS) esters), thiol couplings (maleimides), oxime bond formation (ketones or aldehydes with aminooxy), and Cu(I) catalyzed azide-alkyne Huisgen 1,3-dipolar cycloaddition "click reactions". We will discuss some of the methods in detail in Section 2.3.

1.3 Radionuclide imaging

Apart from planar imaging, SPECT and PET are the two main imaging modalities in nuclear medicine. SPECT imaging is much more widely available than PET imaging and the radionuclides used for SPECT are easier to prepare, financially generally more accessible, and usually have a longer half-life than those used for PET (**Table 1**). Commonly used gamma emitters are: ^{123}I (E_{max} 529 keV, $t_{1/2}$ 13.0 h), ^{111}In (E_{max} 245 keV, $t_{1/2}$ 67.2 h), and ^{99m}Tc (E_{max} 141 keV, $t_{1/2}$ 6.02 h). Compared to SPECT, PET has the possibility to more accurately quantitate the *in vivo* concentration of a tracer labeled with a positron emitting radionuclide, such as for (pre)clinical applications ^{18}F (E_{max} 635 keV, $t_{1/2}$ 1.83 h), ^{68}Ga (E_{max} 1.90 MeV, $t_{1/2}$ 68.1 min), ^{64}Cu (657 keV, $t_{1/2}$ 12.7 h), and ^{124}I (E_{max} 2.13 MeV; 1.53 MeV; 808 keV, $t_{1/2}$ 4.18 days).

PET is independent of the location depth of the reporter probe of interest and is able to detect picomolar concentrations of tracer [7]. This high sensitivity of PET can only be matched to some degree by optical imaging (OI) techniques, but not by MRI, CT or ultrasound (US). In addition, compared to MRI and conventional optical imaging techniques, PET has the advantage of being quantitative. Though, with the introduction of fluorescence mediated tomography (FMT), quantitative measurements are also possible with OI techniques [8].

	Isotope	Half-life (h)	Decay type
γ-emitter (SPECT)	^{99m}Tc	6.02	IT
	^{111}In	67.2	EC, γ
	^{123}I	13.0	EC, γ, e$^-$
β$^+$-emitter (PET)	^{18}F	1.83	β$^+$, EC
	^{64}Cu	12.9	β$^-$, EC
	^{68}Ga	1.14	β$^+$, EC
	^{124}I	76.8	EC, β$^+$, γ
β$^-$-emitter (therapy)	^{90}Y	64.1	β$^-$
	^{177}Lu	161	β$^-$
	^{186}Re	91	β$^-$, EC, γ
	^{188}Re	17.0	β$^-$
	^{131}I	192	β$^-$, γ, e$^-$

Half-life is given in hours, unless stated otherwise. β$^-$ *= negative beta decay,* β$^+$ *= positive beta decay,* γ *= gamma transition, IT = isometric transition, EC = electron capture.*

Table 1.
Half-life and decay type of several radionuclides.

Recent developments also allow semi-quantitative measurements with SPECT, but these developments are not yet widespread and still show higher uncertainties compared to PET.

The spatial resolution of PET and SPECT scanners depends on several factors: the type of isotope (PET or SPECT), the energy of the isotope emissions, and the object being scanned. The type of isotope (positron-emitting or single-photon emitting) has a strong impact, as the image reconstruction techniques for PET are superior to those of SPECT due to physical characteristics in large objects, but this is reversed for small objects. The energy of the isotope emissions is negatively correlated to the spatial resolution: the stronger the energy, the poorer the spatial resolution. The object being scanned has a substantial impact: spatial resolution in mice is vastly superior to that in humans, and even within humans spatial resolution in obese people is worse compared to healthy subjects. Some typical spatial resolutions are: ^{99m}Tc, mouse: 0.5 mm; ^{99m}Tc, human: 10 mm; ^{18}F, mouse: 0.8 mm; ^{18}F, human: 2 mm; ^{68}Ga, human and mouse: both 4 mm. The spatial resolution should be taken into account when designing studies.

2. Peptide and protein synthesis

2.1 Protein production by expression systems

Nowadays, recombinant protein expression is a routine laboratory technology that enables fast and high-yield protein production. The choice of bacterial, yeast, insect or mammalian cellular-based expression system depends on several factors such as, cell growth characteristics, intracellular and extracellular expression, posttranslational modifications, and regulatory issues of proteins used as diagnostics and therapeutics. Recently, even cell-free expression systems using purified RNA polymerase, ribosomes, tRNA and ribonucleotides have been developed [9]. Each expression system has its particular advantages and disadvantages that are relevant for the purpose of use. Several review papers give a good description of the variety of expression systems and their pros and cons. However, for development

of target-specific radiotracers, fluorescent probes, or multimodality molecular imaging agents, chemical protein synthesis is the method of choice because of reasons described below. Therefore, this book chapter does not cover recombinant expression systems further.

2.2 Solid-phase peptide synthesis

Total chemical protein synthesis is an attractive alternative to biological protein production. Chemical peptide synthesis can be divided in: (I) liquid-phase peptide synthesis and (II) solid-phase peptide synthesis. Liquid-phase peptide synthesis is a classical approach to peptide synthesis and since the beginning of the 20th century this technique has developed considerably. Although liquid-phase peptide synthesis has some limitations due to its time consuming nature, solubility issues and the need for lengthy purification procedures, it is still useful for large-scale peptide production and for specialized laboratory applications [10].

Solid-phase peptide synthesis (SPPS) is currently the preferential technique to establish access to synthetic peptides. The general process for SPPS is based on sequential addition of α-amino and reactive side chain protected amino acids to a solid support (resin). The *C*-terminal residue is coupled to the resin and after removal of the N^{α}-protecting group, the next *C*-terminally activated N^{α}-protected amino acid is coupled and so on, until full chain assembly is reached. The most commonly used N^{α}-protecting groups are the acid-labile Boc (*tert*-butyloxycarbonyl) group and the base-labile Fmoc (9-fluorenylmethoxycarbonyl) group. Side chain protecting groups are ideally orthogonal to the N^{α}-protecting group, which means that they are removable under completely different reaction conditions.

The use of synthetic chemistry allows infinite variation of the polypeptide chain by for example incorporation of unnatural amino acids such as β-amino acids, *N*-methyl amino acids, peptoids, stable isotope-labeled amino acids, and D-amino acids. Furthermore, the use of orthogonal amino acid side chain protecting groups during the sequential elongation of the peptide chain in SPPS, allows conjugation of fluorescent tags, chelators, biotin, etc., at single specific sites. Current optimized SPPS chemistry protocols enable effective peptide synthesis of 30–50 amino acids. For the synthesis of peptides and proteins bigger than 30–50 amino acids, various chemical ligation techniques were developed that enable the formation of a peptide bond between two unprotected peptides resulting in larger synthetic proteins with a fully native peptide backbone [11, 12].

