

Design and Application of Novel Supramolecular Theranostic Agents

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“Der Cage hat zwar nicht funktioniert, aber du hattest einen Krapfen”

- Dr. Sandra Dominelli -

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spectrum of the emotional rollercoaster I went through, and I am incredibly grateful that you were always there for me. Thank you for listening, for caring and for cheering me on.

I am very happy and also a bit proud to have finally wrapped up this chapter of my scientific career, but without all the wonderful people I met on the way, the journey would have been only half as much fun.

Abstract

Supramolecular chemistry, the chemistry beyond the single molecule, provides a versatile toolbox to tackle complex challenges in drug design, offering new solutions to the diagnosis and treatment of heterogeneous diseases, as well as to the targeting of specific organs. Within this field, supramolecular coordination complexes (SCCs), and the sub-family of porous metallacages (MCgs) in particular, have attracted attention for biomedical applications due to their straightforward synthesis via coordination-driven self-assembly (CDSA) and modular built up from a plethora of metal nodes and organic ligands. Together with the possibility of ligand functionalization and host-guest chemistry, this enables the design of tailor-made theranostic platforms combining imaging, therapy and drug delivery. Noteworthy, the size of MCgs span the low nanometre scale, making these supramolecules not only attractive for their multifunctionality, but also since they can be fully characterized by classical spectroscopic and analytical methods. Moreover, their pharmacokinetic properties are very different from those of other supramolecular systems (e.g. liposomes) used in biomedicine. Only recently have MCgs entered radiopharmaceutical science, expanding the range of strategies for developing multifunctional radiotheranostic agents.

Hence, this work focused on further advancing the application of radiolabelled metallacages by designing and evaluating $[\text{Pd}_2\text{L}_4]^{4+}$ cages for dual-modality nuclear imaging. The Pd(II)-MCgs feature blood-brain barrier (BBB)-penetrating capability via bioconjugation to a specific peptide (D^{D} PepH3) previously studied for its active transport across the BBB. Two silicon-based fluoride acceptors, the monofunctional (SiFA)BA and the less lipophilic bifunctional (SiFA)SeFe, were incorporated for radiolabelling with fluorine-18, the clinically most relevant isotope for positron emission tomography (PET) imaging. Two *exo*-functionalized ligands (**L1** and **L2**) were synthesized via solid-phase synthesis and subsequent CDSA and yielded the corresponding homoleptic metallacages (**C1**_{hom} and **C2**_{hom}), which were fully characterized by nuclear magnetic resonance (NMR) spectroscopy, reverse-phase high-performance liquid chromatography (RP-HPLC) and high-resolution electrospray ionization mass spectrometry (HR-ESI-MS). Reaction conditions were optimized, and stability studies were conducted in different solvents and concentrations, revealing cage lability in salt-containing solutions. While encapsulation of the chemotherapeutic drug cisplatin and the radiotracer $[\text{}^{99\text{m}}\text{Tc}]\text{TcO}_4^-$, used in single-photon emission computed tomography (SPECT) imaging, was not achieved due to such instability, formation of a host-guest complex with the 'cold' surrogate ReO_4^- was evidenced by mass spectrometry ($[\text{Pd}_2(\text{L2})_4(\text{ReO}_4)\text{H}_8]^{11+}$, $m/z = 655.9501$). Of note, ^{18}F -labelled metallacages were obtained by pre-assembly radiolabelling within 4 hours. Therefore, Kryptofix-free ^{18}F -to- ^{19}F isotopic exchange was applied to generate radiofluorinated ligands (^{18}F -**L1** and ^{18}F -**L2**), which displayed stability toward defluorination; subsequent cage

formation (^{18}F -**C1**_{hom} and ^{18}F -**C2**_{hom}) by CDSA was confirmed by chromatographic analysis. Due to cage instability, the lipophilicity, an important predictor of a tracer's pharmacokinetics, was assessed only for the radiolabelled ligands. As expected, incorporation of lipophilic SiFA synthons led to an unfavourable $\text{Log}D_{\text{pH}7.4}$ (^{18}F -**L1**: 2.53 ± 0.05 ; ^{18}F -**L2**: 2.07 ± 0.07); however, a positive effect of the optimized (SiFA)SeFe in the second-generation ligand was evident. The lipophilicity of ^{18}F -**L1** was additionally examined in the preclinical chick chorioallantoic membrane (CAM) model by PET imaging and *ex ovo* biodistribution studies. Here, high liver uptake and long blood retention were observed, in line with the calculated $\text{Log}D_{\text{pH}7.4}$ values. Beside slight defluorination, satisfactory brain uptake (0.38 ± 0.13 %ID/g 60 min p.i.) and human glioblastoma U87 tumour accumulation (3.94 ± 1.31 %ID/g 60 min p.i.) supported the ligands suitability for brain cancer imaging, although optimization of the lipophilicity is necessary.

Further, heteroleptic metallacages of general formula $[\text{Pd}_2(\text{L})_m(\text{L0})_n]^{4+}$ ($m = 1, 2$; $n = 3, 2$), containing the unfunctionalized ligand **L0**, were synthesized in an attempt to enhance cage stability by reducing the steric demand. The compounds were characterized by mass spectrometry. Heteroleptic assembly resulted in statistical mixtures of supramolecular species separable by reverse-phase high-performance liquid chromatography. Perrhenate encapsulation and ^{18}F -radiolabelling was partially achieved, but isolation of single species proved difficult.

In a second phase of this project, another approach to increase metallacage integrity in physiological conditions and their resilience to post-assembly functionalization was investigated. Specifically, we exploited the use of kinetically more inert metal ions with square planar coordination geometry, such as Pt(II), to achieve MCgs. Thanks to recent progress in the construction of stable lantern-shaped Pt(II)-MCgs, realization of highly functionalized structures now appears within reach and might promote their role in biomedical applications. Thus, two synthetic routes were proposed for the formation of homoleptic and heteroleptic brain-targeted $[\text{Pt}_2\text{L}_4]^{4+}$ cages via post-assembly modification (PAM), including a thoughtful protecting group strategy and photochemical techniques.

The last part of the project has been devoted to the study of ^{18}F -labelled tracers in the CAM model, which provides an ideal environment for tumour growth and makes a promising screening platform for drug candidates due to its low cost and time efficiency compared with mouse models. To further expand the palette of cancer types evaluated so far, AR42J tumour uptake of an sstR2-targeted SiFA-containing tracer ($[\text{F-18}][\text{Ga-67}]\text{Ga}(\text{SiFA})\text{SeFe-rhTATE2}$) was investigated. The accumulation amounted to 10.1 ± 2.5 %ID/g 60 min p.i. and high sstR2 specificity was indicated by minimal uptake in sstR2-negative U87 tumours. Despite optimization of tumour growth on the chick chorioallantoic membrane, it was found that rat-derived cell lines (AR42J) were less tolerated by the chick embryo than human-derived cell

lines (U87), suggesting the use of human-derived sstR2-expressing cell lines for further studies.

To summarize, although considerable effort is still required for clinical translation of radiotheranostic supramolecular coordination complexes, their tunability and multimodality have the potential to enrich future radiopharmaceutical applications.

Kurzzusammenfassung

Supramolekulare Chemie, die Chemie jenseits des einzelnen Moleküls, bietet ein vielfältiges Instrumentarium zur Bewältigung komplexer Herausforderungen im Bereich des Wirkstoffdesigns, darunter neue Lösungen zur Diagnose und Behandlung heterogener Erkrankungen sowie zur Adressierung spezifischer Organe. Innerhalb dieses Feldes haben supramolekulare Koordinationskomplexe und insbesondere die Unterfamilie der porösen Metallakäfige Aufmerksamkeit für biomedizinische Anwendungen auf sich gezogen aufgrund ihrer einfachen Synthese durch koordinationsgesteuerte Selbstassemblierung und ihres modularen Aufbaus aus einer Vielzahl an Metallionen und organischen Liganden. Zusammen mit der Möglichkeit der Ligandenfunktionalisierung und der Gast-Wirt-Chemie ermöglicht dies die Entwicklung maßgeschneiderter theranostischer Plattformen, die Bildgebung, Therapie und Wirkstofftransport kombinieren. Bemerkenswert ist, dass die Größe von Metallakäfigen im niedrigen Nanometerbereich liegt, wodurch diese Supramoleküle nicht nur aufgrund ihrer Multifunktionalität attraktiv sind, sondern auch, weil sie mit klassischen spektroskopischen und analytischen Methoden vollständig charakterisiert werden können. Darüber hinaus unterscheiden sich ihre pharmakokinetischen Eigenschaften stark von denen anderer supramolekularer Systeme (z.B. Liposomen), die in der Biomedizin verwendet werden. Erst seit kurzem halten Metallakäfige Einzug in die Radiopharmazie und erweitern damit das Spektrum der Strategien zur Entwicklung multifunktionaler radiotheranostischer Wirkstoffe. Daher konzentrierte sich diese Arbeit darauf, die Anwendung von radioaktiv markierten Metallakäfigen weiter voranzubringen, indem $[\text{Pd}_2\text{L}_4]^{4+}$ Käfige für die dualmodale nukleare Bildgebung entwickelt und evaluiert wurden. Die Pd(II)-Käfige zeichnen sich durch ihre Fähigkeit aus, die Blut-Hirn-Schranke durch ihre Biokonjugation mit einem spezifischen (α -PepH3) zu passieren, das zuvor hinsichtlich seines aktiven Transports über die Blut-Hirn-Schranke untersucht wurde. Zwei Silizium-basierte Fluoridakzeptoren, der monofunktionelle (SiFA)BA und der weniger lipophile bifunktionelle (SiFA)SeFe, wurden für die radioaktive Markierung mit Fluor-18, dem klinisch relevantesten Isotop für die Positronen-Emissions-Tomographie, eingebaut. Zwei *exo*-funktionalisierte Liganden (**L1** und **L2**) wurden mittels Festphasensynthese hergestellt, und die anschließende koordinationsgesteuerte Selbstassemblierung führte zur Bildung der entsprechenden homoleptischen Metallakäfige (**C1_{hom}** und **C2_{hom}**), die mittels Kernspinresonanzspektroskopie, Umkehrphasen-Hochleistungsflüssigkeitschromatographie und hochauflösender Elektrospray-Ionisations-Massenspektrometrie vollständig charakterisiert wurden. Die Reaktionsbedingungen wurden optimiert sowie Stabilitätsstudien in verschiedenen Lösungsmitteln und Konzentrationen durchgeführt, wodurch eine Instabilität der Käfige in salzhaltigen Lösungen festgestellt wurde. Während die Einkapsulierung des Chemotherapeutikums Cisplatin und des Radiotracers

$[^{99m}\text{Tc}]\text{TcO}_4^-$, der in der Einzelphotonen-Emissionscomputertomographie verwendet wird, aufgrund dieser Instabilität nicht gelang, konnte die Bildung eines Wirt-Gast-Komplexes mit dem ‚kalten‘ Surrogat ReO_4^- jedoch durch Massenspektrometrie nachgewiesen werden ($[\text{Pd}_2(\text{L}2)_4(\text{ReO}_4)\text{H}_8]^{11+}$, $m/z = 655.9501$). ^{18}F -markierte Metallakäfige wurden durch Pre-assembly Markierung innerhalb von 4 Stunden erhalten. Dafür wurde ein Kryptofix-freier ^{18}F -zu- ^{19}F -Isotopenaustausch durchgeführt, um die radiofluorierten Liganden ($^{18}\text{F}\text{-L1}$ und $^{18}\text{F}\text{-L2}$) zu erzeugen, welche Stabilität gegenüber Defluorierung aufwiesen; anschließende Käfigbildung ($^{18}\text{F}\text{-C1}_{\text{hom}}$ and $^{18}\text{F}\text{-C2}_{\text{hom}}$) mittels koordinationsgesteuerte Selbstassemblierung wurde durch chromatographische Analyse bestätigt. Aufgrund der Instabilität der Käfige wurde die Lipophilie, ein wichtiger Prädiktor für die Pharmakokinetik eines Tracers, nur für die radiomarkierten Liganden bewertet. Wie erwartet führte die Einbindung lipophiler SiFA-Synthone zu einem ungünstigen $\text{Log}D_{\text{pH}7.4}$ ($^{18}\text{F}\text{-L1}$: 2.53 ± 0.05 ; $^{18}\text{F}\text{-L2}$: 2.07 ± 0.07); jedoch war ein positiver Effekt des optimierten (SiFA)SeFe im Liganden der zweiten Generation offensichtlich. Die Lipophilie von $^{18}\text{F}\text{-L1}$ wurde zusätzlich im präklinischen Hühner-Chorioallantoismembran-Modell mittels PET-Bildgebung und *ex ovo* Biodistributionsstudien untersucht. Dabei wurden eine hohe Leberaufnahme und eine lange Verweildauer im Blut beobachtet, was im Einklang mit den berechneten $\text{Log}D_{\text{pH}7.4}$ -Werten ist. Neben einer leichten Defluorierung sprachen eine zufriedenstellende Aufnahme im Gehirn ($0.38 \pm 0.13 \text{ \%ID/g}$ 60 min p.i.) und eine Akkumulation in U87-Tumoren ($3.94 \pm 1.31 \text{ \%ID/g}$ 60 min p.i.) für die Eignung des Liganden für die Bildgebung von Hirntumoren, wobei hierfür eine Optimierung der Lipophilie erforderlich ist.

Desweiteren wurden heteroleptische Metallakäfige der allgemeinen Formel $[\text{Pd}_2(\text{L})_m(\text{L}0)_n]^{4+}$ ($m = 1, 2$; $n = 3, 2$) unter Verwendung des unfunktionalisierten Liganden **L0** synthetisiert, um die Käfigstabilität durch Verringerung des sterischen Anspruchs zu verbessern. Die Käfige wurden mittels Massenspektrometrie charakterisiert. Die heteroleptische Assemblierung führte zu statistischen Gemischen supramolekularer Spezies, die durch Umkehrphasen-Hochleistungsflüssigkeitschromatographie getrennt werden konnten. Perrhenat-Enkapsulierung und ^{18}F -Radiomarkierung wurden teilweise erreicht, jedoch erwies sich die Isolierung einzelner Spezies als schwierig.

In einer zweiten Phase dieses Projekts, wurde ein anderer Ansatz zur Erhöhung der Integrität von Metallakäfigen unter physiologischen Bedingungen und ihrer Widerstandsfähigkeit gegenüber Post-Assemblierungsmodifikation untersucht. Konkret nutzten wir die Verwendung von kinetisch inerten Metallionen mit quadratisch-planarer Koordinationsgeometrie, wie z.B. Pt(II), um Metallakäfige zu erzielen. Dank der jüngsten Fortschritte bei der Konstruktion von stabileren laternenförmigen Pt(II)-Käfigen scheint die Realisierung hochfunktionalisierter Strukturen in greifbare Nähe gerückt zu sein und könnte somit ihre Rolle in biomedizinischen Anwendungen fördern. Daher wurden zwei Synthesewege für Hirn-adressierte homoleptische

und heteroleptische $[\text{Pt}_2\text{L}_4]^{4+}$ Käfige über Post-Assemblierungsmodifikation vorgeschlagen, einschließlich einer durchdachten Schutzgruppenstrategie und photochemischen Techniken. Der letzte Teil des Projekts widmete sich der Untersuchung von ^{18}F -markierten Tracern im Hühner-Chorioallantoismembran-Modell, welches eine ideale Umgebung für Tumorwachstum bietet und aufgrund seiner im Vergleich zu Mausmodellen geringen Kosten und Zeiteffizienz eine vielversprechende Screening-Plattform für Arzneimittelkandidaten darstellt. Um die Palette der bisher untersuchten Krebsarten zu erweitern, wurde die Aufnahme eines sstR2-gerichteten SiFA-haltigen Tracers ($[\text{}^{18}\text{F}][\text{}^{\text{nat}}\text{Ga}]\text{Ga}(\text{SiFA})\text{SeFe-rhTATE2}$) durch AR42J-Tumore untersucht. Die Akkumulierung betrug $10.1 \pm 2.5 \text{ \%ID/g}$ 60 min p.i., und die hohe sstR2-Spezifität wurde durch eine minimale Aufnahme in sstR2-negativen U87-Tumoren aufgezeigt. Trotz Optimierung des Tumorwachstums auf der Chorioallantoismembran wurde festgestellt, dass aus Ratten stammende Zelllinien (AR42J) vom Kükenembryo weniger toleriert wurden als aus Menschen stammenden Zelllinien (U87), was die Verwendung von Menschen stammenden sstR2-exprimierenden Zelllinien für weitere Studien nahelegt. Zusammenfassend lässt sich sagen, dass zwar noch erhebliche Anstrengungen für die klinische Translation von radiotheranostischen supramolekularen Koordinationskomplexen erforderlich sind, ihre Abstimmbarkeit und Multimodalität jedoch das Potenzial besitzen, zukünftige radiopharmazeutische Anwendungen zu bereichern.

