Novel [^{18}F]Fluorinated Prosthetic Groups for the Labelling of Peptides for Positron Emission Tomography (PET)

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A dissertation for the degree of Philosophiae Doctor

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“The most exciting phrase to hear in science, the one that heralds the most discoveries, is not "Eureka!" but "That's funny..."

Isaac Asimov

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Dag Erlend Olberg

CONTENTS

ABBREVIATIONS AND SYMBOLS

β^+	Positron
BOC	*tert*-butoxycarbonyl
EOS	End of Synthesis
HPLC	High Performance Chromatography
K222	4,7,13,16,21,24-Hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane
LG	Leaving group
MeCN	Acetonitrile
NMR	Nuclear Magnetic Resonance spectroscopy
nca	Non Carrier Added
PBS	Phosphate Buffer Saline
PTC	Phase Transfer Catalyst
p.i.	Post Injection
RT	Room Temperature
SA	Specific Activity
TBA	Tetrabutylammonium
t-BuOH	*tert*-Butanol
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography

LIST OF PUBLICATIONS

The present thesis is based on the following papers and manuscripts (I-IV) referred to in the text by Roman numerals. Included in the appendix are the supporting information of paper I, II and IV.*

I. Olberg, D.E., Hjelstuen, O.K., Solbakken, M., Arukwe, J., Karlsen, H., and Cuthbertson, A. A novel prosthetic group for site-selective labeling of peptides for positron emission tomography. *Bioconjugate Chem*. **2008**, *19,* 1301-1308.

II. Olberg, D.E., Hjelstuen, O.K., Solbakken, M., Arukwe, J.M., Dyrstad, K., and Cuthbertson, A. Site-specific addition of an ^{18}F-*N*-methylaminooxy-containing prosthetic group to a vinylsulfone modified peptide. *J. Labelled Compd. Radiopharm*. **2009**, DOI: 10.1002/Jlcr.1686 (Article online in advance of print)

III. Olberg, D.E., Cuthbertson, A., Solbakken, M., Arukwe, J.M., Kristian, A., Bruheim, S., Qu, H., and Hjelstuen O.K. Radiosynthesis, biodistribution and preliminary evaluation of a novel [^{18}F]fluorinated *N*-methylaminooxy conjugated to cyclic RGD peptides. Submitted *Eur. J. Nuc. Med. Mol. Imaging*.

IV. Olberg, D.E, Arukwe, J.M., Grace, D., Hjelstuen, O.K., Solbakken, M., Kindberg, G.M., and Cuthbertson A. 6-[^{18}F]Fluoronicotinic acid TFP-ester: A novel single-step prosthetic group for the labelling of biomolecules with fluorine-18. Submitted *J. Med Chem.*

ABSTRACT

Positron emission tomography is a non-invasive imaging modality allowing visualization and quantification of a wide variety of physiological and biochemical processes or of a specific low-density protein target. Some examples are blood flow, glucose consumption, fatty acid metabolism or detection and quantification of cell surface receptors in particular tissues. Within the spectrum of available positron emitters, fluorine-18 is a particularly attractive radionuclide due to its favourable nuclear and chemical properties. One prerequisite to performing an investigation with positron emission tomography (PET) is the availability of suitable radiopharmaceuticals. The selection, preparation, and preclinical evaluation of a new radiopharmaceutical are addressed in particular by the field of radiopharmaceutical chemistry. Currently, [^{18}F]FDG and to a lesser extent other small molecular weight compounds have become important clinical tracers for imaging of malignancies and other disease conditions. So far [^{18}F]FDG, [^{18}F]NaF and [^{18}F]fluorodopa are the only three PET radiopharmaceuticals for 18F-fluorine listed in the USP.

Peptides labelled with ^{18}F have emerged as promising target-specific imaging probes. To date, very few ^{18}F-labelled peptides have been subjected to human studies, compared to other small compound based 18-fluoride tracers. The explanation for this is to a large extent the complicated and low yielding synthesis of ^{18}F-peptides employed so far.

Peptides are labile molecules containing a multitude of functional groups that are not compatible with the conditions where [^{18}F]fluorine is introduced. Peptides are therefore in general labelled indirectly by means of ^{18}F-labeled prosthetic groups also called bifunctional labelling agents. Numerous ^{18}F-prosthetic groups have been described and utilised for labelling of peptides. The number of synthetic steps and the different chemistries for conjugation to the peptide are some of the important properties of a prosthetic group, and renders some of them unsuitable for labelling a wide range of peptides. Also, the synthesis time and ease of production plays an important role as PET involves rapidly decaying isotopes and radiation exposure to PET manufacturing professionals.

In this thesis, a new ^{18}F-prosthetic group based on the site-selective addition of the *N*-methylaminooxy to different sets of model peptides functionalised with Michael acceptors and alkyl halides have been investigated. Two ^{18}F-prosthetic groups were synthesised; one based on a butyl chain and the second with diethylene glycol unit, both modified with the *N*-methylaminooxy functionality and a tosyl group for the introduction of 18-fluorine.

Radiolabelling experiments showed that the diethylene glycol derivative was sufficiently stable, but not the butyl derivative. Both radioactive and non-radioactive experiments with peptides demonstrated that the ^{18}F-prosthetic group reacted in a site-selective manner, and that peptides modified with Michael acceptors such as nitrostyrene, maleimide and vinylsulfone gave better yields and more clean reactions as compared to the alkyl halides. Further investigations of the prosthetic group in conjunction with an RGD peptide modified with either a nitrostyrene or a vinylsulfone moiety *in vitro* and *in vivo* demonstrated that a biologically active peptide can be radiolabelled using this methodology. *In vitro* experiments and *in vivo* studies in osteosarcoma tumour bearing mice gave evidence for that the ^{18}F-*N*-methylaminooxy prosthetic group had good stability. The peptide conjugate bearing the vinylsulfone was found suitable for *in vivo* use, while the nitrostyrene analogue on the other hand was too labile. Finally, a nicotinic acid based system, with direct labelling of active esters was investigated. The 6-[^{18}F]fluoronicotinic-TFP ester proved to be a very suitable prosthetic group that allows labelling of peptides rapidly and in two steps. In conclusion, new and useful ^{18}F-prosthetic groups for labelling of peptides and biomolecules have been successfully developed for use in PET.

1. INTRODUCTION

Imaging techniques that are based on external localisation of administered radioactivity dose allow non-invasive and non-terminal *in vivo* distribution studies. Positron emission tomography (PET) and single photon emission computed tomography (SPECT) are such imaging techniques (Jones, 1996; Lammertsma, 2001; Mountz *et al.*, 2002). Tracing of the *in vivo* localisation of the labelled molecule over time provides information of uptake, distribution, excretion and residence time in tissue (Phelps, 2000). PET and SPECT therefore supply functional information in contrast to the non-radioactive modalities such as CT (X-ray computed tomography), MR (Magnetic resonance tomography) and ultrasound (US). Kinetic modelling obtained by PET or SPECT are difficult to provide by means of other modalities.

1.1 Positron emission tomography

Radionuclides used in PET decay by positron emission (β^+-decay). In the decaying nuclide, a proton is converted to a neutron emitting a positron β^+ simultaneously (McQuade *et al.*, 2005). The positron, being the counter particle to the electron, will almost instantaneously annihilate with an electron producing energy in the form of two gamma photons (511 keV each) as shown in equation 1. The two photons will travel in parallel but in opposite direction. The photons penetrating the tissue are detected by a PET-camera, gamma-detectors organised in a circular array around the body (Le Bars, 2006). Photons registered simultaneously by two opposite detectors within a few nanoseconds will be recorded as an event, and make up a line of response (LOR) shown in Figure 1 (Philip W. Miller *et al.*, 2008). Scattered photons that reach only one of the detectors will be rejected (Phelps *et al.*, 1975). When sufficient events are collected, data can be processed to give information on distribution and quantification of the regional concentration of the tracer.

(1) $${}^{A}_{Z}N_{N} \rightarrow {}^{A}_{Z\text{-}1}Y_{N+1} + \beta^{+}$$

(2) $$\beta^{+} + e^{-} \rightarrow 2\times\gamma \ (511\,\mathrm{keV})$$

Equation 1. *General representation of a positron decaying nuclide (1) and annihilation of positron and an electron (2).*

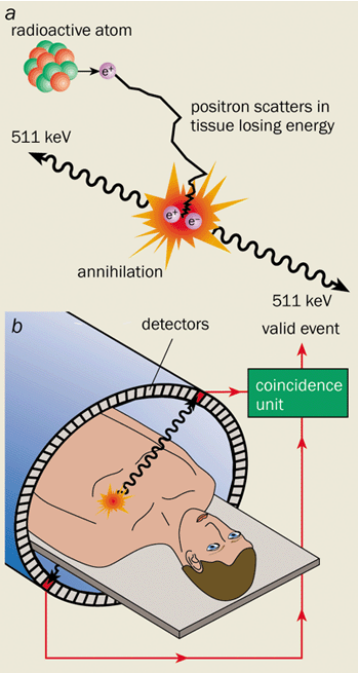


Figure 1. *Schematic presentation of the principle of PET with the positron emission and annihilation event on top (a) and detection by a PET detector surrounding the patient at the bottom (b). (Source physicsworld.com)*

The radionuclides normally used in PET are short lived and can be produced in cyclotrons (particle accelerators) by nuclear reactions by protons or deuterons with high kinetic energy (Papash & Alenitsky, 2008). The positron emitters ^{11}C, ^{13}N, ^{15}O and ^{18}F are some examples of frequently used radionuclides in PET (P. W. Miller, 2009). As can be deciphered from Table 1, the positron emitted from the different nuclides have maximum energies (E_{max}), implying that the positron will travel certain distances before annihilation. Thus, the theoretical imaging resolution obtainable will vary depending on the radionuclide in use.

Table 1*. Some physical properties of common positron emitters used in PET.*

Radionuclide	**Half-life (min)**	**E_{max} (MeV)**	**Max range in water (mm)**
^{18}F	109.8	0.64	2
^{11}C	20.4	0.97	4
^{13}N	9.96	1.20	5
^{15}O	2.04	1.74	8

The radionuclides ^{11}C, ^{13}N and ^{15}O are particularly useful as they can be incorporated into a molecule without altering the pharmacological properties of the original ligand (Gee, 2006; Palmer *et al.*, 1977). However, the application of these nuclides is restricted due to the short half-life, making extended synthesis and long imaging protocols impossible (Varagnolo *et al.*, 2000). In recent time, other PET-nuclides have been gaining increasing interest, in particular ^{68}Ga ($t_{1/2}$ = 68 min) that can be obtained from a generator system. However, requiring a chelator, the use of ^{68}Ga is restricted to biomolecules such as large peptides or proteins, antibodies, aptamers and others where a large modification of the biomolecule is tolerated without perturbing its pharmacological properties (Fani *et al.*, 2008).
The radionuclide used in this thesis is ^{18}F because of its relatively long half-life, widespread use and established production methods (Guillaume *et al.*, 1991).

1.2 Application of PET

The areas of medical PET applications are numerous. It is the most advanced technology currently available for studying *in vivo* molecular interactions in terms of distribution, pharmacokinetics and pharmacodynamics (Frederic Dolle *et al.*, 2008). The ability to measure changes in concentration of a radiolabelled pharmaceutical non-invasively over time in different organs, being healthy or pathological states, is indeed a powerful technique. Assessing parameters such as blood flow, metabolic energy consumption and receptor distribution and density are some of the areas often studied with PET. The field of oncology is possibly the medical discipline benefitting most from PET, particularly in routine clinical use (Mawlawi & Townsend, 2009).
Besides clinical advantages and basic science, PET is increasingly being used for drug development. The major cause for the failures of new drugs is inappropriate pharmakodynamics (Huisinga *et al.*, 2006).
Studies using PET on drug pharmakodynamics and -kinetics, can help speed up the process of drug development by sieving out unwanted candidates at an early time point (Cunningham *et al.*, 2005; Fowler *et al.*, 1999). The injected dose of a PET radiopharmaceutical in terms of mass is very small due to the high specific activity. The risk of the drug provoking any pharmacological or toxicological effects are minuscule and it is therefore possible and safe to perform human PET studies early in the screening process (Vaalburg *et al.*, 1999).