2.3 Site-specific incorporation of chelators and/or fluorescent tags

Functionalization of peptides and proteins still heavily relies on amine or thiol functionalities, present in proteins as lysine and cysteine side chains, respectively. New ligation techniques are emerging that are moving away from amines or use of protected thiols. The functionalization of lysine side chains can be achieved by reacting them with activated esters such as NHS-DTPA or –DOTA that are commercially available (**Figure 2**). The most appropriate derivatives of these chelators for conjugation to a peptide or protein are those which are *t*Bu (*tert*-butyl)-protected at all functional acid groups, except one. This acid group can either be activated *in situ* using a proper coupling agent or be obtained as a preactivated NHS ester in DOTA-tris(*t*Bu)ester NHS ester, NOTA-bis(*t*Bu) ester NHS ester, or DTPA-tetrakis(*t*Bu) ester NHS ester.

The main advantage of these activated ester chelators is their ease of use, while the main disadvantage of this technique is their unspecific labeling. A protein generally contains more than one lysine residue and thus more than one position for chelator conjugation. It is difficult to predict the site of coupling, which will
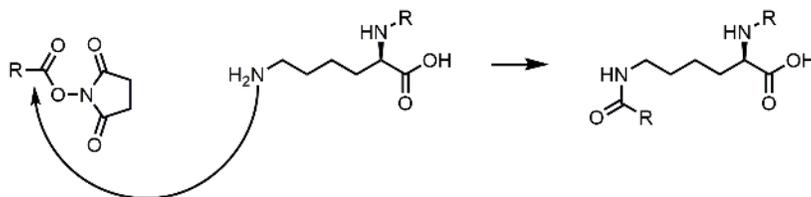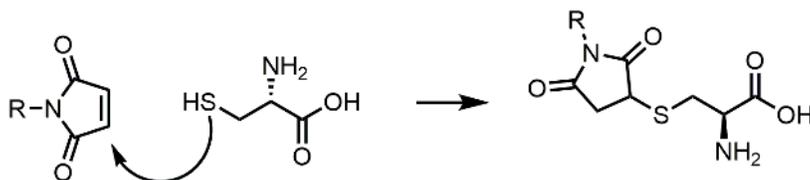
Figure 2.
Conjugation at N$^\varepsilon$-amine group of a lysine residue with an amine reactive NHS ester (N-hydroxysuccinimide ester) resulting in an amide bond.

often lead to heterogenous labeling of the compound of interest. With the use of Boc SPPS this problem can be circumvented by using orthogonally ε-amino Fmoc-protected lysine residues. Deprotection of the Fmoc group can be performed on resin and directly be followed by functionalization of the desired lysine with an NHS-activated label of choice. In case of Fmoc SPPS, orthogonally ε-amino ally-loxycarbonyl (Alloc) protected lysine residues can be used.

Conjugation at cysteine residues can be realized by reactions with maleimide containing compounds or 2-azidoacrylate-derivatives [13]. Similar to the amine reactive NHS esters, commercial compounds with maleimides are widespread. Maleimide-DOTA or –DTPA are coupled to free cysteine-containing proteins (**Figure 3**). Although the reaction is specific and easy to use, maleimides have their disadvantages. The first being the availability of a free cysteine in a protein of interest; the major part of cysteines present in proteins are paired with a second cysteine to form a disulfide bridge. Moreover, these cysteines are often buried within the core of the protein making them inaccessible for maleimides. To overcome this problem an additional cysteine can be incorporated into the protein specifically for labeling. This will, however, lead to problems with oxidative folding of the protein and can lead to improperly folded proteins with loss of activity.

However, this does not mean that thiol reactive compounds cannot be useful in protein labeling. The introduction of an encrypted cysteine that can be deprotected after correct folding of the protein can offer a solution. Recently N^ε-(thiazolidine carboxyl)-lysine was applied for this purpose [14], the thiazolidine carboxylic acid (Thz) that was originally designed to facilitate sequential one-pot native chemical ligation (NCL) [15–17], was used as a handle for late stage site-specific modification of proteins under mild conditions [14]. The encrypted cysteine was introduced on a lysine side chain which after opening under mild conditions with MeONH$_2$ or NH$_2$OH resulted in a free cysteine enabling reactions with maleimide groups (**Figure 4**).

Furthermore, it was shown that this technique is fully compatible with established techniques of peptide synthesis and NCL. The chemokine CCL5 was synthesized from an *N*- and *C*-terminal part that were joined by native chemical ligation. The *C*-terminus contained a lysine with an orthogonal Fmoc protective group. After completion of the synthesis of the *C*-terminus, the Fmoc group was removed and the thiazolidine residue was site-specifically introduced. After cleavage from resin and
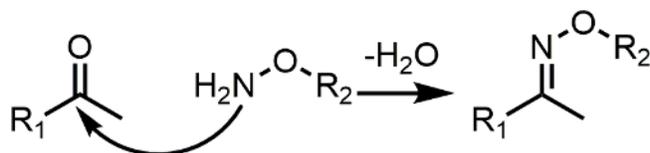