List of Abbreviations

2D/3D	Two/three dimensional
AMBF ₃	Ammonium trifluoroborate
BBB	Blood-brain barrier
CAM	Chorioallantoic membrane
CD	Cyclodextrin
CDSA	Coordination-driven self-assembly
CNS	Central nervous system
CT	Computed tomography
DFO	Desferrioxamine
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
DOTA	2,2',2'',2'''-(1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl)tetraacetic acid)
EPR	Enhanced permeability and retention
FA	Formic acid
[¹⁸ F]FDG	2-deoxy-2-[¹⁸ F]fluoro- <i>D</i> -Glucose
Fmoc-SPPS	Fluorenylmethoxycarbonyl solid-phase peptide synthesis
GSH	Glutathione
HER2	Human epidermal growth factor receptor 2
HOAt	1-Hydroxy-7-azabenzotriazole
HPAC	High-performance affinity chromatography
HR-ESI-MS	High resolution electrospray ionization mass spectrometry
DI-HR-ESI-MS	Direct injection-HR-ESI-MS
LC-HR-ESI-MS	Liquid chromatography-HR-ESI-MS
HSA	Human serum albumin
ID/g	Injected dose per gramme
IE	Isotopic exchange
L	Ligand
L0	2-(3,5-bis(pyridine-3-ylethynyl)phenyl)acetic acid
C0 _{hom}	[Pd ₂ (L0) ₄] ⁴⁺
L1	<i>D</i> PepH3- <i>D</i> Dap((SiFA)BA)-Ebes-L0
C1 _{hom} /C1 _{het}	[Pd ₂ (L1) ₄] ⁴⁺ /[Pd ₂ (L1)(L0) ₃] ⁴⁺
L2	<i>D</i> PepH3-(SiFA)SeFe-Ebes-L0
C2 _{hom} /C2 _{het}	[Pd ₂ (L2) ₄] ⁴⁺ /[Pd ₂ (L2)(L0) ₃] ⁴⁺
LET	Linear energy transfer
Log <i>D</i> _{pH7.4}	Octanol-PBS (pH 7.4) partition coefficient
Log <i>P</i>	Octanol-water partition coefficient
M	Metal centre
MCg	Metallacage

MeCN	Acetonitrile
MEK	Methylethylketone
MIM	Mechanically interlocked molecule
MNP	Metallacage-loaded nanoparticle
MOF	Metal-organic framework
MRI	Magnetic resonance imaging
NHS	<i>N</i> -hydroxysuccinimide
NIR	Near-infrared
NMP	<i>N</i> -Methyl-2-pyrrolidone
NMR	Nuclear magnetic resonance
DOSY NMR	Diffusion ordered NMR spectroscopy
PAM	Post-assembly modification
PBS	Phosphate buffered solution
PDT	Photodynamic therapy
PEG	Polyethylene glycol
PET	Positron emission tomography
PG	Protecting group
PSMA	Prostate-specific membrane antigen
p.i.	Post-injection
RCC	Radiochemical conversion
RCP	Radiochemical purity
RCY	Radiochemical yield
RGD	Arginylglycylaspartic acid
RP-HPLC	Reverse-phase high-performance liquid chromatography
RT	Room temperature
SCC	Supramolecular coordination complex
SiFA	Silicon-based fluoride acceptor
(SiFA)BA	SiFA-benzoic acid
(SiFA)SeFe	SiFA-amino acid
SPECT	Single-photon emission computed tomography
sstR2	Somatostatin receptor 2
TATE	(Tyr ³)-octreotate)
TBTU	<i>N,N,N',N'</i> -Tetramethyluronium-tetrafluoroborate
TFA	Trifluoroacetic acid
TIC	Total ion chromatogram
TLC	Thin-layer chromatography
t _R	Retention time

1 Introduction

When it comes to diagnosis and treatment of malignant diseases, conservative methods often reach their limits. According to the *WHO International Agency for Research on Cancer* 20% of people will develop cancer in their lifetime with approximately 20 million new cases in 2022, making it the third most common cause of disease burden.^[1,2] By 2050, this number is estimated to have increased by 77% to around 35 million cases, mostly driven by lifestyle choices, an aging society and environmental pollution (Figure 1).^[1] Beside improved cancer management in the clinics, innovative strategies in drug design are necessary to tackle this complex, heterogeneous disease. Supramolecular chemistry could offer an elegant solution having shown promising efficiency in drug delivery, targeting, imaging and therapy, including the incorporation of medical radioisotopes.^[3–5] To familiarise with this highly interdisciplinary science, this section aims at clarifying the principles of supramolecular chemistry and its structural subtypes, before introducing the basic concepts of pharmaceutical radiochemistry and its adaptation in supramolecular chemistry.

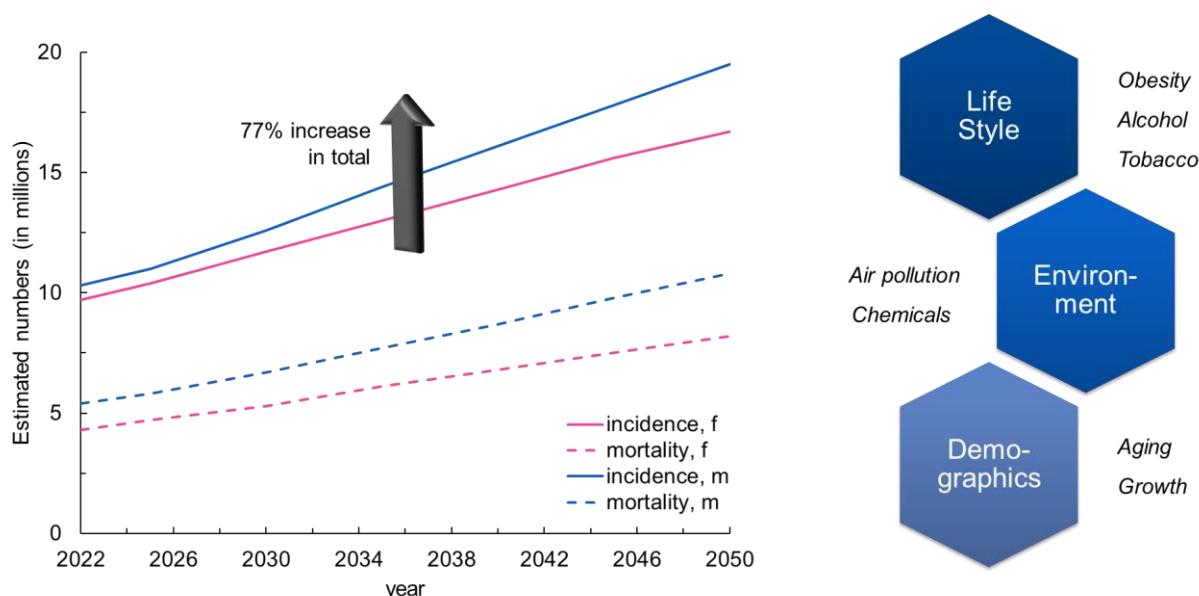


Figure 1: Development of cancer incidents and mortality in the next 25 years in male and female population (graph recreated from *Global Cancer Observatory*^[6]) and the major socioeconomic causes.

1.1 Supramolecular Chemistry

“Supramolecular chemistry may be defined as “chemistry beyond the molecule,” bearing on the organized entities of higher complexity that result from the association of two or more chemical species held together by intermolecular forces.”^[7]

- Jean Marie Lehn (Nobel Lecture) -

As the French chemist and Nobel laureate aptly described above, supermolecules are complex assemblies of individual molecules stabilized by covalent or non-covalent interactions. In fact, they are fundamental to life as many biological entities like the DNA double helix, proteins or cellular vesicles, and processes like mitosis can be understood as supramolecular.^[8] In addition to natural systems, a wide range of artificial platforms with size ranges from < 10 nm up to 1 cm evolved over the past decades (Figure 2). A selection of different types is briefly outlined below; however, it should be noted that also other categorizations of the various classes exist:^[9]

Supramolecular aggregates are one of the broadest classes of supermolecules. As defined by the International Union of Pure and Applied Chemistry (IUPAC), they are “irregular clusters of otherwise individual molecules or particles” and are thus, characterised by a lack of order.^[10] This group includes bioinspired structures like micelles or artificial liposomes that are built from amphiphilic monomers, but also polymeric aggregates depicted in Figure 2. For instance, engineered liposomes are highly biocompatible due to their lipid-based components and are therefore widely studied as drug delivery systems.^[11] Several formulations have already been approved for clinical use by the *US Food and Drug Administration* and the *European Medicines Agency*.^[11]

Reticular systems are infinite, crystalline, two-dimensional (2D) and 3-dimensional (3D) networks of components that are held together by bonds of varying strength and defined by their unique porosity, which enables application in a variety of scientific fields (Figure 2). In ascending order of bond strength these networks include hydrogen-bonded organic frameworks, metal-organic frameworks (MOFs) and covalent organic frameworks.^[12–14] While hydrogen-bonded organic frameworks are less explored due to their limited stability, covalent frameworks and MOFs have been studied amongst others for their catalytic and gas storage ability,^[15,16] as well as for their potential in electrochemistry, including next generation batteries,^[17,18] and biomedicine.^[19,20] For instance, the incorporation of biocompatible oligosaccharides like cyclodextrins (CD) as building blocks into MOFs improved their safety for *in vivo* drug delivery applications.^[21] In fact, due to their potential to host various molecular

guests in the porous cavities for diverse applications this year's Nobel Prize in Chemistry (2025) was awarded to Susumu Kitagawa, Richard Robson and Omar M. Yaghi the development of MOFs.

Mechanically interlocked molecules (MIMs) including structures like rotaxanes, catenanes and molecular knots represent another important class as illustrated in Figure 2. They consist of one or more building blocks that are irreversibly intertwined, creating a so-called mechanical bond, and therefore also referred to as mechanomolecules.^[9] This outstanding characteristic makes MIMs especially appealing for the design of molecular machines, wherein movement through the mechanical bond is induced to perform tasks at the nanoscale.^[22] Their significance in this context was recognized with the Nobel Prize in Chemistry in 2016 of Jean-Pierre Sauvage, Sir J. Fraser Stoddart, Bernard L. Feringa.^[23] A good illustration of this is a rotaxane-based peptide synthesizer able to catalyse the sequential conjugation of β -amino acids, and thereby mimicking the function of a ribozyme.^[24]

Supramolecular coordination complexes (SCCs), as opposed to infinite framework systems, are discrete entities composed of metal nodes (M) and organic linkers (L) and held together by coordination bonds (Figure 2).^[25,26] These Werner-type complexes exhibit versatile 2D and 3D topologies like cycles, helicates and cages, and due to this diversity have been investigated in catalysis^[27–29], sensing^[30], molecular recognition^[31,32] and biomedicine^[33,34] amongst others. As an example, due to their chirality triple-helical Fe(II)-cylinders were investigated in the recognition of various nucleic acid structures including telomeric G-quadruplex DNA.^[35] Stabilization of this sequence led to inhibition of telomerase activity resulting in cell death. Consequently, this affinity could be useful in targeting and eliminating cancer cells. The immense potential of SCCs for applications in nuclear and biomedicine is explained in more detail in the following chapters using the example of metallacages (MCgs), which are defined by their three-dimensional and porous nature and are the research object of this thesis.

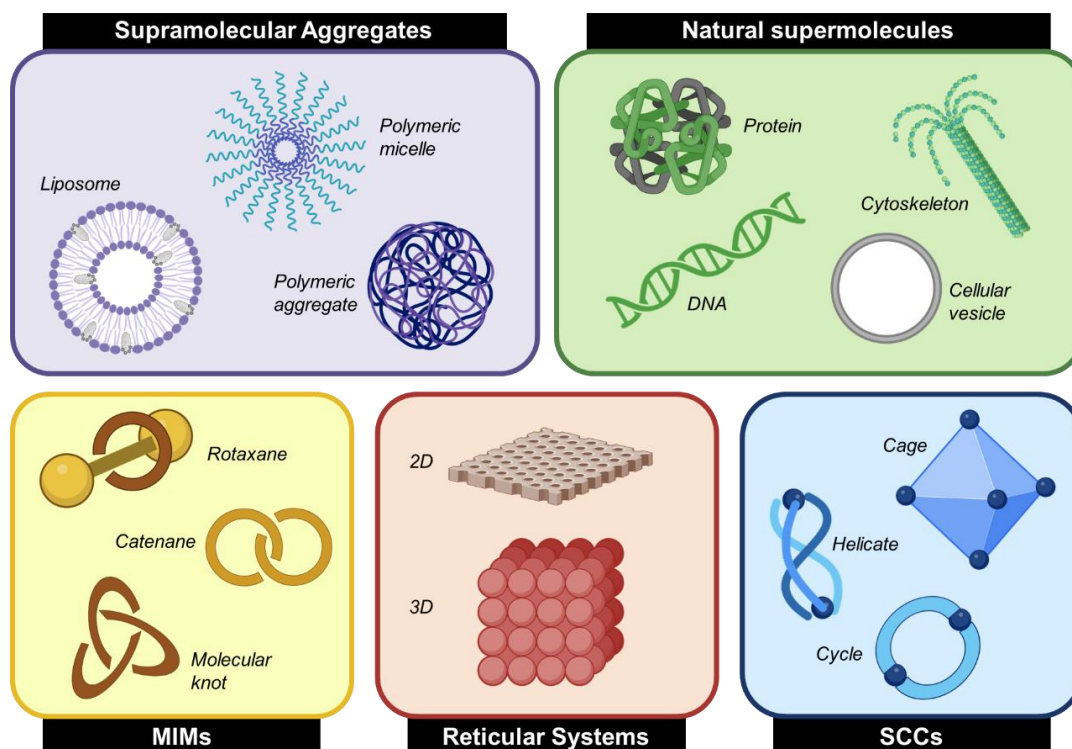


Figure 2: Different classes of supramolecular systems including a selection of exemplary structures. The figure was created in <https://BioRender.com>.

1.2 Metallacages – design and biomedical applications

MCgs are a subtype of SCCs with the general formula $M_x(L)_y(L')_z$. Their unique feature is an internal cavity able to encapsulate guest molecules and is of particular interest for separation, catalysis or drug delivery applications.^[36] The following subchapters focus especially on the synthesis and design principles of MCgs and their functionalization for application in biomedicine.

1.2.1 Design and synthesis of metallacages*

MCgs are constructed by coordination-driven self-assembly (CDSA), a process in which metal nodes and organic ligands (either linear or planar) form coordination bonds with each other to build the symmetrically most favoured structure like tetrahedra, octahedra, prisms, spheres and even more complex geometries.^[37–41] This usually occurs within minutes at mild reaction conditions by simply mixing the ligands and the metal precursor in stoichiometric amounts.

