Preparation of bifunctional ligands for radiometals conjugation to biomolecules

Marinović, Marina

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UNIVERSITY OF ZAGREB
FACULTY OF CHEMICAL ENGINEERING AND TECHNOLOGY
GRADUATE UNIVERSITY STUDY PROGRAMME

Marina Marinović

MASTER THESIS

Zagreb, July 2016.

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MASTER THESIS

Mentors: Assistant Professor, Ján Kozempel, Ph.D.

Full Professor, Silvana Raić-Malić, Ph.D.

Co-mentor: Assistant Professor, Martin Vlk, Ph.D.

Examination committee members:

1. Full Professor, Silvana Raić-Malić, Ph.D.
2. Associate Professor, Danijela Ašperger, Ph.D.
3. Postdoctoral researcher, Andrijana Meščić, Ph.D.

Zagreb, July 2016.

SVEUČILIŠTE U ZAGREBU
FAKULTET KEMIJSKOG INŽENJERSTVA I TEHNOLOGIJE
SVEUČILIŠNI DIPLOMSKI STUDIJ

Marina Marinović

Priprava bifunkcionalnih liganada za konjugaciju s radiometalima u biomolekulama

DIPLOMSKI RAD

Voditelji rada: dr.sc. Ján Kozempel, doc.

dr.sc. Silvana Raić-Malić, red.prof.

Komentor: dr.sc. Martin Vlk, doc.

Članovi ispitnog povjerenstva:

1. dr.sc. Silvana Raić-Malić, redovni profesor
2. dr.sc. Danijela Ašperger, izvanredni profesor
3. dr.sc. Andrijana Meščić, poslijedoktorand

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SUMMARY

The choice of the chelating fragment of bifunctional ligand depends on the nature and oxidation state of the radiometal. There are numerous examples in the literature, which show that the nature of a bifunctional metal complex such as geometry, lipophilicity, overall charge plays a crucial role in determining the biodistribution of targeted radiopharmaceuticals. Furthermore, in designing of novel radiopharmaceuticals energy of the radionuclide should be taken into account.

Cyclen and cyclam based macrocycles are known to form kinetically inert and thermodynamically stable complexes with a wide range of metal ions. Several classes of structurally different phosphonate ligands labeled with β emitting radionuclides such as ^{166}Ho-DOTMP complex are being used clinically for the treatment of painful bone metastases.

The choice of used cyclic chelating agents is also based on the more pronounced thermodynamic stability and kinetic inertness of their lanthanide complexes when compared to that of their acyclic analog. The physical, magnetic and nuclear properties of the lanthanide ions have made them ideal for use in both diagnostic and therapeutic radiopharmaceuticals.

The aim of this work was to prepare bifunctional ligands based on cyclen and cyclam structures, test their labelling conditions with europium as a model radionuclide, in order to develop potential new radiopharmaceuticals.

Through this aim, cyclen and selected tetraphosphonate derivative of cyclam (TETP) were synthesized. Obtained data show that all used ligands, DOTA, TETP, NOTA and TRAP-Pr, form complexes with Eu(III) at 95 ºC and in the pH range from 2 to 6. They also show excellent kinetic stability at pH 4.

Key words: radiopharmaceuticals, chelating agents, DOTA, TETP, europium

SAŽETAK RADA

Izbor kelatnog fragmenta bifunkcionalnog liganda ovisi o prirodi te oksidacijskom stanju radiometala. Postoje brojni primjeri u literaturi, koji pokazuju da priroda bifunkicionalnog metalnog kompleksa kao što je geometrija, lipofilnost, ukupni naboj, ima ključnu ulogu u određivanju biodistribucije ciljanih radiofarmaceutika. Nadalje, u razvoju novih radiofarmaceutika također se treba uzeti u obzir i energija radionuklida.

Poznato je da makrociklički spojevi temeljeni na strukturi ciklena i ciklama tvore kinetički inertne i termodinamički stabilne komplekse sa širokim rasponom metalnih iona. Nekoliko klasa strukturno različitih fosfonatnih liganada obilježenih s β-emitirajućim radionuklidima kao što je ^{166}Ho-DOTMP kompleks se koriste klinički za liječenje bolnih koštanih metastaza.

Izbor korištenih cikličkih kelatnih agensa se također temelji na naglašenoj termodinamičkoj stabilnosti i kinetičkoj inertnosti njihovih kompleksa s lantanidima u odnosu na koje njihove acikličke analoge. Fizikalna i magnetska svojstva iona lantanida čine ih idealnim za primjenu u dijagnostičke i terapijske svrhe radiofarmaceutika.

Cilj ovog rada bio je pripraviti bifunkcionalne ligande temeljene na strukturi ciklena i ciklama, testirati njihove uvjete obilježavanja s europijem kao modelnim radionuklidom, u cilju razvoja novih potencijalnih radiofarmaceutika.

Imajući to na umu, ciklen i odabrani tetrafosfonatni derivat ciklama (TETP) su pripravljeni. Dobiveni podaci pokazuju da svi korišteni ligandi, DOTA, TETP, NOTA i TRAP-Pr, tvore komplekse s Eu(III) pri 95 °C u rasponu pH vrijednosti od 2 do 6, te također pokazuju odličnu kinetičku stabilnost pri pH 4.

Ključne riječi: radiofarmaceutici, kelatni agensi, DOTA, TETP, europij

ABBREVIATIONS

BFC	bifunctional chelating agents
PET	positron emission tomography
SPECT	single photon emission computed tomography
MIRD	medical internal radiation dosimetry
LET	linear energy transfer
MRI	magnetic resonance imaging
CT	computed tomography
NMR	nuclear magnetic resonance
Cyclen	1,4,7,10-tetraazacyclododecane
Cyclam	1,4,8,11-tetraazacyclotetradecane
DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
DOTP	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis(methylphosphonic acid)
H_2DO_2A	1,4,7,10-tetraazacyclododecane-1,7-diacetic acid
H_4DO_2P	1,4,7,10-tetraazacyclododecane-1,7-bis(methylphosphonic acid)
TETA	1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid
TETP	1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetrakis(methylphosphonic acid)
NODASA	1,4,7-triazacyclononane-1-succinic acid-4,7-diacetic acid
NODAGA	1,4,7-triazacyclononane-1-glutaric acid-4,7-diacetic acid
H_4TE_2P	1,4,8,11-tetraazacyclotetradecane-1,8-bis(methylphosphonic acid)
Tacn	1,4,7-triazacyclononane
NOTA	1,4,7-triazacyclononane-1,4,7-triacetic acid
TRAP-Pr	l,4,7-triazacyclononane-l,4,7-tris[methyl(2-carboxyethyl)phosphinic acid
DTPA	diethylenetriaminepentaacetic acid

TLC	thin layer chromatography
CAs	chelating agents
UV	ultraviolet light
ESI-MS	electro spray ionization mass spectroscopy
FT-IR	Fourier transform infrared spectroscopy

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1. INTRODUCTION

Cancer is a generic term for a large group of diseases that can affect any part of the body. Other terms used are malignant tumors and neoplasms. One defining feature of cancer is the rapid creation of abnormal cells that grow beyond their usual boundaries, and which can then invade adjoining parts of the body and spread to other organs, the latter process is referred to as metastasizing. Cancer is a leading cause of death worldwide and accounted for 8,2 million deaths in 2012. Metastases are the major cause of death from cancer [1].

Nuclear medicine is a medical modality that is used to diagnose and treat diseases in a safe and painless way. Nuclear medicine procedures permit the determination of medical information that may otherwise be unavailable, require surgery or expensive and invasive diagnostic tests. The procedures often identify abnormalities very early in the progression of the disease – long before medical problems are apparent with other diagnostic tests. This early detection allows a disease to be treated sooner in its course, when a more successful prognosis may be possible [2].

The history of radionuclide therapy can be traced back to the early 1900s, after the discovery of radioactivity by Henri Becquerel and Marie Curie. In 1903, Alexander Graham Bell suggested placing sources containing radium in or near tumors, and in 1913, Frederick Proescher published the first study on the intravenous injection of radium for therapy of various types of diseases. In the last twenty years, radionuclide therapy has been widely used in various clinical malignant and pain management applications. Radionuclide therapy has the advantage of delivering a highly concentrated absorbed dose to the targeted tumor while sparing the surrounding normal tissues. In addition, the selective ability of radionuclide therapy is advantageous in the treatment of systemic malignancy, such as in bone metastases where whole body irradiation using external beam radiotherapy is impossible. Since the administration of radionuclides is minimally invasive and the duration of treatment is shorter than chemotherapy, targeted radionuclide therapy has become one of the most preferred types of cancer therapy [3].

2. THEORETICAL PART

2.1. Radiopharmaceuticals

The use of specific radiotracers called radiopharmaceuticals for diagnosis or therapy of diseases is a unique capability of nuclear medicine. Radiopharmaceuticals are drugs containing a radionuclide. They are mostly small organic or inorganic compounds with definite composition. Radiopharmaceuticals can also be macromolecules such as monoclonal antibodies and antibody fragments that are not stoichiometrically labeled with a radionuclide. Depending on their medical applications there are two primary classes: diagnostic and therapeutic radiopharmaceuticals.

Receptor-based radiopharmaceuticals are of great interest in molecular imaging and radiotherapy of cancers, and provide a unique tool for specific delivery of radionuclides to the targeted tissues. In general, a target-specific radiopharmaceutical can be divided into four parts: targeting biomolecule (BM), pharmacokinetic modifying (PKM) linker, bifunctional coupling or chelating agent (BFC), and radionuclide.

Almost all radiopharmaceuticals are administered *via* intravenous injection [4].

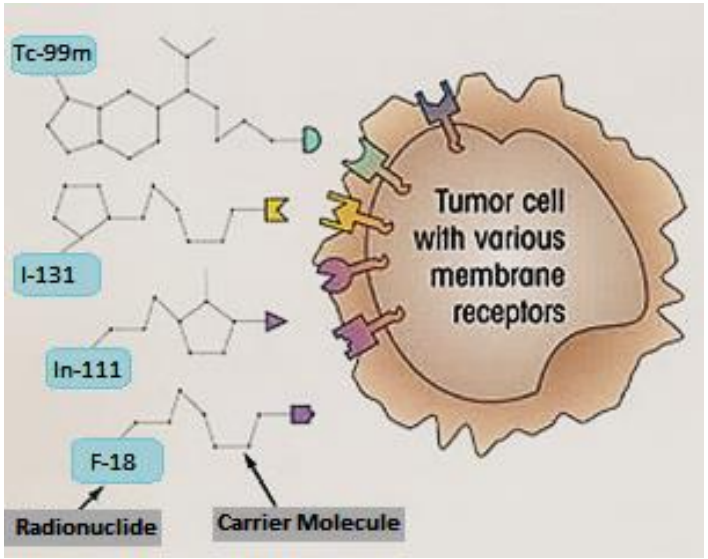


Figure 1. Targeting individual receptors with specific radiopharmaceuticals

2.2. Radionuclides

A radionuclide is a source of radiation [4]. Nature has provided a vast array of radionuclides with emission properties that make them valuable reagents for investigating basic problems in chemistry, biology, and medicine. These properties include α, β, γ, and Auger emissions which are useful for medical diagnostic (γ–scintigraphy, SPECT, PET) and therapeutic applications [5].

