SYNOPSIS

Medical imaging techniques presently play a vital role in the clinical management of the patient disorders and diseases and have witnessed enormous growth over the past few decades. While conventional imaging techniques such as X-rays, CT, MRI, etc. provide anatomical imaging, molecular imaging agents have enabled non-invasive assessment of biological and biochemical processes inside the human body with high sensitivity and specificity. Among the various molecular imaging techniques, nuclear medicine that employs radiopharmaceuticals is the most convincing, as it detects molecular and cellular changes of diseases with high precision.¹

Radiopharmaceuticals are compounds where the molecules have radioisotope attached to it and are used as drugs routinely in nuclear medicine for diagnosis and therapy of various diseases. The choice of radioisotope determines whether the application is diagnostic or therapeutic. Diagnostic radiopharmaceuticals are labeled with either gamma emitting or positron emitting isotopes, and are used to obtain highly precise and detailed morphologic structure of organ or tissues as well as the physiological function of an organ, through the dynamic distribution of the radiotracer. These are used at very low concentrations in the range of $10^{-6}$ to $10^{-8}$ M and are not intended to have any pharmacological effect. Therapeutic radiopharmaceuticals are the molecules designed to deliver therapeutic doses of ionizing radiation at specific diseased tissue or sites.

The chemical composition of a radiopharmaceutical could be considered to consist of a target/carrier molecule, which is generally an organic moiety with a biological role, to which a radioactive isotope of an element is chemically attached either by covalent or coordinate bond (Radiolabeling). The carrier molecule can be a biomolecule such as antibody, protein, drug, etc. or can also be a simple organic molecule, which may not have
any target specificity, but once radiolabeled, due to its overall chemical structure exhibits preferential localization in a specific target organ (metal-essential radiopharmaceuticals). The radioisotopes used for labeling are non-metals, transition metals and lanthanides. Particulate emitting isotopes such as $^{131}$I, $^{32}$P, $^{153}$Sm, $^{90}$Y, $^{177}$Lu, etc. are used for therapeutic purposes whereas some of the radioisotopes most commonly used for diagnostic purposes are $^{18}$F, $^{99m}$Tc, $^{123}$I, etc. ²

Although during the advent of nuclear medicine, $^{131}$I, $^{203}$Hg, $^{198}$Au which emit $\beta$-particles as well as $\gamma$-rays have been used for diagnostic purpose, the images obtained with these isotopes lack the resolution that is possible when single photon emitters such as $^{99m}$Tc or $^{125}$I or positron emitters such as $^{18}$F are used. The technological advances over the past 3-4 decades have resulted in highly precise and well resolved images through Single Photon Emission Computed Tomography (SPECT) and Positron Emission Tomography (PET). ³

The thesis work is aimed at preparation of novel $^{99m}$Tc based SPECT agents, wherein, $^{99m}$Tc is used for the radiolabeling of different carrier molecules and evaluated for biological behavior. The metal, $^{99m}$Tc, exhibits ideal nuclear properties ($t_{1/2} \sim 6$ h, $\gamma$-energy $\sim 140$ KeV) for diagnostic imaging and several other attributes, that makes it the most widely used diagnostic radionuclide (> 80% of all nuclear medicine imaging) and hence earned it the name “Workhorse of Diagnostic Nuclear Medicine”. Further the availability of $^{99m}$Tc from a $^{99}$Mo-$^{99m}$Tc generator facilitates its easy availability on a daily basis at hospital radiopharmacies.

Technetium is a transition metal and can exist in multiple oxidation states (-1 to +7) and hence is amenable for radiolabeling a variety of molecules through various strategies. The most popular labeling strategy followed for its incorporation into a carrier molecule is bi-functional chelating agent (BFCA) approach. A bi-functional chelate is a multidentate ligand (eg: Ethylene diamine tetra-acetic acid), which has appropriate ligating groups for co-
ordinating the metal and also contains a functional group for covalent attachment to the targeting molecule.