By judicious choice of ligands and metal centres, the size, shape and chemical properties of the MCg as well as its cavity can be controlled. Generally, the interplay of both building blocks dictates those parameters. In case of the selected metal ion, the coordination geometry must

* Parts of this subchapter are taken and rewritten from a review published in *Nature Reviews Chemistry* ^[5], to which the author of this work contributed.

be considered as it defines the maximal number of ligands that can be bound. So far, structures featuring transition metals like square planar Pd(II) and Pt(II), octahedral Fe(III), Ru(II), Co(III), Ir(III), Ni(II), Cu(II) and Zn(II) or more recently Au(I) have been investigated.^[42–50] On the ligand side, its denticity, directionality of donor atoms and topology influence the overall shape and nuclearity of the MCg.^[51] Furthermore, the nature of the organic building block determines whether the self-assembly proceeds via edge-directed or face-directed pathways. In the edge-directed assembly, which was pioneered mainly by Stang *et al.*^[52], bitopic ligands are used to define the edges of the resulting polyhedrons, whereas the face-directed assembly, first described by Fujita and co-workers^[53,54] as molecular panelling, relies on planar multitopic ligands that serve as the structural faces of the assembled architectures (Figure 3a). An example of this is the work undertaken by Howlader and Mukherjee, in which the use of linear 1,4-di(*1H*-tetrazol-5-yl)benzene ligands and 1,3,5-tri(*1H*-tetrazol-5-yl)benzene panels resulted in the formation of edge-directed and face-directed Pd₁₂ tetrahedra, respectively (Figure 3b).^[55] Notably, the group of Raymond achieved further advancement of both assembly strategies by studying dynamic behaviour^[56] and chirality^[57] of MCgs, as well as the application of their host-guest chemistry in catalysis.^[29,39,58,59]

The modular design of MCgs via CDSA facilitates the formation of multicomponent and multifunctional architectures.^[60] Compared to covalent strategies, this approach typically offers more accessible and efficient synthetic routes.^[61] However, when more than two components are involved, their assembly needs to be regulated as otherwise, statistical combinations may form, and self-sorting can occur as depicted in Figure 3c.^[62] The latter refers to the preferred formation of certain structures under both thermodynamic and kinetic regime and can be categorized in two groups:^[63] i) narcissistic self-sorting results in homoleptic assemblies made from identical ligands, while ii) integrative self-sorting promotes heteroleptic structures built from different ligands.^[64] For instance, the aforementioned edge- and face-directed Pd₁₂ tetrahedra underwent narcissistic self-sorting when mixing both ligands and the metal precursor (Figure 3b).^[55]

By introducing orthogonal recognition elements or geometrical complementarity in the building blocks, CDSA can be controlled in a way that construction of a desired species is achieved. Heteroleptic MCgs can be obtained by using ligands with matching sizes and shapes, with specific secondary interactions like hydrogen bonds or π - π stacking, or with steric constraints.^[65–67] Furthermore, templating by guest molecules during cage assembly and selective metal-ligand coordination are alternate strategies for directed synthesis.^[65,67,68] The group of Severin investigated the self-assembly of Pd(II)-based MCgs in a combinatorial approach using three dipyriddy ligands with different bent angles (0°, 60°, 120°).^[69] Interestingly, in this study a heterodileptic complex ([Pd₂L₂L'₂]) was formed despite a geometric

mismatch of the ligands as shown in Figure 3d. The selectivity for integrative self-sorting is assumed to originate from an increase in entropy when compared to narcissistic assembly.^[69] It should also be noted that heterometallic MCgs can be designed by exploiting the distinct binding preferences and coordination geometries of different metal ions.^[70] Recently, Thoonen *et al.* reported on a heteronuclear $[\text{Cu}_2\text{PdL}_4]^{2+}$ coordination cage assembled in one step by designing an asymmetric ligand with a Pd(II)-binding pyridyl moiety and Cu(II)-coordinating carboxylate units.^[71]

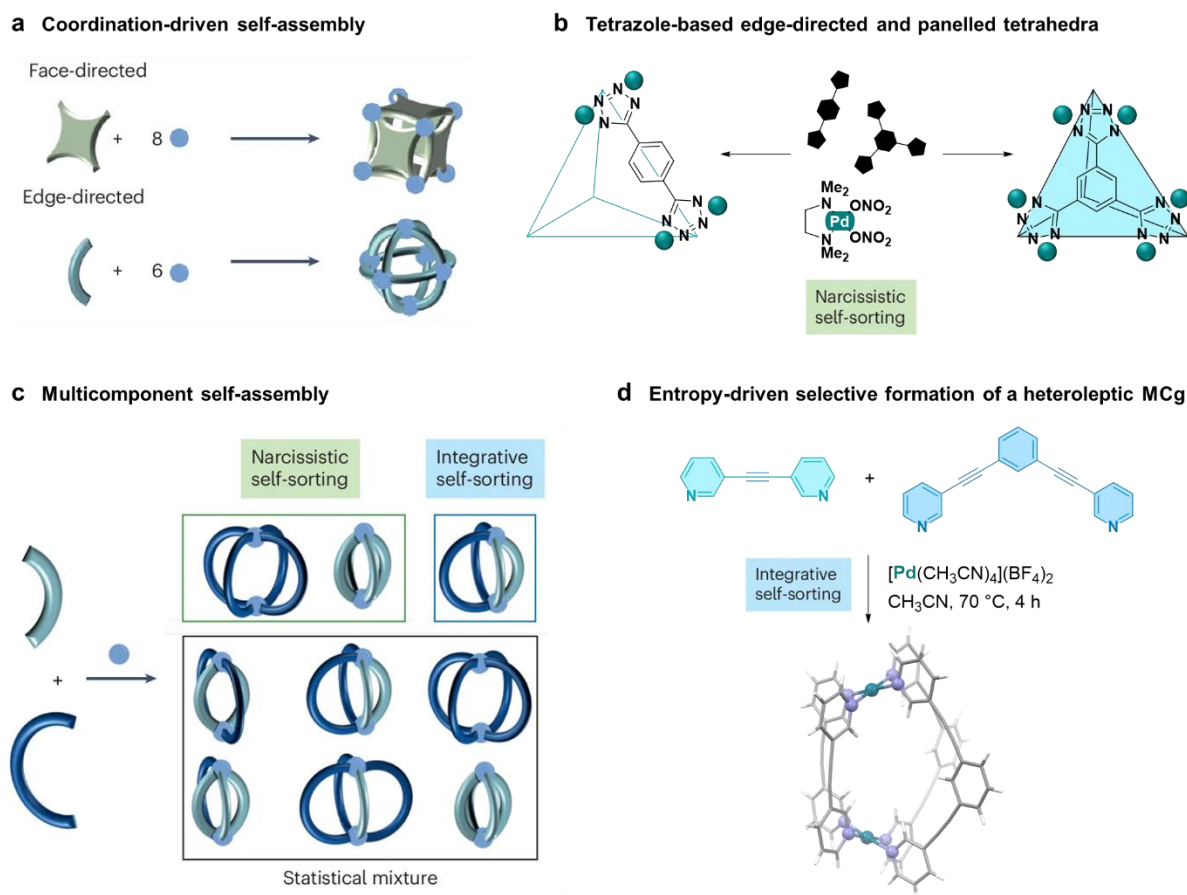


Figure 3: Design and synthetic concepts of MCgs. a) Schematic representation of face-directed and edge-directed CDSA. The blue sphere illustrates a metal ion. b) Narcissistic formation of edge-directed and face-directed Pd_{12} tetrahedra from a mixture of bitopic and tritopic tetrazole-based ligands.^[55] c) Possible building block combinations in multicomponent MCg assembly divided into narcissistic and integrative self-sorting, and statistical mixture. d) CDSA and crystal structure of a heteroleptic MCg featuring dipyrindyl ligands with different bent angles (C = grey, H = white, N = indigo, Pd = cyan). Selectivity for integrative self-sorting is caused by an increase in entropy.^[69] Panels a and c have been reproduced from ref.^[5]. Copyright © 2024, Springer Nature Limited.

In general, the self-assembly pathway of homoleptic and heteroleptic architectures can also be affected by the reaction conditions like solvent, concentration, temperature and pH, which can alter the reactivity and availability of the components as well as the resulting MCg geometry.^[72] A recent study on the design and assembly of brain-targeted $[\text{Pd}_2\text{L}_4]^{4+}$ MCgs revealed the existence of a formation equilibrium wherein full conversion to the cage could

only be achieved with sufficient reactant concentrations in the mM range.^[73] In different works conducted by Fujita and Zhou solvent-controlled CDSA of Pd₃L₆ and Pd₄L₈ tubular MCgs and structural switching between both architectures was observed.^[74,75] While dimethyl sulfoxide (DMSO) promoted the formation of Pd₄L₈ boxes, the assembly of Pd₃L₆ cages was favoured in acetonitrile (MeCN) and H₂O.

Overall, MCgs provide access to a wide variety of geometries and sizes through CDSA, and by judicious selection of components and reaction conditions, rational design of both homoleptic and heteroleptic structures is enabled.

1.2.2 Tuning the properties of metallacages for biomedical applications

The straightforward assembly from modular building blocks as well as the internal cavity make MCgs attractive candidates for novel approaches in biomedicine. Moreover, these platforms offer plenty opportunities for functionalization, enabling fine-tuning for the intended purpose and optimization to meet the physiological requirements of *in vivo* use, as will be discussed below.

Physiological requirements

MCgs are typically synthesized and characterized in organic solvents and not in aqueous solution due to limited solubility of the mostly hydrophobic, aromatic ligands and the potential for water to disrupt the self-assembly process.^[76] However, for biomedical application, sufficient solubility and stability in water are essential and several strategies to enhance these properties have therefore, been explored. While an overall positive or negative charge of the coordination cage may improve solubility, this effect alone is often insufficient.^[76] Integrating water-soluble auxiliary ligands in the design of the MCg, like ethylenediamine that was used for *cis*-capping the metal nodes of supramolecular polyhedrons, have shown to increase the hydrophilicity of the assembled structure.^[77] Additionally, exchanging the cage's counter anion, which originates from the metal precursor used in the CDSA, can lead to pronounced aqueous compatibility. For instance, Nitschke and co-workers reported a porphyrin-faced Fe(II)₈L₆ cube that was soluble in MeCN and upon replacing the triflate anions with sulfates rendered it water soluble (Figure 4a).^[78] Other counterions such as nitrate and acetate have similarly demonstrated beneficial solubilizing effects.^[79–81] In case these adjustments are not applicable, *exo*-functionalization of the MCg, meaning modification of the cage's outside by attaching functional groups to the ligands, offers a viable alternative. This strategy includes the introduction of positively charged substituents like sulfonates or hydrophilic moieties such as sugars, polyethylene glycol (PEG) chains or peptides, all of which have been employed successfully to enhance solubility in aqueous environment.^[82–85] These functional groups can be incorporated prior to CDSA or post-synthetically through post-assembly modification (PAM).

Among the most widely used functionalization techniques are click chemistry and amide coupling, the former of which was applied to tether PEG chains via PAM to an otherwise insoluble dicopper(II) paddlewheel polyhedron as depicted in Figure 4b.^[85]

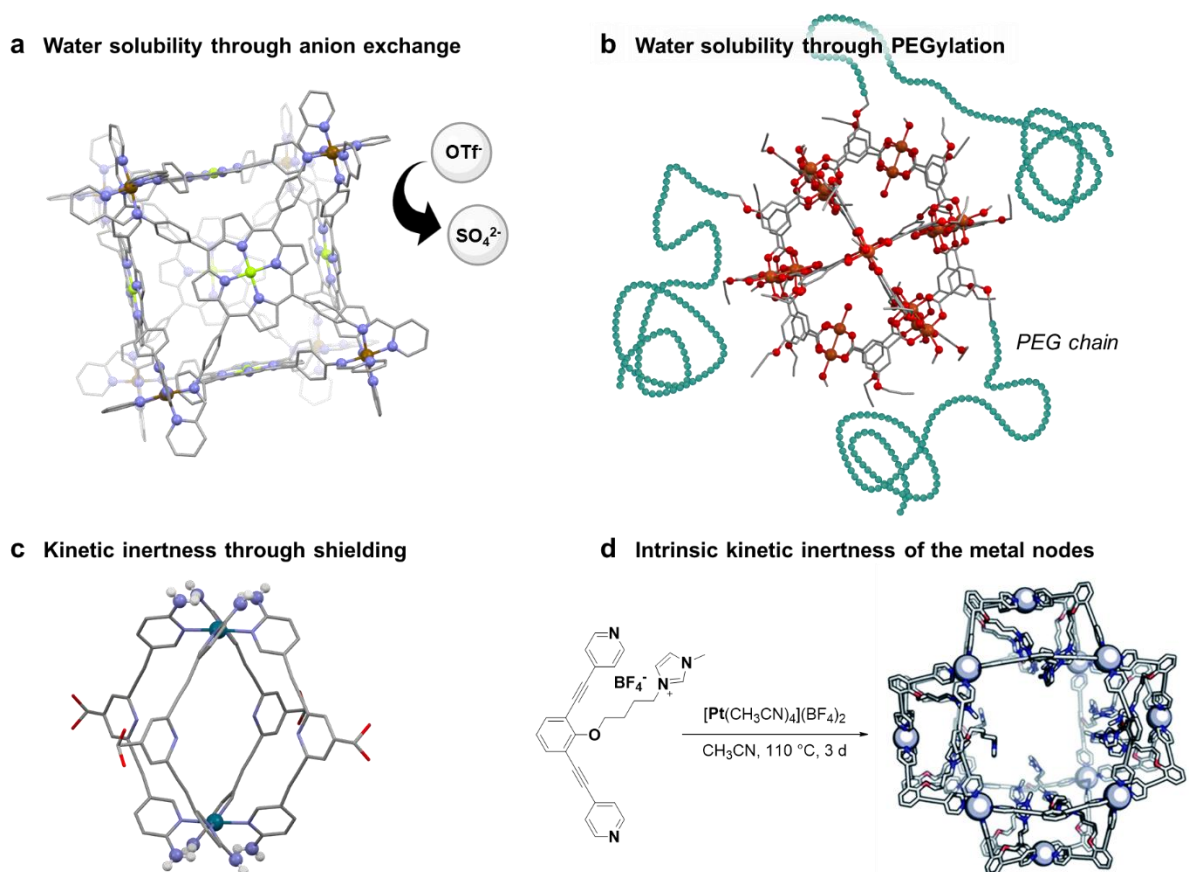


Figure 4: Optimization of physiological properties of MCgs. a) Crystal structure of a porphyrin-based Fe(II)₈L₆ cube, which exhibits enhanced water solubility upon exchanging the counterion.^[78] b) Crystal structure of a Cu(II) paddlewheel cage conjugated to water soluble PEG chains via PAM.^[85] c) Crystal structure of a kinetically inert Pd₂L₄ MCg featuring amino-substituted ligands protecting the Pd-N coordination bond through inter-ligand H-bonds.^[86] d) Molecular mechanics optimized structure and selective synthesis of a robust Pt₁₂L₂₄ cage via ligand *endo*-functionalization. This panel is reproduced from ref.^[87] with permission from the *Royal Society of Chemistry*. Colour code of crystal structures: C = grey, H = white, N = indaco, O = red, Cu = brown-red, Fe = brown, Ni = green, Pd = cyan. Only relevant H-atoms are shown. Panels a and b were created in <https://BioRender.com>.

Another crucial parameter for *in vivo* applications is the stability of the MCg against endogenous nucleophiles like glutathione (GSH), cysteine or histidine. Maintaining structural integrity under physiological conditions is essential to preserve functional efficacy and avoid adverse side effects. The primary degradation pathway for MCgs mainly involves dissociation of the coordination bonds followed by disassembly into the building blocks. To improve the kinetic stability of lantern-shaped [Pd₂L₄]⁴⁺ cages (L = 2,6-bis(pyridine-3-ylethynyl)pyridine), Preston *et al.* introduced modified the ligand by attaching amino groups at the α-position relative to the coordination site (Figure 4c).^[86] This substitution both increased the electron

donating effect of the ligand and introduced intermolecular hydrogen bonding between the amines, which sterically shielded the metal-ligand interface. As a result, the modified cage showed a more robust architecture and exhibited an at least 4-fold increase in half-life in the presence of chloride, cysteine and histidine compared to the unsubstituted analogue (> 2 h vs. < 20 min). However, this modification also compromised the cage's ability to encapsulate the chemotherapeutic drug cisplatin, thus limiting its utility as a drug delivery system.

A more advanced strategy to enhance MCg stability suggests the use of more kinetically stable metal ions to achieve the self-assembly. As an example, Pt(II) complexes are known to be up to five orders of magnitude more kinetically inert toward biological nucleophiles than their isostructural Pd(II) counterparts.^[88] In this context, the group of Reek focuses on the synthesis of $[\text{Pt}_n\text{L}_{2n}]^{2n+}$ cages and reported a strategy to selectively form MCgs with $n = 2-12$ by careful ligand design (Figure 4d).^[87] While the selection of different bent angles favoured different cage geometries through directionality, *endo*-functionalization (modification of the ligand side that points inside the cage cavity) directed the cage assembly through electrostatic and steric repulsion between the *endo*-moieties. The group further studied the lantern-shaped $[\text{Pt}_2\text{L}_4]^{4+}$ ($\text{L} = 3,5\text{-bis(3-ethynylpyridine)phenyl}$) as catalyst in cyclization reactions and for *in vivo* applications.^[89–91] To enable *in vivo* fluorescence imaging in zebrafish embryos, the MCg was conjugated to rhodamine dyes.^[91] Prior to administration, kinetic stability was assessed under physiological conditions. In the presence of chloride, imidazole, GSH and phosphate-buffered saline (PBS, pH 7.4) at 37 °C, no decomposition could be observed for at least 12 h by ^1H nuclear magnetic resonance (NMR) spectroscopy and UV/Vis absorption spectroscopy. In this respect, a significant improvement compared to $[\text{Pd}_2\text{L}_4]^{4+}$ cages was achieved.^[92,93] However, this increased robustness comes at the cost of harsher synthetic procedures and optimized Pt(II) precursors to achieve self-assembly. In a recent study, Preston and co-workers addressed this challenge by investigating $[\text{Pt(3-ClPy)}_4](\text{X})_2$ (3-ClPy = 3-chloropyridine, $\text{X} = \text{BF}_4^-$, NO_3^- , OTf^-) as a new metal precursor.^[94] This complex enabled the efficient synthesis of various cage architectures including homoleptic, heteroleptic, low-symmetry as well as multicavity cages within 1.5 h at 150 °C in aerobic atmosphere using wet solvents, marking a significant advance in the development of kinetically stable $[\text{Pt}_2\text{L}_4]^{4+}$ cages. Regarding other inert metal centres, the class of M_4L_6 tetrahedral MCgs is a notable example. While Lusby and co-workers focused on Co(III)-based tetrahedra featuring pyridyl triazole and quaterpyridine ligands, sufficiently robust to be further assessed for *in vivo* nuclear imaging (see section 1.3.5),^[95,96] the group of Raymond examined tetrahedral architectures with catecholamide ligands and the trivalent metal centres Fe(III) and Ga(III) with respect to applications in catalysis.^[29,39,97] Recently, immobilization of the Ga_4L_6 MCgs on a polymeric matrix was achieved to enable asymmetric heterogeneous catalysis in a continuous-flow setting.^[59] On the other hand, Morrow and co-workers investigated the magnetic resonance

imaging (MRI)-active Fe_4L_6 tetrahedron as an alternative to Gd-based T_1 contrast agents (Figure 5b).^[98] The high kinetic stability of the MCg enabled *in vivo* imaging in ovarian tumour-bearing mice. Interestingly, strong binding to human serum albumin (HSA) was detected, which further stabilized the cage and led to prolonged blood circulation, as well as an increase in the relaxivity due to slowed rotational motion. High imaging contrast at a low administered dose was achieved and biodistribution comparable with a Gd-HSA probe was observed. Similar findings were made with an octahedral Fe_6L_4 MCg, which proved stable towards phosphate, ethylenediaminetetraacetic acid, Zn(II) ions and transferrin.^[99]

Metallacages as multifunctional platforms

Beyond optimizing essential pharmacological parameters such as solubility and stability, MCgs can be designed as multifunctional therapeutics, imaging and drug delivery systems through the strategic use of host-guest chemistry and ligand functionalization.

