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Design and Challenges of Radiopharmaceuticals

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This review describes general concepts with regard to radiopharmaceuticals for diagnostic or therapeutic applications that help to understand the specific challenges encountered during the design, (radio)synthesis, in vitro and in vivo evaluation and clinical translation of novel radiopharmaceuticals. The design of a radiopharmaceutical requires upfront decisions with regard to combining a suitable vector molecule with an appropriate radionuclide, considering the type and location of the molecular target, the desired application, and the time constraints imposed by the relatively short half-life of radionuclides. Well-designed in vitro and in vivo experiments allow nonclinical validation of radiotracers. Ultimately, in combination with a limited toxicology package, the radiotracer becomes a radiopharmaceutical for clinical evaluation, produced in compliance with regulatory requirements for medicines for intravenous (IV) injection.

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Radiopharmaceutical: General Concept

As the name suggests, a radiopharmaceutical is both a pharmaceutical and radioactive. The radioactive label is used either diagnostically as an emitter of electromagnetic radiation (gamma or X-rays), of which detection allows to quantify the concentration of the radiopharmaceutical. Alternatively, the radiolabel can be used for therapeutic applications where the ionizing radiation emitted upon decay of the radionuclide is used to destroy cells. The in vivo distribution of the radionuclide in the whole body, or in specific parts of the body, can be determined by detection of gamma and/or X-rays using a single photon emission computed tomography (SPECT) or positron emission tomography (PET) camera. The contrast of the image will depend on the difference in concentration of the radionuclide in the target tissue relative to that in the surrounding tissue. Static imaging provides an image of the distribution of the radionuclide at a specific timepoint after tracer injection, usually when an optimal target/background ratio is obtained. Dynamic imaging acquires the concentration of the radionuclide as a function of time postinjection and can be combined with arterial sampling and quantification of radiometabolites for exact quantification of pharmacodynamic parameters such as maximum amount of receptors (B_{max}) and dissociation constant (K_D).[1]

The emitted radiation may also be detected intraoperatively using a small detector, for example, for sentinel node identification or localization of occult neoplastic lesions in combination with surgery[2] or isolated limb or lung perfusion leakage monitoring.[3,4] Alternatively, the radiation emitted by the radionuclide can be quantified ex vivo in body fluids (eg, blood or feces using a gamma counter).[5,6]

In analogy with the development of new molecular entities that require druggable targets,[7] the design of novel radiopharmaceuticals requires a "traceable" target. However, not all druggable targets are traceable, for instance, if no molecules with sufficiently high affinity for a target can be identified. Vice-versa traceable targets may not be druggable, as binding of the molecular entity to its target does not necessarily produce a downstream effect. This is the case for molecular entities binding to tau and amyloid.

Further, the traceable target should have sufficiently high differential expression in the target tissue vs the background tissue combined with sufficiently high absolute expression (B_{max}). Moreover, the expression of the target should be related with the (patho)physiological function that one wants to be imaged.

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The structural diversity of small molecules is limited to the "chemical space" that is estimated to 10^{60} for molecules with a molecular mass up to 500 Daltons.[8] The radiochemical space can be interpreted as the collection of different chemical structures in which a radionuclide can be chemically incorporated within a synthesis time compatible with the radionuclide's half-life. The radiopharmaceutical space represents only a small fraction of the radiochemical space, as it is limited to radiochemical compounds that establish in vivo molecular interactions resulting in high contrast images (diagnostic radiopharmaceuticals) or selective irradiation (radionuclide therapy) of the targeted tissue. Besides the requirement of affinity for its target, the radiopharmaceutical also needs efficient access (eg, blood-brain barrier [BBB] penetration for central nervous system (CNS) radiopharmaceuticals and cell membrane penetration for intracellular targets) and clearance form nontarget tissues. The chemical structure of radiopharmaceuticals can often be derived from biochemicals and xenobiotics. Small molecules generally tolerate small chemical modifications for example to allow introduction of a fluorine or iodine atom for labeling with fluorine-18 or radioiodine. Derivatization with a bulky chelator for labeling with radiometals may decrease both affinity and the ability for passive diffusion through biological membranes. However, some neutral technetium-99 complexes are able to cross the BBB ([^{99m}Tc]Tc-ECD, [^{99m}Tc]Tc-HMPAO both used to visualize brain perfusion). [^{99m}Tc]Tc-TRODAT is one of the only examples of a radiometal complex that can be used to visualize a saturable target (dopamine transporter) in brain although with lower contrast compared to SPECT imaging with [^{123}I]I-FP-CIT (DaTscan) or PET tracers for the same target.[9,10]

Larger vector molecules such as peptides, antibodies, and antibody derivatives can be conjugated with bulky bifunctional ligands for radiolabeling with radiometals, typically without compromising their target affinity. Nonmetals such as fluorine-18 can also be used to label larger biomolecules using a prosthetic group-labeling approach or using inorganic approaches.[11-13]

Conventional strategies (eg, conjugation to the side chain ε-amine group of lysine residues) for chelator-protein conjugation or prosthetic group-protein conjugation generate heterogeneous mixtures of bioconjugates. Indeed, antibody-derived fragments usually have several solvent-exposed lysines in their structure making it difficult to control where and how many chelators or prosthetic groups will be conjugated to the protein so that mixtures of polyconjugated proteins are obtained with potentially high within-batch and batch-to-batch variability. Moreover, the occurrence of a lysine residue in or near the antigen-binding site can lead to loss of target affinity upon conjugation. As an alternative, site-specific conjugation can be achieved for example by using maleimide-thiol chemistry, if the protein contains one single cysteine residue. However, many other site-specific conjugation strategies, resulting in a radionuclide-to-protein ratio of one, can be considered.[14,15]

A fundamental and critical component of a radiometal-based radiopharmaceutical is the chelator, the ligand system that binds the radiometal ion in a stable coordination complex so that it can be properly directed to its molecular target in vivo. The properties of an ideal chelator for preparation of a radiopharmaceutical include efficient binding of the radiometal in high yields (quantitative reaction). Preferably within a short reaction time in an aqueous medium at low temperature (conditions compatible with heat-sensitive biomolecules). Fast kinetics of radiometal incorporation (association, k_{ass}) is often also accompanied by fast radiometal release (dissociation, k_{dis}). The rate of dissociation in vivo is what determines the kinetic inertness of a radiometal complex, and these off-rates are highly influenced by the high dilution factor when a radiopharmaceutical (often only micrograms) is diluted into the blood pool and typically occurs via transchelation to serum proteins and enzymes (Fig. 1).[16] Therefore, the stability and kinetic inertness of the chelate should be experimentally determined in vitro as well as in vivo. The consequence of radionuclide loss from a radiopharmaceutical in vivo depends on the properties and biological behavior of the specific radionuclide-ion in question. For example, [$^{67/68}$Ga]Ga^{3+}, [^{18}F]F^-, and [^{89}Zr]Zr^{4+} are known to accumulate in bone, whereas [^{64}Cu]Cu^{2+} is known to accumulate in the liver.

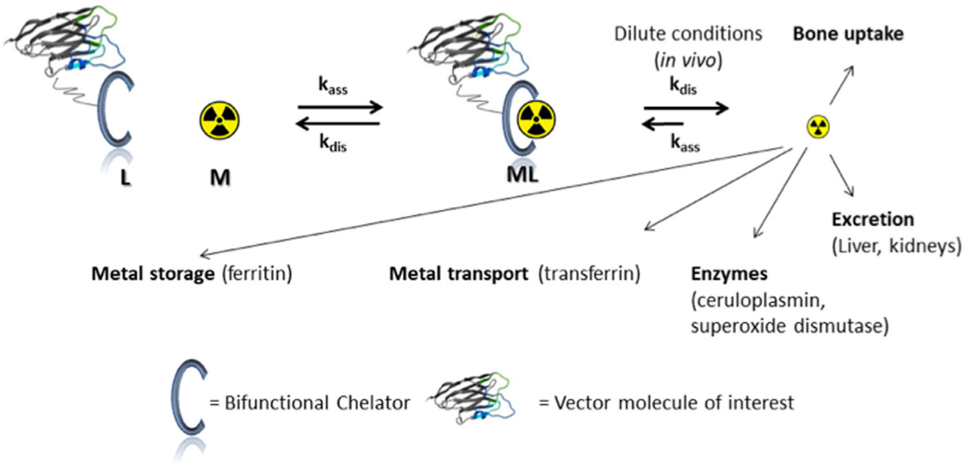


Figure 1 Metal ion coordination kinetics and enhanced in vivo dissociation (k_{dis}) kinetics due to extremely dilute conditions. Possible routes of radiometal ions in vivo (adapted from Eric W. Price and Chris Orvig).[16]

The half-life of the radionuclide should be compatible with the plasma half-life of the vector so that sufficient activity is present in the body at the time that an optimal signal/noise ratio is obtained. This plasma half-life can range from minutes (small organic molecules), hours (peptides, antibody fragments) to even days or weeks (monoclonal antibodies). Longer lived radioisotopes should be selected when an extended time is required to achieve optimal target tissue-to-background ratios. In order to increase the fraction of the injected dose of the radiopharmaceutical binding to its target cells, the plasma half-life of for example radiopeptides can be extended by conjugation to reversible low molecular weight (Mw) albumin binders.[17]

Important to discuss is the difference between residualizing and nonresidualizing radiolabels and its effect on kidney retention and tumor uptake. Most peptides and small proteins (<60 kDa) are cleared from the plasma by the renal elimination pathway. After glomerular filtration these compounds might undergo receptor-mediated reabsorption in the renal tubule cells. This process is mainly mediated by the endocytic megalin and cubulin receptors that are highly expressed on the apical side of proximal tubule cells.[18] Both receptors can interact with protein-based radioligands, followed by cell internalization, transfer to the lysosomal compartment and degradation by proteolytic enzymes into amino acids and other (radio)catabolites. Lipophilic (radio)catabolites are able to diffuse through lysosomal and cellular membranes and thus leak out from cells, and are thus nonresidualizing.[19]

Therefore, radiocatabolites are cleared faster from the kidneys when peptides or small proteins are labeled using nonresidualizing halogens such as fluorine-18, bromine-86, or different iodine radioisotopes. In contrast, the use of residualizing radiometal complexes results in extended retention of radioactivity in kidneys because most stable radiometal complexes are not able to diffuse out of the cells. The residualizing effect is not only important regarding kidney retention but might also occur in target cells of interest (eg, cancer cells) if there is a high rate of internalization. In this case the residualizing effect is beneficial as tumor uptake may be higher and last longer.