Figure 3.
Conjugation at a free thiol moiety present in cysteine using a maleimide resulting in a thioether bond.
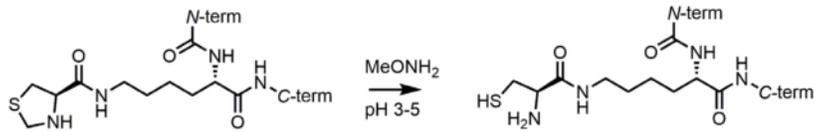
Figure 4.
Deprotection of thiazolidinecarboxyl coupled to N^ε-amine group of a lysine residue using methoxylamine.
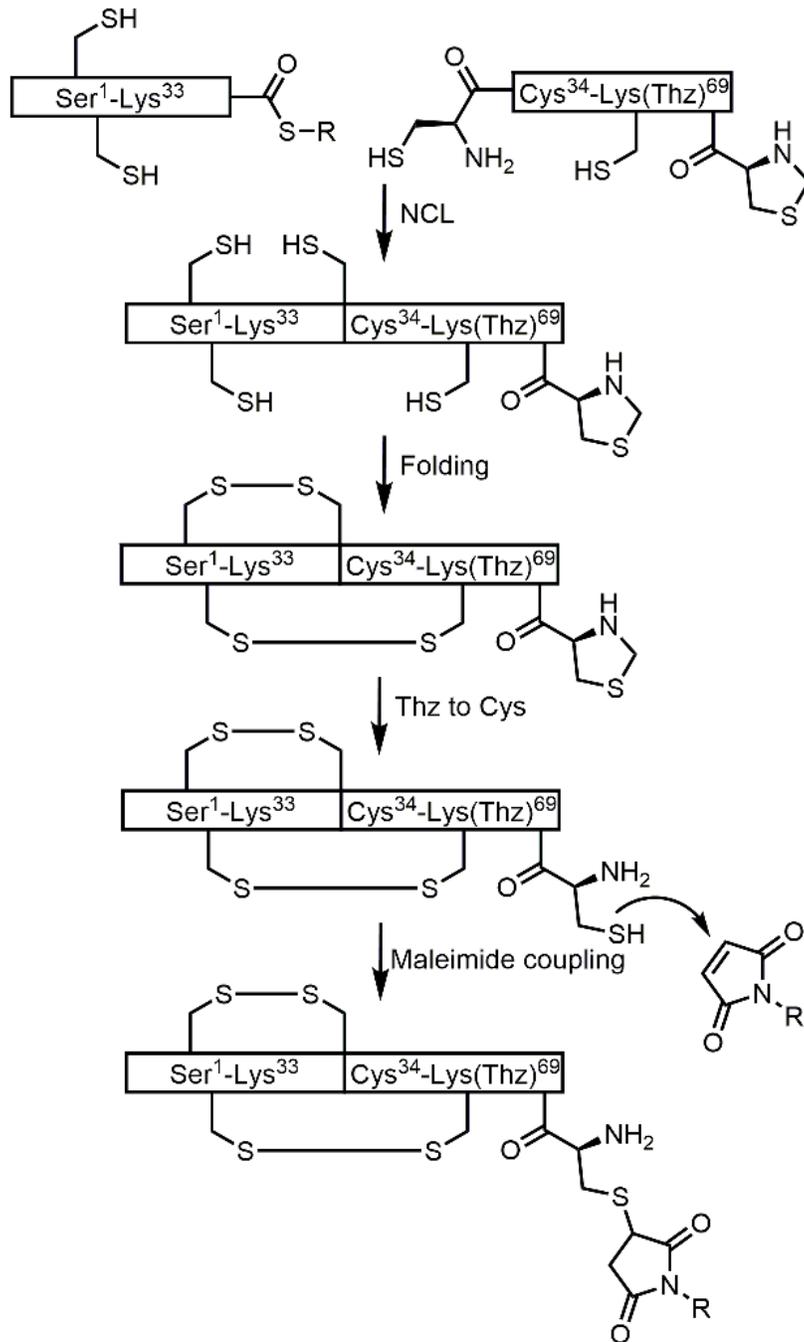
Figure 5.
Schematic representation of the synthesis of chemokine CCL5. In the first step two unprotected peptide fragments are ligated using native chemical ligation. Subsequently, the peptide is folded into an active protein. In the last step the thiazolidine ring is opened while simultaneously the newly formed thiol moiety is modified with a maleimide.

Figure 6.
Reaction of an aminooxy with a ketone which results in an oxime bond.

subsequent purification, the two parts were ligated to obtain the full length protein and subsequently the protein was folded under oxidative conditions. After obtaining the folded protein, the thiazolidine ring was opened by treatment with MeONH$_2$.

Upon treatment with MeONH$_2$ to convert the thiazolidine functionality into a free cysteine and subsequent modification with a maleimide label, unwanted disulfide shuffling can occur in proteins containing disulfide bonds [18]. To circumvent this problem a synchronized protocol for thiazolidine deprotection and maleimide coupling can be best used (**Figure 5**).

A chemoselective conjugation approach that does not use any of the naturally occurring functional groups in proteins is oxime ligation. The reaction is comprised of a ketone or aldehyde reacting with an aminooxy group to yield an oxime bond (**Figure 6**). The reaction can be performed in aqueous media at neutral pH but is faster at slightly acidic pH [19]. The reaction can be catalyzed with aniline or derivatives thereof, to facilitate fast reactions [20, 21].

Although the oxime reaction itself can be performed relatively easy, the more challenging part is the incorporation of a ketone or aminooxy in the peptide/ protein of interest. Since a ketone is virtually inert to most chemical reactions, the ketone is mostly chosen over the aminooxy component to be incorporated in the protein of choice, while the aminooxy component is used to modify the label. The increased attention for the oxime bond in the last decade has led to the development of several methods to incorporate ketones or aldehydes in proteins. An overview of the available techniques was previously reviewed, here we will briefly highlight methods useful in chemical synthesis [22]. Historically, oxidation of peptides/proteins containing an *N*-terminal 2-amino alcohol residue (serine/ threonine) is among the most used to obtain an aldehyde in the form of a glyoxyloyl moiety [23]. Site-specific incorporation using chemical synthesis, however, can be achieved using suitably protected unnatural amino acids such as p-acetylphenylalanine or keto-proline [24, 25]. A large amount of flexibility in both site of introduction and distance from active regions can be achieved with modification of amine moieties with keto-acids [26, 27]. Care has to be taken, however, in the choice of keto-acid as some are less efficient in subsequent oxime formation [28].

In summary, several techniques are available for the modification of proteins to include chelators used for PET and SPECT. Amine and thiol reactive compounds are easy to use through orthogonally protected lysine side chains or through thiazolidine deprotection in pre-folded proteins. Oxime conjugation can be used for chelator incorporation but is also used for covalent radiolabeling approaches, such as introducing ^{18}F-containing prosthetic groups (see for an overview [29]).