Depending on the intended biomedical application the toxicity of MCgs plays a crucial role. When applied as drug delivery vehicles, the MCg design must prioritize the use of non-toxic metal centres and ligands to ensure minimal off-target effects. However, metallacages may exhibit intrinsic cytotoxicity to function as therapeutic agents themselves. For example, based on the well-established antiproliferative activity of cisplatin, Pt(II) metal nodes are frequently incorporated to endow MCgs with chemotherapeutic properties.^[100] In this context, Lippard and co-workers examined an octahedral $[\text{Pt}_6\text{L}_4]^{12+}$ cage featuring tritopic pyridyl ligands (Figure 5a).^[41] The MCg proved potent *in vitro* against ovarian, breast, lung, colon and prostate cancer cell lines with IC_{50} values ($< 14 \mu\text{M}$) comparable to cisplatin and was found to act as a DNA intercalator triggering apoptosis and senescence. Beside chemotherapy, MCgs showed promising activity in photodynamic therapy (PDT), an irradiation-based technique wherein oxidative stress and apoptosis are caused in cancerous tissue by reactive oxygen species that are produced *in situ* via energy transfer from a photosensitizer.^[101] Recently, a Ru(II)-based tetragonal prism with Au(III)-porphyrin faces was developed in our group, which displayed superior phototoxicity in melanoma and gastric cancer cells compared to the apo-MCg and the parent Au(III)-porphyrin complex.^[31] While the production capability of reactive singlet oxygen was similar, the favourable anticancer activity originated from increased cellular uptake and substantial stabilization of G4 quadruplex DNA.

To apply MCgs as diagnostic tools, they need to be detectable by imaging instrumentation. It has already been shown that MRI can be achieved by incorporating MRI-active metal ions like Fe(III) as the MCg metal centre (Figure 5b).^[98,99] On the other hand, for the development of emissive MCgs, three primary strategies have been established.^[102] The first approach involves integrating ligands with intrinsic luminescent properties into the cage framework. Examples include tetraphenylethene, hexaphenylbenzenes or porphyrins, which are suitable

for aggregation-induced emission imaging or fluorescence imaging.^[103–106] A second strategy employs *exo*-functionalization of the cage ligands to attach imaging agents. This has been demonstrated *i.a.* with $[\text{Pd}_2\text{L}_4]^{4+}$ cages ($\text{L} = 3,5\text{-bis}(3\text{-ethynylpyridine})\text{phenyl}$) modified with emissive moieties such as Ru(II) terpyridine or dipyrindine complexes, dipyrromethene dyes, and anthracenes.^[92,93,107,108] The third method relies on encapsulating luminescent molecules within the cage cavity, depending on size and shape complementarity. This strategy has been successfully applied to planar fluorophores like perylene, coumarin or curcumin.^[109–111] A representative example is the morpholine-functionalized supramolecular cube developed for lysosome-targeted cell imaging illustrated in Figure 5c.^[112] The MCg was constructed from tetrapyridyl and tetracarboxylate ligands as well as Pt(II) metal nodes and formed stable host-guest complexes with small molecule dyes emitting across the visible spectrum (blue, green and red). Encapsulation effectively suppressed dye aggregation resulting in enhanced emission. Lysosome-targeting was achieved through the morpholine vector and validated via confocal laser scanning microscopy in NCI-H1299 non-small cell lung cancer cells. Colocalization with LysoTracker confirmed the lysosomal accumulation of the host-guest complexes. It is important to note that incorporating emissive agents into MCgs can result in altered or quenched emission due to “heavy metal” effects.^[102] While this may limit imaging applications, the modulation of the fluorescence properties of MCgs and their host-guest complexes can be a useful tool to study intracellular stability, guest release and interaction dynamics. This was showcased in a comparative study assessing the interaction of the fluorescent anticancer drug doxorubicin with both a $[\text{Pd}_2\text{L}_4]^{4+}$ hydrazone-based MCg and a similar fully organic cage.^[113] The binding affinity and guest release could be investigated spectroscopically by fluorescence quenching titration, wherein the organic cage ($K_{\text{aff}} = 3.2 \times 10^6 \text{ M}^{-1}$) outperformed the metallacage ($K_{\text{aff}} = \sim 10^4 \text{ M}^{-1}$) due to enhanced stability and hydrophobicity.

The host-guest chemistry of MCgs can not only be applied for imaging, but also for delivering drug molecules. The encapsulation approach, often referred to as the *Trojan horse strategy*, is particularly beneficial for therapeutics that would otherwise not be administrable, *e.g.* due to poor solubility or high systemic toxicity like 5-fluorouracil or cisplatin, which can thereby be shielded.^[78,85,114–117] In 2008, this strategy was first proved by Therrien and co-workers.^[114] Their “complex in a complex” consists of a water-soluble Ru-clipped cage featuring 2,4,6-tri(pyridine-4-yl)-1,3,5-triazine panels and square planar metal complexes as guest compounds (Figure 5d). Crowley and co-workers were the first to encapsulate cisplatin in a $[\text{Pd}_2\text{L}_4]^{4+}$ cage ($\text{L} = 2,6\text{-bis}(\text{pyridine-3-ylethynyl})\text{pyridine}$).^[118] The design strategy was aimed to build a cavity that would favour the encapsulation of the metallodrug by establishing interactions with the pyridyl *endo*-N of the ligands. Indeed, the structure of the adduct was confirmed by ^1H NMR spectroscopy, mass spectrometry and X-ray crystallography, which also

revealed the presence of two molecules of cisplatin in the host cavity (Figure 5e). However, the host-guest complex was not preserved in aqueous environment evidenced by ^1H NMR spectroscopy, which indicated cisplatin evacuation upon addition of water.^[118] Later on, Casini and co-workers discovered that the stability of the drug-cage adduct can be improved by modifying the ligand structure, replacing the central pyridine ring with phenyl units ($L = 3,5$ -bis(3-ethynylpyridine)phenyl) to increase the lipophilic character of the internal cavity.^[119] Here, cisplatin encapsulation was favoured over the occupancy of the cavity by water molecules. Notably, most of the reported MCgs and their precursors were non-toxic *ex vivo* in healthy rat liver and kidney tissue, supporting their potential as drug delivery systems.^[84,119] The hydrophobic effect controlling the host-guest binding of MCgs in water finds its ground on previous work by Ward and co-workers, whereby a combination of crystallographic and NMR spectroscopy studies has been used as a basis for analysing enthalpy and entropy contributions to guest binding in a small M_8L_{12} cubic cage host.^[120] Van't Hoff plots for the encapsulation of several lipophilic guest molecules were determined and the thermodynamic parameters dissected. To cancel out binding contributions other than the hydrophobic effect, such as polar interactions or Van-der-Waals forces, sets of similar guests differing only by one methylene group were compared. Interestingly, while guest binding is originally considered an entropy-driven process through the release of ordered water molecules from the MCg cavity into the bulk solution, the calculations suggest the opposite.^[121] Per CH_2 group the incremental enthalpic contribution amounted to 5 kJ/mol whereas the incremental entropic contribution was negligibly small (0-1 kJ/mol).^[120] This finding can be explained by the presence of “high-energy” water molecules in the cavity, whose ability to form hydrogen bonds is restricted by the concave cavity surface.^[122] Crystallographic analysis of the M_8L_{12} cube indicated the presence of 10 water molecules in the cavity with an average of 0.5 hydrogen bonds per molecule less compared with water molecules in the bulk solvent.^[120] Enthalpic stabilization is reached by liberation of these “hydrogen bond-frustrated” water molecules followed by guest accommodation.^[122]

Another important feature of encapsulation is the protection of the guest molecule against metabolic degradation, which can increase drug accumulation in target tissues. Stabilization of the guest within the cage cavity can be achieved through covalent attachment to the ligand in form of a prodrug or via non-covalent interactions like Van-der-Waals forces, hydrogen bonds or π - π stacking.^[34] The latter has been useful in the delivery of planar photosensitizers for PDT, such as porphyrins encapsulated in Ru(II), Rh(III) and Ir(III)-based prisms or Zn(II)-phthalocyanines loaded into supramolecular Pt(II)-barrels.^[43,123,124]

Independently on their desired use, it is necessary to address the challenge of targeting of MCgs, for example to cancer sites. Their relatively small size (low nm scale) is generally insufficient to trigger the enhanced permeability and retention (EPR) effect, which facilitates

passive accumulation in tumour tissue via leaky vasculature.^[125] To enable specific cellular uptake, *exo*-functionalization of the cage ligands with targeting moieties can be employed. To date, various small molecules like morpholine or biotin, as well as selective peptides have been investigated for this purpose.^[73,84,112,126–128] A notable example is a $[\text{Pd}_2\text{L}_4]^{4+}$ cage ($\text{L} = 3,5\text{-bis}(3\text{-ethynylpyridine})\text{phenyl}$) reported in 2018 functionalized with integrin-binding peptides for the targeted delivery of cisplatin.^[84] The peptide-conjugated cages displayed superior binding affinity toward $\alpha\text{v}\beta 3$ and $\alpha 5\beta 1$ integrins in the low nM range in a solid-phase binding assay compared to Cilengitide, a potent RGD (arginylglycylaspartic acid)-based integrin antagonist.^[129] This improved performance can be attributed to the multimerization effect as one MCg presents four integrin-targeting units in a single construct.^[130,131]

Despite the development of increasingly complex and multifunctional supramolecular drug delivery systems, a key challenge remains that is achieving controlled drug release at the target site. One determinant of guest release and retention behaviour is the MCg's portal size.^[132] This matter has been studied on hexaruthenium cages with dioxido ligands of identical length but with altered width, creating cavity entrances of varying size. Fluorescence spectroscopy and molecular modelling demonstrated that smaller openings retained a pyrene fluorophore more effectively, while larger portals facilitated release and host-guest complex formation. Beside passive diffusion through the portals, other release mechanisms include cage disassembly into the building blocks and structural switching to eject the guest molecule.^[133] Both of these processes can be triggered by specific stimuli like light, elevated temperature, altered pH or an increased concentration of reactive oxygen species or nucleophiles like GSH, some of which can often be found in the tumour microenvironment. Such stimuli-responsive design is critical for achieving controlled drug release. An example of this can be found in the MRI-active Fe_4L_6 tetrahedron reported by Morrow *et al.* that encapsulates a Au(I)-based anticancer drug.^[134] Here, strong interactions with HSA stabilized the guest inside the MCg preventing release, which could then be triggered at slightly acidic pH, typical of tumour tissues.

Interestingly, MCgs can also act as cargo within other drug delivery systems like polymeric nanoparticles, resulting in hybrid nanomaterials with enhanced multifunctionality. The combinatorial strategy can help address common limitations of metallacages including solubility, stability, toxicity or drug release. Stang *et al.* developed a hexagonal prism for synergistic photochemotherapy and near-infrared (NIR) fluorescence imaging.^[135] The cage was assembled from disodium terephthalate as well as star-shaped hexakispyridyl ligands and *cis*-(PEt_3)₂Pt(OTf)₂ and was used to encapsulate octaethylporphyrin. In this design, the Pt(II) metal nodes provided chemotherapeutic activity, while the porphyrin guest served both as photosensitizer for PDT and fluorescent imaging agent. Since free porphyrins tend to

accumulate by π - π stacking, which leads to fluorescence quenching, encapsulation was employed to maintain the emissive properties and increase the PDT efficacy. To reduce systemic toxicity and improve stability in physiological conditions, the host-guest complex was loaded into the hydrophobic core of an amphiphilic diblock copolymer, forming a MCg-loaded nanoparticle (MNP). The MNP was further functionalized with integrin-targeting RGD peptides via copper-free azide-alkyne cycloaddition, which enabled active cancer targeting in addition to passive accumulation via the EPR effect. The MNP showed good stability at physiological pH (7.4), but under mildly acidic conditions (pH 5.5) in the presence of guanosine triphosphate 96% of Pt(II) was released within 48 h due to MCg disassembly. This stimuli-responsiveness might be useful in the controlled delivery of drug molecules to both lysosomes and endosomes of cancer cells. *In vivo* NIR fluorescent imaging of the targeted MNP in a A2780cis cisplatin-resistant ovarian cancer mouse model indicated 10-fold higher tumour uptake 24 h post-injection (p.i.) compared to free cisplatin, alongside extended tumour retention. When applied for photochemotherapy, the MNP achieved an 89.2% tumour inhibition rate, outperforming PDT and chemotherapy monotherapies due to the synergistic anticancer effect.

In conclusion, the modular combination of targeting ligands, imaging components and therapeutic agents highlight the versatility of MCgs. Their ability to encapsulate pharmaceutical cargos and to be used to attain multifunctional nanoplatforms make them promising candidates for biomedical applications with unique pharmacokinetic properties compared to other supramolecular systems such as liposomes. While their supramolecular nature enables multimodality, the relatively small size of MCgs in the nanometre range allows for complete characterization using conventional spectroscopic and analytical techniques, an advantageous detail for potential clinical implementation. Moreover, the combinatorial build up and the possibility to construct heteroleptic systems further broadens their applicability by providing tailored designs for simultaneous therapy and diagnostics, forecasting a promising future of SCCs in biomedical research.

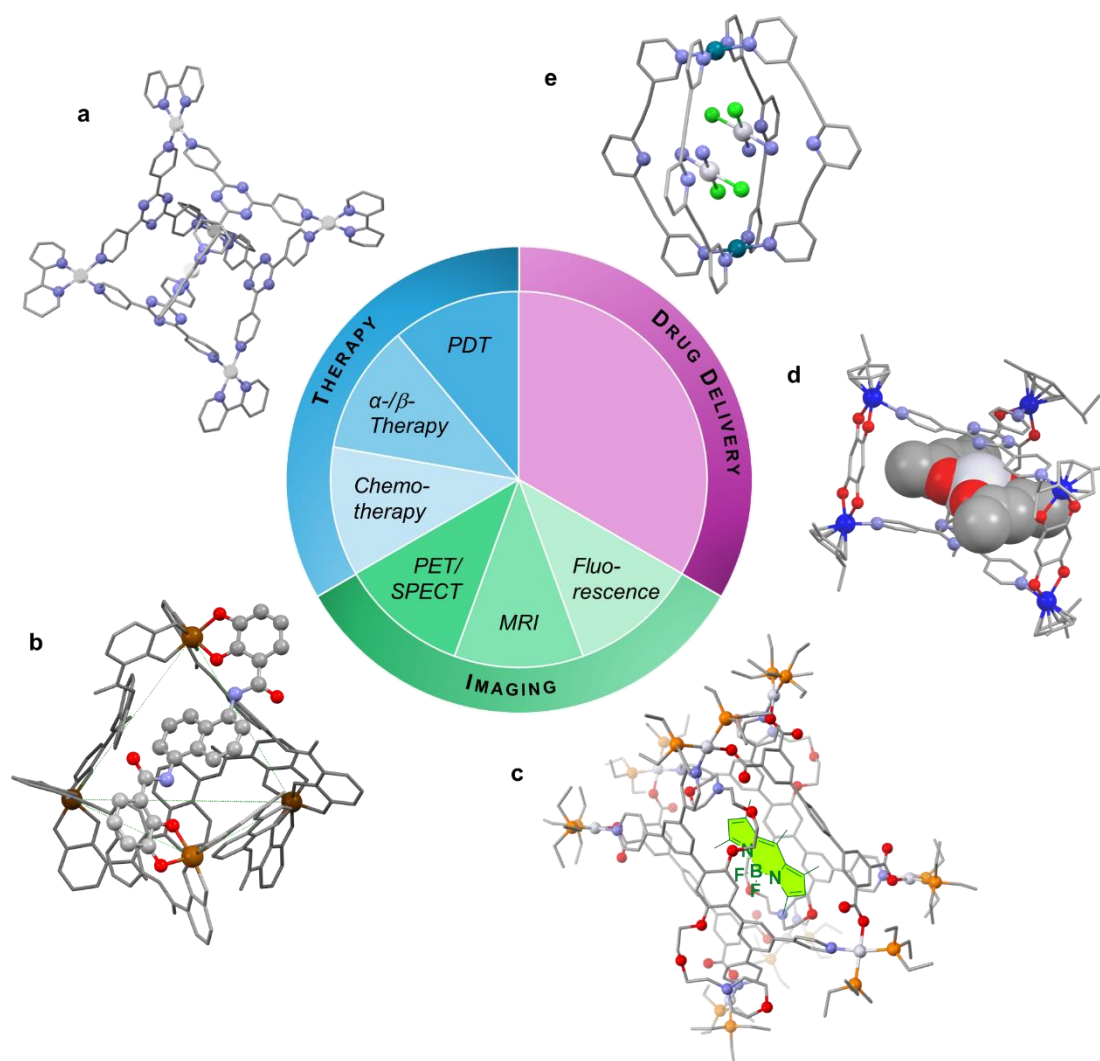


Figure 5: MCgs for biomedical applications. a) $[\text{Pt}_6\text{L}_4]^{12+}$ octahedron with antiproliferative activity *in vitro* against several cancer cell lines.^[41,136] b) Tetragonal MCg featuring MRI-active Fe(III) metal nodes.^[98] c) Morpholine-functionalized Pt(II)-cube encapsulating a small molecule dye for lysosomal imaging.^[112] d) Crystal structure of a Ru(II)-clipped trigonal prism encapsulating a square planar Pt-complex, the first proof-of-concept of the drug delivery ability of MCgs.^[114] e) Crystal structure of a $(\text{cisplatin})_2 \subset [\text{Pd}_2\text{L}_4]^{4+}$ host-guest complex.^[118] Colour code of crystal structures: C = grey, Cl = green, Fe = brown, N = indigo, O = red, P = orange, Pd = cyan, Pt = white, Ru = blue. H-atoms are omitted for clarity.