In general, radiopharmaceuticals are administered intravenously and will quickly distribute over the body and due to physical or chemical interaction they will concentrate in the target tissue or cells. The concentration of the radiopharmaceutical in surrounding tissue, which lacks the interaction mechanism, will be in equilibrium with the plasma concentration that will decrease due to clearance of the radiopharmaceutical from plasma by excretory organs such as liver and kidneys. As for regular medicines for IV administration, radiopharmaceuticals for IV injection need to be sterile, have a low endotoxin level and pH-tonicity.

A small number of radiopharmaceuticals are administered via other routes such as radiopharmaceuticals for lung ventilation study (inhaled) for gastric motility (oral), intracranial shunt patency (injection in shunt reservoir), transarterial radioembolisation (intra-arterial), radiosynovectomy (intra-articular injection and sentinel node visualization [peritumoral injection]).[20-23] Radiopharmaceuticals administered using these alternative routes usually have a physical retention mechanism in the target tissue. These radiopharmaceuticals therefore do not achieve their principal intended action by pharmacologic, immunologic or metabolic means and fall under the new European Union (EU) definition of medical device.[24]

Other radiopharmaceuticals also establish purely physical interactions with their target tissue (eg, [^{99m}Tc]Tc-macroaggregated albumin ([^{99m}Tc]Tc-MAA) for visualization of lung perfusion, [^{51}Cr]Cr-EDTA for glomerular filtration, and [^{15}O]H$_2$O for perfusion) and can thus also be considered as medical devices although they are listed in the European Pharmacopoeia as radiopharmaceuticals. Devices in which the radionuclide is stably bound and which are physically placed on (eg skin) or in the body are considered to be medical devices for brachytherapy.[25-27]

Compared to the global pharmaceutical market, estimated around 1000 billion $ in 2017,[28] the global radiopharmaceutical market was estimated to be 5 billion $ (0.5% of the global pharmaceutical market) in the same year.[29] Radiopharmaceuticals thus only have a limited market potential and their development may be as expensive as regular pharmaceuticals. Therefore, the business model of radiopharmaceuticals is quite challenging and only a limited number of new radiopharmaceuticals are being introduced to the market.

In contrast to the relatively small number of commercially available radiopharmaceuticals, a myriad of radiopharmaceuticals is being evaluated and used clinically in an academic research-oriented setting. Although technetium-99m radiopharmaceuticals in combination with SPECT continues to represent the largest fraction of diagnostic radiopharmaceuticals for routine clinical use, research toward new PET radiopharmaceuticals is by far more prominent than research toward novel technetium-99m radiopharmaceuticals. Big pharma has stepped in the arena of radiopharmaceuticals for targeted radionuclide therapy and given the initial success and the large investment in this area, a spectacular growth of these radiopharmaceuticals is expected in the near future.[30]

Radiopharmaceutical for Diagnosis and Therapy: The Radionuclide Vector Concept

In general, a radiopharmaceutical consists out of three components: a vector molecule, a radionuclide for diagnostic or therapeutic applications and a linker in between. The schematic design is shown in Figure 2. The radionuclide provides

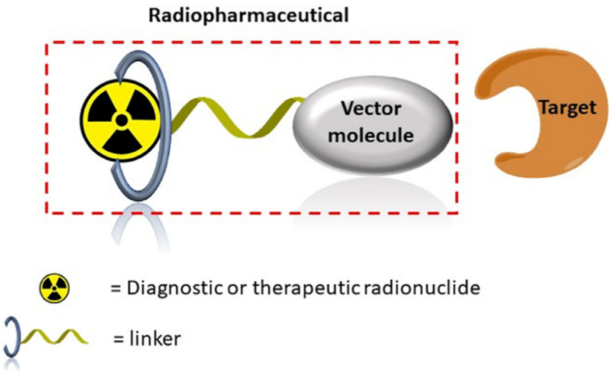


Figure 2 Schematic design of a radiopharmaceutical.

the radiation component (radioactivity), while the vector molecule specifically targets biomolecules expressed in tissues or cells.

The vector molecule can consist of a small inorganic or organic molecule, a peptide, a protein including antibodies, antibody fragments, or a nanoparticle. It is important that the vector molecule maintains high specificity and selectivity for the target after conjugation with the radionuclide. The vector molecules can have high affinity for different type of targets, such as receptor and transporter systems, enzymes, antigens, or misfolded proteins. In some cases, a radiopharmaceutical can consist of a radionuclide in ionic form, such as $[^{131}I]I^-$ for the treatment of thyroid cancer or $[^{89}Sr]Sr^{2+}$ for bone pain palliation. In this case, the radionuclide also fulfils the role of vector molecule.

The linker forms a stable chemical connection between the radionuclide and the vector molecule. In radiometal-based radiopharmaceuticals, a bifunctional chelator is required as linker, which is a chelator with a reactive functional group. This bifunctional chelator ensures a covalent bond with the vector molecule and also forms a stable complex with the radiometal.

The specific decay characteristics of the radionuclide attached to the vector molecule determines if the radiopharmaceutical is used for diagnostic (molecular imaging) or therapeutic (targeted radionuclide therapy) purposes. The availability of a wide range of radionuclides with different characteristics allows to pick the best matching radionuclide for the intended application.

Vector

Basic Concepts

The vector is an essential part of the radiopharmaceutical as it is responsible for the selective interaction with the target tissue leading to a higher concentration of the radionuclide in the target tissue. This provides the image contrast for diagnostic imaging and selective irradiation of the target cells for therapeutic radiopharmaceuticals.

Most vectors have a saturable pharmacologic, immunologic, or metabolic interaction with their target. In this case it is important that only tracer amounts of the radiopharmaceutical are administered in order to avoid saturation of the target and potential (side) effects. For some targets, such as the opioid system, a dose in the order of micrograms, which is in the range of the mass dose administered for a fluorine-18 or carbon-11 labeled radiopharmaceutical, can already lead to pharmacologic effects, as reported for $[^{11}C]$carfentanyl.[31]

The retention in the target tissue can be due to a reversible interaction such as affinity-based receptor or transporter binding and binding to misfolded proteins, governed by equilibrium association and dissociation. After binding to the target, the tracer may however be internalized and retained in the cell, leading to pseudo-irreversible kinetics.

The vector can also be a substrate for an enzyme in which the reaction product is trapped in the target cells (eg, 2-$[^{18}F]$fluoro-2-deoxy-D-glucose ($[^{18}F]$FDG)) or the vector may bind covalently to the enzyme (eg, tracers for Fatty Acid Amide Hydrolase (FAAH))[32], resulting in irreversible tracer kinetics. Binding of tracers in target tissue can also be driven by tissue microenvironmental parameters such as hypoxia, oxidative stress or transmembrane potential.[33-35]

The pretargeting approach allows to use a vector with long plasma half-life in combination with a radionuclide with a short half-life. This is accomplished by a two-step process in which initially the (nonlabeled) vector molecule is injected. After a sufficient waiting time, a radiolabeled click reagent is injected that will find and efficiently react (click) with the vector molecule, resulting in good contrast images with low radiation burden.[36]

Small Molecules

Small molecules are frequently used as vectors for intracellular targets due to their membrane permeability and the ability to design molecules for specific biochemical targets within the cytoplasm and nucleus.[37] A variety of small molecules can be used as vector molecule ranging from biochemicals, amino acids, fatty acids, nucleosides, and xenobiotics to chelates (intrinsic radiopharmaceuticals). In contrast to larger molecules, small molecules are also more likely to pass through the BBB. Table 1 presents a selection of small molecule radiopharmaceuticals.

Peptides and Proteins

Peptides that target specific receptors, expressed on specific cancer entities, are often used as vector molecules for diagnostic radiopharmaceutical applications. Moreover, the overexpression of many peptide receptors on human tumor cells compared to normal tissues makes these receptors attractive targets for peptide receptor radionuclide therapy (PRRT).

Due to their low Mw (peptides are usually classified as containing less than 50 amino acid residues), peptides show rapid diffusion into target tissue, and upon binding of the radioligand, the receptor-ligand complex is often internalized, allowing long retention of radioactivity in tumor cells. Compared to other biological active molecules, peptides are easily synthesized using either an automated peptide synthesizer or by manual synthesis. Further, most peptides have the ability to tolerate harsh chemical (radiolabeling) conditions (pH, temperature, . . .) and known sequences of amino acids can be modified to decrease their in vivo catabolic rate, such as the use of more stable D-amino acids instead of the naturally occurring L-amino acid isomers, the use of pseudopeptide bonds, and the use of cyclic peptides. Other advantages of using peptides as vector molecule are the favorable pharmacokinetics characterized by high concentration in the target tissue, rapid clearance from the blood pool and nontarget tissue, and absence of liver clearance followed by hepatobiliary excretion.