3. Radiolabeling of peptides

A variety of labeling techniques can be applied to peptides and proteins, but according to George De Hevesy's definition of a tracer the radiolabeling procedure should not affect the biological properties, the affinity to the target, or the physicochemical properties (e.g. charge, hydrophilicity, size). In the following part a list

of radiosynthesis techniques for commonly used isotopes is described, omitting isotopes that are used less frequently. For example, ^{11}C is a widespread isotope for labeling small organic compounds, but it is used less frequently in peptides or larger structures and so will therefore not be further discussed here.
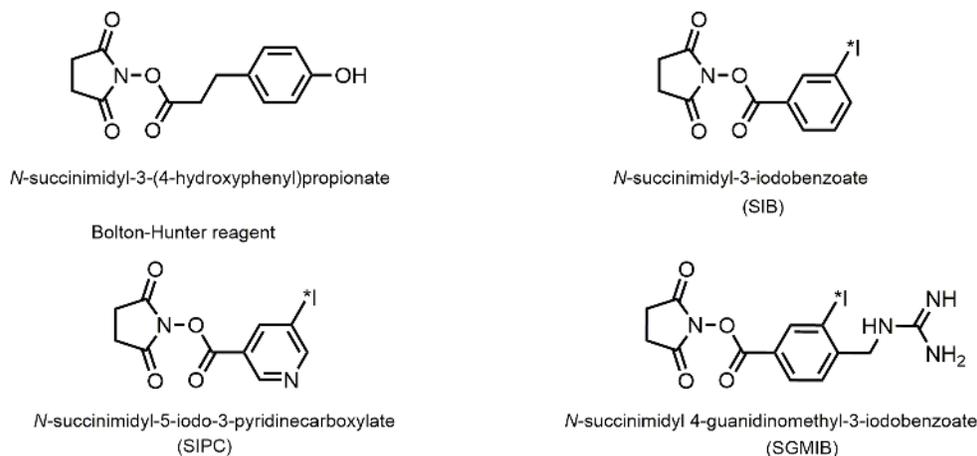
3.1 Radioiodination

Radioiodination of peptides with ^{125}I, ^{123}I, [124]I, or ^{131}I can be performed by either direct labeling or indirect labeling via an auxiliary group. During direct radioiodination, radioactive iodine is incorporated covalently into the side chains of tyrosyl or histidyl residues in the presence of an oxidizing agent such as chloramine-T or iodogen. If no tyrosyl or histidyl residue is available, free amino groups in the peptide may be radioiodinated by auxiliary groups, including *N*-succinimidyl-3-(4-hydroxyphenyl)propionate, known as Bolton-Hunter reagent [30]. Other auxiliary groups for radioiodination of peptides are *N*-succinimidyl-3-iodobenzoate (SIB), *N*-succinimidyl-5-iodo-3-pyridinecarboxylate (SIPC), and *N*-succinimidyl 4-guanidinomethyl-3-iodobenzoate (SGMIB) (**Figure** 7) [31–34].

As none of the radioiodination methods is based on complexation with a chelator, we will not focus on this labeling method in this book chapter further.

3.2 Radiofluorination

For routine PET imaging, fluorine-18 represents the near ideal radionuclide with its half-life of 109.8 min and low β^+-energy (0.64 MeV). Due to this low positron energy, it has a short positron linear range in tissue, leading to particularly high spatial resolution in PET imaging. Furthermore, compared to other short lived radionuclides, such as ^{11}C, its half-life is long enough to allow syntheses and imaging procedures to be extended over hours, enabling kinetic studies and high-quality metabolite and plasma analysis.

Efficient ^{18}F-labeling of peptides and proteins often comprises a multistep process involving labeling and purification of a prosthetic group (synthon) and subsequent conjugation of the ^{18}F-labeled synthon to the peptide/protein with or without activation. If necessary, the ^{18}F-conjugate is purified by a final purification step. Over the years, a variety of prosthetic groups have been developed ranging from amine-reactive groups such as *N*-succinimidyl-4-[^{18}F]fluorobenzoate ([^{18}F]SFB) [35] to chemoselective groups like 4-[^{18}F]fluorobenzaldehyde and [^{18}F]FDG ([^{18}F]

N-succinimidyl-3-(4-hydroxyphenyl)propionate

Bolton-Hunter reagent

N-succinimidyl-3-iodobenzoate
(SIB)

N-succinimidyl-5-iodo-3-pyridinecarboxylate
(SIPC)

N-succinimidyl 4-guanidinomethyl-3-iodobenzoate
(SGMIB)

Figure 7.
The auxiliary groups Bolton-hunter reagent, SIB, SIPC, and SGMIB for the radioiodination of peptides.

fluorodeoxyglucose) [36, 37] that react with an aminooxy- or hydrazine-modified peptides. A special prosthetic group approach that has been explored in ^{18}F-labeling chemistry is the click chemistry methodology. Click chemistry appeared to be an effective method to radiolabel peptides and proteins, because the click reaction is fast, bioorthogonal, chemo- and regioselective, results in relatively high yields and can be performed in aqueous media. This copper(I)-catalyzed azide-alkyne cycloaddition reaction has been exploited in radiopharmaceutical chemistry by several research groups [38–44]. A variant of the copper(I)-catalyzed click reaction is the copper-free click reaction that does not require the use of the cytotoxic metal. Reactions of electron-deficient tetrazines with ring-strained trans-cyclooctenes or norbenes have been investigated [45–49]. Click reactions, Cu(I)-catalyzed and copper-free, appeared to be powerful and versatile reactions for the synthesis of ^{18}F-labeled peptides and proteins.

In spite of the variety of possibilities for introducing ^{18}F, a major drawback of the ^{18}F-labeling methods described above is that they are laborious, (require azeotropic drying of the fluoride and multiple purification steps) and are thus time consuming. In search of a kit-based ^{18}F-labeling method, new ^{18}F-labeling strategies based on fluorine-silicon [50–56], fluorine-boron [57–59], and fluorine-phosphorus [60] have been developed.

A facile chelator-based approach was developed wherein ^{18}F is first attached to aluminum as Al[18]F, which is then complexed in a chelating agent attached to the peptide, forming a stable Al[18]F–chelate peptide complex in an efficient 1-pot process [61].

3.3 Radiolabeling with [68]Ga

Gallium-68 is an interesting positron-emitter, because of its well established radiochemistry and its easy access and availability from commercial available [68]Ge/^{68}Ga-generators ($t_{1/2}$ [68]Ge = 268 days) which renders it independent of an on-site cyclotron. The application of ^{68}Ga-labeled peptides and proteins has attracted considerable interest for molecular imaging, because of its physical characteristics. The high positron emission fraction, 89% through positron emission of 1.9 MeV (max. energy), and half-life of 68 min allows short scanning times with sufficient amounts of radioactivity for high quality images. Generally, DOTA and NOTA are very suitable chelators and are commonly used for [68]Ga^{3+}-complexation. Though, recently TRAP (Tri-azacyclononane-phosphinic acid) and its derivatives revealed to be powerful ^{68}Ga chelators which possess valuable utility in nuclear medicine and molecular imaging [62].