1.3 Characteristics and production of [^{18}F]fluorine

The report on the first production of fluorine-18 originates from 1936 and today more than 20 nuclear reactions are known as production pathways (Schubiger, 2007; Snell, 1937). The success of [^{18}F]fluorine as a PET radionuclide are due to several distinct properties and causes. Fluorine-18 can be produced in high yields, even with low energy cyclotrons (< 16 MeV) (Cai *et al.*, 2008). The half-life of 109.8 allows extended synthesis times and multi-step reactions along with extended PET studies of slow biochemical processes (Ferrieri, 2003). The radionuclide has a low β^+ -energy (0.64 MeV) which allows high resolution images and less radiation burden to patients (Kilbourn *et al.*, 1987). Also, fluorine-18 displays a simple decay for 97% positron emission and 3% electron capture (De Kleijn, 1977). Furthermore, a relatively long half-life makes transportation to off-site facilities feasible. Fluorine-18 can be produced in most cyclotrons by the ^{18}O(p,n)^{18}F reaction by bombardment of ^{18}O-enriched water with protons (Solin *et al.*, 1988). The fluorine-18 is by this method obtained as an aqueous solution in the form of nca [^{18}F]fluoride in high yields and high specific activity (typically above 100 GBq/µmol) (Elsinga, 2002). 18-Fluorine can also be produced as [^{18}F]F_2 from ^{20}Ne or ^{18}O gas targets. A major problem, however, is adsorption of fluorine-18 on the target walls, thus elemental fluorine is added to recover the product while lowering the SA of the 18-fluorine (Schubiger, 2007). Therefore, the method of choice for introduction 18F-fluoride into a molecule is in the form of nca [^{18}F]fluoride and hence [^{18}F]F_2 is only utilised in some reactions where high SA is not required or the chemistry omits the use of [^{18}F]fluoride (Fuechtner *et al.*, 2008). Only nca 18-fluoride was used in studies of this thesis.

1.4 Chemistry with the [^{18}F]fluoride ion

After bombardment of ^{18}O-enriched water, [^{18}F]fluoride is in an aqueous solution which makes it poorly reactive due to the high degree and strength of hydration (Clark, 1980). Therefore, the first step is to remove the major bulk of water. Commonly, [^{18}F]fluoride is adsorbed onto an ion exchange resin, allowing recovery of expensive ^{18}O-enriched water. The [^{18}F]fluoride is then eluted into a reaction vessel with an aqueous weak base followed by azeotropic removal of water with MeCN with addition of a suitable PTC during the process (Block *et al.*, 1987; Palmer *et al.*, 1977). The azeotropic step is repeated 2-3 times and ensures a high degree of “dry” fluoride. However, the truly “naked” fluoride ion is never obtained. The removal of each water molecule of hydration is successively more difficult, and hence trace [^{18}F]fluoride ion will be hydrated to at

least some extent by very small traces of residual water. Reduction in the degree of hydration will correspondingly increase nucleophilicity. In general, a robust and reproducible drying process is required to ensure [^{18}F]fluoride ion with adequate nucleophilicity for difficult reactions (e.g., aromatic nucleophilic substitution reactions), whereas a less strict drying regime may be tolerated for other reactions (e.g., aliphatic nucleophilic substitution reactions) (Cai *et al.*, 2008). After drying, the precursor dissolved in an organic aprotic solvent is added to the fluoride for reaction. Normally, the precursor should not be a source of protons themselves. Recently, reactions with nca 18F-fluoride in protic solvents have been reported (*t*-BuOH), in contrast to the classical thinking that all reactions with the fluoride ion must be conducted in aprotic solvents (Kim *et al.*, 2006). The two major reactions with the fluoride ion are the aliphatic nucleophilic substitution and nucleophilic aromatic substitution. For the introduction of 18-fluoride in to a molecule, "classical" leaving groups (LG) well-known from organic chemistry are regularly employed. A variety of sulfonate esters are used to convert primary and secondary alcohols to excellent LG (Bolton, 2002). Furthermore, halogens are also often used, in particular iodine, bromine and chloride. For aromatic substitutions the trimethylammonium group, nitro and chloride are attractive candidates as leaving groups and to a lesser extent bromine (Angelini *et al.*, 1985; F Dolle, 2005). Conveniently, the trimethylammonium precursors being charged have very different chromatographic properties than its fluorinated product and can offer a very straight forward purification of the reaction mixture on a solid-phase cartridge system (Haka *et al.*, 1989; Poethko *et al.*, 2004). It's worthy of note that homo-aromatic substitutions normally require at least one electron-withdrawing group to achieve good incorporation yields of fluoride, as electron-rich arenes are poorly activated (F Dolle, 2005). Heteroarenes, especially pyridines have also become attractive systems for introduction of fluoride in the last decade, and will be discussed more in depth in chapter 3.4.

One example of a tracer produced by a substitution reaction is [^{18}F]2-fluoro-2-deoxy-D-glucose (FDG) a highly utilised tracer to study glucose metabolism in a number of indications (Adam, 2002; Tewson, 1989). In this thesis the commercially available TracerLab (GE Healthcare) was used in all radiosyntheses and a standard two-cycle azeotropic drying step was used, giving for the major part reproducible results (see article I-IV for details).

1.5 ^{18}F-Prosthetic groups

Far from all molecules can tolerate the harsh conditions for the direct introduction of [^{18}F]fluoride. For small, simple organic molecules it may be sufficient by masking functional groups that may interfere with the fluoride labelling (Okarvi, 2001). Examples are the FDG precursor, mannose triflate, where the hydroxyl functions of the sugar are protected by means of acetyl esters (Hamacher *et al.*, 1986). Other examples are the BOC-protection of amines and trityl-protection of thiols for a large spectrum of small organic ^{18}F-tracers (De Bruin *et al.*, 2005; Glaser *et al.*, 2004). However, for more complex biomolecules such as peptides, proteins and antibodies containing a vast variety of functional groups and acidic protons, this approach is normally not feasible. There are some reports of direct incorporation of [^{18}F]fluoride into a peptide such as bombesin, but a broader application to a variety of peptides using this methodology and further *in vivo* studies are yet to be reported (Mu, 2009). Also, direct labelling of peptides with electrophilic carrier added ^{18}F-fluorination is shown unsuitable for use in receptor studies presumably due to a very low SA (32.8 GBq/mmol) (Ogawa *et al.*, 2003).
For these reasons, peptide and protein labelling with nca [^{18}F]fluoride is accomplished by means of prosthetic groups, also referred to as bifunctional labelling agents. In this indirect methodology, 18-fluoride is introduced into a functionalised compound (the prosthetic group) and coupled to the macromolecule of choice under mild conditions. Currently, a wide spectrum of ^{18}F-prosthetic groups utilising different sets of chemistries are available (Figure 2).

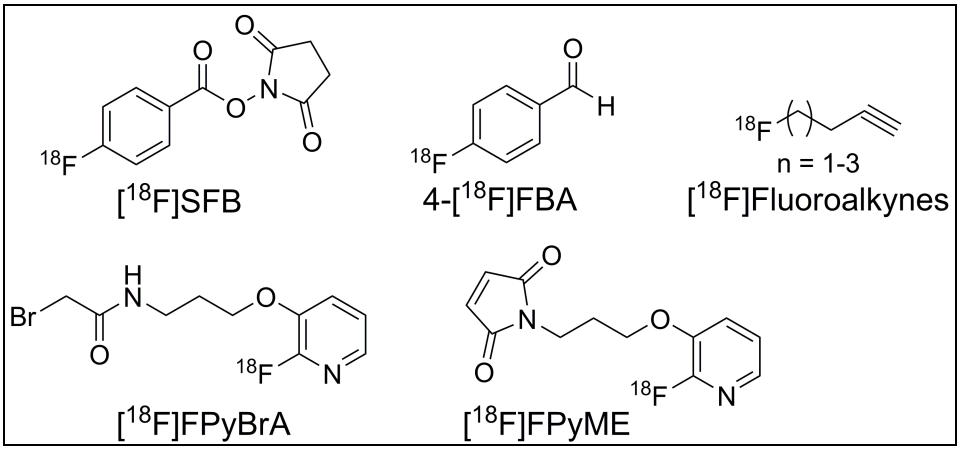


***Figure 2**. Examples of published [^{18}F]fluoride based prosthetic groups exploiting different chemistries. [^{18}F]SFB = N-succinimidyl 4-[^{18}F]fluorobenzoate, 4-[^{18}F]FBA = 4-[^{18}F]fluorobenzaldehyde, [^{18}F]FPyBrA = 2-bromo-N-[3-(2-[^{18}F]fluoropyridin-3-yloxy)propyl]acetamide, [^{18}F]FPyME =1-[3-(2-[^{18}F]fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione.*

Among the first approaches investigated for labelling of peptides was the formation of prosthetic groups based on active esters such as *N*-hydroxysuccinimide and 4-nitrophenyl (Vaidyanathan & Zalutsky, 1992). In particular, *N*-succinimidyl 4-[^{18}F]fluorobenzoate ([^{18}F]SFB) has been used frequently for labelling of peptides and antibodies with free amines in good yields (J. Li *et al.*, 2007; Marik & Sutcliffe, 2007; Neumaier *et al.*, 2008; Tang *et al.*, 2008). However, the synthesis of [^{18}F]SFB is quite tedious and requires 2-3 three steps (Wuest, Koehler *et al.*, 2009). Active esters are not well suited for labelling of macromolecules with more than one free and equally reactive amine groups, leading to potentially a multi-fluorinated peptide and loss of or reduced biological activity (Gill *et al.*, 2009; Thonon *et al.*, 2009). Alkylating prosthetic groups, such as [^{18}F]FPyBrA have been mostly been used to label oligonucleotides and some peptides (Kilbourn *et al.*, 1987; Koslowsky *et al.*, 2008; von Guggenberg *et al.*, 2009). At current, this approach has not gained much attention compared to other methodologies. Chemoselective prosthetic groups are desirable as they allow the use of unprotected peptide- and oligonucleotide-precursors and thus reduce the number of steps for the conjugation to one. This renders the synthesis less complicated and reduces the overall synthesis time (Bruus-Jensen *et al.*, 2006). The free thiol (or sulfhydryl) function is present only in cysteine residues and is not very common in most peptides and proteins. Thiol-reactive agents have therefore been used to modify peptides and proteins at specific sites, providing a means of high regioselectivity in contrast to the carboxylate- and amine-reactive reagents (Berndt *et al.*, 2007). One example is [^{18}F]FPyME, which have been used to radiolabel peptides and proteins in good yields (De Bruin *et al.*, 2005). However, all methods based on sulfhydryl-reactive prosthetic groups involve multi-step preparations (two to four) of the prosthetic group, making them inappropriate for routine synthesis in a clinical setting. In view of the fluorination of peptides described above, there is still need of radiolabelling strategies for faster and simpler production of ^{18}F-peptide tracers. Besides direct labelling which is so far not possible, a 1+1-step labelling method is the best approach. Such an approach involves a one-step fluorination of the prosthetic group which can be conjugated directly to an unprotected peptide in a site-selective manner under mild conditions. Prosthetic groups fulfilling this requirement are e.g. the chemoselective formation of an oxime or hydrazone bound between 4-[^{18}F]fluorobenzaldehyde (4-[^{18}F]FBA) and a peptide functionalised with an aminooxy- or a hydrazino group (Bruus-Jensen *et al.*, 2006; Poethko *et al.*, 2004; Schottelius *et al.*, 2004). This approach has been shown to produce ^{18}F-peptides in up to 40% overall yield and an RGD-peptide based on this methodology is currently under clinical development (Morrison *et al.*, 2009). The aminooxy being highly reactive towards any aldehyde or ketone have the drawback that it needs

careful handling in its unprotected form to avoid side-reactions. Recently, also selective oxime formation between aminooxy-modified peptides and the open aldehyde form of ^{18}F-FDG have been demonstrated, expanding the scope of the aminooxy functionality (Namavari *et al.*, 2009; Wuest, Hultsch *et al.*, 2009). However, major drawbacks are high temperatures and the excess glucose impurities in a clinical FDG formulation which led to disappointing conjugation yields. Yet another promising approach is the reaction of terminal alkynes with azides catalysed with copper(I), the ´click´ reaction or 1,3-dipolar cycloadditon (Glaser & Aarstad, 2007; Marik & Sutcliffe, 2006; Vaidyanathan *et al.*, 2009). The merits of the reaction are in particular high chemoselectivity, regiospecificity, excellent yields as well as mild reaction conditions (Glaser & Robins, 2009). This approach also offer a 1+1-step labelling and certainly show promise for labelling of peptides with ^{18}F although the use of copper as catalyst may be an issue for pharmaceutical productions.