However, an issue often associated with the use of radiolabeled peptides is their high uptake and retention by the kidneys, which is a concern, particularly for radionuclide therapy because of potential radionephrotoxicity.

Interesting examples of peptides that are routinely used for imaging and therapy applications are peptides targeting the somatostatin receptor or the chemokine receptor CXCR4, but many other receptors (eg, bombesin receptor, neurotensin

Table 1 Selection of Small Molecule Radiopharmaceuticals

Radiopharmaceutical	Application	Ref
Biochemical derived		
[^{11}C]CO	Blood volume determination	38
[^{11}C]CH_3COOH	Myocardial metabolism evaluation	39
[^{13}N]NH_3	Myocardial perfusion evaluation	40
[^{15}O]H_2O	Regional perfusion	37
[^{18}F]FDG	Glucose metabolism in brain, heart, and tumors	40
[^{18}F]NaF	Bone imaging agent	37
[^{82}Rb]RbCl	Myocardial perfusion imaging	37
[^{123}I]NaI	Thyroid imaging	37
Amino acid, fatty acid, and nucleoside derivatives		
[^{11}C]methionine	Brain tumor imaging	40
[^{11}C]palmitate	Fatty acid metabolism imaging	41
[^{18}F]FET	Amino acid transport imaging	40
[^{18}F]FLT	Monitoring of cellular proliferation	40
Xenobiotic derivatives		
[^{11}C]carfentanil	Brain μ-opioid receptors imaging	42
[^{18}F]flutemetamol	β-amyloid imaging for Alzheimer's disease	43
[^{123}I]ioflupane	Striatal dopamine transporters imaging for Parkinson's disease	44
[^{123}I]iobenguane	Detection of pheochromocytoma and neuroblastoma tumors	45
Intrinsic radiopharmaceuticals		
[^{99m}Tc]Tc-exametazine	Cerebral perfusion scintigraphy, white blood cell labeling	46
[^{99m}Tc]Tc-bicisate	Cerebral perfusion scintigraphy	46
[^{99m}Tc]Tc-sestamibi	Myocardial perfusion scintigraphy, breast imaging	46
[^{99m}Tc]Tc-tetrofosmin	Myocardial perfusion scintigraphy	46

[^{18}F]FDG = 2-[^{18}F]fluoro-2-deoxy-D-glucose; [^{18}F]FET = *O*-(2-[^{18}F]fluoroethyl)-L-tyrosine; [^{18}F]FLT = 3-[^{18}F]fluoro-3-deoxythymidine.

receptors, luteinizing hormone-releasing hormone receptor, gastrin releasing peptide receptor, etc.) have been targeted using radiolabeled peptides.[47]

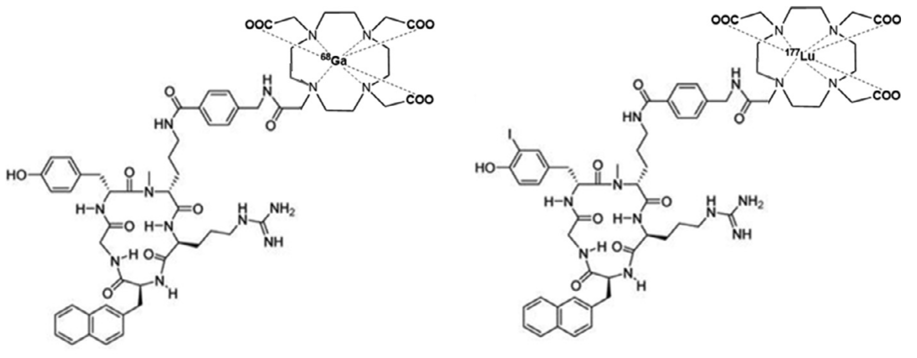
The most commonly used peptide-based radiopharmaceuticals are radiolabeled somatostatine (SST) analogs for the imaging and treatment of neuroendocrine tumors (NETs). SSTRs are variably expressed by a variety of solid and hematological malignancies. NETs represent one of the groups with the highest incidence of SSTR expression. Using radiolabeled somatostatin analogs, the presence of SSTRs on tumor cells may be exploited for molecular imaging and for PRRT in selected cases. Consequently, SSTR imaging and PRRT now occupies a key position in the clinical management of NETs. New developments in SSTR ligands are also strongly driven by the need for improved lesion targeting, especially for tumors with low SSTR expression. This may be achieved by using peptide vectors having a higher binding affinity (K_D) for the SSTR or a broader affinity profile for the different receptor subtypes or by using compounds recognizing a higher number of binding sites (B_{max}), such as the SSTR antagonists.[48]

Increased understanding of the pivotal role of CXCR4 in tumor biology and other diseases has resulted in the development of radiopharmaceuticals for diagnosis, patient selection for CXCR4-targeted therapies, and targeted radionuclide therapy of CXCR4 expressing tumors. In recent years, several CXCR4 imaging agents were developed to meet the clinical need for pretherapeutic quantification of CXCR4 expression and to allow patient selection for CXCR4-targeted therapies. [^{68}Ga]Ga-pentixafor, a cyclic pentapeptide, is the only CXCR4-targeted imaging agent that has found broad clinical applicability so far (Fig. 3).[49] [^{68}Ga]Pentixafor shows high affinity (IC_{50} ^{nat}Ga-pentixafor: 24.8 ± 2.5 nM) and selectivity for human CXCR4 in combination with rapid renal excretion, resulting in high-contrast PET imaging of CXCR4-expressing tissues.[50]

CXCR4-based targeted radionuclide therapy represents a highly promising treatment concept in cancer therapy for patients with otherwise limited or nonexisting treatment alternatives. It is mainly focused on the use of β^--emitters and the ^{177}Lu- and ^{90}Y-version of [^{68}Ga]Ga-pentixafor were produced and evaluated. Unfortunately, these minor structural modifications caused strongly decreased CXCR4-affinity. This led towards the development of [^{177}Lu]Lu-pentixather (Fig. 3), based on a closely related peptide backbone (introduction of 3-iodo-D-Tyr[1] in pentixafor) with high affinity for CXCR4 (IC_{50} ^{nat}Lu-pentixather: 14.6 ± 1.0 nM).[51]

Proteins can also be used as vector molecule for radiopharmaceuticals. An example is the use of radiolabeled human serum albumin (HSA). HSA (HSA, 66.5 kDa) is a heat-sensitive globular protein with a primary sequence made up of 585 amino acid residues, and is the most abundant protein in human plasma (3.5-5 g/dL).[52] Its high solubility, stability, and plasma half-life of approximately 16-18 hours makes HSA the ideal vector for PET blood pool imaging applications.[53]

Intact mAbs (monoclonal antibodies) are considered good candidates for targeted therapy as well as diagnostic purposes, because they provide a versatile platform of probes with outstanding affinity and specificity toward a myriad of antigens. Their large size (150 kDa), which precludes glomerular filtration, combined with Fc-mediated catabolism



$[^{68}Ga]$Ga-pentixafor **$[^{177}Lu]$Lu-pentixather**

Figure 3 Chemical structures of $[^{68}Ga]$Ga-pentixafor and $[^{177}Lu]$Lu-pentixather.

escape, results in a circulation of several days to weeks in blood. This long residence time in blood limits imaging contrast between targeted and nontargeted cells at early time-points after injection. In general, high target-to-background contrast can only be obtained several days after IV injection of the radiolabeled mAbs. As a result of slow kinetics, the use of long-lived radionuclides like zirconium-89 (^{89}Zr, $t_{1/2}$: 3.3 days) or iodine-124 (^{124}I, $t_{1/2}$: 4.2 days) is required, causing a high radiation burden for the patient (20-40 mSv per scan).[54] The availability of a new high sensitive total body PET scanner may however allow to reduce the injected dose and perform clinical ^{89}Zr or ^{124}I immunoPET scanning with an acceptable radiation burden.