1.3 Metallacages as novel radiotheranostic platforms

The attractive properties of self-assembled supramolecular scaffolds have recently also sparked interest in the radiopharmaceutical community as this different type of chemistry offers new routes in the design of radiotracers and might provide solutions for current challenges in the field. The following subsections will give a short overview of therapeutic and diagnostic radiation, treatment options and imaging instrumentation in nuclear medicine as shown in Figure 6 and Table 1 before focusing on the radiolabelling of MCgs and MIMs for radiotheranostic applications.

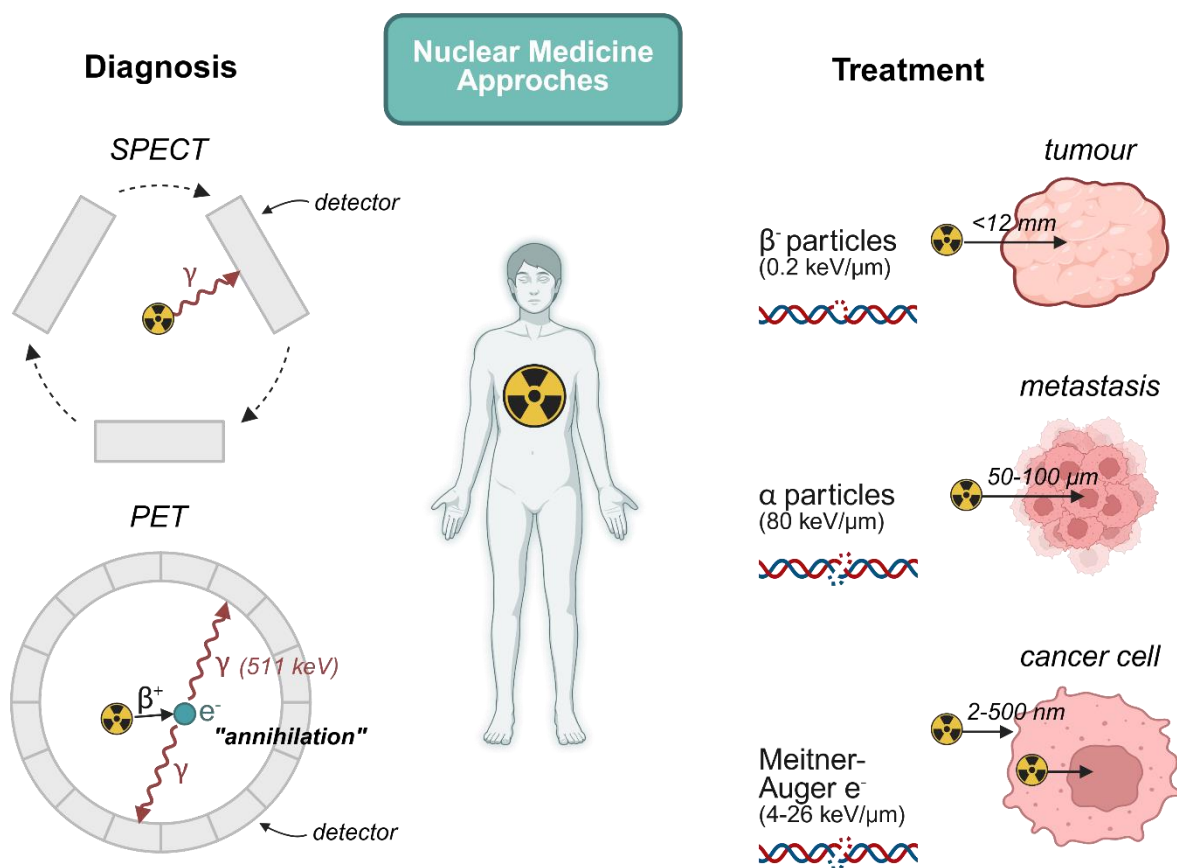


Figure 6: The use of radiation emitted by therapeutic and diagnostic radionuclides in cancer management. The figure was created in <https://BioRender.com>.

Table 1: Selection of medicinal radioisotopes including their half-life and types of emission.^[137–141]

Isotope	$t_{1/2}$	Diagnostic	Therapeutic	Isotope	$t_{1/2}$	Diagnostic	Therapeutic
Ac-225	9.9 d		α	Pb-203	2.2 d	γ	
At-211	7.2 h		α	Pb-212/ Bi-212	10.6 h 60 min		β^- α
Au-198	2.7 d	γ	β^-	Pd-103	17.0 d	γ	Meitner- Auger e^-
Au-199	3.2 d	γ	β^-	Pd-109	13.7 h		β^- , Meitner- Auger e^-
Bi-213	45.6 min		α	Pt- 195m	4.0 d	γ	Meitner- Auger e^-
C-11	20.3 min	β^+		Ra-223	11.4 d	γ	α
Cu-64	12.7 h	β^+	β^-	Re-186	3.7 d	γ	β^-
Cu-67	2.6 d	γ	β^-	Re-188	17.0 h	γ	β^-
F-18	109.7 min	β^+		Sc-44	4.0 h	β^+	
Ga-67	3.3 d	γ	Meitner- Auger e^-	Sc-47	3.4 d	γ	β^-
Ga-68	67.7 min	β^+		Tb-149	4.1 h	β^+ , γ	α
I-123	13.2 h	γ	Meitner- Auger e^-	Tb-152	17.5 h	β^+	
I-124	4.2 d	β^+		Tb-161	6.9 d	γ	β^- , Meitner- Auger e^-
I-125	59.4 d	γ	Meitner- Auger e^-	Tc-99m	6.0 h	γ	
I-131	8.0 d	γ	β^-	Y-86	14.7 h	β^+	
In-111	2.8 d	γ	Meitner- Auger e^-	Y-90	2.7 d		β^-
Lu-177	6.7 d	γ	β^-	Zr-89	3.3 d	β^+	

1.3.1 Diagnostic radiation and instrumentation

Single-photon emission computed tomography (SPECT) and positron emission tomography (PET) are the most widely applied molecular imaging techniques in nuclear medicine (Figure 6). Both are commonly combined with anatomical imaging methods like computed tomography (CT) and MRI to allow for improved diagnostic accuracy.^[137] SPECT imaging requires radionuclides emitting γ -rays (photons) such as technetium-99m, lutetium-177, iodine-123 or indium-111 (Table 1).^[142] The emitted photons are directly detected by γ -cameras that rotate around the patient to reconstruct a three-dimensional image of the tracer distribution (Figure 6).^[143] On the other hand, PET nuclides like fluorine-18, gallium-68, copper-64 or scandium-44 emit positrons (β^+) (Table 1).^[142] The emitted positrons travel a distance depending on the emission energy before hitting electrons leading to an annihilation event, in which two 511 keV photons are released in opposite directions (Figure 6). These photons are detected by circular detectors surrounding the patient, enabling accurate image

reconstruction.^[143] Compared to SPECT, PET generally offers higher spatial resolution as it is dependent on the positron energy, meaning that the lower the energy of the positron, the shorter the tissue penetration before annihilation and thus, the better the image quality. Another advantage is better sensitivity, which arises from the detection of coincident photon pairs from a single decay event instead of filtering out most photons with a collimator in SPECT.^[144]

While PET imaging is usually regarded as the more advanced method, SPECT remains the most widely applied technique in nuclear medicine due to the broad availability and convenience of ^{99m}Tc-based radiotracers. Technetium-99m is readily accessible in the form of [^{99m}Tc]pertechnetate ([^{99m}Tc]TcO₄⁻), which can be eluted from a portable ⁹⁹Mo/^{99m}Tc-generator, making it particularly suitable for routine hospital use.^[145] Additionally, technetium-99m exhibits favourable imaging characteristics, including a half-life of 6.0 h and γ -emission energy of 141 keV that is well suited for the sensitivity range of standard SPECT detectors.^[145] Unmodified [^{99m}Tc]pertechnetate naturally accumulates in the thyroid and is therefore used for functional thyroid imaging.^[146] For broader diagnostic applications, technetium-99m must be conjugated to a targeted vector. This is typically achieved via chelation, exploiting the metal's various oxidation states. Several coordination strategies have been established such as the tricarbonyl approach, the hydrazine-nicotinamide-based ligand system or the oxo method to form stable ^{99m}Tc-labelled complexes for diverse biological targets.^[147] Apart from chelation, supramolecular encapsulation of [^{99m}Tc]pertechnetate could be an alternative radiolabelling strategy that avoids chemical modification of the radionuclide, which will be explained in more detail in section 1.3.5.^[96,148]

In contrast, the workhorse of PET imaging is 2-deoxy-2-[¹⁸F]fluoro-*D*-glucose ([¹⁸F]FDG) with its nuclide fluorine-18. Its low average positron emission energy of 250 keV, high positron branching ratio of 97% and a half-life of 109.7 min render it highly suitable for clinical diagnostics as it offers high resolution imaging while affording sufficient time for synthetic modifications and transport. F-18 is produced in a cyclotron either by irradiation of ¹⁸O-enriched target water to yield [¹⁸F]fluoride for nucleophilic fluorination or via irradiation of gaseous [¹⁸O]O₂ and subsequent extraction with F₂ to generate [¹⁸F]F₂ gas for electrophilic fluorination.^[149] Due to the added F₂ carrier gas [¹⁸F]F₂ is obtained in low molar activities and can only provide radiolabelling products with up to 50% theoretical radiochemical yield (RCY). In contrast, the production of non-carrier added [¹⁸F]F⁻ results in an easy-to-handle aqueous solution with high molar activities and radiofluorination with 100% maximum theoretical RCY, which makes it the preferred source of [¹⁸F]fluorine and nucleophilic fluorination the preferred labelling technique.^[149] Here, nucleophilic aliphatic and aromatic substitutions to form C-¹⁸F bonds are among the most established as exemplified by the synthesis of the metabolic

imaging agent [^{18}F]FDG.^[149,150] However, while the harsh reaction conditions (elevated temperatures, non-physiological pH) can be applied for labelling small molecules, biomolecules and peptide-based radiotracers often require alternative approaches. One such strategy is indirect labelling wherein first a prosthetic group is radiolabelled and then bioconjugated to the tracer, e.g. via click chemistry, oxime ligation, amidation or alkylation.^[151] As this synthetic route involves multi-step reactions and purifications, the relatively short half-life of fluorine-18 could pose a challenge.^[151] Another possible approach is the direct labelling of already incorporated prosthetic groups in the radiotracer under mild conditions. For instance, an enzymatic method applies fluorinase for labelling of adenosine moieties that allows for high chemoselectivity but requires long reaction times and results in low RCY.^[152] A transition-metal mediated technique utilises Ru-activated tyrosine residues for sidechain-specific desoxyfluorination, involving sidechain (de)protection, to obtain ^{18}F -labelled peptides in moderate RCY.^[153,154] A different metal-based strategy is the complexation of [^{18}F]AlF $^{2+}$, which is formed from the reaction of AlCl $_3$ with aqueous [^{18}F]F $^-$, by a cyclic N $_3$ O $_2$ donor chelator such as NOTA (2,2',2''-(1,4,7-triazacyclononane-1,4,7-triyl)triacetic acid) and NODA (1,4,7-triazacyclononane-1,4-diacetate).^[155] The resulting thermodynamically and kinetically inert complexes as well as the great Al-F bond strength (~ 670 kJ/mol) ensure high radiotracer stability *in vivo*.^[155] However, the relatively harsh reaction conditions (~ 100 °C, acidic pH) limit the applicability especially for sensitive biomolecule-containing tracers. Transitioning to N $_2$ O $_3$ open chain chelators that enable [^{18}F]AlF $^{2+}$ complexation at room temperature (RT) could therefore advance the method.^[155,156]

^{19}F -to- ^{18}F isotopic exchange (IE) is an efficient strategy for direct one-step ^{18}F -labelling. This method relies on the introduction of a prosthetic group containing a fluorophilic centre, with boron and silicon being the most widely employed due to the high bond strengths of B-F (766 kJ/mol) and Si-F (570 kJ/mol), respectively.^[157,158] Organotrifluoroborates (BF $_3$ -R) allow for fast IE under aqueous conditions and afford higher RCY and molar activities, owing to the presence of three exchangeable F-atoms per prosthetic group compared to SiF-R.^[158] As the radiolabelling typically requires an acidic environment (pH 2-3) to achieve efficient radiochemical conversion, the procedure can lead to degradation of acid-sensitive compounds.^[158] In addition, the B-F bond is susceptible to hydrolysis, which becomes problematic in *in vivo* settings where defluorination releases free fluorine-18 that accumulates in bone tissue and increases imaging background signal.^[158,159] The introduction of electron-withdrawing substituents on aromatic trifluoroborates and the development of zwitterionic derivatives were strategies to enhance hydrolytic stability.^[159,160] Generally, the incorporation of BF $_3$ units increases tracer hydrophilicity, which in some cases might promote fast excretion and thereby result in low imaging contrast.^[158]

Organofluorosilanes, also referred to as silicon-based fluoride acceptors (SiFAs) are a class of prosthetic groups that enable rapid and efficient ^{18}F -labelling under mild conditions (RT, pH 5-7) via IE without generating radioactive side products. This makes SiFAs especially attractive for radiolabelling of sensitive biomolecules.^[161] First introduced by Schirmmacher *et al.* in 2006, the core SiFA structure consists of a fluorodi-*tert*-butylphenylsilane scaffold, which can be further modified at the phenyl ring with a variety of substituents. Up to now a diverse library of *para*-functionalized SiFA synthons has been developed including SiFA-aldehyde, SiFA-isothiocyanate, SiFA-phenylalanine, SiFA-benzoic acid ((SiFA)BA) as well as heteroaromatic and cyclic analogues (Figure 7).^[162–167] Furthermore, it was found that the *tert*-butyl groups play a crucial role in maintaining the hydrolytic stability of the Si- ^{18}F bond, thereby ensuring the *in vivo* integrity of SiFA-based radiotracers.^[161] Despite their favourable labelling characteristics, a critical limitation of SiFAs is the high lipophilicity that can result in suboptimal pharmacokinetics like increased hepatic retention and therefore low tumour uptake causing off-target radiation and decreased image quality.^[168] Optimization strategies encompass balancing the lipophilic SiFA-moiety with hydrophilic amino acids in the radiotracer or the introduction of charges. One such development is SiFA*lin* (Figure 7), a positively charged SiFA derivative that contributed to improved biodistribution of a neuroendocrine tumour-targeted radiotracer featuring the somatostatin receptor 2 (sstR2)-addressing cyclic peptide TATE ((Tyr³)-octreotate).^[169,170] In our group, an optimized bifunctional amino acid-like SiFA ((SiFA)SeFe, Figure 7) was recently developed, which can be incorporated into a radiopharmaceutical in a bridged configuration.^[171] When introducing it in sstR2-targeted TATE-based radiotracers, the resulting constructs showed decreased lipophilicity compared to their (SiFA)BA counterpart and similar lipophilicity to the clinically established SiFA*lin*-TATE.^[171]

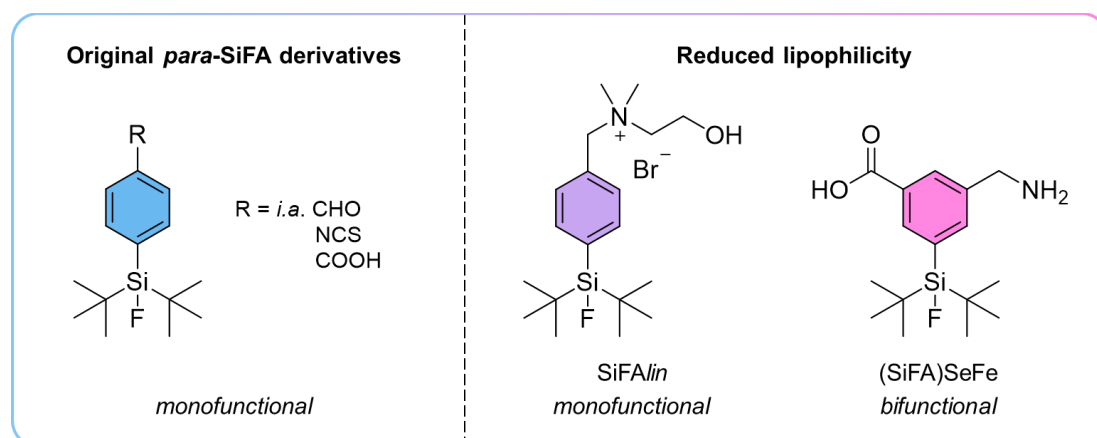


Figure 7: Chemical structures of the first silicon-based fluoride acceptors (SiFAs) as well as of new generation synthons with optimized lipophilicity.