1.6 ^{18}F-Peptides as tracers

Peptides are composed of relative simple components, the amino acids. In contrast to proteins they generally do not possess a well-defined three-dimensional (tertiary) structure and are much smaller in size (up to 10.000 Da) (Blok *et al.*, 1999). Also essential is the difference in production methods, small peptides can easily be synthesised chemically whereas proteins and antibodies often have to be derived from a biological source such as DNA-recombinant techniques. Using e.g. solid-phase based Fmoc-chemistry relatively large peptides (20-30 amino acids) can be synthesised in a short time (Indrevoll *et al.*, 2006). Compared to antibodies and proteins, small peptides distribute more uniformly and penetrate tissues more readily (de Jong *et al.*, 2004). Furthermore, peptides are generally excreted rapidly from the systemic circulation which is an important characteristic of a useful tracer for establishing a target to non-target signal (Tweedle, 2009). However, too rapid metabolism and excretion can result in a peptide tracer that cannot accumulate on the target site. Peptides with a short plasma half-life can often be modified with unnatural amino acids or other chemical modification e.g. amidation of the C-terminal giving them more resistance to enzymatic destruction. Lipophilic peptides tend to show higher hepatobiliary excretion as opposed to hydrophilic peptides that often have predominate renal clearance (Lundqvist & Tolmachev, 2002). These are properties that in most cases can be modified with pharmacokinetic modifiers (PKMs) without perturbing the biological activity of the peptide (Haubner & Decristoforo, 2009). Because of the lack of a tertiary structure, small peptides are less susceptible to loss of integrity through labelling conditions and are less

immunogenic than proteins. However, in some cases the binding affinity for the target can be reduced due to the lack of a well-defined tertiary structure as compared to the native peptide or the intact antibody. Still peptides offer the advantage to be ‘‘as large as necessary, and as small as possible” (Schottelius & Wester, 2009).

As peptides have become increasingly used as agents for therapeutic applications, ^{18}F-peptide tracers may be used for bridging imaging with therapeutic approaches (Edwards *et al.*, 1999; Schubiger, 2007). Amongst the ^{18}F-radiolabelled peptides for PET being extensively studied in recent times are the RGD peptides for $\alpha_v\beta_3$ integrin imaging and octreotide analouges for detection of somatostatin receptors, both for visualisation of solid tumours (Dijkgraaf *et al.*, 2009; H. J. Wester *et al.*, 2003).

1.7 Automation and PET

Conventional manual methods for the synthesis of radiopharmaceuticals using high levels of radioactivity would certainly subject the personnel performing the synthesis to high radiation exposure. Routine manual high activity synthesis would soon lead to unacceptable accumulation of absorbed dose and conflict with the ALARA principle (As low as reasonably achievable) (Sharma *et al.*, 2006). In particular for the PET isotopes such as ^{11}C, ^{13}N, ^{15}O and ^{18}F the amount of radioactivity at start of synthesis requires high levels due to the fast decaying nuclides to give sufficient radiolabelled product for PET scans. For a synthesis of ^{18}F-FDG, it is not uncommon to produce 300 GBq of [^{18}F]fluoride, which gives around 200 GBq of ^{18}F-FDG (Fawdry, 2007). To reduce the exposure burden to personnel, automatic modules have been designed. These modules are placed into so-called hot cells, thick lead compartments shielding the personnel from radiation. Typically the modules are controlled by a remote computer with pre-programmed software, allowing the technologist to operate them. Currently, most commercial modules are intended for ^{18}F-FDG and other relatively simple PET tracers. For the more complicated production of ^{18}F-peptides with a two-step synthesis and final HPLC-purification, no dedicated module is available for routine production. In this thesis the TracerLab FxFN was used for radiolabelling. Although flexible in its nature, fully-automated synthesis of ^{18}F-peptides is difficult to achieve using this module (Speranza *et al.*, 2009). In recent years, modules allowing multi-step synthesis are becoming increasingly available, at least for academic research, allowing automated production of more complex tracers. As it was for ^{18}F-FDG, to further move ^{18}F-peptides from academia to the clinic, the future lies in automation.

2. AIMS AND SCOPE

There are many reports of the application with the vast varieties of ^{18}F-prostetic groups for radiolabelling of peptides and biomolecules. However, they all have their limitations and advantages (Wuest *et al.*, 2008). One prosthetic group is not likely to be suitable for the labelling of the whole diversity of biomolecules in use. A further extension of the ^{18}F-prosthetic group "tool-box" in PET is still warranted.

The overall aim of this thesis was to develop new prosthetic groups for labelling of peptide and other biomolecules with nca [^{18}F]fluorine for PET applications. The PET-radionuclide fluorine-18 was used because of its relatively long half-life, high yielding production method and favourable nuclide characteristics. On the basis of the reported site-selective properties of the *N*-methylaminooxy functionality under mild acidic conditions, it was decided to use this chemistry as the basis of development of new prosthetic groups. Furthermore, during the time period of the work, pyridine systems caught our interest and led to the investigation of the possibility of direct labelling of active esters as more rapid and simpler synthesis of these types of ^{18}F-prosthetic groups. Important consideration parameters for the prosthetic groups in this thesis are; labelling yield, simple synthesis, amenability to automation and site-selectivity. The applicability of the synthesised prosthetic groups in conjunction with relevant peptides *in vitro* and *in vivo* was assessed.

The study is divided into the following milestones:

- Synthesis of a prosthetic group with the *N*-methylaminooxy functionality (paper I)
- Find a suitable functionality covalently linked to a peptide that reacts with the ^{18}F-*N*-methylaminooxy-prosthetic group in a site-selective manner in a time frame acceptable for PET-radiopharmaceutical production (paper I and II)
- Establish a radiosynthesis and purification process (paper I and II)
- Synthesis of a prosthetic group allowing for the direct labelling of active esters (paper III)
- Study the stability of the radiolabelled conjugates in relevant solutions using a biological active peptide (paper III-IV)
- Study *in vitro* and *in vivo* properties and conduct biodistribution studies with the radiolabelled conjugates (paper III-IV)

3. RESULTS AND DISCUSSION

The vast majority of ^{18}F-peptide tracers currently under investigation are aimed at tumour imaging. Examples are $\alpha_v\beta_3$ specific ligands for detection of neovascularisation, somatostatin analogues, VIP (vasoactive intestinal peptide) and bombesin (Cheng *et al.*, 2007; Schottelius *et al.*, 2009; Zhang *et al.*, 2006).

Despite the promising characteristics for peptides labelled with ^{18}F, such as high receptor affinity, rapid excretion and a vast choice of target receptors, these types of ^{18}F-tracers have not yet achieved a clinical breakthrough (H.-J. Wester *et al.*, 2004). Reasons for this may be the success of ^{18}F-FDG as an efficient tracer for detecting and staging tumour, ability to monitor therapy response along with its high yielding automated production process. However, ^{18}F-FDG has its shortcomings and complementary tracers are needed. For example, in endocrine tumours radiolabelled peptides have shown to be superior to ^{18}F-FDG (Gotthardt *et al.*, 2006). In order to make ^{18}F-peptides more attractive for clinical use, their production methods need to be simplified and give higher yields. In this thesis, two approaches have been investigated for this purpose. The first is based on the site-selective addition of an ^{18}F-*N*-methylaminooxy prosthetic group to unprotected peptides, offering the possibility for labelling of complex unprotected peptides with ^{18}F. The second, though lacking the chemoselectivity, is an approach for the synthesis of active ester ^{18}F-prosthetic groups directly in one-step, allowing a less complicated synthesis of the ^{18}F-peptides suitable for use with this type of ^{18}F-bifunctional labelling agent.

Each of the papers (I-IV) and supporting information can be referred to for more elaborate information for synthesis, structures, methods and analytical procedures.

3.1 PAPER I

3.1.1 The *N*-methylaminooxy functionality

N-alkylaminooxy containing amino acids have proved useful for the post-modification of unprotected peptides with reducing sugars, alkylating agents and active esters (Bark *et al.*, 2000; Carrasco & Brown, 2003; Carrasco *et al.*, 2002; Carrasco *et al.*, 2006). This is attributed to the fact that they remain unprotonated and nucleophilic in acidic aqueous solutions (pH 4-5), where other functional groups in peptides are unreactive (except for cysteine). In contrast to the more well-known aminooxy functionality already used for labelling peptides site-specifically with ^{18}F

through oxime-formation, the *N*-alkylaminooxy is unreactive towards aldehydes and ketones although they share the property of being nucelophilic in mild acidic aqueous solutions. Furthermore, by virtue of being extremely reactive towards all aldehydes and ketones, the free aminooxy-group must be handled carefully and the group may require a Boc-protective group during prolonged storage (Hultsch *et al.*, 2009).
In light of this, it was of interest to investigate if this chemistry could be applied for the chemoselective labelling of peptides with ^{18}F.

3.1.2 Synthesis of the ^{18}F-N-metylaminooxy prosthetic groups

Starting from 4-bromo-1-butanol the precursor **1** was synthesised in four steps in 3% overall yield and good purity (>98%). The tosyl leaving group was used as it is relative stable and gives a good UV signal around 250 nm. The nitrogen atom was BOC-protected (Figure 3).

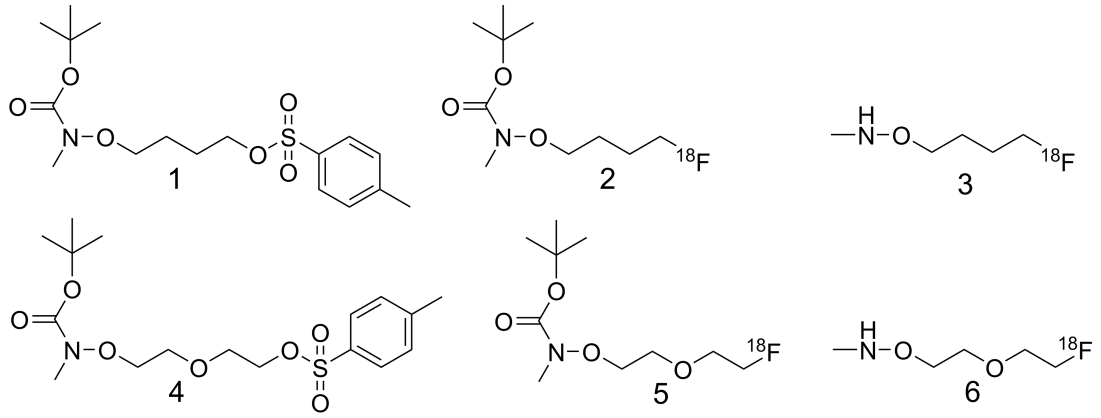


***Figure 3**. Structure of the precursor synthesised, the fluorinated intermediates and the reactive ^{18}F-prosthetic groups.*

Reaction of precursor **1** with Kryptofix 222/KF (2 eq.) in acetonitrile at 70°C gave the desired fluorinated compound **2** in over 50% yield, further treatment with TFA cleaved the Boc-group quantitatively giving the final reactive species **3.** Further radioactive experiments with precursor **1** gave good incorporation yields with ^{18}F in acetonitrile using Kryptofix 222 as PTC. However, after removal of the Boc-group, incubation of **3** in acetate buffer pH 5 at 70°C proved to produce a radioactive side-product eluting in the void volume as analysed by radio-HPLC. This peak was attributed to the release of [^{18}F]fluoride, formed through the cyclisation of the prosthetic group into a favourable six-membered ring. These findings were somewhat surprising as fluorine is

regarded as a poor leaving group in aliphatic substitution reactions. Still, the strong nucleophilic properties of the *N*-methylaminooxy and the favourable six-membered ring conformation proved sufficient to displace fluoride. As a consequence, further investigations with **3** were not conducted. To circumvent this problem, the precursor **4** was synthesised starting from a diethylene glycol. In a similar manner as **1**, precursor **4** was synthesised in 19 % overall yield based on diethylene glycol. Fluorination with Kryptofix 222/KF (2 eq.) in acetonitrile at 70°C gave the desired fluorinated compound **5** in over 50% yield, further treatment with TFA cleaved off the Boc-group quantitatively giving the final reactive species *O*-(2-(2-[^{18}F]fluoroethoxy)ethyl)-*N*-methylhydroxylamine (**6)**. Radiolabelling of precursor **4** with ^{18}F in acetonitrile using Kryptofix 222 gave routinely 60-80% yields and in contrast to **3** the reactive species **6** was not prone to cyclisation in acetate buffer.