Advancements in biotechnology have led to bioengineering of many vector molecules with shorter biological half-lives, and compatible with a short-lived PET radionuclide such as ^{18}F and ^{68}Ga, that can be used as an alternative to mAbs. Examples of suitable vector molecules are 80-kDa minibodies,[55] 55-kDa antigen-binding fragments (Fab),[56] 28-kDa single-chain variable fragment,[57] 18-kDa designed ankyrin repeat proteins,[58] 15-kDa antibody fragments derived from heavy-chain only antibodies (V_{HH} or nanobodies),[59] 7-kDa affibody molecules,[60] 7-kDa albumin-binding domain-derived affinity proteins,[61] and many others.[62]

Particles

Particles have been used for a long time as vectors for radiopharmaceuticals and albumin served as a protein that can be denatured resulting in biodegradable particles with a size distribution that depends on the denaturation conditions. Large particles, such as MAA, 10-100 μm, are retained in the first capillary bed downstream of the injection site and are primarily used to quantify lung perfusion but are also applied for lung shunt estimation in ^{90}Y-radioembolization therapy.[63] MAA particles are usually labeled with ^{99m}Tc, but labeling with ^{68}Ga or ^{18}F for application in PET has also been described.[64,65]

Smaller particles (^{99m}Tc-labeled nanocolloids) are majorly used for surgical planning of sentinel node resection and combination with a fluorescent tag into “bimodal particles” allows for optical surgical guidance toward the sentinel node.[66]

Further, multimodal nanoparticles have been designed to allow in vivo imaging with different modalities such as magnetic resonance imaging (MRI), computed tomography (CT), SPECT, PET, photo acoustic imaging, two photon or fluorescent imaging or ultrasound.[67]

Nanoparticles concentrate in tumor tissue due the enhanced perfusion and retention effect, but can also be targeted by conjugation to an affinity ligand that direct or enhance interaction of the particles with their target. This the case for $[^{99m}Tc]$Tc-tilmanocept particles that are derivatized with mannose, resulting in avid binding to the CD206 receptor on reticuloendothelial cells.[68]

Cells

Autologous cells can also be used as a vector. ^{99m}Tc-red blood cells labeled in vitro or in vivo are used for ventriculography, visualization of hemangiomas, and gastrointestinal bleeding.[69,70] ^{99m}Tc-labeled autologous leucocytes are routinely used to image infections.[71]

PET imaging reporter genes can be expressed in human embryonic stem cells for example using adeno-associated virus technology allowing to determine their biodistribution and viability.[72] Cells transfected with genes encoding for the sodium iodide symporter (hNIS), herpes simplex virus type 1 thymidine kinase (HSV1-TK) or SSTR subtype 2 (SSTR2) can be tracked in vivo with PET after injection of corresponding radiotracers being $[^{124}I]I^-$, $[^{18}F]$FHBG, or $[^{68}Ga]$Ga-DOTATOC, respectively.

Radionuclide

Radionuclides are elements with an excess of nuclear energy, making them unstable. These radioisotopes either have a heavy nucleus ($Z > 83$) or possess an imbalance in proton/neutron ratio, or are in a metastable energy state and will undergo radioactive decay. The excess of energy in the nucleus of the unstable element can result in emission of either particles (α, $\beta^{+/-}$) and/or electromagnetic radiation (gamma ray photons [γ]) and as a secondary effect X-rays, conversion electrons and Auger electrons. Radionuclides occur naturally or are artificially made using cyclotrons, particle accelerators or are generated by decay of other radionuclides and obtained from radionuclide generators. A rapidly expanding number of radionuclides with a broad variety of half-lives, emission types, and energies for application of

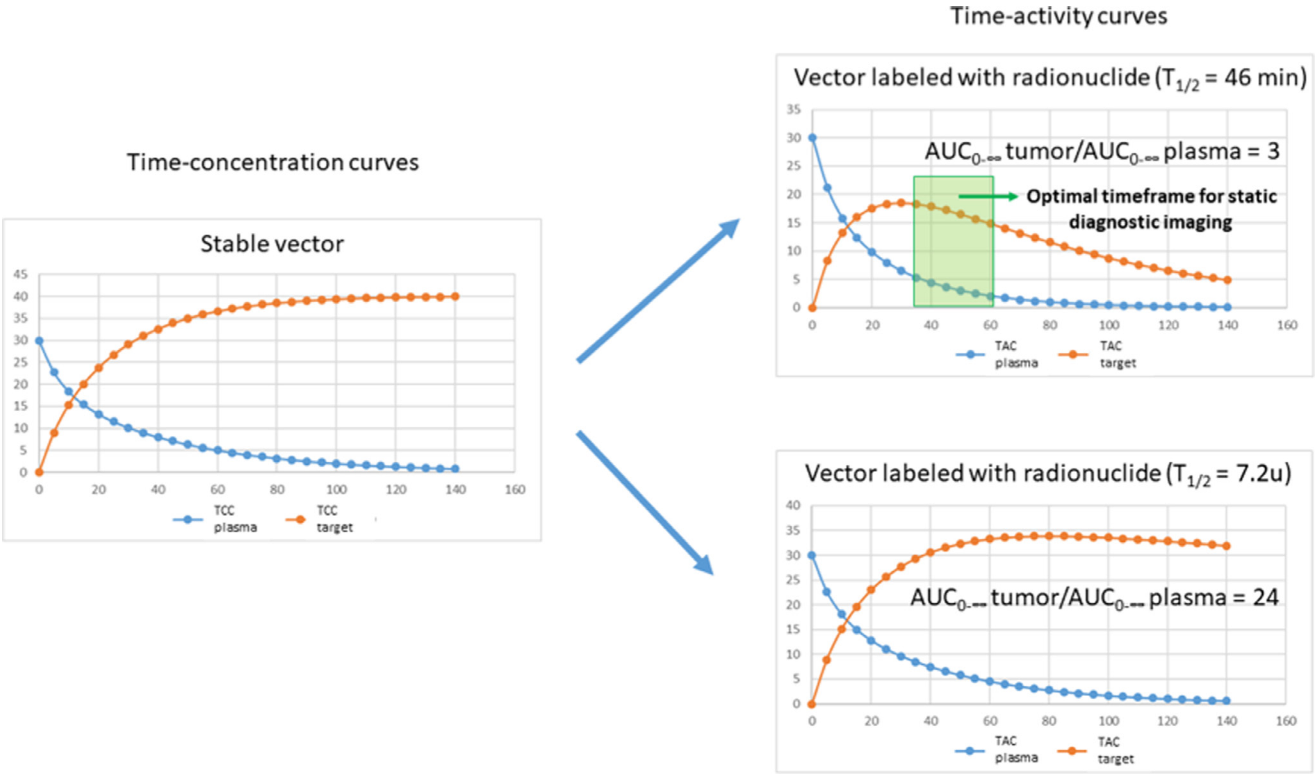


Figure 4 Dependency of the physical half-life of the radionuclide on selectivity of the radiation damage. The nontarget dose is equalized to the AUC (t = 0-∞) of the plasma TAC and tumor dose is equalized to the AUC (t = 0-∞) of the TAC of the tumor. The ratios of both AUC depict the selectivity of the radiation damage. The ratio for the longer lived radionuclide ($T_{1/2}$ = 7.2 hours) is about eight times higher compared to the shorter lived radionuclide ($T_{1/2}$ = 46 minutes). The optimal timeframe for diagnostic static imaging is when the highest target/background ratio is obtained.

radionuclide imaging and therapy studies are routinely produced. The availability of a wide range of radionuclides makes it possible to carefully pick the best matching radionuclide for a certain application. Depending on the decay properties of the radionuclide, it can be used for PET, SPECT, and/or therapeutic applications.

With a few exceptions (such as ^{11}C, ^{13}N, and ^{15}O), most radionuclides used in nuclear medicine are either radiometals or radiohalogens. Radiometals are often used as a complex with a suitable chelator (ligand) that is used as such (eg, most ^{99m}Tc-complexes) or covalently linked to a biologically active targeting molecule, making an active radiopharmaceutical agent. In contrast, radiohalogens are mostly incorporated in a targeting molecule by standard carbon-halogen bond formation.

Important to note, several elements have multiple radioactive isotopes that are useful for diagnostic or therapeutic purposes. Isotopes of a given element have identical chemistry, thus, a single radiopharmaceutical agent can be radiolabeled with different radioisotopes of the same element (eg, $^{86/90}$Y, $^{161/155/149/152}$Tb), while maintaining the same biological behavior and distribution in vivo. As such, pharmacokinetics will not differ and dosimetry can be accurately estimated. Also, diagnostic and therapeutic radiopharmaceuticals with very similar chemical properties (eg, same vector molecule with different radiometal), and thus similar pharmacokinetics, can be used for this purpose. This class of radiopharmaceuticals is called theranostic agents.[73]

The radioactivity which is still present in the patient at the end of a scan does not have any diagnostic value but will contribute to the radiation dose of the patient. From a patient radiation dose point of view, the radionuclide half-life of a diagnostic radiopharmaceutical should thus be as short as possible. The radionuclide half-life should however be long enough to allow radiosynthesis and in vivo distribution toward target tissues. Static imaging should be performed in the timeframe when the highest target/background ratio is obtained (Fig. 4).

The area under the time-activity curve (TAC-AUC) of the tumor site and TAC-AUC of the healthy tissues determines the selectivity of the irradiation of the tumor vs healthy tissue. One should note that the selectivity for radiation damage to malignant tissue is potentially higher for longer lived radionuclides, which is beneficial in a therapeutic setting. This is illustrated in Figure 4, where the plasma and tumor kinetics of a stable, nonradiolabeled vector molecule are showed as well as the TAC of the same vector molecule labeled with a radionuclide with a half-life of 46 minutes (tumor/plasma TAC-AUC = 3) or a radionuclide with a half-life of 7.2 hours (tumor/plasma TAC-AUC = 24).