The versatile chemical nature of $^{99m}$Tc has led to its existence in various core forms such as $^{99m}$Tc-oxo core ($[^{99m}\text{Tc}=\text{O}]^{3+}$), $^{99m}$Tc-carbonyl core ($[^{99m}\text{Tc}(-\text{CO})(\text{H}_{2}\text{O})_{3}]^{+}$), $^{99m}$Tc-Hydrazino nicotinamide core ($[^{99m}\text{Tc}-\text{HYNIC}$]) and so on. A metal core is a substitution-labile technetium complex where a few co-ordinating positions are tightly bound to the metal center while other positions are labile and exhibit a marked reactivity towards ligands having amenable co-ordinating atoms. $^{99m}$Tc exists in different oxidation states in different cores and hence bind with chelates with different donor groups.$^{4}$

The most commonly used $^{99m}$Tc core is $[^{99m}\text{Tc}=\text{O}]^{3+}$ where $^{99m}$Tc exists in $+V$ oxidation state. In the recent past, there has been growing interest in $^{99m}$Tc-nitrido ($[^{99m}\text{Tc}≡\text{N}]^{2+}$) core with $^{99m}$Tc in $+V$ oxidation state, which is isoelectronic to the conventional $[^{99m}\text{Tc}=\text{O}]^{+3}$ core. This core, unlike the oxo core, has the advantage that it is highly stable to redox conditions and pH variations. Further, owing to its excellent stability, this core also enables formation of $^{99m}$Tc labeled biomolecules at high specific activity (with use of relatively lower amounts of ligand molecules in comparison with the $[^{99m}\text{Tc}=\text{O}]^{+3}$ core) which in turn is an advantage for imaging receptors or antibody binding lesions. Recently, several complexes containing this core have exhibited attractive myocardial imaging features and are in clinical evaluation for emerging as new radiopharmaceuticals.$^{5-7}$

$[^{99m}\text{Tc}≡\text{N}]^{+2}$ core is reported to have affinity towards soft donor atoms such as S and P and the core forms complexes with square pyramidal geometry. However, it has been observed that ligand backbone containing four donor groups is not suitable for complexation with this core. The complexes formed with bi-dentate donor ligand are preferred with two ligand molecules involved in co-ordination with the core. The complexes formed in this way are symmetric $[2+2]$ with $^{99m}\text{Tc}≡\text{N}$ occupying the apical position and the other four donors
occupying the basal plane. Dithiocarbamates and xanthates are suitable bi-dentate chelates, which are reported to form stable symmetric complexes with $[^{99m}\text{Tc}\equiv\text{N}]^{2+}$ core.$^{5,8}$

The symmetric labeling approach is well suited for metal essential radiopharmaceuticals. However, for target specific molecules such as proteins, fatty acids, etc. such a labeling approach leads to radiolabeled complexes with two targeting molecules attached to one $[^{99m}\text{Tc}\equiv\text{N}]^{2+}$ core. This in turn results in undesired in-vivo pharmacokinetics, thereby affecting the target uptake and non-target distribution pattern inside the body. To circumvent this problem, the pioneering researchers in the area introduced a long straight chain (seven membered) bi-dentate phosphorus ligand ($\pi$-acceptor), with nitrogen atom as a heteroatom in between (PNP ligand) to complex with $[^{99m}\text{Tc}\equiv\text{N}]^{2+}$ core. PNP ligands, due to the bulkiness and steric effects, do not form symmetric [2+2] core. This new intermediate on reaction with bi-dentate chelating ligands carrying $\pi$-donors as co-ordinating atoms lead to novel type of mixed ligand [2+2] complexes. Complexes prepared using this $[^{99m}\text{TcN(PNP)}]^{2+}$ intermediate and bi-dentate ligands with $\pi$-donor atoms like SS or NS or SO have been reported to be stable. Cysteine, dithiols and dithiocarbamates have been found to be suitable as bi-dentate chelators for complexation with the $[^{99m}\text{TcN(PNP)}]^{2+}$ intermediate.$^{7,9-14}$

The amino acid, cysteine is a useful BFCA to complex with $[^{99m}\text{TcN(PNP)}]^{2+}$ intermediate, through either $\text{NH}_2$ and $\text{S}^-$ or $\text{COO}^-$ and $\text{S}^-$, to form stable asymmetric [2+2] $^{99m}\text{Tc}$-nitrido complexes. The bioactive molecules can be linked to the cysteine moiety and then labeled with $^{99m}\text{Tc}$ via $[^{99m}\text{TcN(PNP)}]^{2+}$ intermediate. The nature of donor groups, decides the overall charge of the asymmetric [2+2] complexes.$^9$
Aim and scope of the work