3.1.3 Reaction of the ^{18}F-N-metylaminooxy prosthetic groups with peptides

Next the strategy was to screen for reactive electrophiles that could be functionalised to a peptide forming a covalent bond to the prosthetic group, preferably with rapid conjugation kinetics. Amongst the groups screened were allylic, benzylic, α-carbonyl bromides and chlorides. Also investigated were maleimide, different sets of acrylates, nitrostyrene and a vinylsulfonamide. The reactive groups were linked to the N-terminal of the model peptide (Lys-Gly-Phe-Gly-Lys) as shown in Figure 4 and reactions with **6** were conducted in acetate buffer pH 5. The reaction mixtures were analysed by LC-MS. As predominately one product was expected from these reactions, additional peaks would indicate side-reactions from two free ε-amines and carboxyl presented in the peptide.

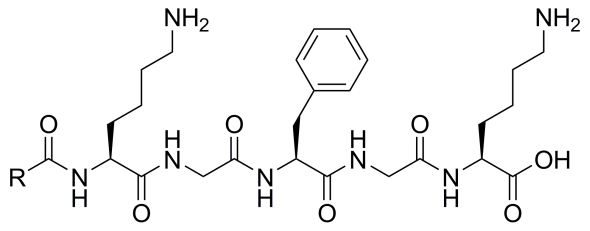


Figure 4*. Model peptide used in the study (R= alkyl halide or Michael acceptor)*

From these investigations it was found that the alkyl halides reacted sluggishly along with less activated acrylates. The vinylsulfonamide gave a clean reaction product, but the reaction was rather slow. More promising were the maleimide and 4-(2-nitrovinyl)benzoyl-functionalized peptides giving clean reactions with one predominant product and relatively rapid kinetics. In particular the nitrostyrene proved to react rapidly, being converted to the desired conjugate in more than 90% yield in less than 10 min at 30°C, in accordance with previous reports (O'Neil *et al.*, 2001). The maleimide required heating at 70°C for 1h to achieve acceptable yields.
As a result of these findings, nitrostyrene and maleimide were selected for full radiochemical assessment.

3.1.4 Radiosynthesis and pre-purification of the prosthetic group before conjugation

The use of acetonitrile and K222/K_2CO_3 proved to give good incorporation yields (60-80%) of ^{18}F into the precursor as analysed by radio-TLC. Considering acetonitrile's favourable properties such as low boiling point and low viscosity, it was concluded that there was no need to investigate additional solvents. A study of reactions times proved that the reaction reached a plateau within 5 min of reaction time, and further heating did not improve yields as shown in Figure 5. The incorporation yields found were comparable with other prosthetic groups using tosyl as leaving group (Glaser & Robins, 2009; Z.-B. Li *et al.*, 2007).

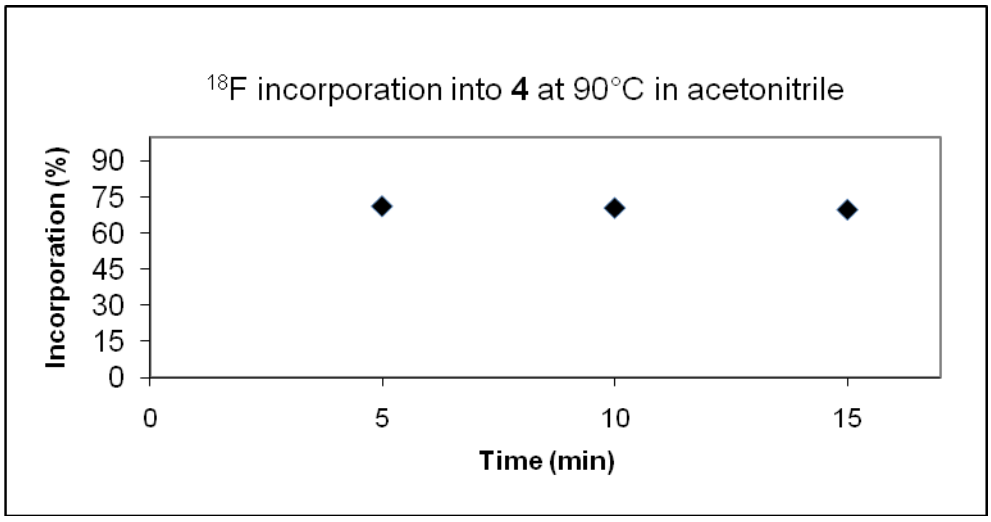


Figure 5. *Incorporation yields of [^{18}F]fluoride with precursor **4** (5 mg, 13µmol)in MeCN at 70 °C as function of time as analysed with radio-TLC (mean of three experiments for each time point*).

A time-consuming and cumbersome HPLC purification step of the prosthetic group should be avoided. Most of the precursor **4** hydrolysed during radiolabelling to the corresponding alcohol that was more hydrophilic than the ^{18}F-labelled compound. Therefore, investigation with non-radioactive compounds using C18 and Oasis HLB Sep-Paks were conducted to see if this approach could be used to separate the alcohol from the fluorinated compound avoiding an HPLC step. This extensive hydrolysis could be explained by additional displacement of the tosylate by the oxygen in position 6 of the precursor (Mcmanus *et al.*, 1990). In comparison, the alkyl precursor **1** did not display the same degree of hydrolysis. It turned out that the Oasis HLB cartridge, based on a poly(divinylbenzene-co-N-vinylpyrrolidone) polymer was most effective. Using 50 ml of 25-30% methanol in water the alcohol impurity could be eluted off the cartridge while retaining the fluorinated compound. A large volume of 50 ml had to be used as the alcohol eluted off the Sep-Pak in a broad band. Furthermore, the fluorinated compound could be eluted off in good purity with 1.5 ml acetonitrile. Applying this system in the radiochemistry process, the major bulk of produced alcohol was removed. After eluting off the ^{18}F-Boc-protected prosthetic group **5**, the protective group and organic solvents were removed by addition of 0.2 ml 2 M HCl in diethyl ether and subsequent heating at 65°C under a stream of nitrogen gas and simultaneously applying vacuum. The total time of this procedure from start of synthesis was 40-45 min. Figure 6 illustrates the recoveries of labelled ^{18}F-product starting from 5 mg (13 µmol) precursor. As can been observed some radioactivity was lost during the evaporation step and was a step difficult to reproduce. However, over 50% (decay corrected) of the starting amount of 18-fluoride was present in form of [^{18}F]**6** after deprotection and evaporation. For the conjugation experiments 3 mg (8 µmol) of precursor was used ensuring minimal carry-over of alcohol. This reduced the yield of [^{18}F]**6** to ≈ 40%.

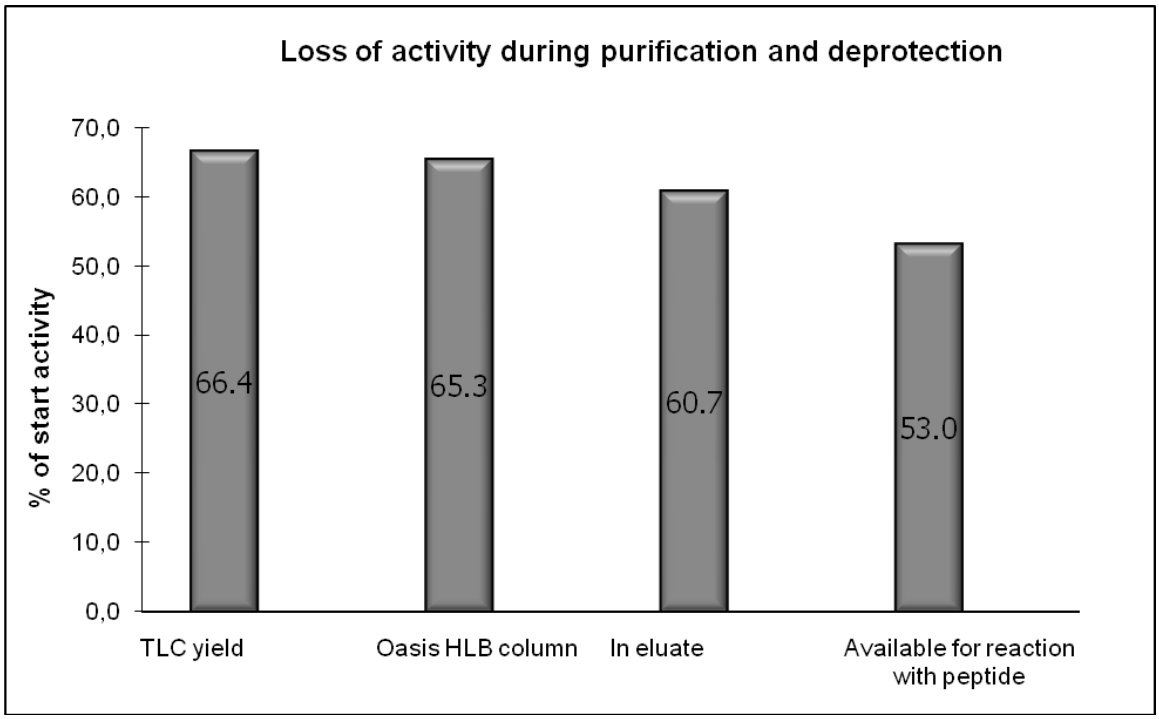


***Figure 6**. Example of typical radioactivity transfers in the synthesis of **6**. TLC yield is incorporation yield analysed by radio-TLC of the crude reaction mixture. Oasis HLB column was activity remaining on column after treatment of 50 ml 25% aqueous methanol. In eluate is activity recovered from Oasis Sep-Pak after elution with 1.5 ml MeCN and last column represents activity remaining after Boc-deprotection and evaporation. The three columns on the right are decay corrected back to start of synthesis.*

3.1.5 Conjugation of the ^{18}F-prosthetic group to model peptides

In analogy with the non-radioactive experiments, conjugation of the prosthetic group [^{18}F]**6** to the two functionalised peptides were done in acetate buffer pH 5 (0.4 M). Using a relatively concentrated buffer ensured that some acid remaining from the evaporation step could be tolerated without pH in the buffer solution dropping to the point where the *N*-methylaminnoxy functionality became protonated and unreactive. A relatively high concentration of 5 mg (8 µmol) peptide in 0.8 ml buffer was routinely used. For the maleimide functionalised peptide incorporation yields of 64-80% of [^{18}F]**6** was achieved within 60 min reaction time at 70 °C. For the nitrostyrene functionalised peptide, incorporation yields in the range 80-89% could be obtained after only 5 min at 30 °C. Both of the ^{18}F-labelled peptides could be purified using semi-preparative HPLC yielding the products in high RCP (>99%) and in 9-12 % yield (non-corrected) based on [^{18}F]fluoride. In particular, the nitrostyrene modified peptide was very well suited with

its fast reaction kinetics. Yields are comparable with other frequently used methodologies for labelling peptides with ^{18}F (Schubiger, 2007).
It was later discovered that the nitrostyrene/*N*-methylaminooxy conjugate did not have the required stability for *in vivo* studies due to instability at physiological pH. This was not observed initially as ^{18}F-peptides were purified with an acidic mobile phase containing 0.1 % TFA, where the conjugate was stable. In these initial studies, SA was not measured. But importantly, radioactive products could be efficiently removed from non-radioactive impurities using a HPLC column.