1.3.2 *Therapeutic radiation in cancer management*

Radiotherapy, the application of ionizing radiation to eliminate malignant cells represents one of the main options in modern cancer treatment alongside surgical removal and chemotherapy.^[137] Radiation can be delivered either externally, using high-energy rays in external beam radiotherapy^[172], or internally via two principal methods: Brachytherapy involves the implantation of radioactive metal seeds directly into the tumour tissue, providing a localized radiation source.^[173] In contrast, radionuclide therapy requires the systemic administration of molecular radiopharmaceuticals.^[174] A more targeted variant of radionuclide therapy is peptide receptor radionuclide therapy, wherein therapeutic radioisotopes are conjugated to peptide ligands that address receptors overexpressed on cancer cells.^[175]

In all targeted radiopharmaceutical applications, whether therapy or imaging, it is essential to match the physical half-life of the radionuclide with the biological half-life of the targeting vector to ensure optimal performance.^[138] Thus, a small molecule vector with fast pharmacokinetics would ideally be paired with a short-lived radioisotope ($t_{1/2}$ = min-hours), whereas biomolecular vectors such as antibodies would require longer lived isotopes ($t_{1/2}$ = hours-days) due to their prolonged blood circulation.^[138]

Therapeutic radionuclides emit ionizing radiation in the form of β -particles, α -particles and/or Meitner-Auger electrons (Figure 6). These radiation types vary in energy deposition and tissue penetration, quantified by linear energy transfer (LET). LET gives the amount of energy per unit distance that is transferred to the tissue (keV/ μ m) and is a critical determinant of the biological damage.^[176]

β -emitting radionuclides such as lutetium-177, yttrium-90, copper-67 or iodine-131 are the most widely used in radionuclide therapy (Table 1).^[137] β -particles (electrons) exhibit relatively low LET values (0.2 keV/ μ m), typically inducing DNA single-strand breaks. Due to their extended penetration depth of up to 12 mm these radionuclides are especially effective in treating larger solid tumours (Figure 6).^[177]

α -particles are $^4\text{He}^{2+}$ -cores with high LET (80 keV/ μ m) capable of causing DNA double-strand breaks.^[177] Their limited pathlength of 50-100 μ m confines their effect to a few cell diameters, making them ideal for targeting small lesions like metastases (Figure 6). A prominent example is actinium-225, which emits four α -particles during its decay series and therefore significantly amplifying its therapeutic potency.^[178]

Radionuclides like gallium-67, indium-111 or iodine-125 that undergo electron capture often release Meitner-Auger electrons as part of this process (Table 1).^[179] Following electron capture, an inner-shell vacancy is created and is filled with an outer-shell electron. Its energy is released and transferred onto another electron, which is thereby ejected from the atom, denominated Meitner-Auger electron. These particles possess high LET (4-26 keV/ μ m) and

extremely short ranges of just 2-500 nm, leading to a highly localized biological effect suited for single cell targeting (Figure 6).^[179] Meitner-Auger electrons induce DNA double-strand breaks and damage the cell membrane but therefore need to be delivered in close proximity to cellular or intracellular targets.^[180,181] Recently, terbium-161 has emerged as a promising radionuclide due its emission of both β^- -particles and Meitner-Auger electrons enabling synergistic therapeutic efficacy, particularly when paired with membrane-targeting biomolecules like somatostatin analogues for the treatment of neuroendocrine neoplasms.^[182]

1.3.3 Theranostic approaches in nuclear medicine

Theranostics refers to the integration of diagnostic imaging and therapy within a single platform, often explained as “see what you treat and treat what you see”.^[183] In the context of radiopharmaceutical chemistry, this concept can be realized through five different strategies:

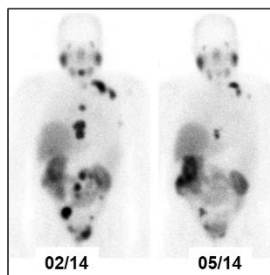
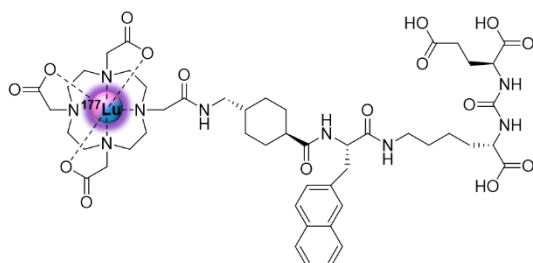
i) “*True*” theranostics defines the use of radionuclides emitting both diagnostic (β^+ or γ) and therapeutic (α , β^- or Meitner-Auger electrons) radiation, enabling imaging and treatment with a single radiopharmaceutical compound.^[137] Representative examples include lutetium-177, iodine-131, copper-64, radium-223, rhenium-188 and terbium-161 amongst others (Table 1).^[142] Here, a milestone was achieved by the *US Food and Drug Administration* approval of [¹⁷⁷Lu]Lu-PSMA-617 (Pluvicto®) in 2022 for therapy and treatment monitoring via SPECT of prostate cancer through targeting the prostate-specific membrane antigen (PSMA) (Figure 8a).^[184,185]

ii) *Isotopically matched pairs* consist of different isotopes of the same element for imaging and therapy such as yttrium-86/90, iodine-124/131 or scandium-44/47 (Table 1).^[142] Although separate compounds are required for each application, their identical chemical structures ensure consistent pharmacokinetics and biological behaviour.^[137] This has been shown in the case of [^{124/131}I]I-metaiodobenzylguanidine that is applied for β^- -therapy of neuroblastomas and a variety of neuroendocrine tumours, and PET dosimetry in the clinics.^[186,187] The long half-lives of the iodine-124/131 pair make them highly suitable also for radioimmunotherapy.^[188] For instance, [^{124/131}I]I-omburtamab is a radiolabelled monoclonal antibody targeting the tumour-specific B7-H3 cell surface glycoprotein and has been assessed in recent phase I clinical trials for PET dosimetry and biodistribution as well as compartmental treatment of central nervous system (CNS), peritoneal and leptomeningeal tumours.^[189–193]

iii) Another possibility to theranostic design is the use of *matched/mixed theranostic pairs* relying on isotopes of different elements. While this approach allows for greater flexibility in radionuclide selection, the radiopharmaceuticals must be carefully designed to ensure comparable pharmacokinetic profiles of both compounds.^[137] A well-known example is Lutathera®, which was approved by the *European Medicines Agency* in 2017 for the treatment of gastroenteropancreatic neuroendocrine tumours. This agent features the sstR2-targeted

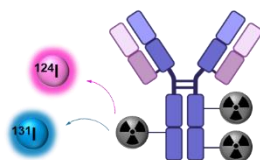
peptide TATE conjugated to the macrocyclic chelator DOTA (2,2',2'',2'''-(1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl)tetraacetic acid) that is compatible with both lutetium-177 for β^- therapy and gallium-68 for PET imaging (Figure 8c).^[194]

a “True” theranostics – Pluvicto®

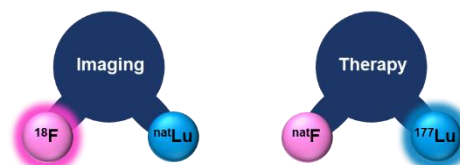


Metastatic prostate cancer

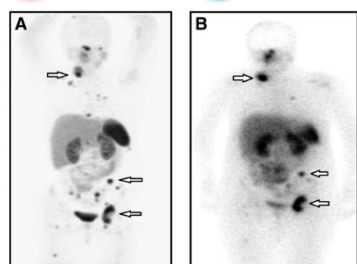
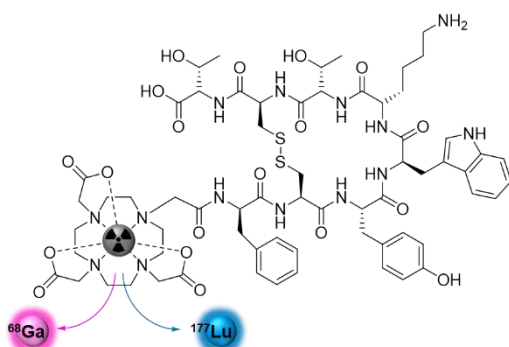
b Isotopically matched pairs – [¹²⁴I/¹³¹I]-Omburtamab



d Radiohybrids



c Matched/mixed theranostic pairs – Lutathera®



Relapsed neuroblastoma

e Matched radiopharmaceuticals – [^{99m}Tc]Tc-MDP/[²²³Ra]RaCl₂

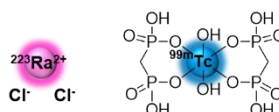


Figure 8: Theranostic strategies in radiopharmaceutical design (therapeutic nuclides are highlighted in blue, diagnostic nuclides in pink). a) Chemical structure of [¹⁷⁷Lu]Lu-PSMA-617 (Pluvicto®) as well as SPECT/CT images before and 3 months after treatment of a patient with metastatic prostate cancer.^[185] b) Illustration of [¹²⁴I/¹³¹I]-omburtamab investigated in phase I clinical trials for imaging and treatment of B7H3-expressing tumours. c) Chemical structure of [⁶⁸Ga]Ga/[¹⁷⁷Lu]Lu-DOTA-TATE (Lutathera® with ¹⁷⁷Lu) as well as corresponding PET/CT (A) and SPECT/CT (B) scans in a patient with relapsed sstR2-expressing neuroblastoma (lesions are indicated by arrows).^[194] d) Chemical structure of a sstR2-addressing radiohybrid tracer that can be used for imaging when labelled with ^{nat}Lu/¹⁸F and for therapy when labelled with ¹⁷⁷Lu/^{nat}F.^[171] e) Structures of [^{99m}Tc]Tc-methyl diphosphonate (MDP) and [²²³Ra]RaCl₂ applied in SPECT imaging and α -therapy of bone metastases. The scans in panel a were adapted from ref.^[185] © 2015, Springer-Verlag Berlin Heidelberg; the scans in panel c were adapted from ref.^[194] © SNMMI.

iv) *Radiohybrids* are a special class of matched/mixed theranostic pairs, in which one radiopharmaceutical structure offers two distinct binding sites as shown in Figure 8d allowing for the incorporation of diagnostic and therapeutic radionuclides without altering the pharmacokinetics.^[195–197] To ensure efficient radiolabelling, the binding sites should be specific and not compete for the radionuclides used. For instance, a sstR2-targeting radiohybrid ligand was designed in our group with a (SiFA)SeFe moiety for ^{18}F -labelling and DOTA for ^{177}Lu -complexation, combining PET imaging and β^- therapy (Figure 8d).^[171] In a different work, this concept was used to target the fibroblast activation protein, a biomarker expressed in the tumour microenvironment of various cancer types, thereby expanding its potential clinical applications.^[198]

v) The fifth strategy uses two completely *matched radiopharmaceuticals* for imaging and therapy that are defined through different targeting motifs and radionuclides.^[137] While a similar pharmacokinetic profile cannot be ensured with this method, it provides an adequate solution when the design of one compound doesn't allow the implementation of the second radionuclide. This is evident in the treatment of bone metastases, in which the α -emitting [^{223}Ra]RaCl₂ (Xofigo®) is administered, while imaging is performed with the clinical standard [$^{99\text{m}}\text{Tc}$]Tc-methyl diphosphonate (Medronate®) as depicted in Figure 8e.^[199,200]

1.3.4 The promise of self-assembled supramolecular radiopharmaceuticals

Conventional metal-based radiopharmaceuticals typically consist of four components: a radionuclide, its chelator and a linker that conjugates it to a targeting vector (Figure 9a).^[201] So far, achieving radiotheranostic application has mainly relied on the careful selection of suitable radioisotopes, as described in the previous subsection. However, multifunctional approaches, wherein various radioactive and non-radioactive imaging techniques and therapeutic strategies are combined, are more difficult to implement due to the limited flexibility of the classical design.^[5] Additionally, fine-tuning pharmacokinetics or introducing stimuli-responsive elements is challenging as such modifications that are often undertaken at the linker sequence may negatively affect target affinity and other physicochemical and biological properties.^[5]

Supramolecular chemistry offers a promising alternative by providing a huge playground for radiopharmaceutical design with modular building blocks and extensive options for functionalization and encapsulation. This potential will be illustrated in the following sections with focus on MCgs and MIMs (Figure 9b).

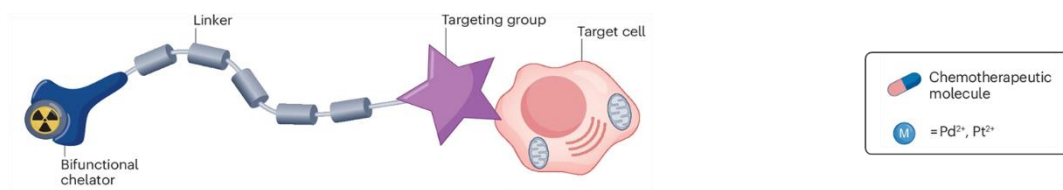
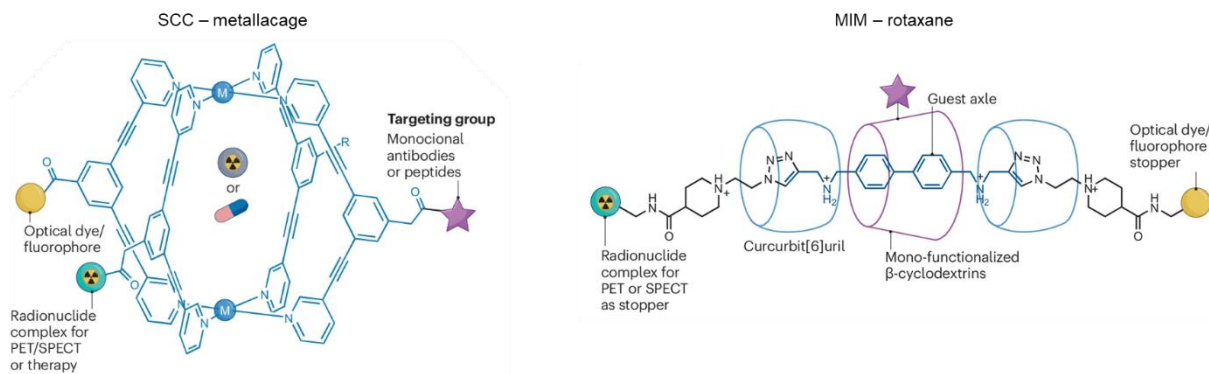
a Classical metal-based radiotracer**b Supramolecular radiopharmaceuticals**

Figure 9: Schematic representation of a) classical metal-based radiotracer design versus b) the supramolecular approach. The figure was adapted from ref.^[5]. Copyright © 2024, Springer Nature Limited.

1.3.5 Radiolabelled metallacages – design and applications*

Although radiolabelling of MCgs is just in its initial stages, the successful integration of various radioisotopes for diagnostic and therapeutic purposes already highlights their potential in radiotheranostics.^[5] In general, radionuclide incorporation into MCgs can be achieved via four principal strategies as illustrated in Figure 10a:

- I. By *exo*-functionalizing the ligand with a prosthetic group or chelator for radiolabelling,
- II. By encapsulating the radionuclide within the cavity through host-guest interactions,
- III. By complexing a radiometal in the form of a metalloligand and
- IV. By complexing a radiometal as the metal node in the self-assembly.

The selected strategy also dictates at what stage during cage synthesis the radiolabelling can be performed – either prior to self-assembly, in a one-pot fashion or following CDSA. This subchapter provides an overview of radiolabelled MCgs structured according to these design concepts.

* This subsection is taken and rewritten from a review published in *Nature Reviews Chemistry*^[5], to which the author of this work contributed.