Both physical and biochemical characteristics are important for a therapeutic radionuclide. The considerations for physical characteristics include the physical half-life, type of emissions, energy of the radiation(s), daughter product(s), method of production, and radionuclide purity. The biochemical characteristics include tissue targeting, retention of radioactivity in the tumor, *in vivo* stability, and toxicity. The most important factor to be considered when choosing a therapeutical radionuclide is the effective half-life, which is the net half-life considering both physical half-life (T_p) and biological half-life (T_b) within the patient's body or organs. The determination of effective half-life (T_e) is explained in the medical internal radiation dosimetry (MIRD) calculation method, which is summarized as:

$$T_e = \frac{T_p\, T_b}{(T_p + T_b)} \qquad (1)$$

A suitable range of the physical half-life for therapeutic radionuclides is between 6 h and 7 d. A very short physical half-life limits the delivery flexibility and is very impractical, while a long half-life will retain the radiation dose and expose the patient for a longer period. On the other hand, the biological half-life depends on the used tracer. In addition, the tracer should have sufficient retention so that radiation can be delivered to the tumor efficiently. If the biological half-life is too short, the radionuclide will be discharged with a significantly high activity, resulting in the need for extensive radioactive waste management. Therefore, for an efficient radiation delivery, a balanced optimal biological and physical half-life should be chosen, which results in an optimal effective half-life.

For therapeutic purposes, radiations with high linear energy transfer (LET), such as α and β particles, are preferred. These types allow very high ionization per range. Some β-emitting radionuclides decay with γ-radiation. This associated γ-radiation could be advantageous if the energy and intensity are within the diagnostic range, as it provides the ability to visualize distribution of the radiopharmaceutical within the patient's body using gamma scintigraphy methods. Depending on the type of tumor, the energy and intensity of the

emitted radiation should be chosen so that the energy and intensity of non-penetrating radiation (i.e., α and β particles) are high enough compared with the penetrating radiation (i.e., γ radiation, X-rays, or Auger electrons), if present. The other factor to be considered is the daughter product of the radionuclide. If the daughter nuclide is not stable, it may contribute to the total amount of absorbed dose. An ideal radiopharmaceutical should be able to decay into a stable daughter product, such as ^{153}Sm, which fully decays into stable ^{153}Eu. **Table 1** shows a summary of physical characteristics of the commonly available therapeutic radionuclides [3].

Table 1. Physical characteristics of commonly available therapeutic radionuclides

Radio-nuclide	Physical half-life	Decay mode	Max E_{β^-} (keV) [% intensity]	β^- range in soft tissue (mm) min	max	Daughter nuclide	Clinical indication
^{32}P	14,26 d	β	1710	2,6	7,9	^{32}S	Polycythemia vera, cystic craniopharyngioma
^{89}Sr	50,53 d	β	1496 [100,0]	2,4	8,0	^{89}Y	Painful bone metastasis
^{90}Y	64,10 h	β	2280,1 [100,0]	3,6	11,0	^{90}Zr	Hepatic metastasis, PVNS, RIT for NHL
^{117m}Sn	13,60 d	IT	130*,150*	0,22	0,27	^{117}Sn	Bone tumor treatment
^{131}I	8,02 d	β	606 [89,3]	0,4	2,4	^{131}Xe	Hyperthyroidism, thyroid cancer, RIT for NHL and neuroblastoma
^{153}Sm	46,50 h	β	808,2 [100,0]	0,7	3,1	^{153}Eu	Painful bone metastasis, synovitis
^{169}Er	9,40 d	β	350	0,3	1,0	^{169}Tm	Synovitis
^{177}Lu	6,73 d	β	497,8 [100.0]	0,28	1,7	^{177}Hf	Synovitis, RIT for various cancer treatments
^{186}Re	3,72 d	EC, β	1069,5 [92,5]	1,2	3,6	^{86}Os (unstable)	^{186}V Painful bone metastasis, painful arthritis
^{223}Ra	11,44 d	α	5979,2α		<10μα	^{219}Rn (unstable)	Palliative treatment of bone metastasis

The use of these metallic radionuclides has necessitated the development of metal chelating agents to effectively provide a handle over their behavior. These chelating agents have been termed “bifunctional chelating agents” since they have a metal binding moiety function and a chemically reactive functional group.

There are a number of fundamental criteria that have to be taken into account in the design of bifunctional chelating agents for such applications, for example stability of the metal complex. Clearly, the consequences of loss or dissociation of the radionuclide are associated with toxicity in the case of therapeutics and poor image qualities for diagnostics. Fundamental coordination chemistry criteria such as: charge, matching cavity size of the chelating agent with the ionic radius of the radionuclide, providing the appropriate chelate denticity or number of donor binding groups and providing donor binding groups of appropriate chemical character are all key elements. Two additional properties are also critical to consider: the rate at which the metal complex forms and the rate of dissociation. All of these criteria are interrelated [5].

2.3. Positron emission tomography (PET)

In recent years, Positron Emission Tomography (PET) has become a practical, high performance clinical imaging modality for visualization of biological process within the living system. The growth in PET imaging over the past decades has mainly been fueled by the success of [^{18}F]fluorodeoxyglucose (FDG), which has become an indispensable tool for cancer diagnosis as well as for monitoring response to therapy in various types of malignancies [6].

PET is a non-invasive, *in vivo* imaging technique that uses relatively short-lived positron-emitting radioisotopes either in their pure form or as part of a larger molecule designed and synthesized with the isotope either incorporated within or appended onto the structure. The phenomenon of emitting a positron, which is defined as the antimatter particle to an electron, allows the unstable isotope to shed some of its unnecessary positive charge transforming the atom into a more stable atomic form. Once a positron has been emitted, it will travel a small distance, dictated by the amount of energy the particle was emitted with, from its origin and then encounter an electron, which are plentiful in comparison to the number of positrons. Once this occurs, the two particles will come together and destroy each other in an event termed annihilation. This process converts the total mass of both the positron and electron to pure energy in the form of two 511 keV photons of light traveling 180º away from each other.

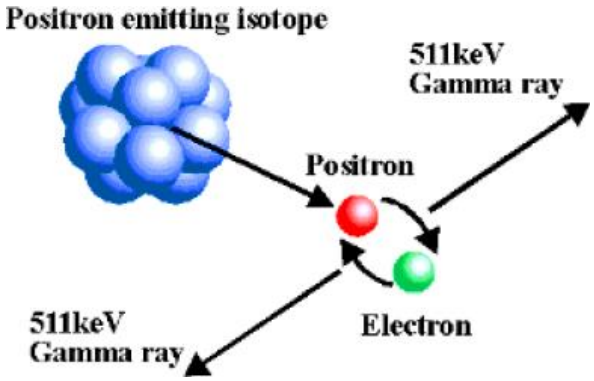


Figure 2. Schematic showing positron emission and subsequent annihilation with an electron

Detection of these photons is traditionally accomplished by placing the subject into a ring of detectors that have been programmed to differentiate between actual positron decay, i.e. two photons striking detectors on opposite sides of the ring, and background radiation that may be coming from a variety of sources. In an effort to probe the physiological processes occurring within an organism, radioisotope-containing drugs are injected into the organism of interest and then given time to localize in the areas for which it was designed to target. Once localization has occurred, the amount of radiation within the targeted type of tissue or the tissue containing a greater amount of the molecular species of interest will be much larger than the surrounding tissue; this is referred to as contrast [7].

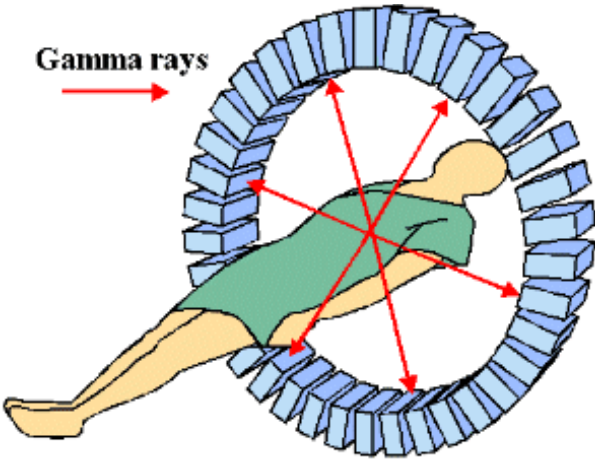


Figure 3. Schematic showing a PET scanner detecting divergent γ rays

The use of traditional short-lived isotopes is restricted to probing rapid biological processes and events occurring over the duration of several hours or days. Due to the ongoing development of novel biological targeting vectors such as antibodies, peptides and proteins, which are characterized by a wide range of biological half-lives ranging from hours to days, nonstandard PET radioisotopes have recently been produced and investigated as they provide a wide range of physical half-lives that are more compatible with the biological half-life of particular targeting vectors for the design of novel and more beneficial radiopharmaceuticals. Among those nonstandard isotopes, radiometals such as zirconium, yttrium, indium, gallium and copper have received increased attention [6].

Table 2. Commonly used PET radionuclides, their half-lives, range, positron energy, and production source

Isotope	Half-life (min)	Positron Energy (MeV)	Positron range	Production Source
^{82}Rb	1,26	3,15	1,7	generator
^{15}O	2,03	1,70	1,5	cyclotron
^{13}N	9,97	1,19	1,4	cyclotron
^{11}C	20,3	0,96	1,1	cyclotron
^{18}F	109,8	0,64	1,0	cyclotron
^{64}Cu	768	0,66	n/a	cyclotron
^{68}Ga	67,72	1,90	2,9	generator

PET has been widely used in both basic research and clinical settings for imaging tissue pharmacokinetics, tumor response, cell proliferation, gene expression and the status of receptors or tumors along with the diagnoses of heart disease, epilepsy, and stroke [7].

2.4. Magnetic resonance imaging (MRI)

Tomography of magnetic resonance (Magnetic Resonance Imaging, MRI) has gained great importance in the last three decades in medicinal diagnostics as an imaging technique with a superior spatial resolution and contrast. The most important advantage of MRI over the competing radio-diagnostic methods such as X-Ray Computed Tomography (CT), Single-Photon Emission Computed Tomography (SPECT) or Positron Emission Tomography (PET) is definitely no use of harmful high-energy radiation. MRI is a diagnostic medical technique used to identify pathological regions within the body. Physical principles of MRI rely on monitoring different distribution and properties of water in the examined tissue and also on a spatial variation of its proton longitudinal (T_1) and transversal (T_2) magnetic relaxation times [8].

In the absence of a magnetic field, the magnetic moments of proton nuclei are randomly oriented, but on application of a strong external magnetic field, they precess around an axis which is aligned parallel or antiparallel with the direction of the applied field. This creates two states, a low energy state which is aligned with the field and a high energy state opposed to the field, where the energy difference between the two states is given by:

$$\Delta E = h\nu \qquad (2)$$

Where h is Planck's constant, $6{,}626 \times 10^{-34}$ Js, and ν is Larmor or resonance frequency. This frequency is unique for protons and proportional to the applied magnetic field. The lower energy state is more populated than the higher one. A pulse of electromagnetic radiation at the Larmor frequency is then used to excite nuclei from the lower to the higher energy state. Subsequently, these protons relax back to the lower state by emission of a pulse of electromagnetic radiation, and it is this pulse that can be detected, measured and converted into a signal. More accurately, it is the difference in the total absorbed (nuclei excited) and emitted (nuclei relaxing) electromagnetic radiation that is measured, so the signal intensity is proportional to the population of the two energy states. The macroscopic property being visualised on a magnetic resonance image is the distribution and concentration of water in body tissues. This gives a contrast between tissue of different types, such as bone and skin, but also differentiates between healthy and malignant tissues [9]. The contrast obtained in images is commonly due to different relaxation rates of protons found in different tissues. Contrast enhancement can be achieved via the use of so-called contrast agents, which have the

ability to shorten either T_1 or T_2 relaxation times [7]. An example of an image generated in this way is shown in Figure 4.