Based on the symmetric [2+2] and asymmetric [2+2] labeling approaches, an attempt has been made to prepare a significant number of $^{99m}$Tc labeled preparations using $[^{99m}\text{TcN}]^{2+}$ core, for their potential use as myocardial and hypoxic imaging agents. The significance of the work is to evaluate the versatility of $[^{99m}\text{TcN}]^{2+}$ core by modifying the labeling approach and correlating the change in the in-vivo biological behavior. Also, the importance of this $[^{99m}\text{TcN}]^{2+}$ core for target specific radiopharmaceuticals has been explored by labeling different biomolecules for varied biological applications.

CHAPTER 1 Introduction

This chapter deals with the basic concepts of radiopharmaceutical research and its development. Right from the selection of target/ carrier molecule, choice of radionuclide and its production, to the chemistry to be followed for its incorporation in a carrier molecule is described. Apart from the chemical synthesis of radiopharmaceutical, the quality control parameters required for the evaluation of a new radiolabeled formulation are discussed. This includes all the in-vitro and in-vivo biological testing required before the clinical trial of the radiopharmaceutical for in-vivo patient use. The chapter ends highlighting the aim and the scope of the work carried out in the present thesis.

CHAPTER 2 Symmetric [2+2] complexes

This chapter gives an idea about the simple derivatization desired in the lead molecule for the introduction of $[^{99m}\text{TcN}]^{2+}$ core, to obtain symmetric [2+2] complexes with suitable biological characteristics. The chapter has been divided into two parts, where syntheses of
three symmetric $[^{99m}TcN]$-complexes suitable for myocardial imaging are presented. There are two categories of myocardial agents used for diagnostic imaging viz. myocardial perfusion imaging and myocardial metabolic imaging. The first part deals with symmetric [2+2] complexes prepared using dithiocarbamate ligands for perfusion imaging, whereas the next part deals with xanthate symmetric [2+2] complex for metabolic imaging.

The perfusion agents are based on the blood flow through the myocardium and the difference in uptake of the blood in the cardiac muscle is used as a measure for the detection of cardiovascular diseases. The $^{99m}$Tc based perfusion agents used clinically are $^{99m}$Tc-Methoxy Iso butyl Isonitrile (MIBI) and $^{99m}$Tc-6,9-bis(2-ethoxyethyl)-3,12-dioxa-6,9 diphosphatetradecane (Tetrofosmin). These positively charged agents available clinically are far from ideal and get retained in the myocardium for a long time which poses logistic problems to the patient. In this context, $[^{99m}TcN(NOEt)_2]$, a neutral complex, has been shown to hold promise as a good myocardial perfusion imaging agent and is currently under clinical evaluation. However, the results obtained using $[^{99m}TcN(NOEt)_2]$ are still not as ideal as one may desire and hence the search for an improved agent forms a relevant field of research. In this respect, two new $[^{99m}TcN]$-dithiocarbamate complexes were synthesized and evaluated for their biological performance. The results of bio-distribution are compared with the standard agent $[^{99m}TcN(NOEt)_2]$.

The work involved synthesis of two dithiocarbamate ligands tertiary butyl dithiocarbamate (TBDTC) and methoxy isobutyl dithiocarbamate (MIBDTC) followed by their radiolabeling with $[^{99m}TcN]^2+$ core. The rationale behind the synthesis of these two ligands is their structural similarity with the clinically used ligands for perfusion imaging, tertiary butyl isonitrile (TBI) and methoxyisobutyl isonitrile (MIBI). The bio-distribution results of the two complexes showed reasonable uptake in the myocardium. However, the
results of TBDTC and MIBDTC complexes in comparison to the standard agent NOEt were sub-optimal.

In the second part of the work, \([^{99m}TcN]\)-fatty acid-xanthate symmetric complex was prepared and evaluated as a marker for metabolic cardiac imaging. Fatty acids are the main source of energy for the normal myocardium. A diseased myocardium undergoes a change in the fatty acid metabolism, leading to altered uptake and clearance characteristics, thereby making them useful target biomolecules for cardiac imaging. The metabolic markers currently used for myocardial imaging are \(^{123}I\) labeled fatty acids viz. \(^{123}I\)-Iodophenyl pentadecanoic acid and \(^{123}I\)-beta-methyl iodo phenyl pentadecanoic acid.\(^{15}\) However, the limited availability of \(^{123}I\) via cyclotron contribute to unfavorable logistics, and hence the quest for \(^{99m}Tc\)-labeled fatty acid for metabolic imaging continues to form a relevant field of research.