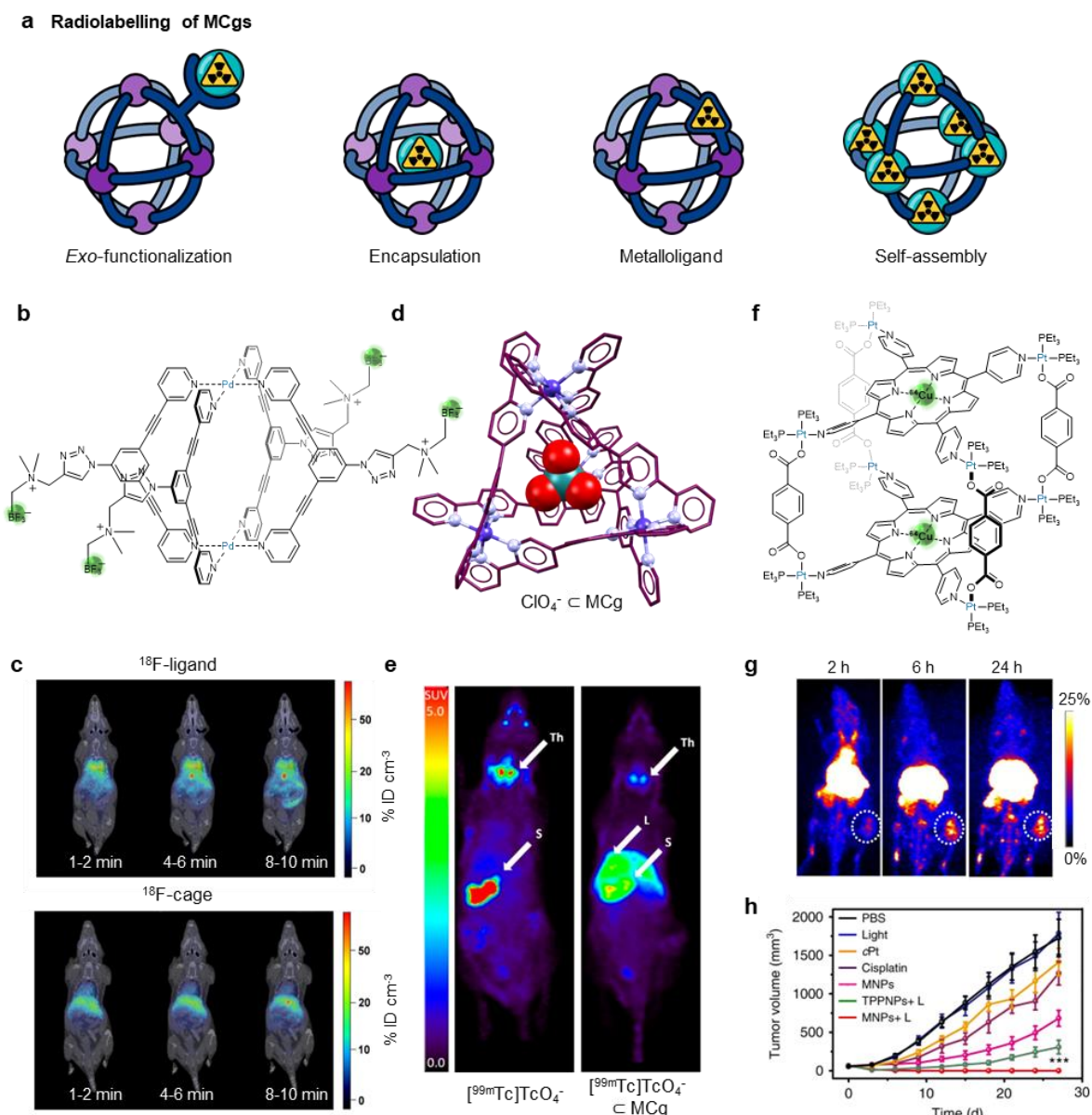


Figure 10: Radiolabelled MCgs. a) Strategies to obtain radiolabelled MCgs. b) $[\text{Pd}_2\text{L}_4]^{4+}$ cage exo-functionalized with AMBF_3 units for ^{18}F -labelling.^[202] c) *In vivo* PET/CT images of the ^{18}F -ligand compared to the radiolabelled AMBF_3 -cage in BALB/cJRj mice showing distinct biodistribution profiles.^[202] d) Crystal structure of an oxoanion $\subset \text{Co}_4\text{L}_6$ host-guest complex.^[96] e) *In vivo* SPECT images of $[\text{99mTc}]\text{TcO}_4^- \subset [\text{Co}_4\text{L}_6]^{12+}$ (2 h p.i.) compared to free $[\text{99mTc}]\text{TcO}_4^-$ (1 h p.i.) indicating reduced thyroid (Th) and stomach (S) uptake, but increased liver accumulation (Lv).^[96] f) Pt(II)-based porphyrin-panelled MCg for trimodal imaging, here complexing copper-64 for PET imaging. For stability and targeting, the MCg is loaded into polymeric nanoparticles (MNP).^[106] g) *In vivo* PET images of the ^{64}Cu -labelled MNP in U87MG tumour-bearing mice.^[106] h) *In vivo* tumour growth inhibition in U87MG tumour-bearing mice after treatment with MNPs for synergistic photochemotherapy (dark blue line) compared to other treatment regimes.^[106] Illustrations of panel a were kindly provided by Dr. Iñigo Irribarren. Panels b-h were reprinted from ref.^[5]. Copyright © 2024, Springer Nature Limited.

Exo-functionalization

Exo-functionalization of ligands with radiolabelling moieties is a practical approach to construct radioactive SCCs. For instance, Cosialls *et al.* engineered a lantern-shaped $[\text{Pd}_2\text{L}_4]^{4+}$ MCg featuring 3,5-bis(3-ethynylpyridine)phenyl ligands for PET imaging (Figure 10b).^[202] The ligands were modified via click chemistry to introduce zwitterionic ammonium trifluoroborate (AMBF_3) groups for ^{19}F -to- ^{18}F isotope exchange, a prosthetic group that enables kit-like, single-step preparation of the radiolabelled precursor in the clinics. The AMBF_3 approach enables a kit-like, single-step preparation of the radiolabelled precursor in the clinics and thus, enabling on-site assembly of the MCg prior to administration.^[203] CDSA of the MCg and subsequent encapsulation of the chemotherapeutic drug cisplatin were validated by NMR and mass spectrometry confirming that ligand functionalization neither disrupted self-assembly nor intervened with host-guest chemistry, which is consistent with findings from non-functionalized analogues.^[118] Notably, radiolabelling of the ligand prior to assembly proved essential for achieving high radiochemical purity (RCP) of 95% of the ^{18}F -labelled $[\text{Pd}_2\text{L}_4]^{4+}$ cage. *In vivo* PET imaging in mice revealed distinct biodistribution profiles for the free ligand and the cage, supporting the structural integrity of the MCg under physiological conditions (Figure 10c). Conversely, the cisplatin-loaded host-guest complex exhibited biodistribution similar to that of the free ligand, suggesting reduced stability of the MCg upon drug loading.

The exo-functionalization strategy was further applied to develop homoleptic $[\text{Pd}_2\text{L}_4]^{4+}$ cages for imaging and therapy of neuroendocrine tumours. In the work of Deiser *et al.*, the ligand was conjugated to the sstR2-targeting peptide TATE.^[127] Furthermore, a DOTA chelator was incorporated for complexation with lutetium-177 for β^- -therapy and SPECT imaging.^[137] Due to the steric demand of the functionalized ligand, self-assembly yielded a $[\text{Pd}_2\text{L}_2]^{4+}$ metallacycle rather than a 3D cage, thereby precluding guest encapsulation. ^{177}Lu -labelling was carried out at the ligand stage and optimization was necessary to prevent Pd^{2+} chelation during CDSA. The radiolabelled metallacycle was obtained with modest RCY of around 6% but high RCP (96%) and was substantially stable (> 50%) in human serum over 24 hours at 37 °C. Compared with the clinically established ^{177}Lu -DOTA-TATE (Lutathera®), the metallacycle maintained high *in vitro* affinity for the sstR2 receptor, but exhibited increased lipophilicity and enhanced binding to human serum albumin, which might affect biodistribution. To overcome limitations in guest encapsulation, a heteroleptic strategy was explored, in which a 1:3 mixture of functionalized (L) and unmodified (L0) ligands yielded a $[\text{Pd}_2(\text{L})(\text{L}0)_3]^{4+}$ cage.

Both examples show nicely how modification of the ligands can be applied to create radiolabelled Pd(II)-based MCgs. Their capacity to additionally encapsulate small theranostic agents opens the road for multimodal applications. However, future research should first aim to optimize their stability and biological parameters. Here, substitution of Pd(II) with Pt(II) in the

MCg scaffold, which forms kinetically more inert coordination bonds, could improve structural integrity under physiological conditions and facilitate covalent attachment to large biomolecules post-assembly.^[204]

Encapsulation

Early studies in the 2000s demonstrated the potential of organic aza-cages for environmental applications, particularly in the separation of oxoanions such as [^{99m}Tc]pertechnetate from wastewater.^[148,205,206] Building on this foundational work, the groups of Lusby and Archibald developed a tetrahedral [Co₄L₆]¹²⁺ metallacage from quaterpyridine ligands for the encapsulation of the [^{99m}Tc]TcO₄⁻ anion for SPECT imaging as depicted in Figure 10d.^[96] Host-guest complex formation was confirmed by ¹H NMR spectroscopy and single-crystal X-ray diffraction, employing the non-radioactive analogues ReO₄⁻ and ClO₄⁻. In biological media, including human serum, gradual release of [^{99m}Tc]TcO₄⁻ was observed and attributed to slow disassembly of the MCg rather than direct exchange with competing anions. To enhance the cage's stability, electron-donating amino substituents were incorporated into the quaterpyridine ligands, promoting the Co(III) oxidation state via increased σ-donation. These modifications yielded a more robust host-guest complex, exhibiting significant inertness in the presence of GSH and in human serum. Biodistribution SPECT imaging studies were performed in healthy mice comparing the free [^{99m}Tc]pertechnetate and the [^{99m}Tc]TcO₄⁻ ⊂ [Co₄L₆]¹²⁺ (Figure 10e). Unlike [^{99m}Tc]pertechnetate, which accumulates in the thyroid and stomach, the encapsulated anion showed reduced uptake in these tissues and enhanced liver accumulation. The elevated hepatic uptake indicates a limitation of the current design, suggesting that future iterations must address the lipophilicity of the MCg to mitigate liver retention. Nonetheless, these results demonstrate that encapsulation can effectively modulate the pharmacokinetics of the radiolabelled species, potentially enabling redirection to alternate biological targets. The host-guest radiolabelling strategy was extended to address delivery across the blood-brain barrier (BBB), a highly selective membrane that safeguards the central nervous system by tightly regulating molecular transport from the bloodstream and will be discussed in more detail in section 3.1.1. Woods *et al.* *exo*-functionalized a [Pd₂L₄]⁴⁺ cage with PepH3, a peptide sequence derived from the Dengue virus type-2 capsid protein, which facilitates translocation across the BBB.^[128,207] The peptide-conjugated bispyridyl ligand was synthesized by solid-phase peptide synthesis, which was followed by successful cage formation and encapsulation of ReO₄⁻ as evidenced by ¹H NMR spectroscopy. The resulting host-guest complex displayed high stability in aqueous and buffered solutions over 24 h but was prone to fast disassembly in the presence of GSH. An *in vitro* BBB translocation assay confirmed the capability of the MCg to cross the barrier two times more effectively than the unfunctionalized cage. Based on this encouraging result, [^{99m}Tc]TcO₄⁻ ⊂ [Pd₂L₄]⁴⁺ was prepared for *in vivo* evaluation using a

one-pot assembly method, subsequent cartridge purification and quality control by radio thin-layer chromatography (TLC). Brain uptake measured at 5 min p.i. showed radioactivity levels comparable to antibody-based tracers, suggesting the promise of this platform for nuclear brain imaging.^[207] Future optimization may involve the development of heteroleptic MCgs bearing additional targeting moieties for selective tumour recognition like glioblastoma-specific ligands, and encapsulation of therapeutic radioisotopes.

The unique porous nature of MCgs allows for encapsulation of radioactive guests and so far, the diagnostic [^{99m}Tc]TcO₄⁻ has successfully been investigated. Under the prerequisite of size and shape complementarity between the cargo and the internal cavity, a huge plethora of guest molecules remains to be explored. For instance, by exchanging [^{99m}Tc]TcO₄⁻ with the β⁻-emitting [¹⁸⁸Re]ReO₄⁻ a mixed theranostic pair may be created.^[208,209] More importantly, encapsulation could be a promising strategy in nuclear medicine to make use of radioisotopes like the α-emitter radium-223, for which only few suitable chelators exist.^[138,210,211] For this, further studies on the binding affinity and encapsulation efficiency of MCgs under physiologically relevant conditions, namely at pH 7.4, in buffered serum-containing environments, and at nanomolar concentrations are necessary, but challenging to analyse due to limited sensitivity of conventional techniques such as NMR.

Complexation

As previously stated, complexation may be used to incorporate a radiometal in MCgs either through a metalloligand or by acting as the metal node of the assembly. Stang, Chen and co-workers adopted the metalloligand approach to design a metallacage for radiotheranostic applications combining tri-modality imaging and photochemotherapy.^[106] The system is based on CDSA of 5,10,15,20-tetra(4-pyridyl)porphyrin panels and disodium terephthalate linkers coordinated to *cis*-(PEt₃)₂Pt(OTf)₂ nodes (Figure 10f). Coordination of [⁶⁴Cu]Cu²⁺ and Mn²⁺ within the cores of the porphyrin metalloligand confers additional PET and MRI imaging capabilities, alongside intrinsic NIR fluorescence. To ensure *in vivo* stability, the MCg was encapsulated within hollow polymeric nanoparticles functionalized with integrin-targeting RGD peptides. This nanocarrier not only enhances blood circulation time and tumour accumulation via the EPR effect but also supports imaging via three complementary modalities. NIR fluorescence imaging in U87MG tumour-bearing mice confirmed sustained tumour accumulation of the MCg-loaded nanoparticle. *In vivo* MRI with the Mn²⁺-based MCg showed excellent signal-to-noise ratio 24 h p.i. and high longitudinal relaxivity, comparable to the clinical T₁-contrast agent Gd-DTPA (diethylenetriaminepentaacetic acid). Similarly, PET imaging using the ⁶⁴Cu-labelled MCg demonstrated significant tumour uptake at 24 h p.i., although with elevated hepatic retention (Figure 10g). For therapeutic applications, the porphyrin panel acts as a photosensitizer for PDT, while the *cis*-platinum node contributes to

chemotherapeutic functionality. *In vitro* studies on U87MG cells revealed that the supramolecular architecture increases singlet oxygen generation by preventing π - π stacking of the free porphyrins and thereby improving the efficacy of PDT. *In vivo*, the MCg-loaded nanoparticles exhibited strong antitumour response across multiple tumour models following a single round of PDT due to synergistic anticancer effects as indicated by long-term tumour growth inhibition (Figure 10h). The capacity for real-time therapeutic monitoring via NIR fluorescence, PET and MRI emphasizes the theranostic potential of this supramolecular platform. Prospective developments may include the use of copper-64 for β^- and Auger electron emission in therapy.^[212] However, reducing the observed liver accumulation remains an unresolved challenge, and covalent tethering of the MCg to the polymeric carrier could prevent early release and improve pharmacokinetics.

In a more foundational work on coordination complexes, the group of Spreckelmeyer investigated the assembly of radiolabelled heterobimetallic Au(I)-based coordination polyhedrons.^[213] The formation of $[M\{\text{Au}_2\text{L}_2\}]^+$ and $[M\{\text{Au}_3\text{L}_3\}]$ complexes with L = 2,6-dipicolinoylbis(N,N-diethylthiourea) or 2,6-dipicolinoylbis(N,N-dimorpholylthiourea) and M = Ga^{3+} , Lu^{3+} , Y^{3+} , In^{3+} , Tb^{3+} and La^{3+} results in a molecular structure, in which the ligands are arranged around the metal ion M and in case of the trileptic compound build a distorted tricapped trigonal prism. Radiometallation with the therapeutic isotope $[^{177}\text{Lu}]\text{Lu}^{3+}$ and the PET isotope $[^{68}\text{Ga}]\text{Ga}^{3+}$ afforded the ‘hot’ analogues $[^{177}\text{Lu}][\text{Lu}\{\text{Au}_3\text{L}_3\}]$ and $[^{68}\text{Ga}][\text{Ga}\{\text{Au}_2\text{L}_2\}]^+$ with the radionuclides located in the polyhedron centre, whereas formation with the $^{198}\text{Au}(\text{I})$ precursor resulted in the $[^{198}\text{Au}][\text{Ga}\{\text{Au}_2\text{L}_2\}]^+$ complex featuring the radionuclide as the metal nodes of the polyhedron. Unfortunately, instability of the coordination compounds in the presence of human serum albumin precluded *in vivo* studies, which requires optimization of the heterobimetallic system. However, the potential to incorporate up to two different radionuclides for therapy and imaging offers an interesting design for multimodal theranostics. In addition, the β^- and γ -emitting radionuclide gold-198 is primarily used in the form of implantable grains for brachytherapy and is currently under investigation as nanoparticles for targeted radiotherapy.^[214–216] Thus, based on Spreckelmeyer’s work, further exploration of true ^{198}Au -labelled MCgs may advance the generation of supramolecular theranostics and unlock the full potential of this relatively underexplored radiotheranostic metal.