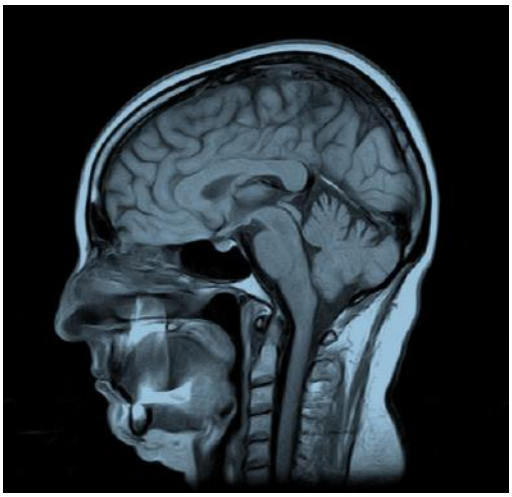


Figure 4. A regular MRI brain scan

2.5. Bifunctional chelating agents

The use of metal or radiometal complexes in medicine as therapeutic or diagnostic agents is an area of growing interest and commonly requires bifunctional chelators (BFC). Bifunctional chelating agents are small molecules containing a chelating unit, able to strongly coordinate a metal ion, and a reactive functional group, devised to form a stable covalent bond with a suitable biomolecule. For this reason, BFC are used in diagnostic imaging, molecular imaging, and radiotherapy of cancer [10].

The choice of BFC is largely determined by the nature and oxidation state of the radiometal. Different radiometals require BFC with different donor atoms and chelator frameworks. An ideal BFC is able to form a stable radiometal chelate with high thermodynamic stability and kinetic inertness [4].

The optimal BFC should ideally fulfill the following requirements:

- *Stability/inertness:* The BFC should form thermodynamically stable and kinetically inert complexes to prevent any ligand exchange reactions or hydrolysis *in vivo*.
- *Rapid complexation kinetics*: Radiolabelling of the BFC should be efficient and rapid at low temperatures and low concentration at a pH that is suitable for biological targeting vectors.

- *Selectivity*: The BFC should selectively bind the radiometal of interest to avoid low specific activities during radiolabelling, e.g. due to the presence of other trace metals (decay products).
- *Versatile conjugation chemistry*: Flexibility in the conjugation of the BFC to functional groups of targeting vectors allows optimization of pharmacokinetics by adjusting the polarity of the overall conjugate.
- *Accessibility*: The preparation of the BFC should be straight-forward, quick and cost-effective, and be scalable to the preparation of multigram quantities of product with as few reaction steps as possible [6].

All of these properties provide some information that can be used to suggest potential *in vivo* suitability. Serum stability can be a very useful tool and model that serves to predict and eliminate from contention the bifunctional chelating agents that are unsuitable for *in vivo* applications. None of these properties or models is predictive of actual *in vivo* stability of the metal complex. To assess real *in vivo* stability of the metal complex, evaluation in an appropriate animal model is necessary. The definition of appropriate animal model is variable, however it should clearly reflect the ultimate intended biological application. As yet, no *in vitro* model system replicates all of the ongoing processes and components of a living organism so the therapeutic efficacy of a macromolecule cannot be predicted from *in vitro* results [5].

However, the success of a BFC is not guaranteed even when all above listed requirements are completely fulfilled because the nature of a BFC can have a profound impact on the pharmacokinetics of a radiopharmaceutical such as receptor binding and clearance from non-target tissues. The two most important parameters of a BFC that influence the *in vivo* properties of radiopharmaceuticals are the overall charge and the lipophilicity of the corresponding BFC metal complex. Obviously, the nature of the BFC metal complex can play a crucial role in determining the biodistribution of targeted radiopharmaceuticals, and thus, further research in the field of BFCs is warranted for a better understanding of these findings [6].

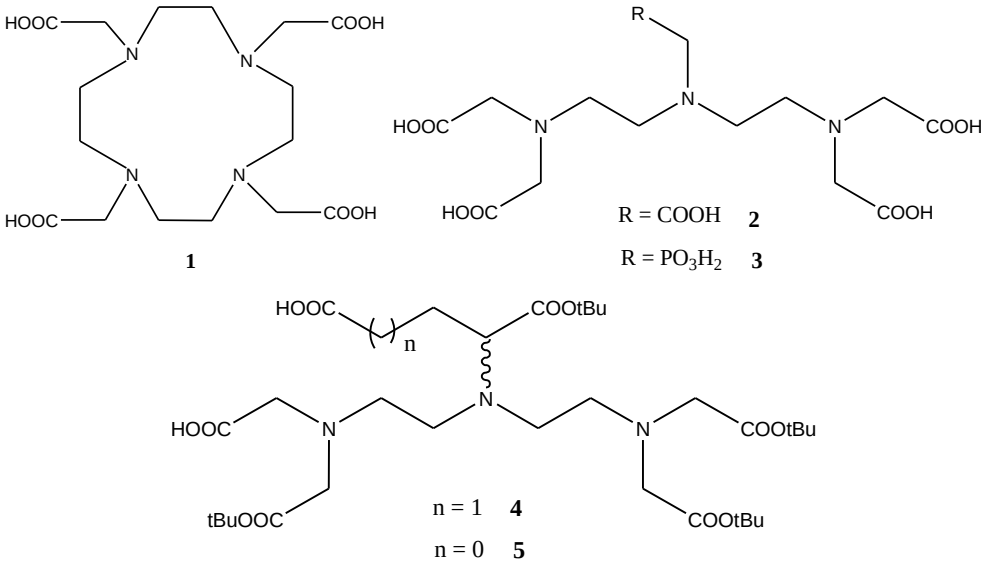
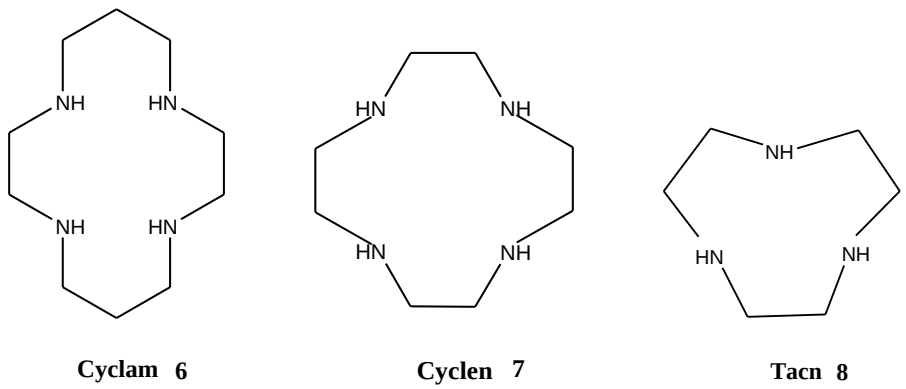


Figure 5. Common chelating agents and bifunctional derivatives

2.6. Polyazamacrocycles

Polyazamacrocycles have become a large and variable group of important organic substances because they represent irreplaceable ligands in complexes with transition metals, lanthanides and actinides, or they are chemical and structural bases of different anion receptors. One common group are tetraazamacrocycles, namely cyclam ***6*** (1,4,8,11-tetraazacyclotetradecane) and cyclen ***7*** (1,4,7,10-tetraazacyclododecane). Another important group are triazamacrocycles based on structure of tacn 8 (1,4,7-triazacyclononane). They have all found many applications as contrast agents for Magnetic Resonance Imaging (MRI), in optical imaging, Positron Emission Tomography (PET), Single-Photon Emission Computed Tomography (SPECT) and as radiotherapeutics [11].



Cyclam 6 **Cyclen 7** **Tacn 8**

Figure 6. Structures of cyclam 6, cyclen 7 and tacn 8

2.6.1. Factors for strong binding of metal ions

The two major areas of molecular organization, as related to coordination chemistry, are complementarity and constraint factors. To construct the ultimately stable metal/ligand complex, both of these factors should be maximized.

Complementarity may be described as a 'first-order' factor for complex stability; it is a requirement of stable complexes, but can only be improved to a finite level. Constraint, on the other hand, might be described as a 'second-order' factor. Constraint is concerned with ligand rigidity and complexity, factors of seemingly infinite variability.

These parameters can be manipulated to produce large jumps in complex stability compared with ligand systems that lack them, if care is taken to maintain the difficultly achieved complementarity relationships. The components of constraint are topology (here meaning the interconnectedness of ligand donor atoms) and rigidity (how fixed in space those donor atoms are with respect to each other). Among the two, topology is the most well studied, variously described in the chelate, macrocyclic, and cryptate effects.

The linking of two donor atoms together results in a chelate. Surprisingly, such a linkage results in a large increase in the binding constants with metal ions as compared with the separate donor groups. The common thermodynamic rationalization for the chelate effect points out the increase in entropy associated with chelate binding as compared with the binding of separate monodentate donors. Cyclam and cyclen each contain four nitrogen donors, and form metal ion complexes which are more thermodynamically stable than complexes comprising four monodentate ligands [12]. This can be explained by an increase in entropy of a system such as that shown in Figure 7. As the four monodentate ligands are displaced from the metal ion by the polyamine ligand, there is an increase in disorder; there are two molecules on the left hand side of the reaction but five on the right, giving a favorable entropic increase in stability [9].

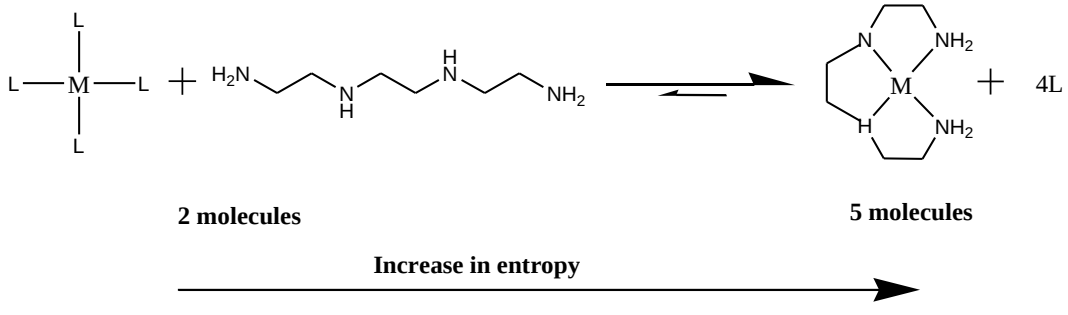


Figure 7. Entropy in the chelate effect

A second explanation of the chelate effect is the increased effective concentration of the second donor, because its distance from the metal ion is fixed by the link to the bound first donor. This distance is short compared with an unlinked second donor, whose average distance from the metal ion will depend primarily on its concentration (Figure 8). A variation of this rationalization of the chelate effect is that the formation of the second M-N bond is abnormally fast, compared with an unlinked second donor.

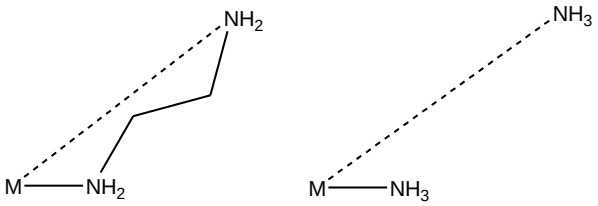


Figure 8. Tethering effect leading to increased effective concentration of the ligand

As might be expected, tying successive donor atoms together produces tri- or tetradentate chelates and further increases the metal complex stability as a function of the number of chelate rings. But, an impressively large additional stabilization occurs from linking the terminal donor atoms into a ring; this phenomenon was termed the macrocyclic effect [12]. The cyclam and cyclen macrocycles provide four nitrogen donor atoms for complexation of a host metal cation, however, substitution of the secondary amines can be undertaken to increase the number of donor atoms. This gives these macrocycles and their derivatives the ability to act as ligands for a range of metal cations which require coordination numbers between four and eight, including transition metals and lanthanoids [9].

2.6.2. Ligands based on cyclam and cyclen

Functionalized macrocycles are interesting in many respects. On the one hand, they are ideal ligands for studying changes in structures and stability when donor groups are introduced as pendant side chains. On the other hand, these compounds allow studying of metal-promoted reactions since the substrate, covalently attached through the side chain of the macrocycle, can be brought in close vicinity of the metal ion so that the formation of pseudo metal-substrate complex takes place [13].