The work involved synthesis of a xanthate derivative of 15-hydroxy pentadecanoic acid, its radiolabeling with \([^{99m}TcN]\)^{2+} core and subsequently its bio-evaluation in Swiss mice. The complex showed undesirable \textit{in-vivo} pharmacokinetic behavior due to the bulkiness of the final complex. This restricts the use of this complex for the aforementioned purpose.

**CHAPTER 3 Asymmetric [2+2] neutral complex**

This chapter highlights the usefulness of \([^{99m}TcN]\)^{2+} core for labeling target specific biomolecules. The symmetric [2+2] labeling approach is not suitable for target specific molecules such as proteins, fatty acids, etc. as the complexes formed are bulky with unfavorable \textit{in-vivo} biological characteristics. This poses a problem for the labeling of target specific molecules via the base core. This problem was addressed by formation of asymmetric complexes, wherein only one biomolecule was complexed with \([^{99m}TcN]\)^{2+} core.
Biomolecules could be suitably derivatized with bi-dentate ligands containing donor atoms such as SS/ NS/ OS leading to final asymmetric \([^{99m}\text{TcN(PNP)}]\)-complexes having single bioactive moiety per \(^{99m}\text{Tc}\) metal centre.

The present chapter uses 16 carbon fatty acid biomolecule, as a representative example, for the present labeling strategy. The radiolabeled fatty acids, as discussed in chapter 2, are known targets for myocardial metabolic imaging. In the present case, long chain acid has been terminally linked with an amino group of cysteine (BFCA) in a four step synthetic procedure. The target ligand, 16 carbon fatty acid-cysteine conjugate, was then radiolabeled with \([^{99m}\text{TcN}]=2^+\) core, in combination with bi-dentate PNP6 ligand (bis-phosphine ligand), leading to final asymmetric complex. The nature of BFCA cysteine donor groups involved in final complexation with \([^{99m}\text{TcN(PNP)}]=2^+\) intermediate are (SH, COOH) which results in a neutral complex. The final complex after purification was used for carrying out the biological studies.

The biological studies involved in-vitro (cysteine challenge studies, serum stability studies and octanol/ water partition coefficient determination) and in-vivo bio-distribution studies. The results of bio-distribution have been compared with the standard agent \(^{125}\text{I}-\text{Iodophenylpentadecanoic acid (IPPA)}\). Here, \(^{125}\text{I}\) was used as the radiolabel instead of clinically used \(^{123}\text{I}\). The latter isotope is not available in India so \(^{125}\text{I}\) was used as a rationale substitute for the present work. The results of the bio-distribution studies of the complex in Swiss mice showed low myocardial uptake alongwith rapid washout, compared with \(^{125}\text{I}-\text{IPPA}\), thereby, limiting its utility for the metabolic imaging. However, the initial uptake and washout kinetics as observed from non-target organs such as blood, liver and lungs are superior compared to the standard agent. Thus, the present study gives an insight into the amenable synthetic derivatization that can be carried in biomolecules, for the preparation of \([^{99m}\text{TcN}]\) fatty acid complexes with excellent non-target clearance characteristics.
Apart from 16 carbon fatty acid-cysteine conjugate, two PNP ligands were also synthesized as a representative example of such categories of ligands. The synthesized PNP ligands were used for the preparation of $[^{99m}\text{TcN}(\text{PNP})]^{2+}$ intermediate and characterized by HPLC.

**CHAPTER 4 Asymmetric [2+2] charged complex**

In this chapter, the versatility of $[^{99m}\text{TcN}]^{2+}$ core is explored by varying the charge and lipophilicity of the final asymmetric complex. The $[^{99m}\text{TcN}(\text{PNP})]^{2+}$ intermediate is having an overall charge of +2. This intermediate leads to *in-vivo* inert complexes on complexation with bi-dentate ligands having SS/ NS/ OS donor groups. The amino acid cysteine is a useful bi-functional chelator for this intermediate, where a biomolecule can be linked to either an amino or the carboxylic acid functionality of cysteine and leaving either (SH, COOH) or (SH, \(\text{NH}_2\)) groups, respectively, available for co-ordination. The nature of bi-dentate donor groups influences the charge of the complexes. Thus, when cysteine is used as the BFCA, then structural analogues of the final $[^{99m}\text{TcN}]^{2+}$ complexes carrying different charges can be prepared by changing the mode of the conjugation of cysteine with the carrier biomolecule.