3.2 PAPER II

3.2.1 Site-specific addition to vinylsulfone modified peptide

During the screening studies the sulfonamide moiety displayed a very clean reaction with the *N*-methylaminooxy group yielding only one product. However the reaction kinetics were sluggish even at 70 °C. It was expected that a vinylsulfone group bearing a carbon atom adjacent to the sulfone as opposed to a nitrogen atom would be more reactive as Michael acceptors (Reddick *et al.*, 2003). This would perhaps increase the reaction kinetics but still maintain the favourable properties of the reaction, such as a very clean conversion to the conjugate. With the same model peptide modified with vinylsulfonyl acetic acid reaction with the non-radioactive prosthetic group **6**, more than 90% of the peptide was converted to the conjugate within 60 min at 70 °C with few side products (Figure 7).

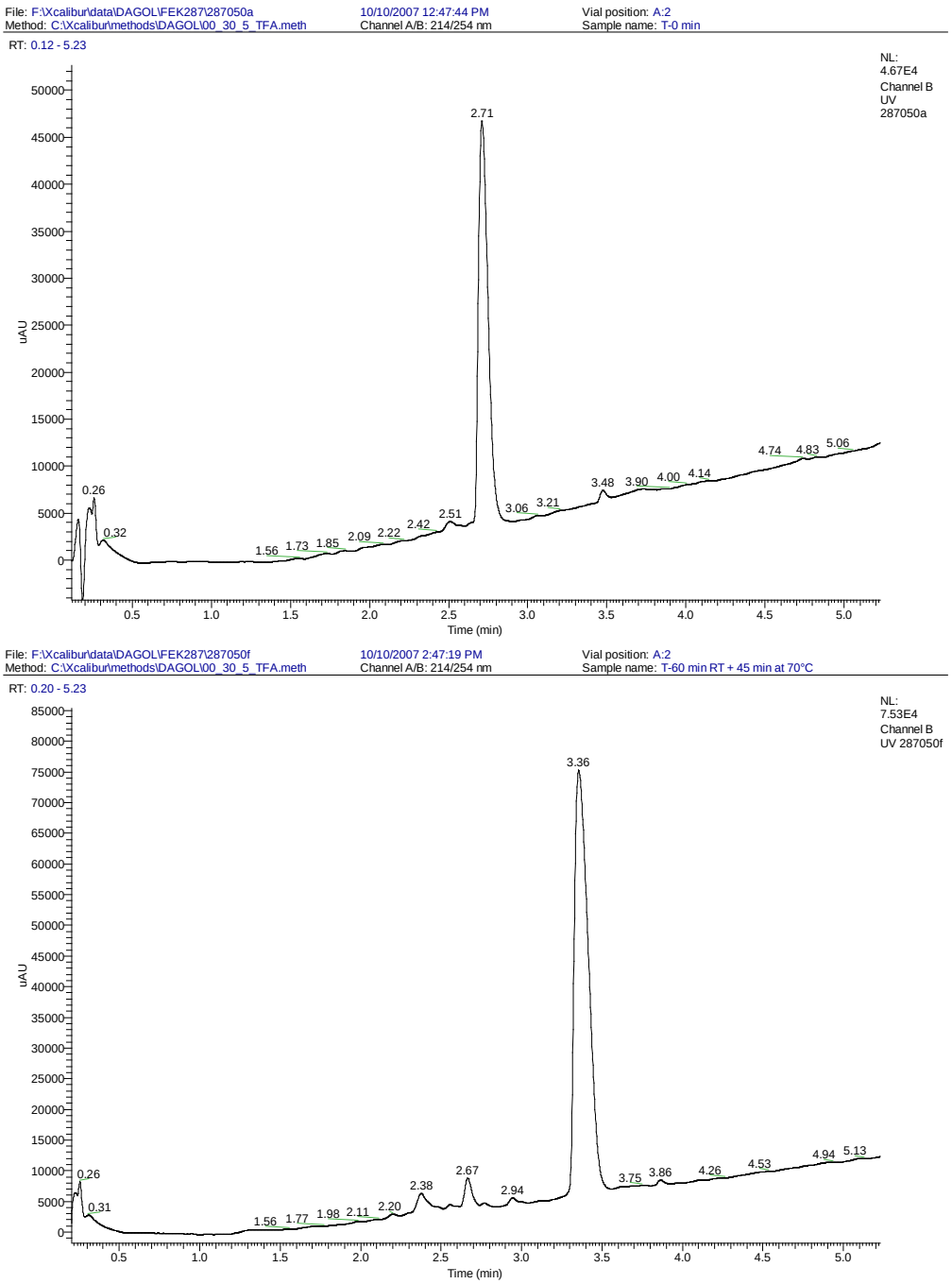


Figure 7. *Reaction if the non-radioactive N-methylaminooxy prosthetic group with vinylsulfone modified model peptide in acetate buffer pH 5 at 70 °C Above chromatogram: Start of reaction. The major peak is starting peptide. Lower chromatogram: Above reaction after 60 min. The major peak is conjugate.*

In the following radioactive experiments with this system, conjugation yield dependence on peptide concentration and purification method of the prosthetic group were studied in more detail. The reaction of the prosthetic group with the vinylsulfonyl modified peptide is shown in Figure 8.

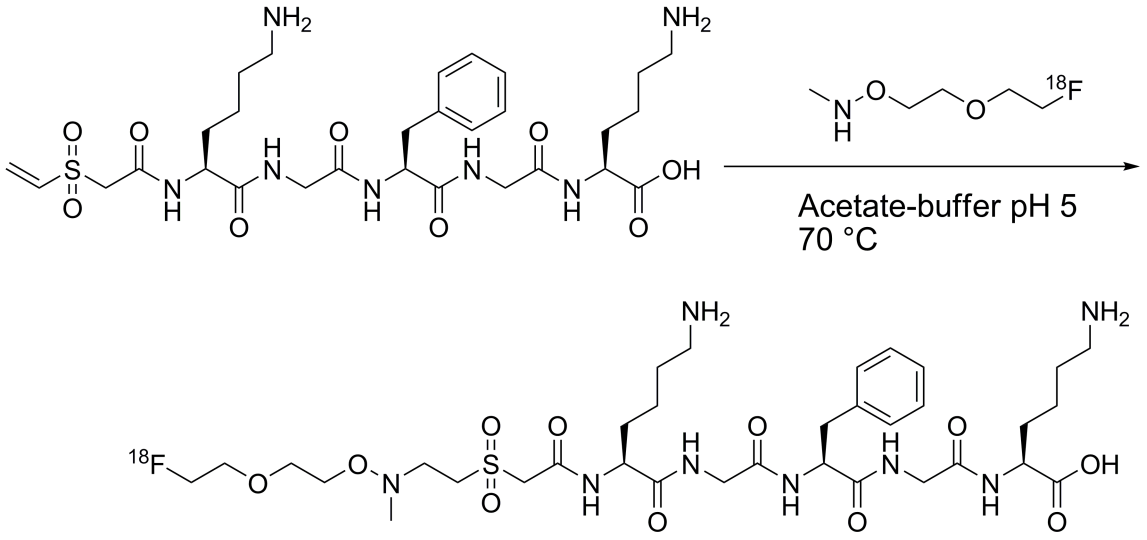


Figure 8. *Reaction of [^{18}F]**6** with the vinysulfonyl modified model peptide.*

The Sep-Pak based purification step was compared with a HPLC purification step where all side-products had been removed prior to conjugation giving a "non-carrier" labelled prosthetic group. In a chemometric design study the effect of the radioactivity level was investigated in conjunction with reaction time and peptide concentration. As typical for reaction of ^{18}F-prosthetic groups with peptides, the conjugation yield was highly dependent on peptide concentration. Moreover, the results showed that HPLC purified prosthetic group gave only marginally better incorporation yields of the ^{18}F-*N*-methylaminooxy prosthetic group as compared with the Sep-Pak approach, 84% and 76%, respectively, using 7.5 mM peptide after 70 min. When 3 and 0.75 mM peptide were used, yields were reduced to 40 and 15 percent, respectively.

This proved that the Sep-Pak approach indeed was capable of removing the major bulk of hydrolysed precursor. Spiking the HPLC purified reaction mixture with known amounts of alcohol by-product showed to a have a pronounced effect on conjugation yields, dropping to around 50 % with addition of 1µmol of alcohol. Higher concentrations of alcohol reduced the yield further. Varying the amount of [^{18}F]fluoride 10-fold had no effect on the percent incorporation yields and is expected as peptide concentration is several magnitudes of orders higher than [^{18}F]fluoride, and the reaction follows a pseudo-first order kinetics (Philip W. Miller *et al.*, 2008; Rengan *et al.*, 1993). Interestingly, the sulfonyl conjugate showed a shorter retention time on a reversed phase HPLC column compared with the maleimide and nitrostyrene conjugates from paper I, which can be beneficial in terms of route of elimination for a tracer. More hydrophilic peptide tracers are known to show predominately renal clearance as opposed to

more hepatobiliary clearance for more hydrophobic tracers (Ogawa *et al.*, 2003). A further improvement implemented in this part of the work was eluting and removal of the Boc-protected ^{18}F-labelled prosthetic group trapped on the Oasis Sep-Pak. Using a mixture of 1 ml dichloromethane (or acetonitrile) with 0.5 ml 2 M HCl in diethyl ether the labelled product was eluted back to the reaction vessel where evaporation and Boc-cleavage was effected. This procedure saved space in the Tracerlab FxFn system used and brought the set-up one step closer to automation, see Figure 9 for overview of the process.

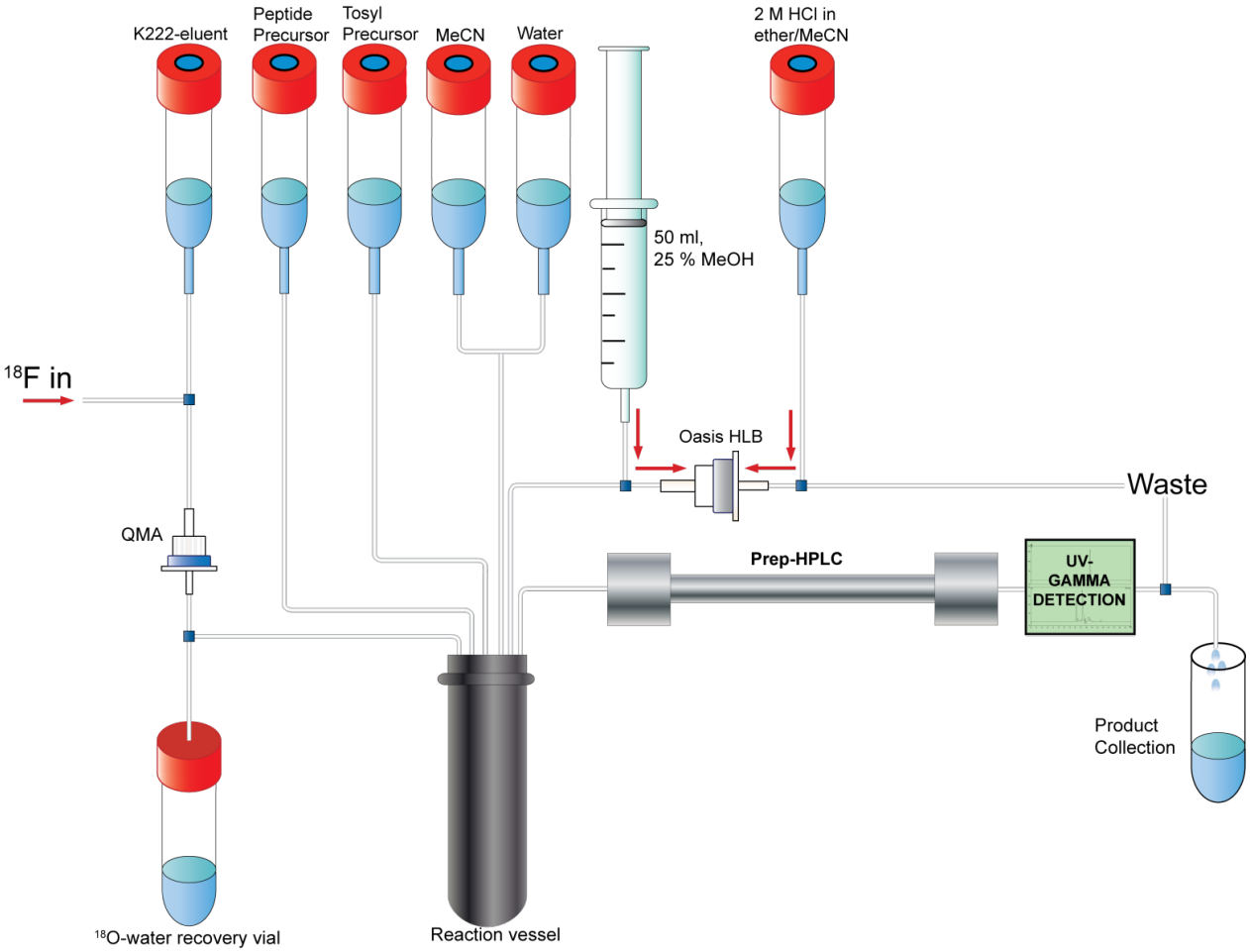


***Figure 9**. Schematic presentation of the radiosynhesis process for the ^{18}F-peptide in paper II.*

3.3 PAPER III

3.3.1 Synthesis of cyclic RGD peptides

In order to study the *in vivo* applicability of the new ^{18}F-*N*-methylaminooxy prosthetic group ([^{18}F]**6**), an RGD peptide was used as model and studied in tumour xenograft bearing mice. The cyclicRGD peptide (NC100717) bearing a free lysine was first modified with two cysteic acids moieties ensuring good solubility of the peptide precursor in the relatively concentrated acetate buffer (0.4 M) used in the conjugation reaction. The cysteic acid moieties would also function as pharmacokinetics modifiers being unprotonated under physiological conditions, giving the peptide a very hydrophilic character that favoured renal excretion (Harris *et al.*, 2006). Furthermore, the peptide was functionalised with 4-[(*E*)-2-Nitrovinyl]benzoic acid or 3-vinylsulfonylpropionic acid as Michael type acceptors for reaction with [^{18}F]**6** as shown in Figure 10.