Polyazamacrocycles with coordinating pendant arms form very stable complexes with a wide range of metal ions. The ligands encapsulate metal ions in the macrocyclic cavity and the complexes often exhibit both thermodynamic and kinetic stability. Convenient properties of the complexes have been explored for use in applications such as contrast agents in magnetic resonance imaging or for labelling of biological substances with metal radioisotopes for diagnostic and therapeutic purposes. For the latter of these uses, a metal ion is coordinated by a suitable bifunctional ligand, ensuring, as a result of strong metal binding, no deposition of harmful radioisotopes in the body, while also allowing conjugation of the complex to a biomolecule by means of another reactive group. Biomolecules such as small peptides, monoclonal antibodies or their fragments, and biotin can be labelled through a reactive group placed on the macrocycle rim or on a carbon atom of a pendant arm, as well as directly through an acetate pendant group with the formation of an amide functionality [14].

The study of the polyazamacrocycles with pendant arms has been focused mainly on acetates, phosphonates, phosphinates or carboxamides. Besides these groups, the ligands could contain also other pendant groups, for example, alcohol, phenol, or esters of carboxylate, phosphinate or phosphonate. Many structurally modified analogues have been prepared (Figure 9) such as the well-known N-substituted derivatives with a different number of pendant arms [15].



R = R' = H	cyclen	**7**			
R = R' = COOH	DOTA	**1**	R = R' = H	cyclam	**6**
R = R' = $CH_2PO_3H_2$	DOTP	**9**	R = R' = CH_2COOH	TETA	**12**
R = H, R' = CH_2COOH	H_2DO_2A	**10**	R = R' = $CH_2PO_3H_2$	TETP	**13**
R = H, R' = $CH_2PO_3H_2$	H_4DO_2P	**11**	R = H, R' = $CH_2PO_3H_2$	H_4TE_2P	**14**

Figure 9. Structural representations and abbreviations of selected macrocycles derived from cyclen 7 (left) and cyclam 6 (right)

Numerous analogues of DOTA 1 and TETA 12 have been developed over the past few decades for the efficient chelation of transition-metal ions, including those for potential medical applications [16].

2.7. Azamacrocycles in Positron emission tomography (PET) and Magnetic resonance imaging (MRI)

Among the various available PET radioisotopes, $^{64/67}Cu$ and ^{68}Ga offer many advantages over other traditionally used isotopes. With a half-life of 12,7 hours, ^{64}Cu is compatible with *in vivo* kinetics to investigate biodistribution and metabolism of compounds of interest using PET imaging. Additionally, this long half-life radioisotope has been shown to be useful for tracking cell migration and their cellular fate *in vivo*.

The challenge in the implementation of metal radioisotopes lies in the design of chelating agents that have the ability to not only retain the metal *in vivo*, but either become the targeting agent or be attached to a known targeting agent without adversely affecting the targeting properties or kinetics [7]. A range of such bifunctional ligands has been investigated for $^{64/67}Cu^{2+}$. Typically they are comprised of complexing agents based on the open-chain polyaminocarboxylate, tetraaza-, polyaminocarboxylate and polyaminophosphonate macrocycles and the hexaza-cage class.

The second radiometal that holds great promise for the design of novel radiopharmaceuticals for PET is gallium-68 ($t_{1/2}$ = 68 min). Gallium(III) strongly binds to highly ionic, nonpolarizable hard Lewis bases such as oxygen and nitrogen donors and forms thermodynamically stable complexes with carboxylate, phosphonate, hydroxamate, and amine functionalities [6].

Chelating agents for carrying ^{68}Ga radionuclide can be designed for therapeutic and targeted applications. The coordination position of Ga in the periodic table is such that it can bind strongly with macrocyclic/cyclic systems similar to lanthanides [17]. This is a direct consequence of the increased kinetic inertness of macrocycles compared to acyclic ligands due to the macrocyclic effect minimizing transchelation or loss of metal *in vivo*, which is favorable for radiopharmaceutical applications. The most prominent representatives of this category are ligands based on polyaza-macrocycles such as tacn 8 and cyclen 7, specially their bisphosphonate ligands. These ligands improved bone imaging and diagnosis and some of them were published as novel bone-seeking agents [6].

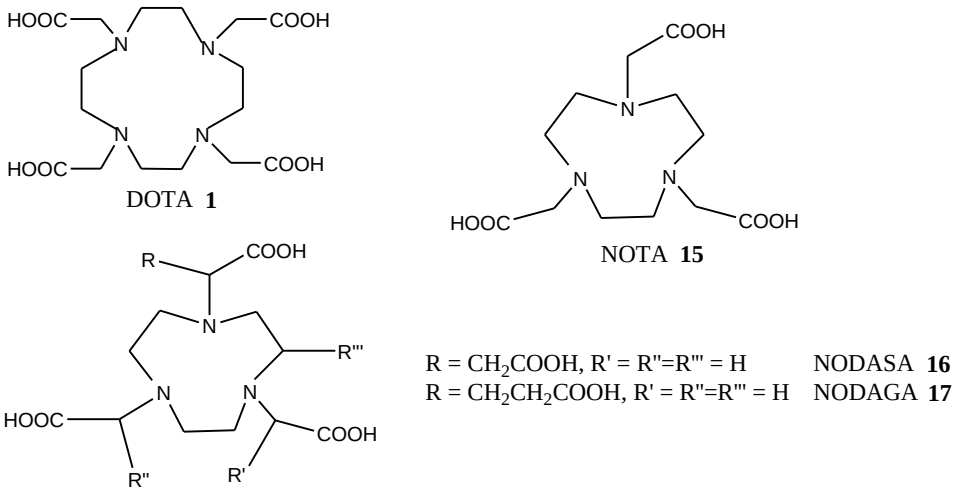


Figure 10. Macrocyclic ligands commonly used for ^{68}Ga radiolabeling

From the physical point of view, there are two major families of chelating agents classified according to the relaxation process they predominantly accelerate, T_1-CAs (paramagnetic) and T_2-CAs (superparamagnetic). Whereas T_1-CAs induce a positive contrast, i.e. a ^{1}H NMR signal of the affected tissue increases, compounds affecting the T_2 relaxation cause lowering of a local proton signal and, thus, they show a "negative enhancement" pattern.

At present, the most widespread family of the T_1-CAs consists of complexes with the Gd(III) ion. Although there are other candidates in the lanthanide series [europium(III) or terbium(III)] that have a high magnetic moment, the intrinsic relaxation time of the electron-spin state of the cation has to be long enough for efficient transfer of magnetic information to the bulk water. Thus, the prominent position of the Gd(III) ion relies not only on the high magnetic moment (7,9 BM) given by seven unpaired *f*-electrons, but also on the totally symmetric electronic state, which makes the electronic relaxation time much longer than for other Ln(III) ions. However, the main problem of the medical utilizations of heavy metal ions like the Gd(III) ion is a significant toxicity of their "free" (aqua-ion) form [18]. Therefore they need to be chelated to a ligand in order to be used *in vivo.* Common chelating agent designs that have been exploited are based on incorporating a cyclen macrocycle unit, DOTA 1 and a linear chelator diethylenetriaminepentaacetatic acid DTPA 2. They are both known to form lanthanide ion complexes of high stability. Gadolinium contrast agents are by far the most popular choice of contrast agent, with greater than 10 million MRI studies done each year [19]. Clinically used contrast gadolinium agents are shown in Figure 11.

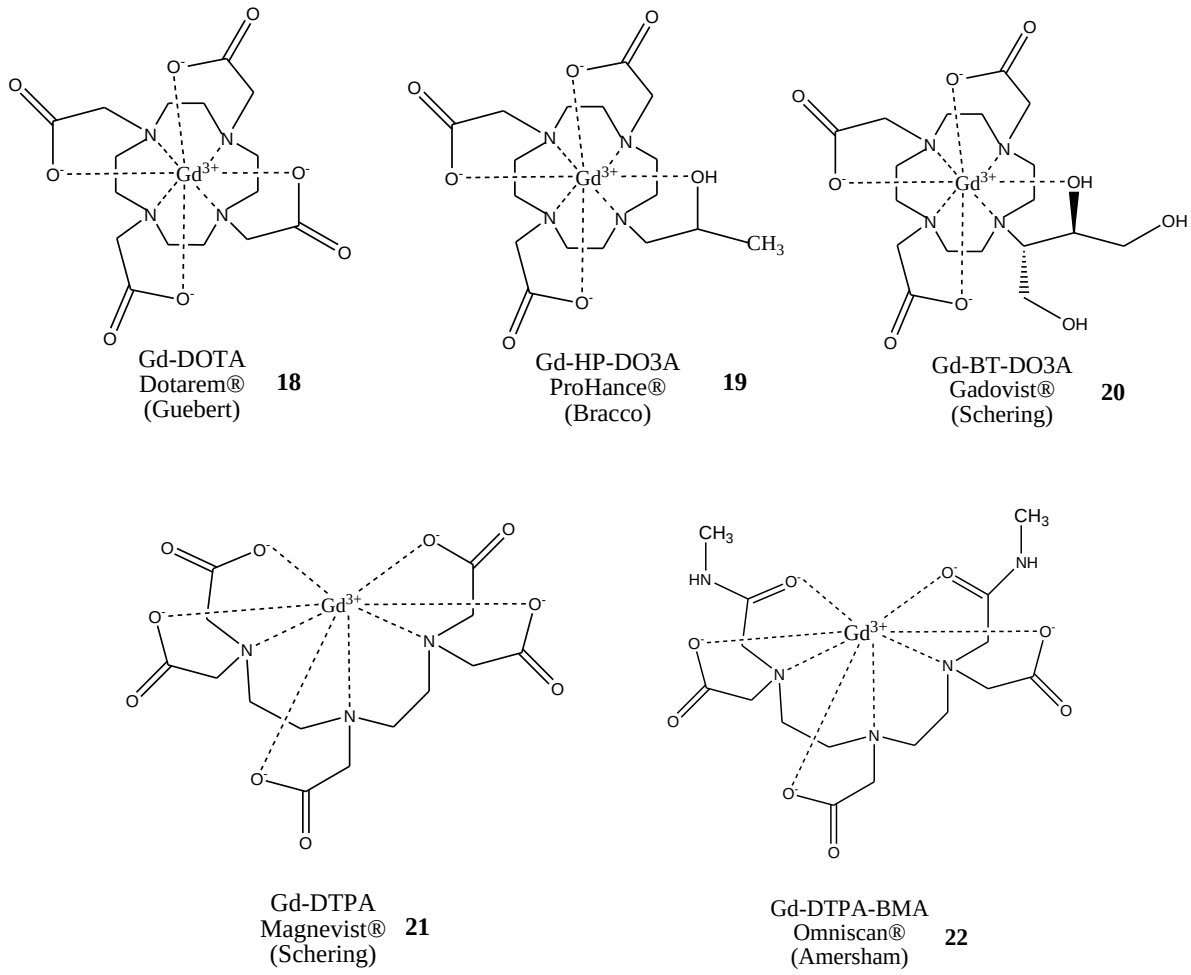


Figure 11. Clinically used gadolinium contrast agents

2.8. Lanthanide azamacrocycle complexes

The lanthanides are a family of highly electropositive metals in period 6, composed of a series of fifteen elements in the *f*-block that correspond to filling of seven 4*f* orbitals (Table 3).

Table 3. Lanthanide metals

$_{57}$**La**	$_{58}$**Ce**	$_{59}$**Pr**	$_{60}$**Nd**	$_{61}$**Pm**	$_{62}$**Sm**	$_{63}$**Eu**	$_{64}$**Gd**	$_{65}$**Tb**	$_{66}$**Dy**	$_{67}$**Ho**	$_{68}$**Er**	$_{69}$**Tm**	$_{70}$**Yb**	$_{71}$**Lu**

The chemistry of the lanthanides is dominated by their +3 oxidation state. Lanthanides have low charge densities, which leads to their compounds being predominantly ionic in character [19].