In the present work, the effect of charge has been evaluated by synthesizing a uni-positively charged structural analogue of the neutral complex reported in chapter 3. The biodistribution results of the two complexes have been compared together with $^{125}\text{I}$-IPPA to assess the effect of charge on the *in-vivo* pharmacological behavior and to evaluate the potential of charged fatty acid complex for metabolic cardiac imaging.

A 16-cysteiny1 hexadecanoic acid conjugate was synthesized in a six step synthetic procedure starting with 16-bromohexadecanoic acid. The ligand on reaction with $[^{99m}\text{TcN}]^{2+}$ core together with PNP6 ligand, formed the required positively charged complex. The complex after HPLC purification was used for *in-vivo* studies in Swiss mice. In terms of
absolute uptake, the positively charged complex performed better than the neutral analogue reported earlier. The positively charged fatty acid complexes, prepared using $[^{99m}\text{TcN}(\text{PNP})]^{2+}$ core seems to be better candidates for the development of myocardial metabolic tracers than their neutral counterparts.

The lipophilicity in an asymmetric $[^{99m}\text{TcN}]$-complex can be varied by changing the lateral alkyl groups present on the phosphorus and nitrogen atoms in PNP ligand. Also, the lipophilicity can be altered by introducing a linker between a BFCA and the biomolecule. In the present work, the latter approach was followed for the synthesis of a series of ligands with variable lipophilicities. The synthesis of ligands with different linkers was achieved by conjugating the commercially available fatty acids of different chain length (11, 12, and 15 carbons) with the acid group of cysteine in a similar fashion as that carried out for unipositively charged 16 carbon fatty acid complex. These were then used to obtain unipositively charged asymmetric $[^{99m}\text{TcN}]$-fatty acid complexes. The results of bio-distribution of the three positively charged 11, 12 and 15 carbon fatty acid complexes were compared with those obtained with 16 carbon complex, to see the effect of lipophilicity. The fatty acid complexes showed steady increase in the initial myocardial uptake values with increase in chain length, however, associated with slower clearance from the non-target organs.

Thus, the present study reviews the flexibility of $[^{99m}\text{TcN}]^{2+}$ core for labeling similar molecules with varied chemical nature and in-vivo biological characteristics.

**CHAPTER 5** $[^{99m}\text{TcN}]^{2+}$ core for other imaging applications

In this chapter, $[^{99m}\text{TcN}]^{2+}$ core is used for labeling a nitro-triazole derivative, a known hypoxia marker. This chapter highlights the usefulness of $[^{99m}\text{TcN}]^{2+}$ core for labeling molecules with different in-vivo diagnostic applications other than the myocardial imaging discussed in previous three chapters.
The search for an ideal hypoxia-imaging agent requires high in-vivo stability of the labeled preparation, rapid accumulation of activity in hypoxic regions of tumors, sufficient retention times therein, and rapid clearance of activity from other non-target tissues to provide better contrast between lesion and background. However, the clearance of the activity from the blood with time must be optimum, so as to allow sufficient time for the accumulation of activity at the target hypoxic site.

Among the different $[^{99m}\text{TcN}]^{2+}$ tagging methods, the use of asymmetric approach was followed which yields complexes with high in-vivo inertness and excellent non-target clearance characteristics. Asymmetric [2+2] neutral complex was formed in a manner, similar to that discussed in chapter 3. Sanazole, a nitrotriazole derivative was coupled to a cysteine residue in a four step synthetic procedure, which was then radiolabeled using $[^{99m}\text{TcN(PNP)}]^{2+}$ core. The product after HPLC purification was used for carrying out the in-vivo bio-distribution studies in swiss mice bearing fibrosarcoma tumor. The complex showed low uptake which remained constant over the limited period of study. Though, retention of activity is observed in tumor, it could not be fully ascertained due to low uptake values. However, the rapid clearance of activity from the background organs, favors the use of this core for similar applications.

References