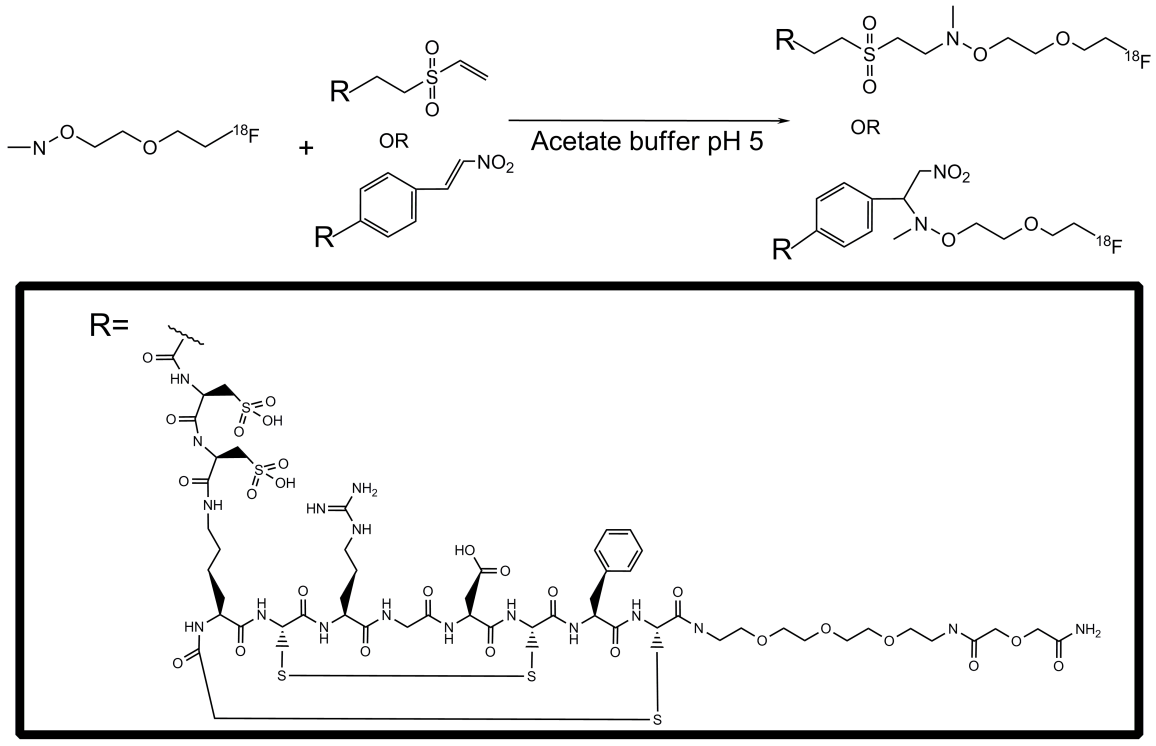


***Figure 10**. Structure of the RGD peptide precursor and conjugation product with [^{18}F]**6**.*

3.3.2 Integrin receptor-binding affinity

The affinity for the $\alpha_v\beta_3$ integrin of unlabelled derivatives was determined via competitive binding assay with ^{125}I-echistatin. Binding of ^{125}I-echistatin to $\alpha_v\beta_3$ was competed by the two conjugates in a concentration-dependent manner. The K_i values were 0.8 nM for the nitrostyrene conjugate and 3.0 nM for the vinylsulfone. The low K_i affinities suggest that the modification of the peptide NC100717 had minimal effect on the receptor binding. The affinity for the nitrostyrene conjugate should be interpreted with care, since it was shown to be very labile at physiological pH.

3.3.3 Radiosynthesis, log P and *in vitro* stability

In the radiosynthesis process, 3 mg (8 µmol) of tosyl precursor and 2 mg (1.2 µmol) peptide precursor were used with the same Sep-Pak purification protocol as described above. Due to the fast kinetics of the nitrostyrene, 15 min at 40°C was the conjugation conditions for this system. The vinylsulfone was less reactive and required 1 h at 70°C to achieve sufficient yields to allow for biodistribution and microPET studies. Overall yields were 2-7% after formulation (decay corrected) with 2 h synthesis time for the nitrostyrene conjugate and 165 min for the vinysulfonyl conjugate, respectively. Non-radioactive reference compounds were used to generate a standard curve using HPLC and from this the SA was calculated after radio-HPLC analysis of the formulated ^{18}F-conjugates. The specific activities for the preparations used in the biodistribution studies were in the range 40-50 GBq/µmol. The SA found is not in the high range compared to other methodologies, but still in a range acceptable for tumour imaging (Schirrmacher *et al.*, 2006). Furthermore, a fully automated radiosynthesis process would allow a faster production and the use of higher quantities of starting activity leading to higher SA. The starting quantities of [^{18}F]fluoride used in the radiosynthesis for the biodistribution studies were 3-5 GBq.
Stability of the two ^{18}F-labelled RDG peptides was studied in mouse plasma. After formulation the ^{18}F-productes were incubated at 37 °C over a period of two hours and aliquots were collected at 30 min, 1h and 2 h and analyzed by radio-HPLC. The nitrostyrene was highly unstable in the mouse plasma and also at physiological pH. A likely reason for this is the elevated acidity of the proton on the carbon adjacent to the nitro group, that becomes increasingly unprotonated at higher pH and hence the nitrogen of the *N*-methylaminooxy gets pushed out by the negative charge (Kresge, 1974).

3.3.4 MicroPET and biodistribution studies

Dynamic microPET studies with this labile nitrostyrene conjugate was tested in three mice under the hypothesis that the conjugate would show increased stability in tumours, as they are known to be more acidic than surrounding tissue. These studies resulted in very poor images with no visual detection of tumour and further studies with the conjugate were not conducted.
The vinylsulfone conjugate proved to be very stabile in the plasma with hardly any degradation detectable after 2 h. As the nitrostyrene proved unstable in mice plasma, log P experiments were conducted only for the vinylsulfone peptide. The octanol/water partition coefficient (log P) was measured to be -2.61±0.01, demonstrating a hydrophilic character.
Initially, biodistribution and microPET was conducted in mice under anaesthesia (isoflurane) from time of injection to time of sacrifice. This protocol proved to give slow excretion rates resulting in very poor contrast and images, demonstrating the effect anaesthesia can have on tracer kinetics (Fueger *et al.*, 2006). Under anaesthesia, nude mice are also more prone to hypothermia slowing down the circulation. As a result of these observations mice were injected with tracer without anaesthesia and allowed to remain so until sacrifice or microPET. The tumour model used was an osteosarcoma (OHS) derived from humane tissue (Fodstad *et al.*, 1986). After injection of approximately 1 MBq of tracer into the tail vein, the mice were sacrificed after 5 min and 120 min (n= 3-4). In a similar experiment, mice were also co-injected with non radiolabelled cyclic RGD peptide (10 mg/kg) and sacrificed 120 min p.i. The organs were wet-weighted and counted in an automatic gamma counter from which the percent id/g in organs could be derived. In parallel with these studies, mice were injected with 4-7 MBq. Static PET images were acquired for 15 minutes in mice under isoflurane anaesthesia 105 min post injection. As above this was also performed with co-administration of 10 mg/kg cyclic RGD peptide.
Biodistribution studies show initial high uptake of the ^{18}F-vinylsulfonyl labelled peptide in kidneys (14 % ID/g), liver (6.5 % ID/g) and lungs (6 % ID/g) 5 min p.i. which all decreased with time. Predominantly renal clearance resulted in low muscle and blood values 120 min post injection, 0.68 and 0.18 % ID/g, respectively. The initial activity accumulation in the osteosarcoma mass was between 6.5 and 4 % ID/g 5 min p.i., decreasing to about 3.5% ID/g 120 min p.i. At 120 min p.i., most organs showed lower activity uptake than tumour. Liver, gut and kidneys revealed a similar activity concentration as the tumour. Low activity accumulation in the bone suggested little or no defluorination *in vivo*. Altogether, this translated into high tumour to background ratios [e.g., tumour:blood, 19.0 tumour:muscle: 5.0]. Co-injection of the osteosarcoma bearing mice with 10 mg/kg of the $\alpha_v\beta_3$-selective peptide NC100717 reduced the

tumor:blood ratio at 120 min p.i. from 19 to 5 and the tumour:muscle ratio from 5 to 3 suggesting that the uptake in tumour is $\alpha_v\beta_3$ mediated. In comparison with other studies of ^{18}F-labelled RGD peptides in mice bearing OHS xenografts, such as ^{18}F-galacto-RGD, considered a "gold standard" amongst ^{18}F-RGD tracers (Schottelius *et al.*, 2009), the ^{18}F-vinylsulfonyl demonstrated very similar tumour to organ ratios as ^{18}F-galacto-RGD shown in Figure 11. Radioactivities in the blood were slightly higher after 120 min with the vinylsulfonyl-RGD compared to ^{18}F-galacto-RGD (0.18 vs. 0.13) and may explain to some degree the higher activity levels in blood rich organs. This may be attributed to the lower log P of ^{18}F-galacto-RGD than the ^{18}F-peptide studied in this thesis, - 3.17 and – 2.61, respectively (Haubner *et al.*, 2004).