They form complexes of higher coordination numbers ranging from 7 to 12. This is attributed to the large size of these metal ions together with the ionic nature of the metal-ligand bonding. Macrocyclic ligands form stable complexes with lanthanides and actinides and hence they enable to explore the coordination chemistry of these metal ions. Azamacrocyclic complexes of lanthanides are currently attracting a lot of attention as radiopharmaceuticals, in radioimmunotherapy, in other medical applications, such as radioimmunoscintigraphy (γ-scintigraphy) and positron emission tomography, and as contrast-agents in magnetic resonance imaging [20].

Macrocyclic complexes with gadolinium(III), europium(III) and terbium(III) are being investigated as luminescent biological probes and are extensively used for studying metal ion sites in macrocyclic complexes and in biological systems. Some of them have been suggested as markers in cytology and immunology [19].

2.8.1. Europium azamacrocycle complexes

Europium is a silvery-white metal. It is the softest, least dense, and most volatile member of the lanthanide series.

Of the fourteen major radioactive isotopes, only four have half-lives long enough to be of potential interest. Three of these four long-lived isotopes, (europium-152, europium-154, and europium-155) have half-lives ranging from 5 to 13 years, and they decay by emitting a β particle. Europium-152 also decays by electron capture. A significant amount of energy in the form of γ rays accompanies the decays of europium-152 and europium-154. The half-lives of all other europium isotopes are less than four months.

Table 3. Radioactive properties of key europium isotopes

Isotope	Half-life (*yr*)	Specific activity (*Ci/g*)	Decay mode	Radiation energy (*MeV*)		
				Alpha (α)	Beta (β)	Gamma (γ)
Eu-150	34	70	EC	-	0,044	1,5
Eu-152	13	180	β, EC	-	0,140	1,2
Eu-154	8,8	270	β	-	0,290	1,2
Eu-155	5	470	β	-	0,063	0,061

Europium-152, europium-154, and europium-155 are produced primarily as fission products. Europium-152 can also be produced by neutron activation. When a fissile nuclide such as an atom of uranium-235 fissions, it asymmetrically splits into two large fragments which can include the three europium isotopes and two or three neutrons. The fission yield of europium-155 is about 0,03 %. That means about 3 atoms of europium-155 are produced per 10,000 fissions. The yields of the other two isotopes are much lower. In order to control this fission reaction, isotopes that can absorb excess neutrons are used in a nuclear reactor control rods. Europium-151 is a very good neutron absorber and neutron activation of this stable isotope produces europium-152 [21].

The γ -ray spectrum of the activated europium sample is shown in Figure 12. Downward and upward arrows indicate the respective ^{152}Eu and ^{154}Eu decay lines [22].

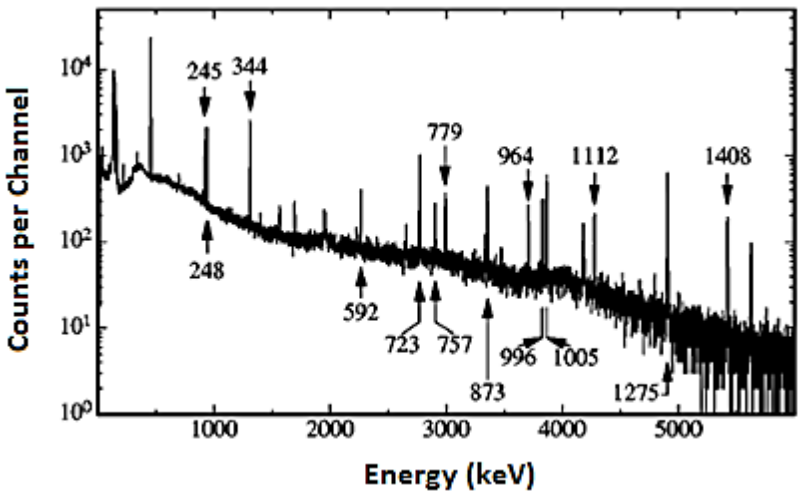


Figure 12. The γ-ray spectrum of the activated europium sample

The primary use of europium is in nuclear reactor control rods, because of its effectiveness in absorbing neutrons. Other uses have been limited because it is rare and thus very expensive.

Europium-doped plastics have been used as laser materials, and europium oxide serves as a phosphor activator [21].

Europium is toxic and needs to be chelated to a ligand in order to be used *in vivo.* Common chelator designs that have been exploited are based on a cyclen macrocycle DOTA 1, TETP 13 and a linear chelator DTPA 2. They all form high stability complexes with europium. [19].

2.9. Current issues and future prospects of therapeutic radionuclides

Drug delivery is an important part of targeted radionuclide therapy because merely developing an effective anticancer agent is not sufficient unless it is delivered to the site of action with an adequate dose. Conventional drug development has focused different routes of administration, mostly oral or injectable, but this is no longer an effective strategy. Nanotechnology, advanced polymer chemistry, and biomedical engineering have contributed to the development of novel methods of drug delivery that target specific tissues without causing too much collateral damage. Innovative methods of cancer treatment, e.g., cell and gene therapies, require innovative drug delivery concepts.

3. EXPERIMENTAL PART

3.1. General procedure

Ligands Cyclen and TETP were synthesized according to published procedures [23] [24]. Other three ligands used in this work (DOTA, NOTA, and TRAP-Pr) were obtained from the Department of Inorganic Chemistry, Faculty of Science Charles University in Prague.

TLC analysis of the ligands and intermediates during their synthesis were performed on Merck silica gel 60 TLC plates F254 and visualized by using UV light (365 nm) and iodine. Solvent system used for TLC was: $CHCl_3$:MeOH = 10:5.

Measurments of radiochemical yields were also performed on silica gel 60 TLC plates F254. Radioactivity was measured on Bioscan AR-2000 Radio-TLC Imaging Scanner (Canberra Packard).

Products were dried by lyophilization on a vacuum line with an internal branch connected to a low-temperature cooler Huber TC50E with rotary oil pump LAVAT NRA 04/21 classic, dual-chamber vacuum pump. For cooling, a Dewar flask containing liquid nitrogen was used.

For identification of synthesized compounds, NMR spectra were recorded on Bruker Avance II 300 (^{1}H at 300,13 MHz and ^{13}C at 75,45 MHz) in $^{2}H_2O$. Chemical shifts are expressed in *parts per million* (ppm, δ) relative to an internal standard. For ^{1}H NMR, dioxane was used as an internal standard, while chemical shifts of ^{13}C NMR spectra were referenced relative to δ (dioxane) = 66,66 ppm.

Samples were measured using FT-IR spectrometer iS50, on a diamond crystal with ATR technique. The spectra were measured at a resolution of 2 cm^{-1} in the mid-IR spectral region (MIR) from 4000 to 400 cm^{-1}, and further evaluated in the program Omnic 9.1.

Electro spray ionization mass spectroscopy (ESI-MS) analysis was carried out using Finnigan MAT SSQ 7000 in MeOH or H_2O. Results are shown as M^+, M^- ions, and possible few fragments.

Chemicals and solvents were obtained from commercial sources and directly used without further purification:

- triethylenetetramine (60 % aqueous solution), 1,2-dibromoethane (purity 98 %), hydrazine hydrate (78-82 % aqueous solution) glyoxal (40% aqueous solution), N,N-dimethylformamide (purity 99,8 %), diethoxymethane (purity 99,7 %), 1,4,8,11-tetraazacyclotetradecane (purity 98 %), phosphorous acid (purity 99 %)
- ethyl alcohol (purity 96 %), methyl alcohol (purity 99,8 %), potassium hydroxide, ammonium hydroxide (25 % aqueous solution), paraformaldehyde (36-38 % aqueous solution), citric acid anhydrous and pure (Lachner)
- conc. hydrochloric acid (37 % aqueous solution) (Acros Organics)
- Dowex 50 WX 8 (Serva)
- iodine, sodium citrate (Lachema)
- activated carbon (20-40 mesh)

Ultrapure water (Milli-Q, 18,2 MΩ·cm at 25 °C) was produced by Direct-Q3 water purification system MILLIPORE and used in all radiolabelling experiments.

Stock solutions of the ligands were prepared by dissolving the solids (1 mg) in a 1 mL of ultrapure water (Υ = 1 mg/mL). The buffer solutions were freshly prepared.

3.1.1. Hydrochloric acid/Potassium chloride buffer

Stock solutions:

A: 0,2 M solution of hydrochloric acid (83,3 μL conc. HCl in 4,92 mL of ultrapure water)
B: 0,2 M solution of potassium chloride (74,5 mg in 5 mL of ultrapure water)

Table 5. x mL of A + y mL of B, diluted to a total of 5 mL

x	**y**	**pH**
0,265	0,05	2

3.1.2. Citrate buffer

Stock solutions:

A: 0,1 M solution of citric acid (4,80 g in 250 mL of ultrapure water)

B: 0,1 M solution of sodium citrate (7,35 g in 250 mL of ultrapure water)

Table 6. x mL of A + y mL of B, diluted to a total of 5 mL

x	y	pH
2,330	0,175	3
1,650	0,850	4
1,025	1,475	5
0,475	2,075	6

3.2. Preparation of the compounds

1,4,7,10-tetraazacyclododecane (Cyclen 7)

10 g of triethylenetetramine (0,068 mol) was dissolved in 200 mL of ethanol and mixed with 8 mL of 40% glyoxal in water (0,068 mol) at room temperature. After 24 hours of stirring, the solvent was distilled off in a vacuum, and an orange colored oil, which was taken up in 80 mL of dimethylformamide and mixed with 18 mL (39,2 g = 0,209 mol) of 1,2-dibromoethane, was obtained. After 24 hours of stirring at 40 °C, it was concentrated by evaporation in a vacuum; the residue was taken up in 80 mL of ethanol and acidified to about pH 3-4 with 37 % aqueous hydrochloric acid. 41 mL (41,9 g = 1,31 mol) of hydrazine hydrate was added to this reaction solution at room temperature, and it was heated under reflux for 30 hours. The reaction solution was set at pH 13 with solid potassium hydroxide and was subsequently concentrated by evaporation in a vacuum, taken up once more in 20 mL of ethanol, and the solvent was removed. The residue was mixed with 1 g of activated carbon and 25 mL of formaldehyde diethylacetal. It was heated under reflux for some time before the hot solution is filtered through a membrane. After the solution is cooled, the product was isolated by filtration washed with methanol and purified on an ion exchange column (Dowex 50 WX8, H^+ form, 200-400 mesh, elution with water and followed by a 3 % aqueous ammonia solution). The product was obtained as a yellow oily (5,4 mg; 0,03 mmol; 0,05 %).

ESI-MS: (positive) m/z: 173 $[M+H]^+$.

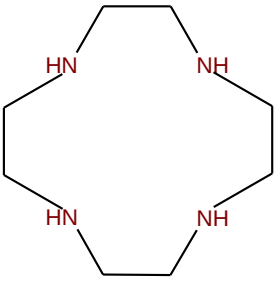
^{1}H and ^{13}C NMR spectra of the synthesized cyclen correspond to one described in the literature.