Before targeted radionuclide therapy can be initiated, sufficient expression of the target in the malignant tissue has to be confirmed. This can be done by first performing a PET or SPECT-scan, after injection of the diagnostic sister of the therapeutic radiopharmaceutical. If the diagnostic radiopharmaceutical can truthfully predict the pharmacokinetics of its

Table 2 Characteristics of Commonly Used SPECT Radionuclides[76,77]

Radionuclide	$T_{1/2}$	Nuclear Reaction	Mode of Decay*	Decay Product	Energy (keV)**
^{67}Ga	3.26 d	^{67}Zn(p,n)^{67}Ga ^{68}Zn(p,2n)^{67}Ga	EC (100%)	^{67}Zn	93
^{67}Cu	3 d	^{68}Zn(γ,p)^{67}Cu	β^- (100 %) γ (52%)	^{67}Zn	185
^{99m}Tc	6.06 h	^{99}Mo/^{99m}Tc-generator	IT (89%)	^{99}Tc	140
^{111}In	2.83 d	^{111}Cd(p,n)^{111}In ^{112}Cd(p,2n)^{111}In	EC (100%)	^{111}Cd	245
^{123}I	13.2 h	^{123}Xe/^{123}I generator ^{124}Xe(p,pn)^{123}I	EC (100%)	^{123}Te	159
^{153}Sm	47 h	^{152}Sm(n, γ)^{153}Sm	β^- (100%) γ (29%)	^{63}Eu	103
^{159}Gd	20 h	TNB on Gd_2O_3	γ (11.4%)	^{159}Tb	363

*abundance in %, EC, electron capture, IT, internal transition, TNB, thermal neutron bombardment.
**Energy of main γ-ray emitted.

therapeutic sister, the diagnostic scan also allows to accurately calculate the dosimetry. The pair of tracers can thus be used in a true "theranostic" approach.[73]

Diagnosis: SPECT Radionuclides

SPECT is based on the detection of single photons emitted upon decay of a radionuclide by rotating detectors, providing a 3D image representing the distribution of the radionuclide in the body.[74] Generally, energy levels between 100 and 200 keV are required for adequate imaging. Commonly used SPECT isotopes are depicted in Table 2. Isotopes used for SPECT are typically radiometals, requiring complexation chemistry. One exception is iodine-123, which can be incorporated in to small molecules via radio-iodination techniques. The predominant radioisotope used in SPECT imaging is the metastable form of technetium (^{99m}Tc, $T_{1/2} = 6$ hours). This isotope can be easily generated via a molybdenum-technetium generator.

^{99m}Tc can be incorporated into various vector molecules via complexation chemistry using a kit-based radiolabeling method. The ^{99m}Tc-complexes are routinely used in the clinic for evaluation of cardiovascular diseases, renal excretion, identification of tumor lesions, and CNS disorders.[75]

Diagnosis: PET Radionuclides

PET is based on detection of two 511 keV photons that are emitted in opposite directions upon annihilation of a positronium, that results from combination of an electron and a positron, the latter being emitted from a neutron deficient nucleus.[78]

Typical radionuclides, used for PET imaging, are listed in Table 3. These radionuclides can be divided in pure PET radionuclides (^{11}C, ^{13}N, ^{15}O, ^{18}F, ^{68}Ga, and ^{82}Rb) with high branching factors for β^+-decay and non-pure radionuclides (^{64}Cu, ^{76}Br, ^{86}Y, ^{89}Zr, ^{124}I, and ^{152}Tb) where β^+-decay is generally not the main decay route or where the nuclides do not decay directly to a ground state.[79] The short half-life of ^{15}O, ^{13}N, and ^{82}Rb limits the time available for radiochemical conversion so that only a limited number of radiopharmaceuticals labeled with these isotopes have been reported.[80] Most PET-radionuclides are produced using a cyclotron, except for ^{82}Rb and ^{68}Ga which are available from a generator and ^{152}Tb which can be produced by high-energy proton-induced spallation of Tantalum foil targets. Hereafter, an additional purification step is required to separate ^{152}Tb from other formed isotopes (^{149}Tb and ^{155}Tb).[81]

Therapy: Alpha, Beta, and Auger Electron Emitters

The goal of targeted radionuclide therapy is to deliver therapeutic doses of ionizing radiation to specific disease sites for cure, disease control, or pain palliation.

The biological effect of radionuclide therapy obtained by three mechanisms:

1) Interaction of ionizing radiation with water in which chemically active free radicals (hydroxyl radical) are formed that can react with biomolecules (phospholipids, proteins, RNA, DNA, etc.), thereby irreversibly damaging the cells.
2) Direct interaction of ionizing radiation with DNA in which single-strand breakage or double-strand breaks can occur. Therefore, therapeutic radionuclides must emit particulate radiation with relatively short path lengths thereby depositing the radiation energy at a short distance to spare surrounding non-target tissues. Single-strand breaks can be repaired while double-strand DNA breaks are far more difficult to repair. When repairing DNA strand breaks, repair errors can also occur, causing mutations.
3) During treatment of a tumor, the immune system can be activated against certain antigens of the tumor so that other tumor cells (possibly at a distance from the treated tumor) can be attacked by the immune system, this is known as the abscopal effect.[83]

Therapeutic radionuclides can be divided in three subgroups; β^--, Auger-, and α-emitters. Within each radionuclide subgroup, there are multiple radionuclides with a variety of half-lives, particle range in soft tissue and chemical properties available which allows to select the best radionuclide for a specific therapeutic application. Availability and

Table 3 Characteristics of Commonly Used PET Radionuclides[79,81,82]

Radionuclide	$T_{1/2}$	Nuclear Reaction	Mode of Decay*	Decay Product	Energy (MeV)	Average Range of β^+ in H_2O (mm)
^{11}C	20.4 min	^{14}N(p,α)^{11}C	β^+ (100%)	^{11}B	0.960 (β^+ E_{max})	1.2
^{13}N	10.0 min	^{16}O(p,α)^{13}N	β^+ (100%)	^{13}C	1.199 (β^+ E_{max})	1.8
^{15}O	2.0 min	^{14}N(d,n)^{15}O	β^+ (100%)	^{15}N	1.732 (β^+ E_{max})	3.0
^{18}F	109.8 min	^{18}O(p,n)^{18}F ^{20}Ne(d,α)^{18}F	β^+ (97%) EC (3%)	^{18}O	0.634 (β^+ E_{max})	0.6
^{64}Cu	12.7 h	^{64}Ni(p,n)^{64}Cu	β^+ + EC (61%) β^- (39%)	^{64}Ni ^{64}Zn	0.653 (β^+ E_{max}) 0.3293-1.675 0.5794	0.7
^{68}Ga	67.6 min	^{68}Ge/^{68}Ga-generator	β^+ (89%) EC (11%)	^{68}Zn	1.899 (β^+ E_{max}) 0.227-2.821	3.5
^{76}Br	16.0 h	^{76}Se(p,n)^{76}Br	β^+ (55%) EC (45%)	^{76}Se	3.382 (β^+ E_{max}) 0.599	
^{82}Rb	1.3 min	^{82}Sr/^{82}Rb-generator	β^+ (100%)	^{82}Kr	3.378 (β^+ E_{max})	7.1
^{86}Y	14.7 h	^{86}Sr(p,n)^{86}Y	β^+ (32%) IT (68%)	^{86}Sr	1.221, 1.545, 1.988 ($\beta^+_{1,2,3}$ E_{max}) 0.433-1.920	1.9, 2.8, 3.7
^{89}Zr	3.3 days	^{89}Y(p,n)^{89}Zr	β^+ (23%) EC (77%)	^{89m}Y	0.902 (β^+ E_{max}) 0.909	1.3
^{124}I	4.2 days	^{124}Te(p,n)^{124}I	β^+ (26%) EC (74%)	^{124}Te	2.138, 1.535 ($\beta^+_{1,2}$ E_{max}) 602	4.4, 2.8
^{152}Tb	17.5 h	^{143}Nd(^{12}C,5n)^{152}Tb ^{144}Nd(^{12}C,4n)^{152}Tb ^{145}Nd(^{12}C,3n)^{152}Tb Spallation on Ta§	β^+ (17%) EC (83%)	^{152}Gd	2.9678 (β^+ E_{max})	

*Abundance in %, EC, electron capture, IT, isomeric transition.

§= proton-induced spallation on Tantalum.

Table 4 Characteristics of Frequently Used Radionuclides for Radionuclide Therapy[47,85]

Radionuclide	$T_{1/2}$	Main Emissions*	Production
^{225}Ac	10 d	α	^{229}Th/^{225}Ac generator
^{223}Ra	11.4 d	α	^{227}Ra/^{223}Ra generator
^{213}Bi	45.6 min	α, β^-, γ	^{225}Ac/^{213}Bi generator
^{211}At	7.2 h	α, EC	Cyclotron ^{209}Bi(α,2n)^{211}At
^{90}Y	64 h	β^-	Nuclear reactor ^{90}Zr(n,p)^{90}Y
^{177}Lu	6.6 d	β^-	Nuclear reactor ^{176}Lu(n,γ)^{177}Lu
^{67}Ga	78 h	EC	^{67}Zn(p,n)^{67}Ga ^{68}Zn(p,2n)^{67}Ga
^{99m}Tc	6 h	IT	^{99}Mo/^{99m}Tc-generator
^{111}In	67 h	EC	^{111}Cd(p,n)^{111}In ^{112}Cd(p,2n)^{111}In

*EC, electron capture; IT, internal conversion.

production cost are also important parameters that need to be considered. Typical radionuclides, used for targeted radionuclide therapy are listed in Table 4. All of these are radiometals except for the radiohalogens ^{131}I and ^{211}At.[84]

During β^--decay, a neutron is converted into a proton in neutron rich nuclei, thereby emitting an electron (β^--particle) and an antineutrino. β^--particles have maximum kinetic energies of 0.3-2.3 MeV with corresponding ranges of around 0.5-12 mm in soft tissue. The range of β^--particles, depends on their energy but covers a large number of cells (10-1000). This results in a useful therapeutic property that has been termed the "crossfire effect," ensuring sufficient dose delivery to kill cells in a large tissue mass, including cells that do not express the binding target. This can be beneficial for the treatment of bulky, heterogeneous cancers. The disadvantage of this crossfire effect is potential damaging of surrounding healthy tissue leading to collateral damage. Although β^--particle emitting radionuclides are effective for medium to large size tumors, they are less effective in treating smaller metastatic tumor lesions. The relatively low linear energy transfer (LET, expressed in energy lost per unit distance travelled) of β^--particles (~0.2 KeV/μm) causes mostly repairable DNA damage including chemical modifications, protein crosslinks but especially single strand breaks which are relatively easy repaired by specialized enzymes.[85]