DOTA has a larger cavity than NOTA and, thus, needs higher ring distortion for complexation of [68]Ga^{3+}. Therefore, higher temperatures are required for ^{68}Ga-DOTA complex formation compared to ^{68}Ga complex formation with NOTA. Typically, DOTA-conjugated peptides are radiolabeled with ^{68}Ga at 90–100°C, whereas NOTA-conjugated peptides can be labeled at room temperature [62].

Generally, ^{68}Ga is obtained by eluting a [68]Ge/^{68}Ga generator with a 0.01–1 M HCl solution. The eluate can be added directly to the NOTA- or DOTA-conjugated compound dissolved in a suitable buffer system such as HEPES (4-(2-hydroxy-ethyl)piperazine-1-ethanesulfonic acid), phosphate, or ammonium acetate. It is important that the resulting pH of the reaction mixture is a pH value <4 to prevent formation of colloidal hydroxide [[68]Ga(OH)$_3$]$_n$ which begins at a pH value above 4.

3.4 Radiolabeling with [111]In

The SPECT isotope indium-111 is a frequently used radionuclides in diagnostic nuclear medicine. It has a physical half-life of 67 hours and is produced by a

cyclotron. The principle photons are 173 keV (89%) and 247 keV (94%). The most commonly used chelators for ^{111}In-labeling of peptides and proteins are DTPA and DOTA. DTPA chelates ^{111}In at room temperature with sufficient efficiency and stability. This DTPA chelator is used in the commercially available somatostatin analog OctreoScan®. DOTA forms a more stable complex with ^{111}In, but requires heating of the reaction mixture which can lead to protein denaturation, especially for larger proteins. Labeling of DTPA- and DOTA-conjugated peptides and proteins is a one pot, one step procedure in which the compound is incubated with [111]InCl3 at a pH between 4 and 6. Ammonium and sodium acetate buffers are commonly used as buffer for labeling of DTPA- and DOTA-conjugated compounds with [111]In. However, it was shown that ^{111}In-labeling efficiency and specific activity of DTPA- and DOTA-conjugated peptides was significantly improved in MES (2-(*N*-morpholino)ethanesulfonic acid) and HEPES buffer compared to acetate buffers [63]. The enhanced labeling efficiency appeared to be due to the reduced competitive chelation of cadmium, the decay product of [111]In. These observations made MES and HEPES the buffers of choice for ^{111}In-labeling.

3.5 Radiolabeling with [99m]Tc

Technetium-99 m is the most widely used isotope due to its ideal half-life of 6 hours, its low cost, and excellent imaging characteristics. Similar to ^{68}Ga, ^{99m}Tc can be obtained from a generator ([99]Mo/^{99m}Tc, with a half-life of 66 hours for [99]Mo) which is easily shipped, allowing worldwide use. Since the 1960s, ^{99m}Tc has been used in a variety of applications, including cancer research and cardiac assessment. [99m]Tc is a nearly pure gamma-emitter (88%), with the remaining 12% yielding internal conversion electrons. The application is therefore restricted to SPECT imaging (although research into therapeutic applications is also performed).

Possibilities for coupling of ^{99m}Tc to small organic compounds, peptides or proteins are nearly unlimited, in part due to the many oxidation states that ^{99m}Tc can have, ranging from +I to +VII. The isotope is obtained from the generator in a pH-neutral and isotonic saline solution, ensuring computability with nearly any peptide or protein.

One solution to radiolabel proteins is, instead of chemically modifying the protein, synthesizing it with an additional hexa-histidine chain at the *N*-terminus (His-tag). This addition mostly does not interfere with recognition sites and allows for site-specific labeling of [99m]Tc (in the form of [99m]Tc(CO)$_3$) [64]. This is a two-step reaction, where [99m]TcO$_4$- (Technetium pertechnetate, as it is eluted from the generator) is first converted to [99m]Tc(CO)$_3$ (thereby changing the oxidation state from +VII to +I, under relatively harsh conditions) and subsequently coupling [99m]Tc(CO)$_3$ to the His-tag.

An alternative for ^{99m}Tc-labeling of peptides and proteins is conjugating them with the bifunctional chelator HYNIC (6-hydrazinonicotinic acid). HYNIC has been introduced to radiolabel an IgG antibody with ^{99m}Tc for infection imaging [65]. HYNIC is an established and appropriate BCA for ^{99m}Tc-labeling, because it allows rapid and efficient labeling of proteins. In addition, ^{99m}Tc-labeled HYNIC-conjugates can be produced with high specific activities. However, conjugation of HYNIC to a peptide of protein can be complex as the hydrazine group of HYNIC is highly nucleophilic and, when unprotected, undergoes unwanted side reactions with electrophiles. Protecting the hydrazine group of HYNIC-conjugated compounds with for example Fmoc, Cbz, and Boc, has been investigated by several research groups and is still subject of current research [66–71].

Since the HYNIC group can only coordinate to a metal through 2 donor groups at most, it is unable to saturate the technetium coordination sphere. To complete

the coordination sphere, an additional coligand, such as EDDA (ethylenediamine diacetic acid), tricine (*N*-[Tris(hydroxymethyl)-methyl]glycine), or nicotinic acid is required. However, the choice of the coligand has significant influence on the pharmacokinetics, and thus, biologic properties of the radiotracer [72]. Because the chemistry of HYNIC conjugation to a peptide or protein remains a challenge and the coordination mode of the ^{99m}Tc-HYNIC complex is still undefined, it is not possible to give a standard HYNIC-conjugation and ^{99m}Tc-radiolabeling protocol. For a review about ^{99m}Tc-HYNIC coordination chemistry see Meszaros *et al*. and references herein [73].

4. Conclusions

Improvements in effective nuclear imaging are not only dependent on progression in imaging equipment and technology, but is also strongly dependent on the availability of powerful probes with optimal pharmacokinetic and imaging characteristics. The diversity of methods for syntheses of peptides and proteins and the variety of possibilities for modification, stabilization, labeling (radiolabeling and labeling for other modalities), and construction of more complex multivalent and multimodal constructs, make radiolabeled peptides and proteins a flexible class of tracers and meaningful molecules for nuclear imaging of several diseases such as cancer, thrombosis, infection, and inflammation in pre-clinical and clinical research.

Radiotracers enable early diagnosis and thus early treatment of disease and they enable better stratification of patients with disease-stage-adapted therapy instead of escalating to the most aggressive and costly therapy. Moreover, radiolabeled compounds are used to monitor the therapeutic effect of drugs and are used as therapeutic radiopharmaceuticals when labeled with either β^- - or α-emitting radionuclides such as ^{90}Y, ^{186}Re, ^{188}Re, ^{131}I, ^{177}Lu and ^{211}At and ^{213}Bi, respectively.