1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetrakis(methyl phosphonic acid) (TETP 13)

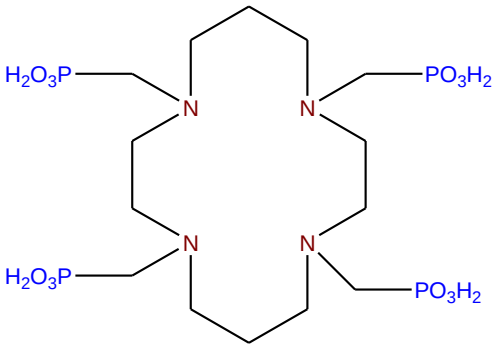
1,4,8,11-tetraazacyclotetradecane (Cyclam) (200 mg, 1 mmol) and phosphorous acid (656 mg, 8 mmol in 0,25 mL HCl) were mixed in water (0,7 mL). The mixture was heated to 40 °C while being stirred, and 36 % aqueous paraformaldehyde (0,57 mL g, 6 mmol) was added in small portions over 5 min. The mixture was further heated at this temperature for 5 hours. The reaction mixture was purified on a cation exchange resin (Dowex 50 WX8, H^{+} form, 200-400 mesh, elution with water and followed by a 3 % aqueous ammonia solution). The product was eluted in the first water fraction which contained the pure ligand in a form of white crystalline solid (254,3 mg; 0,44 mmol; 44, 1%)

ESI-MS: (positive) m/z: 581 $[M+5H]^{+}$, (negative) m/z: 573 $[M-3H]^{-}$.
^{1}H and ^{13}C NMR spectra of the synthesized phosponate derivative of cyclam correspond to spectra described in the literature.

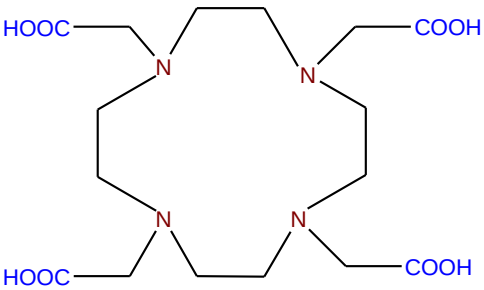
3.3. Structures of the compounds



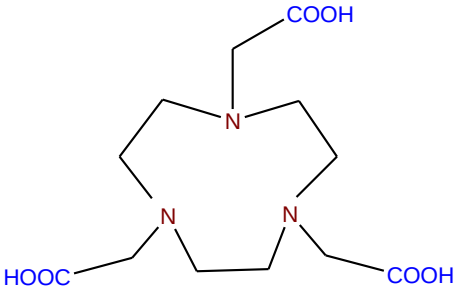
CYCLEN 7



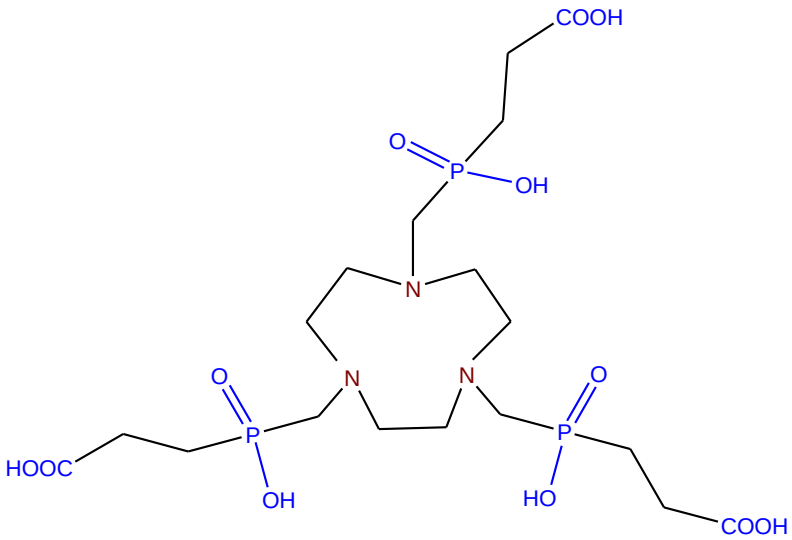
TETP 13



DOTA 1



NOTA 15



TRAP-Pr 23

3.4. General procedure for labelling with ^{152,154}Eu

Labelling with Eu was performed in hydrochloric acid/potassium chloride (pH 2) and citrate (pH 3-6) (400 μL) buffer solutions by adding 17 nmol of ligand as a stock solution with a concentration of 1 mg/mL. Then 20 μL of stock solution of Eu (152 000 cps/mL in 0,1 M HCl) was added and the solution was shaken in a heating block for 30 min at 95 °C. After cooling, pH was adjusted to 6-7 by adding small amounts of 1 M aq. NaOH solution.

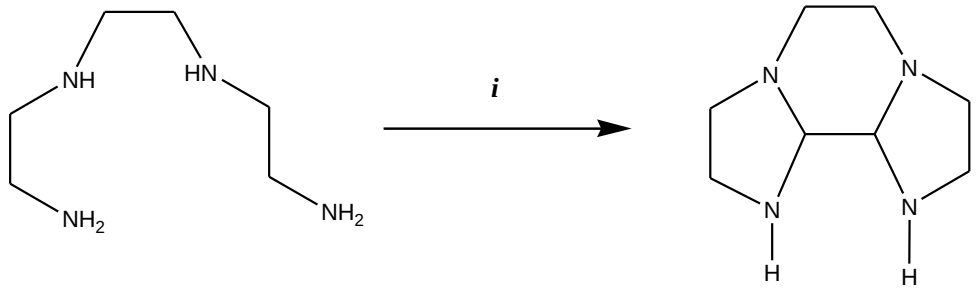
Radiochemical yields were determined by silica TLC (Merck) using a solvent mixture consisting of 2 parts of A (conc. aq. HCl:acetone:water = 0,1:1:1) and 1 part of B (pure acetylacetone). Free Eu migrates as an acetylacetonate complex. In-cage complexes stay on the baseline (Eu-complexes: Rf = 0,0-0,1; Eu-acac.: Rf = 0,5-0,6).

4. RESULTS AND DISCUSSION

4.1. Synthesis

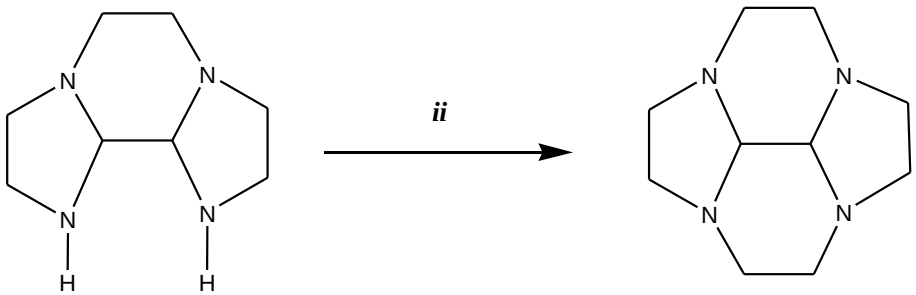
This thesis work was focused on obtaining possible azamacrocycle ligands based on cyclen and cyclam structures for their ^{152,154}Eu-labelling studies. For this purpose cyclen and TETP ligands were prepared.

Cyclen was obtained through a four-step reaction. First step included Mannich condensation of triethylenetetraamine with glyoxal at room temperature in a polar protic solvent (ethanol). After 24 h of stirring, a tricyclic compound was obtained as a first intermediate (Sheme 1).



Sheme 1. Reagents and conditions: (i) EtOH, glyoxal, r.t., 24 h

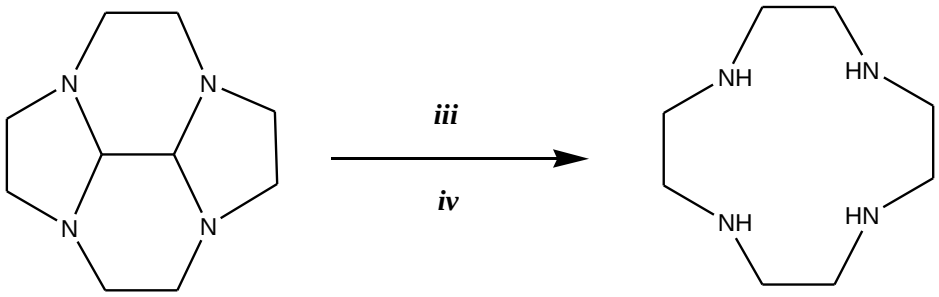
Tricyclic compound was converted into a tetracyclic intermediate by alkylation of two secondary amine-nitrogen atoms with a 1,2-dibromoethane. This step was carried out in polar aprotic solvent, DMF at 40 °C (Sheme 2).



Sheme 2. Reagents and conditions: (ii) 1,2-dibromoethane, DMF, 40 °C, 24 h

Removal of the ethylene bridge that connects four nitrogen atoms was achieved by treating tetracyclic intermediate with hydrazine hydrate in ethanol, at pH 3 to 4. The cyclen that was obtained after 30 h of stirring at reflux temperature was released from the cyclene salt by adding potassium hydroxide as a base.

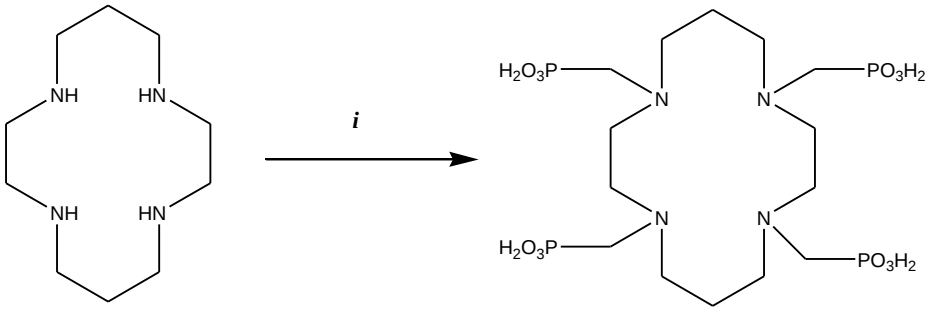
The last step included isolation of the cyclen by crystallization in formaldehyde diethylacetal.



Sheme 3. Reagents and conditions: (iii) EtOH, hydrazine hydrate, HCl, reflux, 30 h
(iv) KOH, EtOH, active carbon, formaldehyde diethylacetal

Ion exchange chromatography after the last step is necessary in order to obtain a very pure product. The complete synthesis was carried out in less than four days, but the pure product was obtained in a low yield.

With modification of the synthesis procedure from literature [24], TETP ligand was prepared by Mannich condensation. The preparation protocol is extremely short; there is just one step starting from commercially available 1,4,8,11-tetraazacyclotetradecane (Cyclam) as a secondary amine. The amine reacts with formaldehyde, in this case paraformaldehyde, and phosphorous acid. Amino alkylation was achieved under mild conditions at 40 ºC in hydrochloric acid and water as solvents. To obtain a pure ligand for further labelling studies, the product mixture was purified on Dowex cation exchange column. The final product was obtained in a yield of 44,1 %. Synthesis of TETP is shown in Sheme 4.



Sheme 4. Reagents and conditions: (i) H_3PO_3, HCl, H_2O, H_2CO, 40 ºC, 5h

4.2. ^{152,154}Eu-labelling studies

According to the general procedure (Chapter 3.4.), labelling of the ligands DOTA, TETP, NOTA and TRAP-Pr was performed with ^{152,154}Eu at various pH values (pH 2-6) at constant temperature (95 ºC) and constant concentration of the ligands. This procedure allows a very precise adjustment of pH with different buffer solutions and is therefore well studied for the investigation of labelling properties.

Radiochemical yields were determined by thin layer radio chromatography. The results of complexation of ^{152,154}Eu with DOTA, TETP, NOTA and TRAP-Pr, at pH 4 (95 ºC, 30 min), are shown in Figures 13, 14, 13 and 15.