α-decay is a radioactive decay in which a nucleus emits an alpha particle (a helium nucleus), consisting of two protons and two neutrons. Due to the short penetration depth of α-particles (50-100 μm, corresponding to a diameter of 2-10 cells), they are able to selectively destroy tumor cells and cause minimal damage to the surrounding healthy tissue. Alpha-particle emitters are more suitable in treatment of tumors with small diameters and where their localization within the tumor is more spatially homogeneous. They are characterized by a high LET (~100 keV/μm) and thus a high relative biological effectiveness (biological damage caused by ionizing radiations compared with a reference radiation). The high LET of alpha-particles leads to a high frequency of double-stranded DNA breaks, and thus irreparable damage, causing the high kill rate of α-emitting radioisotopes both in hypoxic as well as in normoxic tumor cell environments.[85]

Radionuclides with multiple alpha-emitting daughters like ^{225}Ac ($t_{1/2}$= 10.0 days) or ^{223}Ra ($t_{1/2}$ = 11.4 days) might have a promising future in targeted radionuclide therapy. However, toxicity is clearly still an issue due to the recoil energy the daughters experience upon alpha decay. This energy is in most cases at least 100 keV, more than 1000 times larger than the binding energy of any chemical compound. This recoil energy will cause chemical bond rupture, causing subsequent release of the daughter radionuclides, which are often themselves alpha emitters. Since the daughter nuclides are no longer bound to the vector molecule there is a risk for uncontrolled redistribution of recoiled atoms causing considerable toxic effects to healthy tissues.[86]

Auger electrons have a short range in tissue (subcellular, order of nanometers) and are very cytotoxic due to the Auger electron cascades. In this case, an electron fills a vacant position on a more inner electron shell. Next, an X-ray is emitted and its energy is transferred to an electron of the outer shell, called the Auger electron, which is than ejected from its orbit. Auger electron-emitting radionuclides have potential for the therapy of small cancers because of their high level of cytotoxity, low-energy, high LET (4-26 KeV/μm), and short-range biological effectiveness. Because of their short range, they are generally strongly dependent on their close proximity to DNA, and thus it is required that the Auger electron-emitting radionuclides enter the cell nucleus to allow efficient cell killing.[47]

Design of New Radiopharmaceuticals

Basic Requirements

When a tracer is administered, the general pharmacokinetic principles of distribution, metabolism, and excretion apply.[87] Most radiopharmaceuticals are administered intravenously and thus absorption can be neglected.

The radiopharmaceutical will be distributed over the body with the bloodstream and accumulate in the target tissue as a result of the specific interaction of the vector with its target. At the same time the radiopharmaceutical will be cleared from plasma so that the target-to-background ratio will sufficiently increase within the timeframe imposed by the (short) half-life of the radionuclide.

For oncological application, renal clearance and urinary excretion are preferred as hepatobiliary clearance followed by slow transfer through the GI tract results in high background activity levels in the abdomen potentially interfering with sensitive tumor detection in this area. Diagnostic radiopharmaceuticals for CNS are usually lipophilic and often cleared from plasma by hepatobiliary clearance but this is of no concern as it does not interfere with brain imaging.

For a radiotracer interacting through equilibrium binding, the bound-to-free ratio at (pseudo)-equilibrium will be driven by the B_{max}/K_D ratio which should be at least >4.[88]

For quantitative (CNS) PET, complex dynamic imaging studies will continuously record brain TACs during extended periods (hours) starting from the time of tracer injection. This allows in vivo quantification of the target expression or determination of drug dose-occupancy relation.[1,89] For this purpose, tracers with reversible brain kinetics within the scan period are generally preferred. In such studies, only a limited number of subjects are scanned but repeated PET scans in the same subjects may be required and radiation dosimetry will limit the number of repeated studies. In this case radiolabeling with a short half-life radionuclide such as carbon-11 has a clear advantage.

In clinical routine, high throughput and low scanning burden to the patient is important and the use of a short static scan is generally preferred. In this case, sufficient time between radiotracer injection and scanning is required to obtain a sufficient target-to-background ratio again within the timeframe imposed by the (short) half-life of the radionuclide used. Here, radiopharmaceuticals with (pseudo)-irreversible kinetics are preferred as it is the case for most diagnostic radiopharmaceuticals used for oncology studies. When large numbers of patients need to be scanned the use of radionuclides that can be produced in high activity amounts (eg, ^{18}F) and have a (relative) long half-life will allow to inject multiple patients with one production batch, which will reduce the effective cost per dose of the radiopharmaceutical.

For radiopharmaceuticals for radionuclide therapy, also (pseudo)-irreversible tumor kinetics within the radionuclide lifespan are preferred to maximize selective irradiation of the tumor tissue.

When intracellular targets are targeted, radiotracers need to cross the cell membrane. In this case, tracer lipophilicity needs to be sufficiently high, but not too high to avoid non-specific interaction with the phospholipid double membrane layer reducing the signal-to-noise ratio.[90] The BBB and the blood-testis barrier, consist of closely aligned endothelial cells interconnected with tight junctions and require even more rigid properties to allow free diffusion.[91,92] The BBB is one of the biggest hurdles to overcome in CNS imaging as besides a structural barrier, it also incorporates drug efflux transporters such as P-glycoprotein and multidrug resistance protein 1[93]. A rule of thumb implies that CNS radiotracers should be uncharged at physiological pH, have a logD value ranging from 2 to 3, a topological polar surface area value under 90 $Å^2$ and a Mw under 500 Da to be able to efficiently pass BBB or blood-testis barrier by passive diffusion.[90]

PET or SPECT detectors cannot discriminate between radiation emitted by a radionuclide associated with the parent radiopharmaceutical or with radiometabolites. Analogously, radiation emitted from therapeutic radionuclides will be harmful independent whether the radionuclide is associated with the parent or a radiometabolite. Radiotracer metabolism arising from interaction with metabolic enzymes generally accelerates tracer blood clearance, decreasing background noise associated with radioactivity in blood.[94]

Radiometabolites may however also exhibit specific binding to the same or a different molecular target complicating image interpretation (diagnostic radiopharmaceuticals) or increasing off-target radiation dose (therapeutic radiopharmaceuticals). The fraction of radiometabolites in plasma and target tissue (eg, brain) can be determined using radio high performance liquid chromatography (radioHPLC) or radio thin layer chromatography (radioTLC).[94]

The low amount of injected tracer (picomoles to nanomoles, sum of radioactive and stable molecules) avoids pharmacologic effects.[95] The molar activity (MA) is a measure of the radioactivity per total amount (in mole) of the tracer (GBq/μmol or Ci/μmol) and is of importance in studies targeting saturable targets (eg, receptors, transporters, and enzymes).[96] A low MA can result in underestimation of the target concentration, as the presence of unlabeled tracer may occupy a significant fraction of the saturable target.

On the other hand, when targeting tumors with diagnostic or therapeutic radiopeptides or labeled antibodies, tumor uptake vs MA may be bell shaped. If too few molecules are administered (high MA), a large fraction of the radiotracer is trapped during its first pass effect by high-affinity low-capacity binding sites in nontarget organs such as the liver, and will not reach their target. Selective use of a range of MA values can be a means to find an optimal MA that represents a compromise between, for example, a saturable elimination and receptor saturation.[97]

The Radiochemical and Radiopharmaceutical Space

It is evident that a suitable radiolabeling procedure needs to be developed that robustly provides the desired radiopharmaceutical with a sufficient yield, high purity, and MA within a short production time.

For ^{11}C- and ^{18}F-radiolabeled compounds the radiochemical space has increased considerably during the last decade thanks to novel synthetic approaches that allow efficient introduction of the radionuclide on positions on chemical structures that were considered impossible previously.[98-102] For a detailed overview concerning ^{11}C- and ^{18}F-radiochemistry we refer the reader to r*adiopharmaceutical chemistry*.[103-106]

For radiometals, novel chelators are continuously being added to the armamentarium improving chelation efficiency, stability or pharmacokinetics of the corresponding radiotracers.[107] In addition to this, click chemistry and the use of prosthetic groups have also introduced robust avenues to obtain radiolabeled compounds.[108]

Validation Path: In Silico, In Vitro, and In Vivo

Identification of a new PET imaging target generally starts by searching for an unmet clinical diagnostic need, in which the expression and/or ligand binding affinity of a protein (such as receptor, transporter, and enzyme) is altered compared to the healthy state. Here, the biological function and the localization of the aberrant entity are important.[109]

Next, literature can be explored to identify (a library of) compounds or scaffolds with high affinity for the target. This library can be screened in vitro against recombinant protein, cell lysate, or tissue homogenate in which affinity of the compound for the target can be determined.[110] Affinity is indeed a crucial parameter for tracers since the bound-to-unbound ratio, depends on the maximum density of receptors/dissociation constant (B_{max}/K_D) ratio. If the target resides at an intracellular location or is surrounded by a blood barrier with tight junctions, often additional structural modifications need to be made to increase membrane permeation. Even if these requirements are met, tracers can still fail as imaging agents as other parameters, such as efflux transporter affinity, high nonspecific binding, off-target binding, or undesirable kinetics (too slow or to fast) can limit the applicability. To maximize chances of lead identification, in silico molecular docking studies can be implemented in the high-throughput screening of potential scaffolds, with an additional benefit that it is very cost friendly.[111]

The use of predictive in vitro and in silico algorithms is also implied in CNS PET tracer development.[112] These in silico and in vitro assays are based on molecular and physicochemical parameters, including LogP, LogD, Mw, topological polar surface area, number of hydrogen bond donors, and pKa. Further, passive permeability assays can be conducted on cells harboring efflux transporters (P-glycoprotein or multidrug resistance protein 1) to identify substrates for these receptors. The development of in silico, distribution, metabolism, and excretion calculation algorithms can further aid in lead identification of CNS PET tracers. [112,113]

In Vitro: Cell Binding Internalization, Tissue Binding, and Blocking

A promising tracer candidate has to be evaluated for its specificity for the target under investigation. This can be performed on cells or tissue which express the target. To assess the specificity of binding, the tracer is incubated with or without a nonstructurally related compound with known affinity for the target. This is generally known as blocking or competition study in which the blocking agent is usually present in molar excess (eg, 100 μM) compared to the radiotracer, and competitively decreases the fraction of bound radiotracer.