The growth of the world population and the overall rise in life expectancy in the last decades will increase the demand for radiopharmaceuticals, including basic research into and the development of new radiopharmaceuticals.

Conflict of interest

The authors declare no conflict of interest.

Author details

Ingrid Dijkgraaf[1,2*], Stijn M. Agten[1], Matthias Bauwens[2]
and Tilman M. Hackeng[1]

1 Department of Biochemistry, Maastricht University, The Netherlands

2 Department of Radiology and Nuclear Medicine, Maastricht University Medical
Centre, Maastricht, The Netherlands

*Address all correspondence to: I.Dijkgraaf@maastrichtuniversity.nl

IntechOpen

References

[1] Weiner RE, Thakur ML. Radiolabeled peptides in diagnosis and therapy. Semin Nucl Med. 2001;31:296-311.

[2] Powell MF, Grey H, Gaeta F, Sette A, Colón S. Peptide stability in drug development: A comparison of peptide reactivity in different biological media. J Pharm Sci. 1992;81:731-735.

[3] Okarvi SM. Peptide-Based Radiopharmaceuticals: Future Tools for Diagnostic Imaging of Cancer and Other Diseases. Medicinal Research Reviews. 2004;24:357-397.

[4] Wester HJ, Schottelius M, Poethko T, Bruus-Jensen K, Schwaiger M. Radiolabeled Carbohydrated Somatostatin Analogs: A review of the Current Status. Cancer Biother Radiopharm. 2004;19:231-244.

[5] Okarvi SM. Recent developments in ^{99m}Tc-labelled peptide-based radiopharmaceuticals: An overview. Nucl Med Commun. 1999;20:1093-1112.

[6] Price EW, Orvig C. Matching chelators to radiometals for radiopharmaceuticals. Chem Soc Rev. 2014;43:260-290.

[7] Weber WA. Positron emission tomography as an imaging biomarker. J Clin Oncol. 2006;24:3282-3292.

[8] Jaffer FA, Weissleder R. Molecular Imaging in the Clinical Arena. JAMA. 2005;293:855-862.

[9] Rosenblum G, Cooperman BS. Engine out of the chassis: cell-free protein synthesis and its uses. FEBS Letters. 2014;588:261-268.

[10] Wentworth P Jr. Recent developments and applications of liquid-phase strategies in organic synthesis. Trends Biotechnnol. 1999;17:448-452.

[11] Dawson PE, Kent SBH. Synthesis of native proteins by chemical ligation. Ann Rev Biochem. 2000;69:923-960.

[12] Kulkarni S, Sayers J, Premdjee B, Payne RJ. Rapid and efficient protein synthesis through expansion of the native chemical ligation concept. Nat Rev Chem. 2018;2:0122.

[13] Ariyasu S, Hayashi H, Xing B, Chiba S. Site-Specific Dual Functionalization of Cysteine Residue in Peptides and Proteins with 2-Azidoacrylates. Bioconjug Chem. 2017;28:897-902.

[14] Van de Vijver P, Suylen D, Dirksen A, Dawson PE, Hackeng TM. Nepsilon-(thiaprolyl)-lysine as a handle for site-specific protein conjugation. Biopolymers. 2010;94:465-474.

[15] Dawson PE, Muir TW, Clark-Lewis I, Kent SB. Synthesis of proteins by native chemical ligation. Science. 1994;266:776-779.

[16] Hackeng TM, Griffin JH, Dawson PE. Protein synthesis by native chemical ligation: expanded scope by using straightforward methodology. Proc Natl Acad Sci U S A. 1999;96:10068-10073.

[17] Bang D, Kent SB. A one-pot total synthesis of crambin. Angew Chem Int Ed Engl. 2004;43:2534-2538.

[18] Van de Vijver P, Schmitt M, Suylen D, Scheer L, Thomassen MC, Schurgers LJ, Griffin JH, Koenen RR, Hackeng TM. Incorporation of disulfide containing protein modules into multivalent antigenic conjugates: generation of antibodies against the thrombin-sensitive region of murine protein S. J Am Chem Soc. 2012;134:19318-19321.

[19] Cordes EH, Jencks WP. General Acid Catalysis of Semicarbazone Formation. J Am Chem Soc.1962;84:4319-4328.

[20] Dirksen A, Hackeng TM, Dawson PE. Nucleophilic catalysis of oxime ligation. Angew Chem Int Ed Engl. 2006;45:7581-7584.

[21] Rashidian M, Mahmoodi MM, Shah R, Dozier JK, Wagner CR, Distefano MD. A highly efficient catalyst for oxime ligation and hydrazone-oxime exchange suitable for bioconjugation. Bioconjug Chem. 2013;24:333-342.

[22] Agten SM, Dawson PE, Hackeng TM. Oxime conjugation in protein chemistry: from carbonyl incorporation to nucleophilic catalysis. J Pept Sci. 2016;22:271-279.

[23] Gaertner HF, Rose K, Cotton R, Timms D, Camble R, Offord RE. Construction of protein analogues by site-specific condensation of unprotected fragments. Bioconjug Chem. 1992;3:262-268.

[24] Wang L, Zhang Z, Brock A, Schultz PG. Addition of the keto functional group to the genetic code of *Escherichia coli*. PNAS. 2003;100:56-61.

[25] Choudhary A, Kamer KJ, Shoulders MD, Raines RT. 4-ketoproline: An electrophilic proline analog for bioconjugation. Biopolymers. 2015;104:110-115.

[26] Sohma Y, Kent SB. Biomimetic synthesis of lispro insulin via a chemically synthesized "mini-proinsulin" prepared by oxime-forming ligation. J Am Chem Soc. 2009;131:16313-16318.

[27] Kochendoerfer GG, Chen SY, Mao F, Cressman S, Traviglia S, Shao H, Hunter CL, Low DW, Cagle EN, Carnevali M, Gueriguian V, Keogh PJ, Porter H, Stratton SM, Wiedeke MC, Wilken J, Tang J, Levy JJ, Miranda LP, Crnogorac MM, Kalbag S, Botti P, Schindler-Horvat J, Savatski L, Adamson JW, Kung A, Kent SB,

Bradburne JA. Design and chemical synthesis of a homogeneous polymer-modified erythropoiesis protein. Science. 2003;299:884-887.

[28] Agten SM, Suylen D, Ippel H, Kokozidou M, Tans G, van de Vijver P, Koenen RR, Hackeng TM. Chemoselective oxime reactions in proteins and peptides by using an optimized oxime strategy: the demise of levulinic acid. Chembiochem. 2013;14:2431-2434.