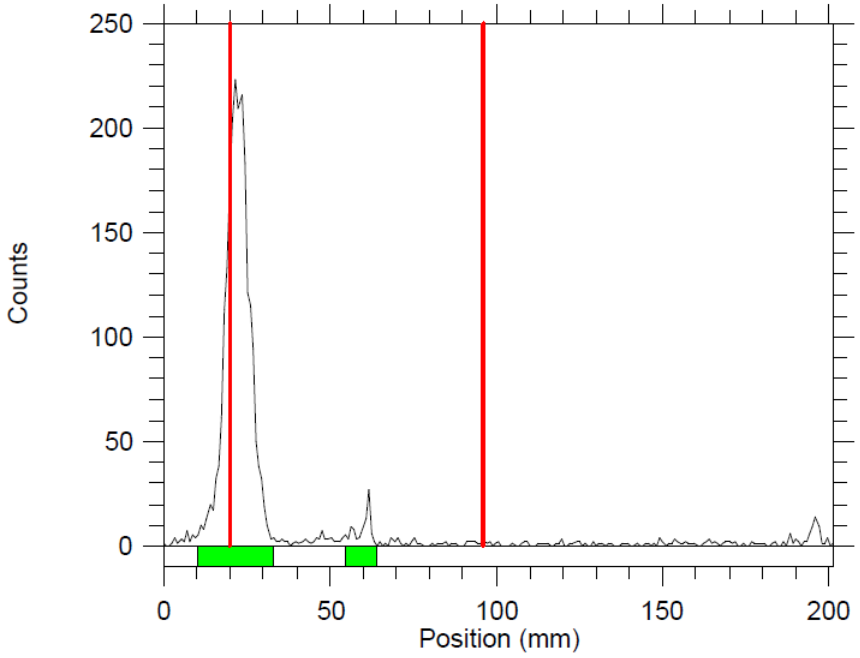


Figure 13. Radio-chromatogram of ^{152,154}Eu-DOTA complex

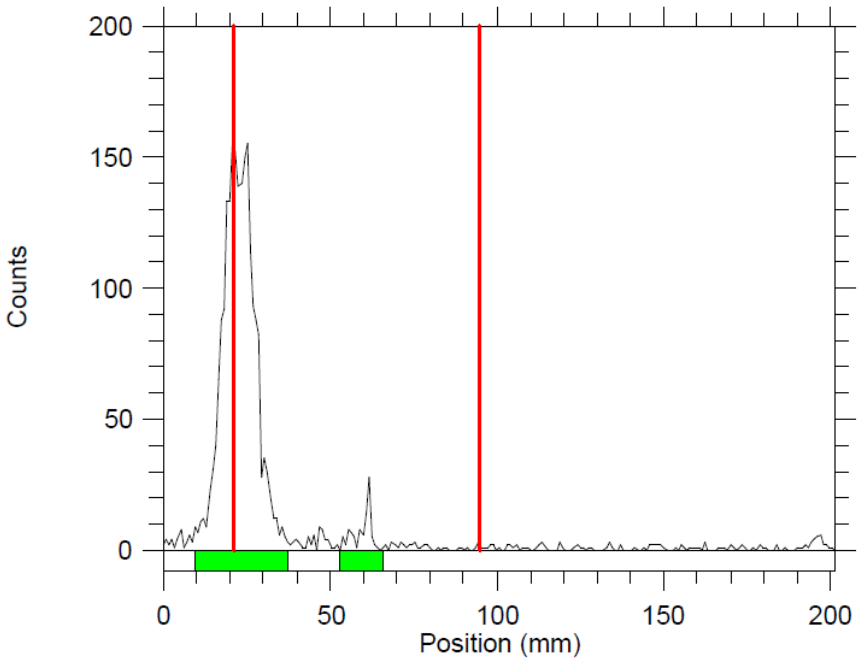


Figure 14. Radio-chromatogram of ^{152,154}Eu-TETP complex

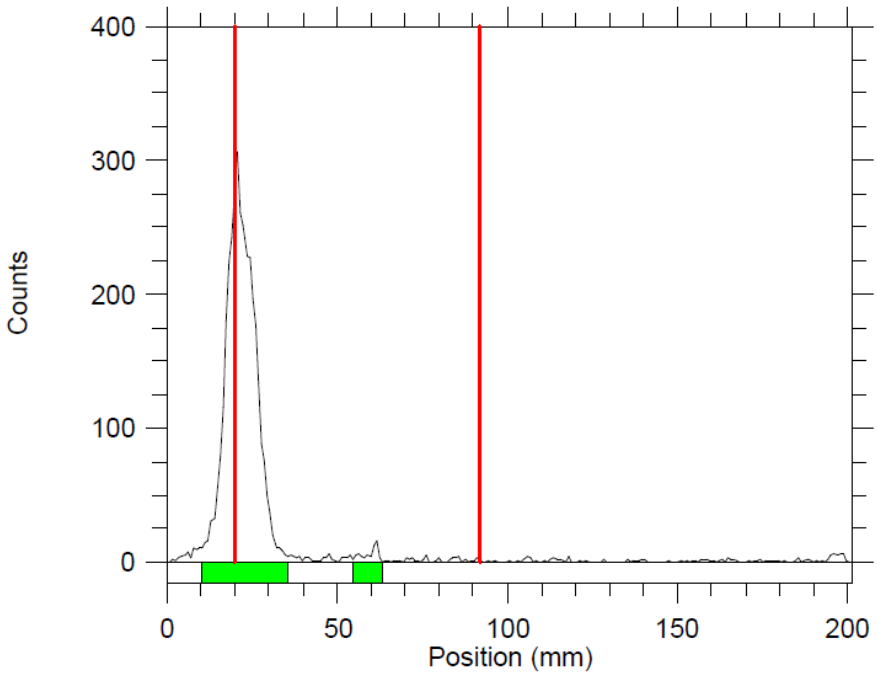


Figure 15. Radio-chromatogram of ^{152,154}Eu-NOTA complex

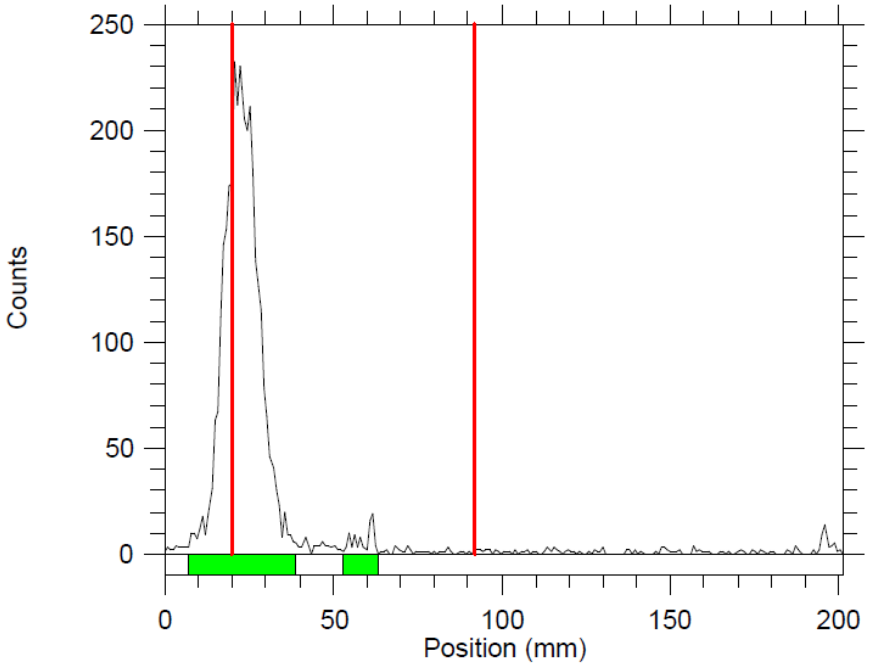


Figure 16. Radio-chromatogram of ^{152,154}Eu-TRAP-Pr complex

The radio-chromatograms show that formed DOTA, TETP, NOTA and TRAP-Pr ^{152,154}Eu-complexes stay on the baseline (Rf = 0,0-0,1), while free europium in form of ^{152,154}Eu(acac)$_3$ complex stays at Rf = 0,52-0,54.

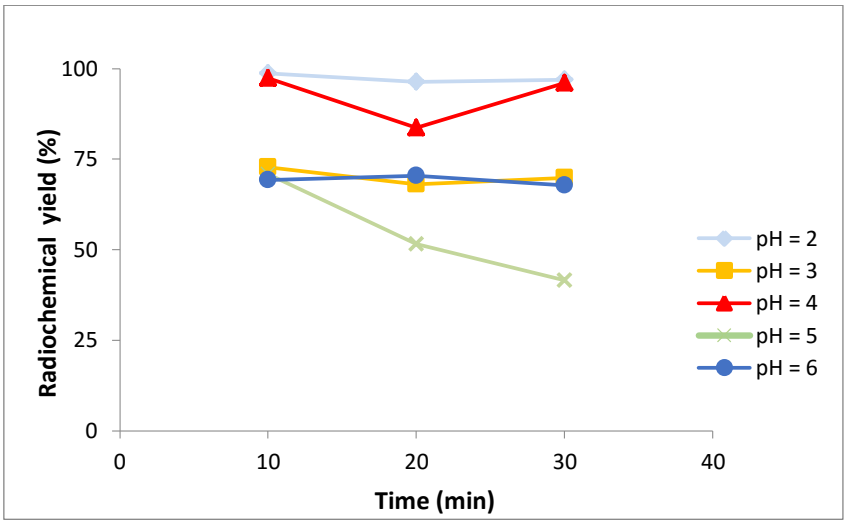


Figure 17. Radiochemical yields (%) of complexation of ^{152,154}Eu with DOTA ligand (19, 8 nmol) as a function of time (min) in the pH range from 2 to 6 at 95 ºC

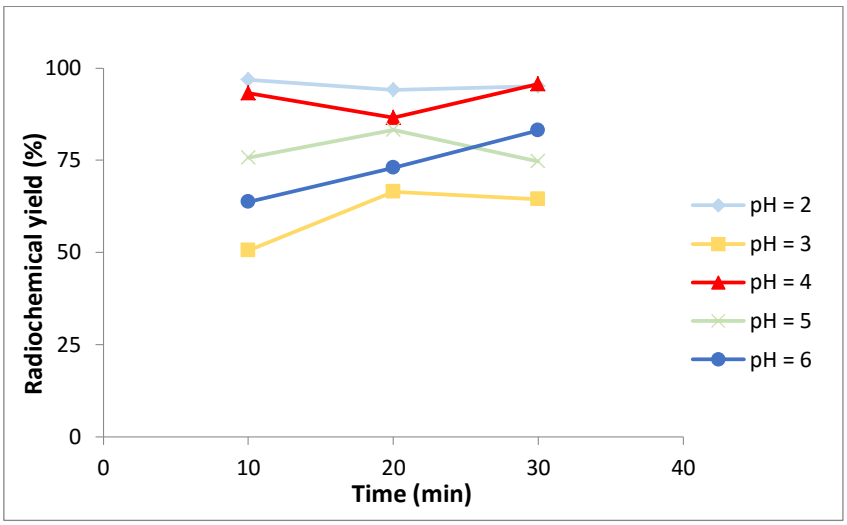


Figure 18. Radiochemical yields (%) of complexation of ^{152,154}Eu with TETP ligand (17,4 nmol) as a function of time (min) in the pH range from 2 to 6 at 95 ºC