To evaluate the efficacy of therapeutic radiopharmaceuticals, a cell viability assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay) assay) and cell survival assay (clonogenic assay) can be conducted, using the appropriate control experiments.[114] Cellular experiments also allow to determine the rate of internalization of receptor-bound radiopharmaceuticals. Internalization studies can be performed at 37°C and 4°C, as internalization normally does not occur at 4°C. High internalization rate might be a strategy in targeted radionuclide therapy to tackle the problem of uncontrolled redistribution of recoiled atoms when using radionuclides with multiple alpha-emitting daughters.[86]

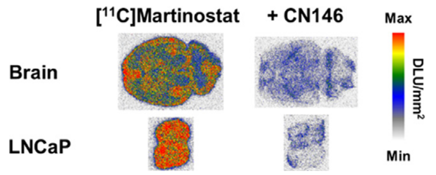


Figure 5 In vitro autoradiography study on rat brain and murine grown LNCaP tumor sections. Slices are incubated with [^{11}C]martinostat (radiotracer used for visualization of histone deacetylase) with/without structural unrelated inhibitor CN146 (100 μM). The residual signal in the "blocked" tissue indicates nonspecific binding.

In vitro autoradiography studies (Fig. 5), allow for initial estimation of specific binding of the radiotracer to the tissue of interest (eg, brain, tumor, heart,...). Hereby, only a limited amount of radioactivity is required, resolution is relatively high, confounding factors such as BBB passage or metabolism are omitted and relevant human tissues can be used.

In Vivo Animal Models: Knock-Out, Vector-based Models

After determination of in vitro specific binding, a radiotracer can be advanced to in vivo studies, where the pharmacokinetic properties can be further evaluated. Noninvasive imaging with smaller PET or SPECT scanners (μPET, μSPECT) allows visualization of the tracer distribution. Further, dynamic imaging can be combined with arterial sampling and quantification of radiometabolites for exact quantification of pharmacodynamic parameters such as B_{max} and K_d.[1]

Pharmacokinetics of radiotracers that are labeled with radionuclides that do not allow SPECT or PET imaging can be determined by ex vivo biodistribution studies in which radioactivity is quantified using gamma or scintillation counting.[115]

In vivo blocking studies can also be conducted, where small animals are pretreated with structural unrelated compounds, to assess the in vivo binding specificity. A displacement experiment, where a blocking agent is administered after injection of the radiopharmaceutical, can be used to determine whether the radiopharmaceutical binds reversible or irreversible to its target.

To assess tracer binding to targets only expressed in certain disease states (eg, neuroinflammation or cancer), specific animal models can be generated. To evaluate the properties of a radiopharmaceutical for oncological application, rodents can be inoculated with auto- or xenograft-tumor cells. Subsequently, these animals can be scanned to determine tumor uptake and selectivity for the malignant tissue over healthy tissues.[116]

To evaluate the therapeutic efficacy of a therapeutic radiopharmaceutical, tumor-bearing mice can be injected with the therapeutic radiopharmaceutical in a single dose, or in a repeated dose interval scheme. Tumor growth can be evaluated before and during treatment using a diagnostic sister radiopharmaceutical, or physically using a Vernier caliper. Further, [^{18}F]FDG μPET scans can be performed to quantify functional tumor tissue. Finally, weight, survival, and toxicity to the liver and kidneys should be evaluated and blood values should be monitored.

Animal models of some CNS disorders can be generated by stereotactic injection of cytotoxic agents (eg, lipopolysaccharide) in brain where the corresponding contralateral site can be used as control.[117] Similarly, cardiovascular disorders can be studied in animals where stroke or hypertension is induced via surgical procedures or cytotoxic agents.[118]

Also transgenic animal models which lack expression of the target (knock-out)[119] or overexpress a disease-related protein can be used to evaluate the radiotracer.[120,121]

An elegant way to establish target overexpression can be performed using viral vectors. These are derived from replicating viruses and are used to transfer genes into cells. The viral genome has been replaced by the gene of interest. When this vector is introduced into the target tissue, the target gene will be introduced, without propagation of the viral vector. The contralateral side can be used as a control by injecting a nontarget expressing viral vector (Fig. 6).[122] As a last step toward first-in-man studies, PET studies on nonhuman primate studies can be conducted as they more closely resemble the human organism.[109]

Specific Challenges of Radiopharmaceuticals

Production

The production of a radiopharmaceutical consists of (a) production of the radionuclide (b) incorporation of the radionuclide in the radiopharmaceutical and (c) purification and reformulation (Fig. 7). For diagnostic radiopharmaceuticals, the production time should be as short as possible to avoid loss of activity due to decay during the production process.

Radionuclides are generally obtained from a nuclear reactor (generally β^- emitters for radionuclide therapy), a cyclotron (generally β^+–emitters or nuclides decaying by electron capture) or a radionuclide generator (eg, ^{99}Mo/^{99m}Tc; ^{68}Ge/^{68}Ga; ^{89}Sr/^{89}Rb). The nuclear data for production of relevant radionuclides have recently been reviewed by Qaim et al.[123]

Incorporation of the radionuclide in the desired radiopharmaceutical can be achieved by "kit preparation" in which the generator eluate is added to a vial (labeling kit) that contains all necessary ingredients required to chemically bind the radionuclide to the vector molecule. Besides the vector molecule as such, the labeling kit may also contain reducing agents (eg, $SnCl_2$ in the case of ^{99m}Tc-labeling kits), buffers and antioxidants. Chelation of radiometals is particularly suited for kit preparation as it is compatible with an aqueous environment, proceeds quickly and provides high yields, the latter allowing to omit a purification step. Kit preparation is common for ^{99m}Tc-radiopharmaceuticals but has recently also been developed for ^{68}Ga-radiopharmaceuticals.[124] The manipulations required for kit preparation are almost identical to reconstitution of IV medication, but the kit preparation involves a chemical reaction whereas the reconstitution only results in merely dissolving the active ingredients.

Since the kit-based radiopharmaceutical is intended for IV injection, its preparation should avoid microbiological contamination. Therefore, the use of a shielded laminar air

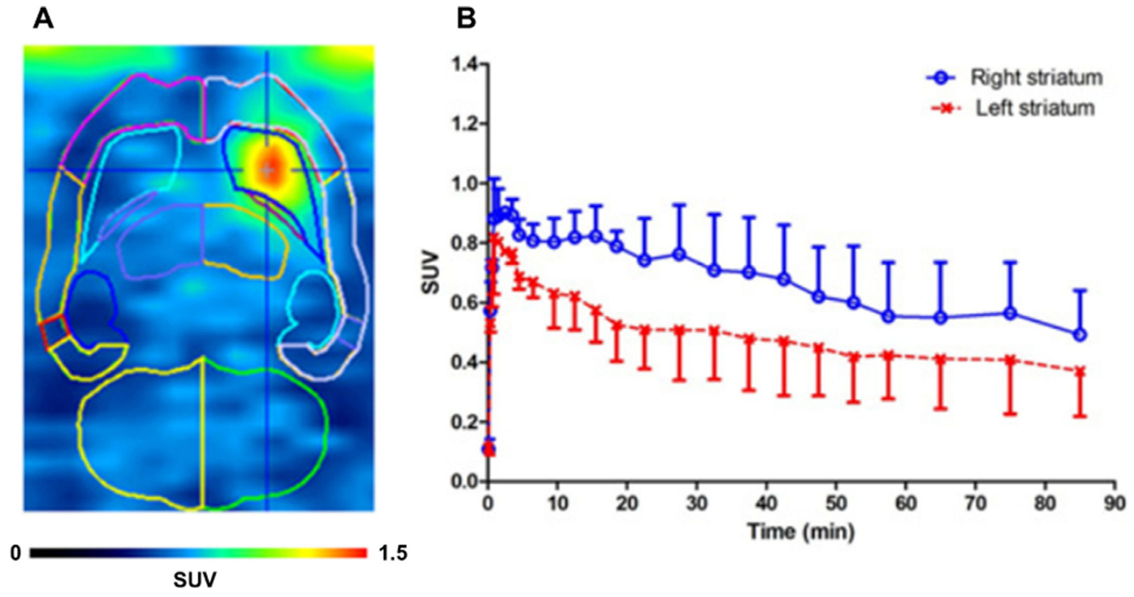


Figure 6 Example of μPET imaging with [^{11}C]JNJ-54173717 of a viral vector rat model where the P2 × 7 receptor is overexpressed in the right striatum. (right striatum, rAAV_3flag-hP2 × 7R, and left striatum, rAAV_3flag- GFP). (A) Averaged baseline image of a 90-minute dynamic scan. (B) Corresponding time-activity curves. Data were normalized for injected radioactivity and body weight of animal (standardized uptake values [SUV]). Error bars correspond to SD. Figure adapted from ref.[122]