[29] Li XG, Haaparanta M, Solin O. Oxime formation for fluorine-18 labeling of peptides and proteins for positron emission tomography (PET) imaging: A review. J Fluor Chem. 2012;143:49-56.

[30] Bolton AM, Hunter RM. The labeling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. Biochem J. 1973;133:529-538.

[31] Khawli LA, Kassis AI. Synthesis of ^{125}I labeled N-succinimidyl p-iodobenzoate for use in radiolabelling antibodies. Nucl Med Biol. 1989;16:727-733.

[32] Zalutsky MR, Narula AS. A method for radiohalogenation of proteins resulting in decreased thyroid uptake of radioiodine. Appl Radiat Isot. 1987;38:1051-1055.

[33] Garg PK, Zalutsky MR. N-succinimidyl 5-(trialkylstannyl)-3-pyridinecarboxylates: A new class of reagents for protein radioiodination. Bioconjugate Chem. 1991;2:50-56.

[34] Vaidyanathan G, Affleck DJ, Li J, Welsh P, Zalutsky MR. A polar substituent-containing acylation agent for the radioiodination of internalizing monoclonal antibodies: N-succinimidyl 4-guanidinomethyl-3-[^{131}I] iodobenzoate ([^{131}I]SGMIB). Bioconjugate Chem. 2001;12:428-438.

[35] Vaidyanathan G, Zalutsky MR. Labeling proteins with fluorine-18 using N-succinimidyl 4-[18F]fluorobenzoate. Int J Rad Appl Instrum B. 1992;19:275-281.

[36] Hultsch C, Schottelius M, Auernheimer J, Alke A, Wester HJ. (18)F Fluoroglucosylation of peptides, exemplified on cyclo(RGDfK). Eur J Nucl Med Mol Imaging. 2009;36:1469-1474.

[37] Namavari M, Cheng Z, Zhang R, De A, Levi J, Hoerner JK, Yaghoubi SS, Syud FA, Gambhir SS. A novel method for direct site-specific radiolabeling of peptides using [18F]FDG. Bioconjug Chem. 2009;20:432-436.

[38] Marik J, Sutcliffe JL. Click for PET: Rapid preparation of [18F] fluoropeptides using CuI catalyzed 1,3-dipolar cycloaddition. Tetrahedron Lett. 2006;47:6681-6684.

[39] Ramenda T, Kniess T, Bergmann R, Steinbach J, Wuest F. Radiolabelling of proteins with fluorine-18 via click chemistry. Chem Commun (Camb). 2009;48:7521-7523.

[40] Glaser M, Arstad E. "Click labeling" with 2-[18F]fluoroethylazide for positron emission tomography. Bioconjugate Chem. 2007;18:989-993.

[41] Thonon D, Kech C, Paris J, Lemaire C, Luxen A. New strategy for the preparation of clickable peptides and labeling with 1-(azidomethyl)-4-[(18) F]-fluorobenzene for PET. Bioconjugate Chem. 2009;20:817-823.

[42] Li ZB, Wu Z, Chen K, Chin FT, Chen X. Click chemistry for (18) F-labeling of RGD peptides and microPET imaging of tumor integrin alphavbeta3 expression. Bioconjugate Chem. 2007;18:1987-1994.

[43] Maschauer S, Einsiedel J, Haubner R, Hocke C, Ocker M, Hübner H, Kuwert T, Gmeiner P, Prante O. Labeling and glycosylation of peptides using click chemistry: A general approach to (18)F-glycopeptides as effective imaging probes for positron emission tomography. Angew Chem Int Ed. 2010;49:976-979.

[44] Li Y, Liu Z, Harwig CW, Pourghiasian M, Lau J, Lin KS, Schaffer P, Benard F, Perrin DM. (18) F-click labeling of a bombesin antagonist with an alkyne-(18) F-ArBF(3) (−): In vivo PET imaging of tumors expressing the GRP-receptor. Am J Nucl Med Mol Imaging. 2013;3:57-70.

[45] Arumugam S, Chin J, Schirrmacher R, Popik VV, Kostikov AP. [18F]azadibenzocyclooctyne ([18F] ADIBO): A biocompatible radioactive labeling synthon for peptides using catalyst free [3+2] cycloaddition. Bioorg Med Chem Lett. 2011;21:6987-6991.

[46] Hausner SH, Carpenter RD, Bauer N, Sutcliffe JL. Evaluation of an integrin αvβ6-specific peptide labeled with [18F]fluorine by copper-free, strain-promoted click chemistry. Nucl Med Biol. 2013;40:233-239.

[47] Campbell-Verduyn LS, Mirfeizi L, Schoonen AK, Dierckx RA, Elsinga PH, Feringa BL. Strain-promoted copper-free "click" chemistry for 18F radiolabeling of bombesin. Angew Chem Int Ed. 2011;50: 11117-11120.

[48] Selvaraj R, Liu S, Hassink M, Huang CW, Yap LP, Fox JM, Li Z, Conti PS. Tetrazine-trans-cyclooctene ligation for the rapid construction of integrin αvβ3 targeted PET tracer based on a cyclic RGD peptide. Bioorg Med Chem Lett. 2011;21:5011-5014.

[49] Knight JC, Richter S, Wuest M, Way JD, Wuest F. Synthesis and evaluation of an 18F-labelled norbornene derivative for copper-free click

chemistry reactions. Org Biomol Chem. 2013;11:3817-3825.

[50] Schirrmacher E, Wangler B, Cypryk M, Bradtmoller G, Schafer M, Eisenhut M, Jurkschat K, Schirrmacher R. Synthesis of p-(di-tert-butyl[(18)F]fluorosilyl)benzaldehyde ([(18)F]SiFA-A) with high specific activity by isotopic exchange: a convenient labeling synthon for the (18)F-labeling of N-amino-oxy derivatized peptides. Bioconjug Chem. 2007;18:2085-2089.

[51] Schirrmacher R, Wangler C, Schirrmacher E. Recent developments and trends in F-18-radiochemistry: syntheses and applications. Mini-Rev Org Chem. 2007;4:317-329

[52] Mu L, Hohne A, Schubiger PA, Ametamey SM, Graham K, Cyr JE, Dinkelborg L, Stellfeld T, Srinivasan A, Voigtmann U, Klar U. Silicon-based building blocks for one-step ^{18}F-radiolabeling of peptides for PET imaging. Angew Chem Int Edn Engl. 2008;47:4922-4925.