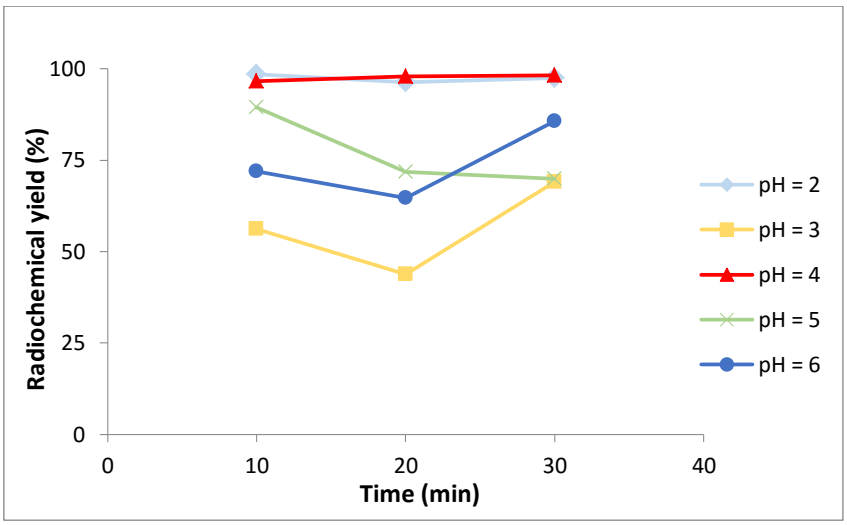


Figure 19. Radiochemical yields (%) of complexation of ^{152,154}Eu with NOTA ligand (18,2 nmol) as a function of time (min) in the pH range from 2 to 6 at 95 ºC

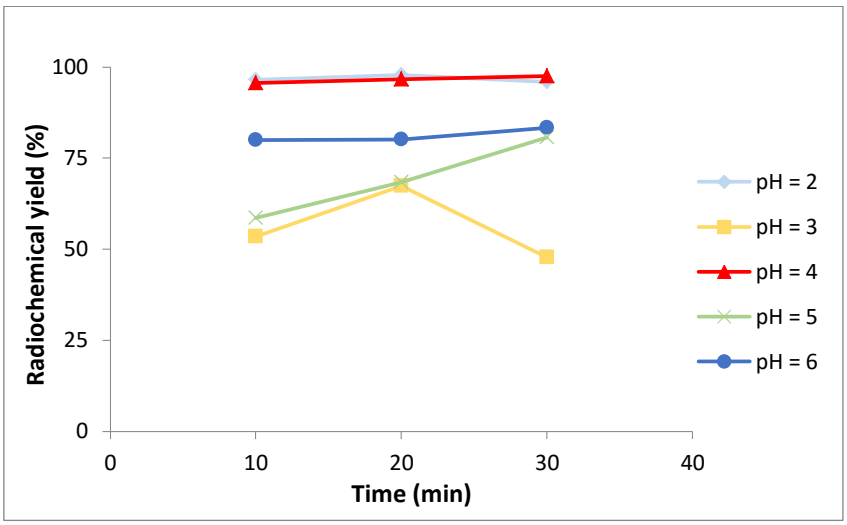


Figure 20. Radiochemical yields (%) of complexation of ^{152,154}Eu (%) with TRAP-Pr ligand (17,1 nmol) as a function of time (min) in the pH range from 2 to 6 at 95 ºC

The results presented above confirm strong dependence of radiochemical yields of ^{152,154}Eu complexation with DOTA, TETP, NOTA and TRAP-Pr on the pH value. The complexation can be described as a two-step process. The intermediate out-of-cage complex is formed instantly and the metal ion is coordinated only through oxygen atoms of phosphonate and carboxylate groups, at a low pH, whereas macrocyclic amines are protonated. In the rate-determining step, ring nitrogen atoms lose proton(s) and the metal ion simultaneously moves into the macrocycle cavity.

Radiochemical incorporations of more than 95% are achieved by dissolving nanomolar concentration of the ligands in a solution containing a specified volume of $EuCl_3$ solution at pH 2 and also at pH 4. Potassium chloride/hydrochloric acid buffer was used to adjust pH at 2 and citrate buffer was used for adjusting pH values from 3 to 6. The lowest yields for TETP, NOTA and TRAP-Pr were observed at pH 3, while for DOTA the lowest yields were at pH 5.

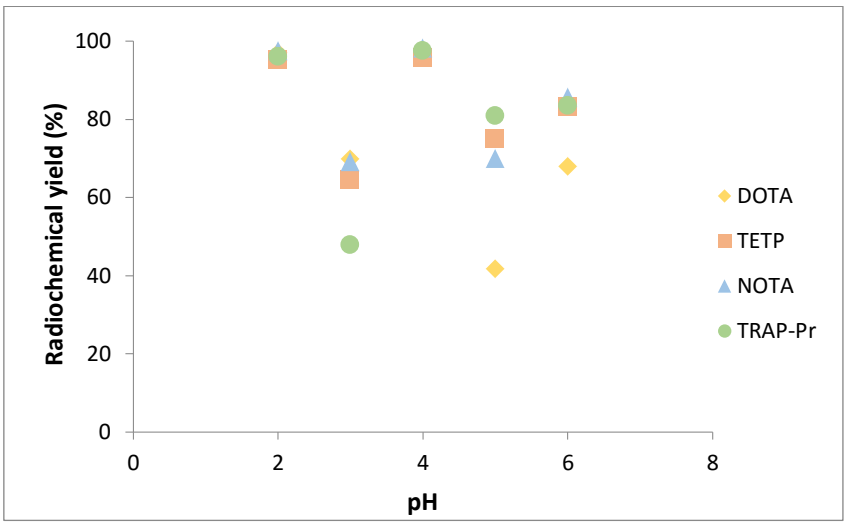


Figure 21. Radiochemical yields (%) of complexation of ^{152,154}Eu (%) with DOTA (19,8 nmol), TETP (17,4 nmol), NOTA (18,2 nmol) and TRAP-Pr (17,1 nmol) ligands as a function of pH (95 ºC, 30 min)

Variation of pH at a constant temperature of 95 ºC revealed that all four ligands exhibit almost the same coordinating abilities. After 30 minutes, radiochemical yields follow the order pH 3 < pH 5 < pH 6 < pH 4 ~ pH 2. In literature [25], it was found that the trivalent cation of europium which is stable in aqueous solutions forms complexes with citrate anion. At pH 3, Eu(III)-citrate complex is identified as a neutral charge (1:1) complex, $EuHCit^{0}$. Eu(III)-citrate complex at pH 4 to 5,5 is found to be a 1:2 complex $Eu(HCitH)Hcit^{2-}$. In this complex, one hydrogen citrate $HcitH^{2-}$ and one citrate anion $Hcit^{3-}$ bind to the Eu^{3+} ion. Creation of Eu^{3+} complexes with citrate anion is the main cause of lower radiochemical yields at pH 3 and 5.

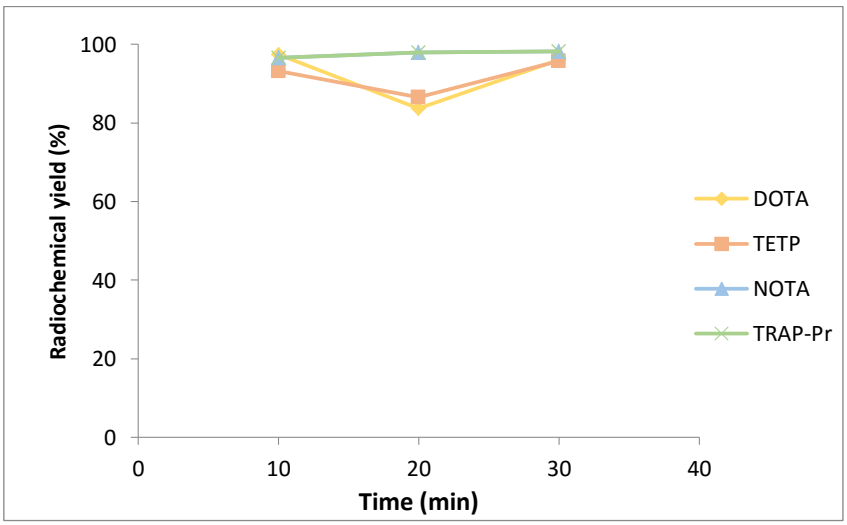


Figure 22. Radiochemical yields (%) of complexation of ^{152,154}Eu (%) with DOTA (19,8 nmol), TETP (17,4 nmol), NOTA (18,2 nmol) and TRAP-Pr (17,1 nmol) ligands as a function of time (min) at 95 ºC

The best yields, after 30 minutes, for ligands DOTA (96,0 %), TETP (95,7%), NOTA (98,2 %) and TRAP-PR (97,5 %) were achieved at pH 4. Citrate buffer was used to adjust the pH to 4. This pH was chosen for kinetic studies in order to ensure realistic comparison of the ligands, since it was found to be optimal for all four ligands.

The results of the kinetic studies of DOTA, TETP, NOTA and TRAP-Pr are summarized in Table 5.

Table 4. Time course of complexation of ^{152,154}Eu with with DOTA (19,8 nmol), TETP (17,4 nmol), NOTA (18,2 nmol) and TRAP-Pr (17,1 nmol) ligands in 400 μL of citrate buffer solution at pH 4 (0-95 ºC)

Time (min)	0	1	2	3	4	5	6	7	8	9	10	15	30	45	60
Yield (%) DOTA	95	95	91	91	90	91	89	81	91	84	89	90	89	89	89
Yield (%) TETP	94	95	95	93	87	89	86	90	89	87	93	92	93	93	92
Yield (%) NOTA	93	90	87	85	82	83	86	90	84	85	83	85	83	92	86
Yield (%) TRAP-Pr	93	89	89	89	91	92	92	87	92	91	89	74	83	83	85

Direct comparison of radiochemical yields of the ligands show that they are able to incorporate ^{152,154}Eu instantaneously (>92 %) at room temperature. These ligands form kinetic stable complexes and they all achieve radiochemical yields between 83 and 95 % within 60 minutes. Yield of 74,4 %, which is out of range, can be considered as experimental error caused of constant pipetting small amounts of ligands.

5. CONCLUSIONS

The synthesis of TETP ligand was fast, simple, and scalable. Starting from commercially available 1,4,8,11-tetraazacyclotetradecane (Cyclam) the final product was obtained by Mannich condensation. The synthesis of cyclen appears to be more problematic and all attempts gave poor yields. Cyclen was obtained through a four-step reaction in a low yield and further synthesis of potential phosphonate or carboxylate derivatives of cyclen could not be implemented. Both cyclen and TETP ligand were purified on dowex exchange column to obtain a pure ligand. The structures of synthesized compounds were confirmed by ^{1}H and ^{13}C NMR, FT-IR spectroscopy and mass spectrometry.

Ligands DOTA, TETP, NOTA and TRAP-Pr showed efficient complexation with Eu^{3+}. Almost quantitative complexes with radiochemical yields over 95 % are formed at pH 2 and pH 4 within 30 min at 95 ºC. Complexation at pH 3 and 5 is partially decelerated by the presence of citrate anion from the buffer solution. Competition between formation of citrate and macrocyclic complexes results in lower yields at pH 3 and pH 5. Lower radiochemical yields at pH 6 are caused by more extensive deprotonation of phosphate and carboxylate functional groups at higher pH leading to their stronger interaction with Eu(III) ion and stabilization of the out-of-cage complexes.

DOTA, TETP, NOTA and TRAP-Pr form kinetically stable complexes with $^{152,154}Eu$ at pH 4. These ligands are also able to form complexes instantaneously at room temperature.

All of the ligands are particularly suitable for innovative tracer design since they can be derivatized by amide formation without affecting the integrity of the complexation site. For potential use *in vivo* further studies are needed.

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7. CURRICULUM VITAE

Marina Marinović was born on 02/04/1993 in Slavonski Brod, Croatia. She finished both elementary school and high school in Orahovica. In 2011 she has been enrolled in Faculty of Chemical Engineering and Technology (Zagreb), course of Applied chemistry. In 2014 she finished undergraduate study programme obtaining a bachelor degree in a field of organic chemistry by giving a presentation „1,3-dipolar cycloaddition of terminal alkynes and azides“. In 2014 she continued her studies as a graduate student in the same Faculty, course of Applied organic chemistry. In 2015 she participated in II. Symposium of chemistry students and she was awarded with Dean’s prize for remarkable student scientific work.