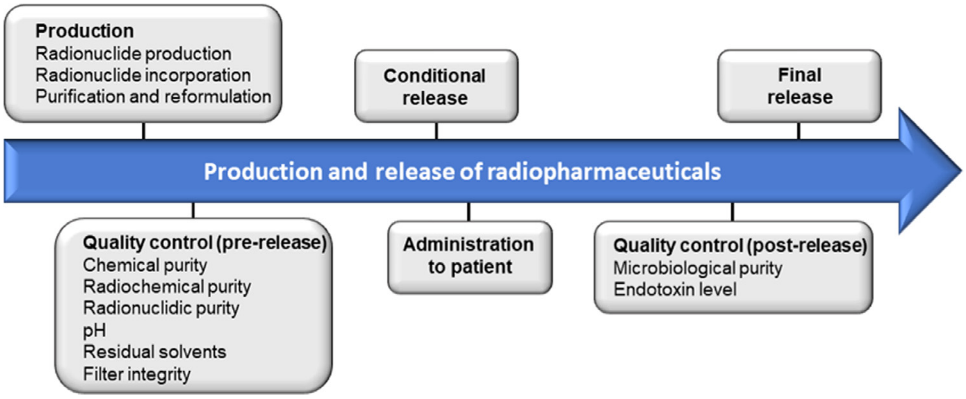


Figure 7 Schematic overview of production and release of radiopharmaceuticals.

flow hood is recommended and staff should be trained in aseptic processing practices and validated by media fill simulations.[125]

For more complex radiosynthesis procedures, as it is generally the case for ^{18}F- and ^{11}C-radiopharmaceuticals, automated synthesis modules can be used. These modules allow complex multistep synthesis followed by cartridge or HPLC purification. Automated modules can consist of systems with fixed tubing or with disposable cassettes, the latter reducing the risk of cross contamination between subsequent production batches. Automated modules provide the flexibility to produce different radiotracers, each requiring specific production program sequences and purification methods. In addition, high reproducibility and robustness of the radiosynthesis procedures are obtained and moreover, radiosynthesis procedures can easily be exchanged between different users of the same type of module.

Purification is usually required to remove the vast excess of precursor organic solvents and reagents (eg, kryptofix 222) from the radiolabeled reaction product. Purification using disposable cartridges is simple and fast and has also been combined with solid phase supported radiolabeling.[126] Compared to cartridge purification, HPLC purification usually affords better resolution which may be required to separate the chemically similar precursor from the radiopharmaceutical but has a higher level of complexity.

Care should be taken to avoid decreasing the chromatographic resolution between the radiopharmaceutical and impurities caused by the combination of large injection volumes and potentially high elution strength of the reaction mixture solution. If biocompatible mobile phases containing organic solvents such as ethanol are used, the isolated peak can be simply diluted to an appropriate concentration of ethanol.[127]

In case class I or class II solvents such as acetonitrile are used for preparative HPLC, a reformulation of the isolated peak using solid phase extraction chromatography and desorption with ethanol will be required. HPLC columns used for purification of radiopharmaceuticals should be rinsed after each use and stored in a solvent that inhibits microbial growth.[128]

In view of the small mass amounts of the produced radiopharmaceuticals, radiosynthesis methods have successfully been miniaturized applying microfluidic and microliter droplet technology.[129,130]

The last step in the production consists of sterile filtration of the radiopharmaceutical solution using a disposable membrane filter which is chemically compatible with formulation solution and does not exhibit excessive "sticking" of the small mass amounts of the radioactive compound. To avoid sticking of lipophilic compounds to the filter membrane, the solution with a high concentration of the radiopharmaceutical can be filtered prior to dilution to a lower ethanol concentration. As the radiopharmaceutical will be conditionally released prior to obtaining the results of the sterility test (see below), a filter integrity test ("bubble point test") should be performed postfiltration.[131]

Stability and Quality Control

Quality control of radiopharmaceuticals includes assay of the chemical, radiochemical, radionuclidic, and microbiological purity of radiopharmaceuticals (Fig. 7). Yet again, the quality control process needs to be established fast, to avoid loss of activity of the radiopharmaceutical due to decay.

The radiochemical purity of ^{99m}Tc-radiopharmacetucals is usually determined using (instant) TLC ([i]TLC). For radiopharmaceuticals, (i)TLC allows fast and precise quantification of the radiochemical purity by either cutting and counting the (i)TLC strips or determining the activity distribution using dedicated scanning or autoradiography systems or by quantification of Cerenkov luminescence.[132,133] Besides its low cost and simplicity radio-(i)TLC has the advantage that all (except for volatile) radiolabeled compounds will be detected, also those that stay on the application point.

HPLC provides a higher resolution compared to (i)TLC and allows to use combinations of different types of serial detectors (eg, UV-VIS, fluorescence, electrochemical, evaporative light scattering, radioactivity, MS,...) so that the chemical and radiochemical purity can be determined simultaneously. The simultaneous assay of radioactivity and mass of the radiopharmaceutical allows to determine its MA. Validation of an HPLC method can include determination of the recovered activity, as radiolabeled compounds that are not eluted from the column will remain undetected, resulting in an overestimation of the radiochemical purity. As an example, ^{18}F-fluoride was found to be only partially recovered from C_{18}-columns eluted with acidic mobile phases.[134]

Compared to radio-HPLC, the analysis time can be reduced and the detection sensitivity can be increased by using radio-UPLC (ultrahigh pressure liquid chromatography) analysis or microfluidic quality control systems.[135,136]

Radiolysis is the decomposition of the radiopharmaceutical induced by direct or indirect effect of radiation and is a specific issue for radiopharmaceuticals. Radiolysis depends on the activity concentration, the MA, the chemical structure of the radiopharmaceutical and the position of the radiolabel. Radiolysis can occur at any stage during or after production of the radiopharmaceutical. The stability of the radiopharmaceutical in the formulation solution at a specific radioactivity concentration should be verified to define its shelf-life. Ascorbic acid/sodium ascorbate and ethanol can be used as free radical scavengers, effectively reducing radiolysis.[137] The presence of ascorbic acid will however generate large UV peaks in HPLC QC chromatograms and its effect on the pH of the formulation should be verified.

In case organic solvents or heavy metals were used during synthesis or purification, their residual level in the formulation solution can be quantified using gas chromatography or inductively coupled plasma mass spectrometry, respectively. The level of organic solvents in the injection volume of the radiopharmaceutical should be below the daily exposure limits specified by ICH Q3C-R7[128] and ICH Q3D[138], respectively.

Other radionuclides present in the radiopharmaceutical are termed radionuclidic impurities and are usually generated as impurities of the radionuclide production process, or alternatively, they can be generated during the decay as daughter nuclides of the principle radionuclide (eg ^{212}Pb).[139] Radionuclides that emit gamma rays can be identified by a multichannel gamma spectrometer coupled to a sodium iodide crystal or a Ge(Li)detector.[140]

As most radiopharmaceuticals are administered intravenously, their microbiological purity is of high importance. The sterility of the radiopharmaceutical solution is determined by direct inoculation of appropriate growth media and inspection of the growth media at 7 and 14 days after incubation. The results of the sterility tests will thus only be available after administration of the radiopharmaceutical (Fig. 7), hence the importance of validation of the production process especially with regard to sterility. Endotoxins can be quantified in the injection solution using a gel-clot or rapid photometric method.[141]

From Tracer to Radiopharmaceutical: Regulatory Challenges

Advancing from preclinical studies towards clinical application, where the radiotracer becomes a radiopharmaceutical, requires several regulatory hurdles that needs to be overcome, as recently reviewed by Kolec et al.[142]

ICH M3 R2 provides guidance with regard to the nonclinical safety studies that need to be performed prior to first-in-man studies.[143] For PET tracers, the microdosing approach can be used, in which only limited toxicology testing (one species, single administration of a 1000-fold higher dose) is required.

In order to proceed to first clinical evaluation, a clinical trial application needs to be drafted and submitted for review by the local ethical committee and the competent authorities. In the European Union (EU), the clinical trial application consists of an investigators brochure (an investigational medicinal product dossier (similar to the Investigational New Drug Application (IND) in the United States (US)) and the study protocol.[144,145] Further, radiopharmaceuticals are regulated by medicine agencies and nuclear agencies. United States Pharmacopeia (USP) and European Pharmacopoeia monographs are available for a limited number of radiopharmaceuticals but also provide general guidance. In the EU, novel radiopharmaceuticals for clinical studies need to be produced in agreement with Good Manufacturing Practice (GMP) requirements (see below).

A specific EU regulation guideline for Good Manufacturing Practice (GMP) compliant production of radiopharmaceuticals can be consulted on the European Medicines Agency (EMA) website (Eudralex Volume 3 Radiopharmaceuticals)[146] as well as on the Pharmaceutical Inspection Co-operation Scheme Annex 3).[147] For the US, the Federal Drug Agency (FDA) published specific regulations on production of PET radiopharmaceuticals.[148] The international atomic energy agency (IAEA) recently published a document providing practical support for the introduction of new radiopharmaceuticals to clinical use.[149]

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