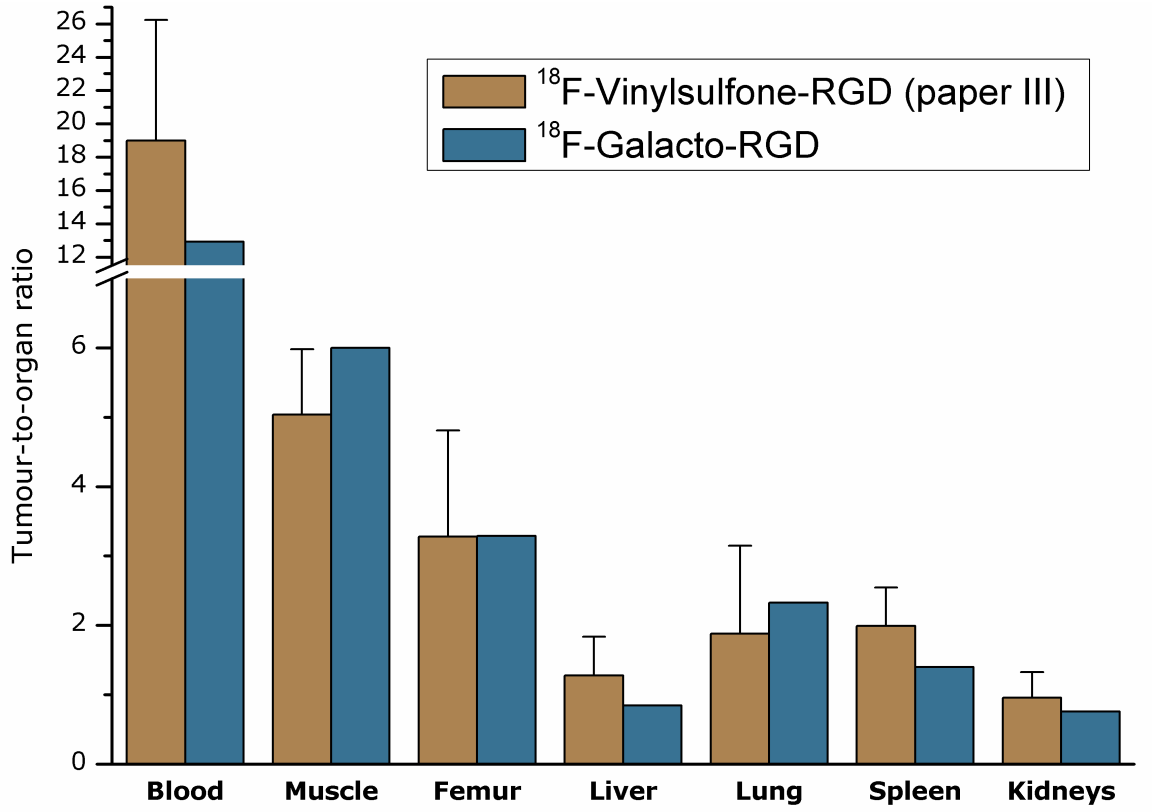


Figure 11. *Comparison of the tumour to organ ratios between the 18F-vinylsulfone-RGD studied in this thesis and 18F-galacto-RGD 120 min p.i. The ^{18}F-galacto-RGD data are also derived from OHS bearing mice (Haubner et al., 2001).*

The image (Figure 12) of an OHS bearing mouse obtained by the animal scanner demonstrates the high tumour to background ratio found in the biodistribution studies and allowed clear visualisation of the tumour. In contrast, the same experiment using a mouse with co-injected

cyclic RGD peptide showed no increased uptake in tumour compared to background. These experiments show that the prosthetic group [^{18}F]**6** could be useful for labelling of peptides functionalised with a vinylsulfone moiety with ^{18}F for use in PET. The modification of the RGD peptide with the cysteic acids may also be beneficial as PKMs for other ^{18}F-labelled peptides as it increases the hydrophilicity and leads predominate renal clearance (Blok *et al.*, 1999).

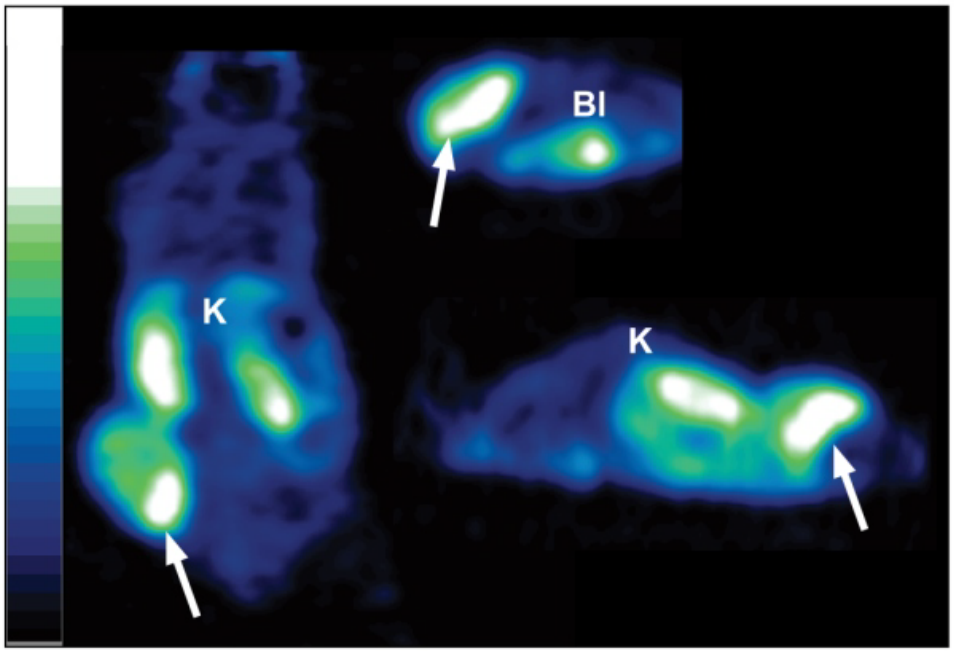


Figure 12*. Coronal (left), transaxial (upper right) and sagittal (lower right) microPET images (15 min static single frame) beginning after 105 min of a mouse bearing a s.c. human OHS tumour injected with 5.2 MBq ^{18}F-peptide. K = kidneys Bl = bladder. White arrow indicate tumour.*

3.4 PAPER IV

3.4.1 Active esters in PET

^{18}F-labelled active esters is a widely used and efficient method for the incorporation of 18-fluorine into peptides and proteins. The synthesis of this type of ^{18}F-prosthetic groups normally require two to three steps and often a HPLC purification step prior to conjugation, making the overall synthesis of the ^{18}F-labelled peptides challenging to automate. Attempts to label active esters directly with ^{18}F have so far resulted in poor yields, probably due to the harsh conditions

needed for the introduction of [^{18}F]fluoride resulting in side-reactions and degradation of the ester (Johannsen *et al.*, 1999; Lang & Eckelman, 1994).

3.4.2 [^{18}F]Fluropyridines

Pyridine systems have been shown to be very susceptible for nucleophilic aromatic substitution with ^{18}F in 2- and 4-positions with respect to the nitrogen in the pyridine system (Dolci *et al.*, 1999). These two positions are in particular activated due to the inductive and mesomeric withdrawal of electrons by the nitrogen, stabilising the negatively charged intermediate in a nucelophilic attack (Schubiger, 2007). For the 3-position the intermediate is not stabilised to the same extent, and for practical purposes substitution at this position can be considered not to occur. Due to this, fluorination of the 2- and 4-position has been the main focus of investigations the recent years (Abrahim *et al.*, 2006; Inkster *et al.*, 2008). Furthermore, compared to homoaromatic substitutions, nucelophilic substitution at the 2- and 4-positon of the pyridines do not require an additional electron-withdrawing demonstrating the elevated of pyridine systems (Frederic Dolle *et al.*, 2008). From investigations with 6-chloronicotinic acid ethyl ester with ^{18}F/K222 complex in acetonitrile at 80 °C for 40 min that showed high incorporation yields (≈90%) as analysed by radio-HPLC. It would be of interest to see if an active ester applied to a similar system could be useful for direct labelling with ^{18}F.

3.4.3 Synthesis of precursors and the 6-[^{18}F]fluronicotinic acid active esters

Two precursors were attempted synthesised from 6-chloronictinic acid, both being active esters. The first step was esterification with *N*-hydroxysuccinimide (NHS) or tetraflurorphenol (TFP), both obtained in good yields. The trimethylammonium is an excellent leaving group in nucleophilic aromatic substitutions, and it was desirable to substitute the chloride with this group. Treating the 6-chloronicotinic acid esters in THF with trimethylamine expelled the chloride in a nucleophile aromatic substitution reaction giving the desired trimethylammonium precursor. In this step, the NHS-ester underwent extensive decomposition resulting in poor yields. As this was not the case for the TFP ester, further studies was only conducted with the TFP-precursor. As the trimethylammonium precursor was obtained as chloride salt and was poorly soluble in acetonitrile, it was reacted with trimetylsilyl triflate thus obtaining the precursor as the triflate

salt. Structure of the precursor **7** and is fluorinated product **8** (^{18}F-Py-TFP) are shown in Figure 13.

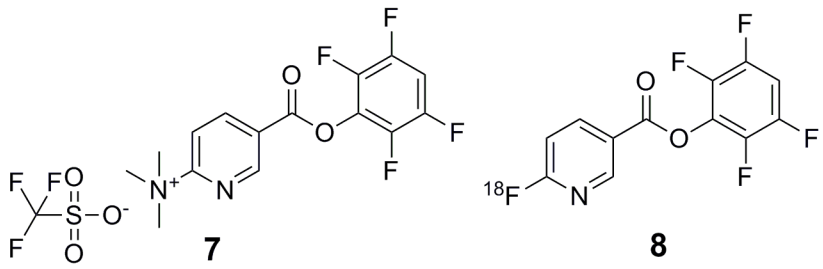
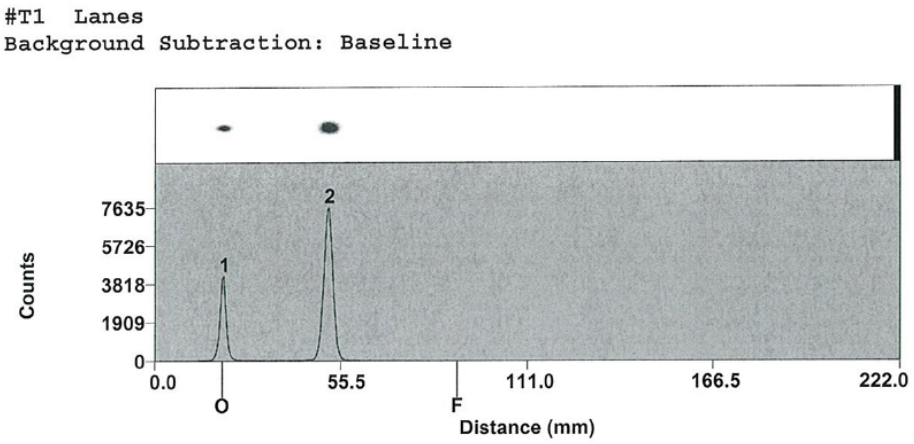


***Figure 13**. Structure of precursor **7** and the ^{18}F-fluorinated prosthetic group **8** (^{18}F-Py-TFP)*

Non radioactive labelling experiments of the precursor **7** with KF/K222 in acetonitrile proved to give the fluorinated compound **8** in good yields still retaining the intact ester as analysed by NMR and LC-MS. Conjugation experiments with the fluorinated active ester **8** in phosphate buffer pH 9 with a RGD peptide bearing a free lysine further demonstrated good acylating properties. The conjugate was formed in over 90% yield in less than 30 min at RT as analysed by LC-MS. Radiolabelling with [^{18}F] of precursor **7** gave the target compound **8** in acetonitrile using K^{18}F/K222 complex at room temperature, but in low yields. Further experiments with $KHCO_3$ and K222 improved yields (60-70 % from radio-TLC) but gave low recovery of ^{18}F from the reaction vessel. The best conditions were found to be TBA-HCO_3 as PTC in *t*-BuOH/acetonitrile (8:2) at 40°C for 10 min. This gave incorporation yields of 66.3±5.1 % as analysed with radio-TLC (n =4). A radio-TLC of the crude reaction mixture using these conditions is shown in Figure 14.



ID	Gross Counts	Baseline Subtract	Net Counts	Net CPM	Net % Sum Regions	Net % Total	Lane Rf	Label
Lane #1	Origin=20.00 Front=90.00							
1-1	19.112	0	19.112	63.706,66	27.7	27,1	0.007	
1-2	49.939	0	49.939	166.463,33	72.3	70,9	0.457	
Lane	70.435	0	70.435	234.783,33		100,0		
Unres	1.384	0	1.384	4.613,33		2,0		

***Figure 14**. Radio-TLC of the crude reaction mixture using TBA-HCO_3 in t-BuOH/MeCN at 40°C for 10 min. Peak 1 is [^{18}F]fluoride, 2 is[^{18}F]**8** (Ethyl acetate/n-hexane 1:1).*

3.4.4 Purification and peptide labelling

After radiolabelling the 18F-fluorinated compound **8** could be purified on Oasis MCX Sep-Pak before conjugation to the peptide. Experiments with the Sep-Pak purified [^{18}F]**8** with an RGD peptide (NC100717) in phosphate buffer pH 9/DMSO/MeCN in different concentrations demonstrated the significance of peptide concentration on incorporation yields. Using 0.5 mg (0.4 µmol in 1 ml) peptide over >90% conversion was achieved after 30 min at 40 °C, with 2 mg (2.4 µmol) over 95% conversion was achieved after 10 min as analysed by radio-HPLC. Structure of peptide and conjugate are shown in Figure 15.