[53] Hohne A, Mu L, Honer M, Schubiger PA, Ametamey SM, Graham K, Stellfeld T, Borkowski S, Berndorff D, Klar U, Voigtmann U, Cyr JE, Friebe M, Dinkelborg L, Srinivasan A. Synthesis, ^{18}F-labeling, and in vitro and in vivo studies of bombesin peptides modified with silicon-based building blocks. Bioconjug Chem. 2008;19:1871-1879.

[54] Iovkova L, Wangler B, Schirrmacher E, Schirrmacher R, Quandt G, Boening G, Schurmann M, Jurkschat K. Para-functionalized aryl-di-tert-butylfluorosilanes as potential labeling synthons for (18)F radiopharmaceuticals. Chemistry. 2009;15:2140-2147.

[55] Rosa-Neto P, Wangler B, Iovkova L, Boening G, Reader A, Jurkschat K, Schirrmacher E. [^{18}F]

SiFA-isothiocyanate: a new highly effective radioactive labeling agent for lysine-containing proteins. Chem Biochem. 2009;10:1321-1324.

[56] Wangler B, Quandt G, Iovkova L, Schirrmacher E, Wangler C, Boening G, Hacker M, Schmoeckel M, Jurkschat K, Bartenstein P, Schirrmacher R. Kit-like ^{18}F-labeling of proteins: synthesis of 4-(di-tert-butyl[^{18}F]fluorosilyl) benzenethiol (Si[^{18}F]FA-SH) labeled rat serum albumin for blood pool imaging with PET. Bioconjug Chem. 2009;20:317-321.

[57] Ting R, Harwig C, auf dem Keller U, McCormick S, Austin P, Overall CM, Adam MJ, Ruth TJ, Perrin DM. Toward [18F]-labeled aryltrifluoroborate radiotracers: in vivo positron emission tomography imaging of stable aryltrifluoroborate clearance in mice. J Am Chem Soc. 2008;130:12045-12055.

[58] Liu Z, Pourghiasian M, Radtke MA, Lau J, Pan J, Dias GM, Yapp D, Lin KS, Bénard F, Perrin DM. An organotrifluoroborate for broadly applicable one-step ^{18}F-labeling. Angew Chem Int Ed Engl. 2014;53:11876-11880.

[59] Liu Z, Radtke MA, Wong MQ, Lin KS, Yapp DT, Perrin DM. Dual mode fluorescent (18)F-PET tracers: efficient modular synthesis of rhodamine-[cRGD]$_2$-[(18)F]-organotrifluoroborate, rapid, and high yielding one-step (18)F-labeling at high specific activity, and correlated in vivo PET imaging and ex vivo fluorescence. Bioconjug Chem. 2014;25:1951-1962.

[60] Studenov AR, Adam MJ, Wilson JS, Ruth TJ. New radiolabelling chemistry: synthesis of phosphorus-[F-18]fluorine compounds. J Labelled Compd Rad. 2005;48: 497-500.

[61] McBride WJ, Sharkey RM, Karacay H, D'Souza CA, Rossi EA, Laverman P, Chang CH, Boerman OC, Goldenberg DM. A novel method of ^{18}F

radiolabeling for PET. J Nucl Med. 2009;50:991-998.

[62] Notni J, Šimeček J, Hermann P, Wester HJ. TRAP, a powerful and versatile framework for gallium-68 radiopharmaceuticals. Chemistry. 2011;17:14718-14722.

[63] Brom M, Joosten L, Oyen WJ, Gotthardt M, Boerman OC. Improved labelling of DTPA- and DOTA-conjugated peptides and antibodies with ^{111}In in HEPES and MES buffer. EJNMMI Res. 2012;2:4.

[64] Alberto R. New Organometallic Technetium Complexes for Radiopharmaceutical Imaging. Top Curr Chem. 2005;252:1-44.

[65] Abrams MJ, Juweid M, tenKate CI, Schwartz DA, Hauser MM, Gaul FE, Fuccello AJ, Rubin RH, Strauss HW, Fischman AJ. Technetium-99m-human polyclonal IgG radiolabeled via the hydrazino nicotinamide derivative for imaging focal sites of infection in rats. J Nucl Med. 1990;31:2022-2028.

[66] Greenland WE, Howland K, Hardy J, Fogelman I, Blower PJ. Solid-phase synthesis of peptide radiopharmaceuticals using Fmoc-N-epsilon-(hynic-Boc)-lysine, a technetium-binding amino acid: application to Tc-99m-labeled salmon calcitonin. J Med Chem. 2003;46:1751-1757.

[67] Schwartz DA, Abrams MJ, Hauser MM, Gaul FE, Larsen SK, Rauh D, Zubieta JA. Preparation of hydrazino-modified proteins and their use for the synthesis of [99mTc]-protein conjugates. Bioconjugate Chem. 1991;2:333-336.

[68] Rajopadhye M, Harris TD, Yu K, Glowacka D, Damphousse PR, Barrett JA, Heminway SJ, Edwards DS, Carroll TR. Synthesis, evaluation and Tc-99m complexation of a hydrazinonicotinyl conjugate of a gp IIb/IIIa antagonist cyclic peptide for the detection of deep vein thrombosis. Bioorg Med Chem Lett. 1997;7:955-960.

[69] Edwards DS, Liu S, Harris AR, Poirier MJ, Ewels BA. [99mTc]-labeling of hydrazones of a hydrazinonicotinamide conjugated cyclic peptide. Bioconjugate Chem. 1999;10:803-807.

[70] Harris TD, Sworin M, Williams N, Rajopadhye M, Damphousse PR, Glowacka D, Poirier MJ, Yu K. Synthesis of stable hydrazones of a hydrazinonicotinyl-modified peptide for the preparation of [99mTc]-labeled radiopharmaceuticals. Bioconjugate Chem. 1999;10:808-814.

[71] Surfraz MB, Biagini SC, Blower PJ. A technetium intermediate specifically promotes deprotection of trifluoroacetyl HYNIC during radiolabelling under mild conditions. Dalton Trans. 2008;22:2920-2922.

[72] Babich JW, Fischman AJ. Effect of "co-ligand" on the biodistribution of [99mTc]-labeled hydrazine nicotinic acid derivatized chemotactic peptides. Nucl Med Biol. 1995;22:25-30.

[73] Meszaros LK, Dose A, Biagini SCG, Blower PJ. Hydrazinonicotinic acid (HYNIC) -Coordination chemistry and applications in radiopharmaceutical chemistry. Inorg Chim Acta. 2010;363:1059-1069.