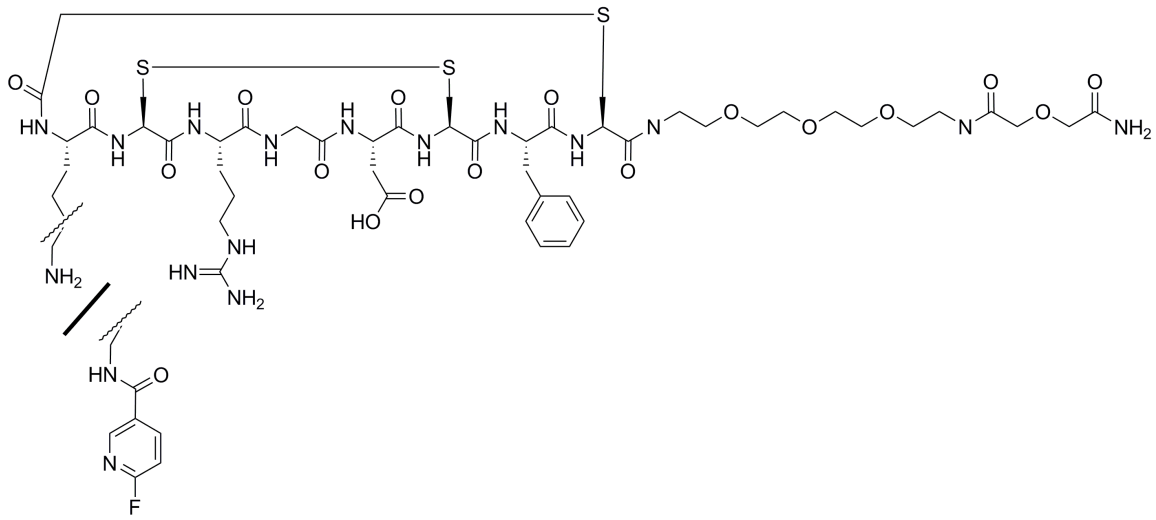


***Figure 15**. Structure of the RGD peptide NC100717 and the conjugate formed with **8**.*

In order to study the above process in a scenario more similar to a large scale production, a semi-automated synthesis was established. With 2 mg of the above peptide and Sep-Pak purification of the ^{18}F-labelled prosthetic group **8** isolated yields of 22±6 % (decay corrected) within 90 min could be achieved for the ^{18}F-peptide conjugate. Radiochemical purity was >99% with the radiolabelled product easily separable from unreacted peptide and other impurities with reverse phased HPLC.

This study demonstrates that direct labelling of an active ester system with [^{18}F]fluoride is feasible in good yields. The TFP esters are known to be more resistant to hydrolysis than other esters such as pentafluorophenyl (PFP) and NHS, still being very reactive comparable with other activated esters. Moreover, the highly activated pyridines system allowed for reaction with [^{18}F]fluoride at room temperature. This approach might serve as an alternative to [^{18}F]SFB, commonly used for labelling of peptides and macromolecules. The rapid one-step synthesis and Sep-Pak purification of [^{18}F]**8** are properties that should render this system suitable for automation.

4. MAIN CONCLUSION

The following conclusions are supported by the present project:

- The *N*-methylaminooxy is suitable as functional group for the site-specific conjugation to unprotected peptides decorated with alkyl halides and Michael type acceptors in mild acidic aqueous environment.
- ^{18}F-prosthetic groups based on the *N*-methylaminooxy can be produced in good yields with nca [^{18}F]fluoride and be conjugated to unprotected peptides in moderate yield
- ^{18}F- *N*-methylaminooxy prosthetic group conjugated to a vinysulfone modified RGD peptide could be produced in sufficient yields and acceptable specific activity for imaging of $\alpha_v\beta_3$ expression in xenograft bearing mice using microPET.
- ^{18}F- *N*-methylaminooxy prosthetic group displayed little or no defluoridation *in vivo*.
- Direct labelling of active esters with nca [^{18}F]fluoride in good yields is feasible using a highly activated pyridine system.

5. FUTHER PERSPECTIVES

A new methodology for labelling of peptides site-selectively with [^{18}F]fluoride has been described in this project. Although the *N*-methylaminooxy prosthetic group is capable of site-selective ligation with suitably modified peptides, such as vinylsulfone, the reaction kinetics are slow compared with the half-life of ^{18}F-fluorine. To render it a truly attractive choice, other Michael acceptors allowing faster reactions should be sought, allowing faster kinetics and use of less peptide precursor. Another alternative could be to investigate catalyst for the reaction.
The *in vivo* studies conducted with the ^{18}F-*N*-methylaminooxy-RGD conjugate indicated little or no defluoridation *in vivo*, analysis of blood, liver, urine and faeces should be conducted to assess the *in vivo* stability further.
An attractive methodology for the labelling of peptides with 18F-fluorine is through ^{18}F-bifunctional labelling agents based on acylation with activated carboxylic acid. In most instances this is done with prosthetic groups such as [^{18}F]SFB. [^{18}F]SFB requires 2-3 steps for its synthesis and frequently a HPLC step prior to conjugation to peptides. The ^{18}F-Py-TFP prosthetic group allows for a simpler peptide labelling process with respect to [^{18}F]SFB, and seems as an attractive

alternative. To elucidate its potential for use *in vivo* studies further investigations are a prerequisite. *S*tudies with [^{18}F]fluoropyridines have not to our knowledge indicated *in vivo* instability with these systems .

For both of the above prosthetic groups, further implementation in to a fully automated system would be interesting for studies with higher radioactive levels.

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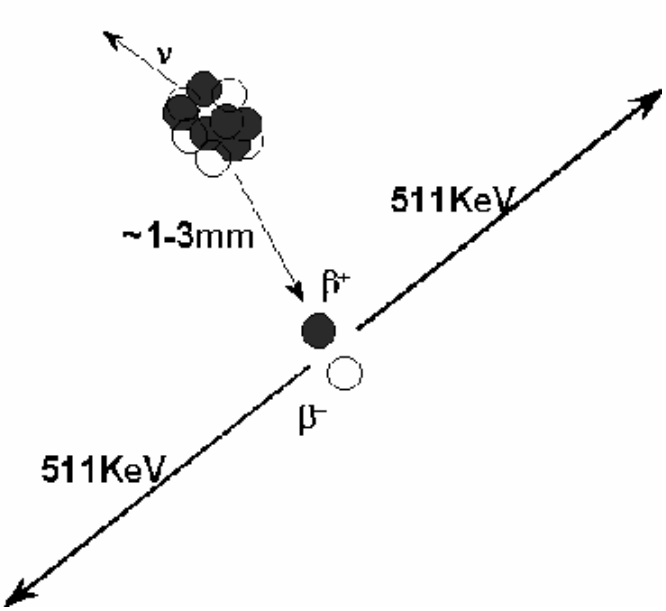

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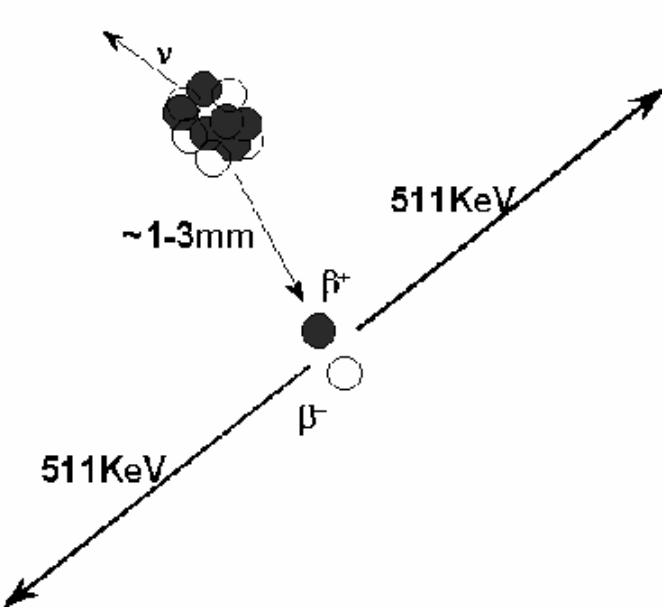

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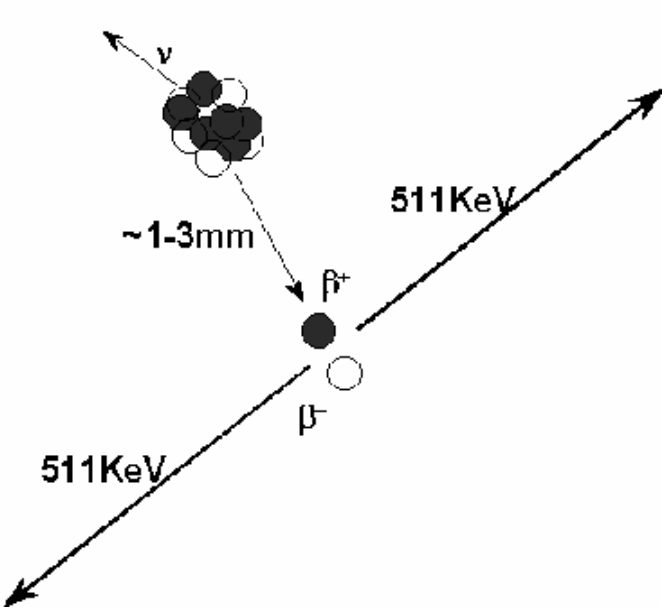

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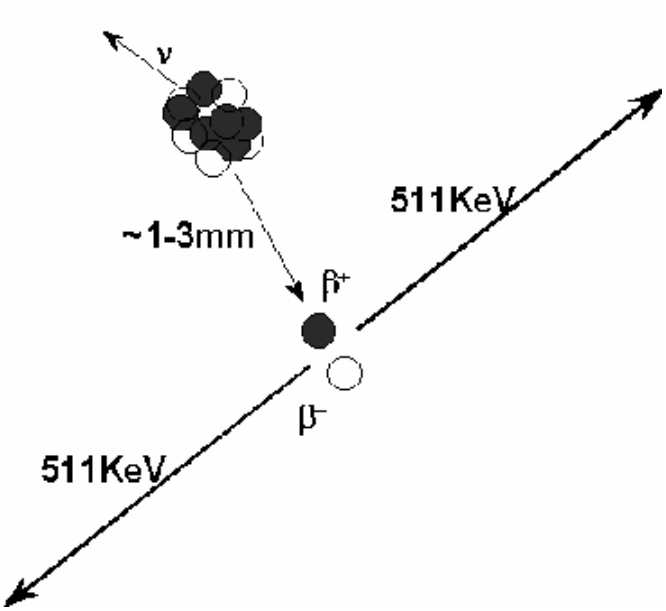

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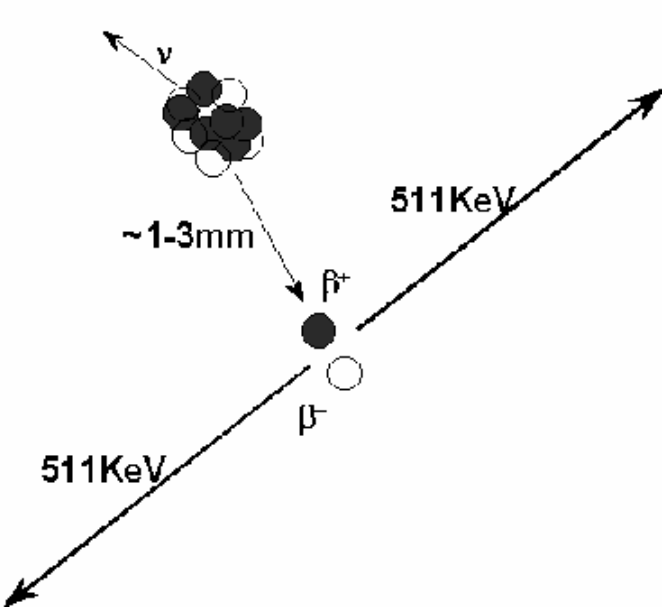

Appendix