Integrating a radionuclide in the cage scaffold as a metal node or by using a metalloligand is a challenging task as the basic fabrication of the MCg is directly affected. While in the exo-functionalization or the encapsulation strategy the cage framework already exists, new design concepts need to be explored to find ligands with functionalities that are suitable to incorporate the radiometal and form a stable MCg at the same time. The incorporation of organometallic bonds in the cage structure may offer new opportunities in this respect.

In summary, radiolabelled MCgs are still in their infancy, and further optimization is required to enhance their suitability for radiopharmaceutical applications. Key aspects are improving their structural stability under physiological conditions and fine-tuning biological parameters such as lipophilicity. Additionally, the development of heteroleptic architectures should be prioritized to enable multifunctional designs and the scope of radioactive guest molecules should be investigated. Despite these current limitations, MCgs represent highly promising candidates for radiotheranostic applications, owing to their modular assembly, structural versatility and capacity for precise functionalization.

1.3.6 *Excursus: radiolabelled mechanically interlocked molecules**

Beside metallacages, also MIMs have been successfully radiolabelled and applied for radiotheranostic applications. This subchapter shortly reflects on their advantages and disadvantages using the example of rotaxanes.

Rotaxanes are dumbbell-shaped structures consisting of three main components as illustrated in Figure 11a: a linear guest molecule (axle) that is threaded through a host macrocycle (rota) and bulky end-groups (stoppers) at the axle termini preventing the disassembly.^[9] This mechanical bond can be generated by different synthetic pathways including ring closure around the preformed stoppered axle (clipping) and covalent capping of the rota-axle complex with stopper molecules (Figure 11a).^[5,217] Coordination knotting is a variant, in which chelators are installed at the axle termini and upon complexation of a metal ion form bulky knots.^[218] A fourth option is active templating that describes the conjugation of two half axles bearing stoppers catalysed by the macrocycle.^[217]

For radiopharmaceutical applications, rotaxanes can be functionalized at the stoppers and the macrocycle with metal radionuclide chelators, targeting vectors and imaging moieties.^[5] Furthermore, the mechanical bond endows them with unique pharmacokinetic properties.^[219] It is well known that classical radiolabelled monoclonal antibodies suffer from high dosimetry burden due to the long blood half-life and the slow distribution and excretion.^[220] Holland and d'Orchymont demonstrated that tethering of an antibody and a radionuclide to separate compartments of a rotaxane enables radionuclide clearance via metabolic cleavage of the macrocycle and thereby mitigating the aforementioned concern.^[221]

* This subsection is taken and rewritten from a review published in *Nature Reviews Chemistry* ^[5], to which the author of this work contributed.

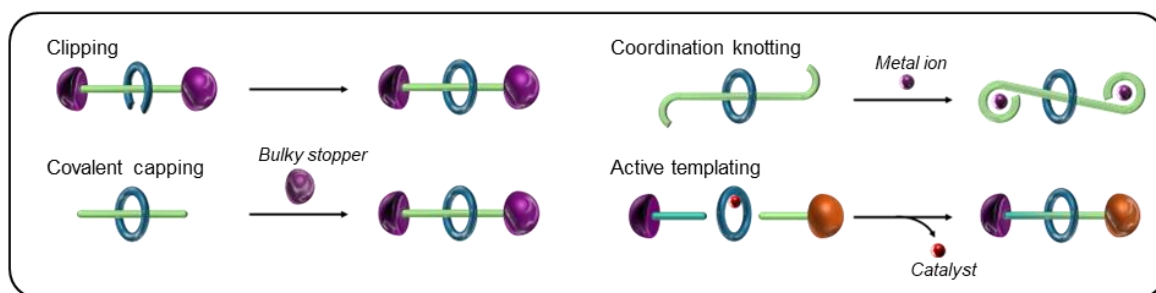
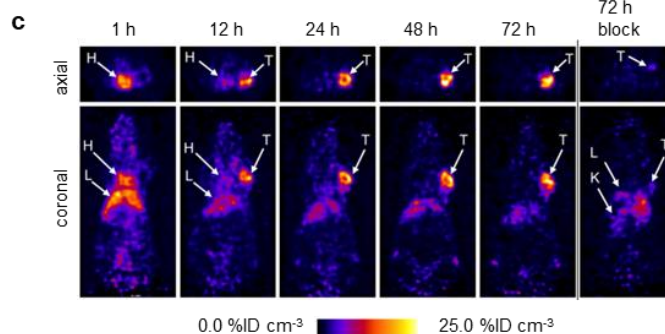
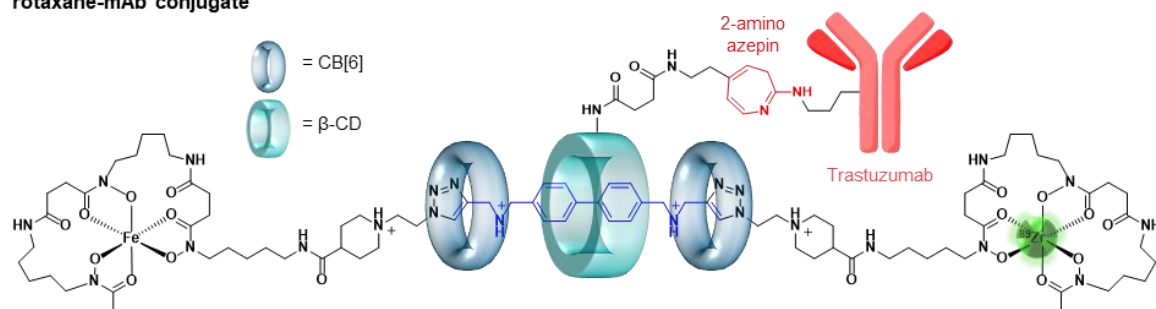
a Synthesis of rotaxanes**b rotaxane-mAb conjugate**

Figure 11: Rotaxanes for radiopharmaceutical applications. a) Strategies to synthesize rotaxanes.^[5] b) HER2-addressing ⁸⁹Zr-labelled [4]rotaxane-trastuzumab conjugate assembled via photoradiochemistry. The photoconjugated group 2-aminoazepin is highlighted in red. CB[6] = cucurbit[6]uril.^[222] c) *In vivo* PET imaging of the rotaxane-antibody adduct in a xenograft SK-OV-3 ovarian cancer model over the course of 72 h, including a blocking control for specificity determination. Relevant tissues are labelled (heart = H, liver = L, kidney = K, tumour = T).^[222] Panel a was adapted with permission from ref.^[5]. Copyright © 2024, Springer Nature Limited. Panel c was adapted with permission from ref.^[222]. © 2022 Angewandte Chemie published by Wiley-VCH GmbH.

Even though rotaxanes are promising theranostic platforms, deficient solubility in aqueous environment and elaborate synthesis pose quite the challenge, which is why only few examples exist.^[221–223] For instance, Holland and d'Orchymont developed an alternative method for synthesizing coordination-knotted rotaxanes as molecular imaging agents (Figure 11b).^[222] In this approach, cooperative-capture self-assembly was applied to construct a pseudo[4]rotaxane intermediate, in which the axle was formed by azide-alkyne cycloaddition between a linear diphenyl bialkyne and two desferrioxamine (DFO)-azide chelators.^[224,225] The assembly was conducted in aqueous solution and accelerated by the enclosure of the components within a macrocyclic ensemble consisting of one β -CD and two cucurbit[6]uril

units. The complete [4]rotaxane was generated by knotting metalation of the terminal DFO units with the PET-active radionuclides [^{68}Ga] Ga^{3+} and [^{89}Zr] Zr^{4+} , affording high RCY and RCP. The axle capping was essential for the formation of a strong mechanical bond leading to high water stability of the rotaxane. To enable targeted imaging of breast cancer, the β -CD unit was functionalized with a photoactivatable aryl azide enabling photochemical conjugation to trastuzumab, a monoclonal antibody addressing the overexpressed human epidermal growth factor receptor 2 (HER2).^[222] ^{89}Zr -labelling and light-induced antibody attachment were achieved simultaneously by photoradiochemistry.^[226,227] Notably, the resulting rotaxane underwent degradation *in vivo*, releasing radiolabelled metabolites either through hydrolysis of the β -CD moiety or cleavage of the 2-aminoazepin linkage. As demonstrated in cellular binding assays with SK-OV-3 ovarian cancer cells, HER2 binding affinity was preserved in the supramolecular construct. Moreover, PET imaging and biodistribution studies in xenograft models revealed prolonged tumour retention and highly specific uptake (Figure 11c). Finally, comparative evaluation against a fully covalent ^{89}Zr -labelled trastuzumab-azepin-DFO conjugate highlighted the mechanical bond's contribution to improved dosimetry and excretion kinetics.^[222]

2 Aim of the Work

Supramolecular chemistry provides a powerful toolbox for the design of multifunctional theranostic agents, yet remains underrepresented within the field of radiopharmaceuticals. The first part of this doctoral work aimed to demonstrate the potential of metallocages (MCgs) as radiotheranostic platforms by incorporating radionuclides via both host-guest chemistry and ligand functionalization, focusing on blood-brain barrier (BBB)-penetrating $[\text{Pd}_2\text{L}_4]^{4+}$ -type cages (Figure 12). Therefore, two generations of MCg ligands were designed and synthesized, integrating a BBB-shuttle peptide together with a silicon-based fluoride acceptor (SiFA) synthon; the latter being either the classical monofunctional (SiFA)BA or the less lipophilic bifunctional (SiFA)SeFe moiety. Subsequent coordination-driven self-assembly (CDSA) of homo- and heteroleptic cages was investigated and optimized alongside stability studies in different conditions. Furthermore, host-guest chemistry studies were performed with the chemotherapeutic drug cisplatin, the SPECT tracer $^{99\text{m}}\text{Tc}$ pertechnetate and its non-radioactive surrogate perrhenate to explore MCgs' encapsulation properties. For PET imaging applications, ^{18}F -radiolabelling of the MCg ligands and formation of ^{18}F -labelled homo- and heteroleptic MCgs was pursued. Full characterization of non-labelled and radiolabelled ligands and cages was conducted using reverse-phase high-performance liquid chromatography (RP-HPLC), thin layer chromatography (TLC), NMR spectroscopy, diffusion ordered NMR spectroscopy (DOSY NMR) and high-resolution electrospray ionization mass spectrometry (HR-ESI-MS). Moreover, *in vitro* studies to address key pharmacological parameters such as lipophilicity, HSA binding and defluorination stability were carried out, while the *in vivo* behaviour was assessed in the chick chorioallantoic membrane (CAM) model by *in ovo* PET imaging and *ex ovo* biodistribution studies.

While Pd(II)-MCgs are affected by a certain degree of kinetic instability and can be subjected to ligand exchange reactions which limit both their functionalization post-assembly and their stability in complex physiological environment, Pt(II)-based MCgs demonstrate enhanced stability due to more kinetically inert metal-ligand bonds. However, the self-assembly of PtCgs requires anhydrous conditions, elevated temperatures and long reaction times. Recently, strategies to make $[\text{Pt}_2\text{L}_4]^{4+}$ cages synthetically more accessible have been made available, including work from our group. Therefore, in the second part of this project, two potential synthetic routes for the preparation of brain-targeted homoleptic and heteroleptic Pt(II)-based cages was also developed.

In parallel to the cage design studies, the CAM model was further evaluated as a cost-effective preclinical screening platform that has the potential to fill the gap between *in vitro* and *in vivo* cancer research. Here, the focus was set to investigate its suitability in tumour-specific uptake of a sstR2-addressing radiohybrid tracer, which includes model optimization, biochemical

analyses, as well as *in ovo* PET imaging and *ex ovo* biodistribution studies. This latter part of the project has been conducted in collaboration with the group of Prof. Tim Witney (*King's College London*, UK).

Altogether, this work aimed to deepen the understanding of functionalized MCgs as versatile, multimodal radiotheranostic platforms and to explore innovative and ethical methodologies for their preclinical evaluation.

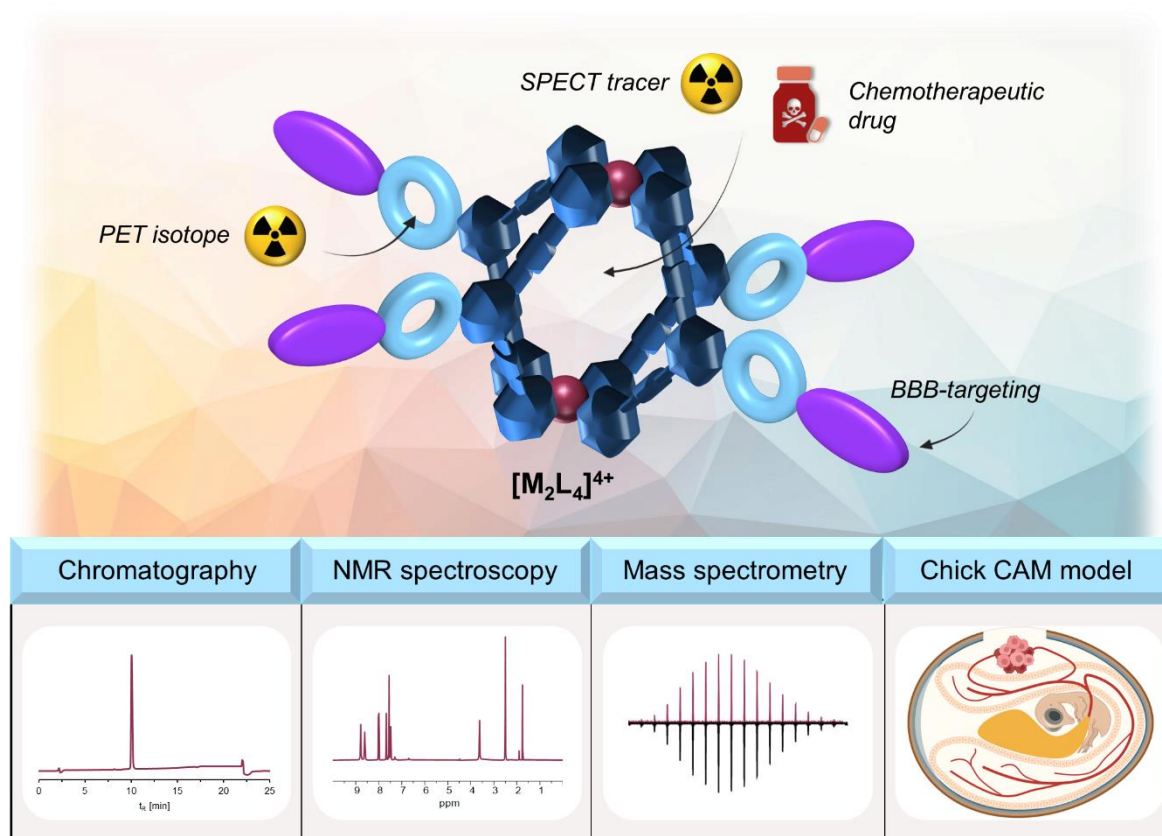


Figure 12: Graphical summary of the aim of the work.

3 Results and Discussion

The outcome of this work is divided into three main research lines focusing on: i) the development of Pd(II) metallacages (MCgs) for dual-modality nuclear imaging targeted to the brain, ii) a brief section on the design strategies for Pt(II)-MCgs, as well as iii) a methodological chapter on the chick chorioallantoic membrane (CAM) model for ^{18}F -imaging and biodistribution studies.

3.1 Brain-targeted palladium(II) homoleptic and heteroleptic metallacages*

Before going into detail about the synthesis and evaluation of brain-targeted $[\text{Pd}_2\text{L}_4]^{4+}$ cages, the importance of the blood-brain barrier (BBB), the challenges it poses for drug development, as well as solutions to overcome it will be highlighted in the next section.

3.1.1 The blood-brain barrier – an obstacle for drug delivery

The blood-brain barrier is a central component of the neurovascular unit and forms a critical interface between the bloodstream and the neural tissue, which mediates communication between the central nervous system and the periphery.^[228] Its unique structure is primarily built from brain capillary endothelial cells that are held together by narrow tight junctions and further interact with pericytes, astrocytes, neurons and immune cells via the basal lamina (Figure 13a).^[229,230] By strictly regulating the passage of *i.a.* nutrients, ions or enzymes, the BBB safeguards brain homeostasis and ensures accurate neuronal activity.^[229] At the same time, its low permeability prevents the entry of harmful substances like pathogens or neurotoxins and thus, fulfils a protective function as well.^[231] However, this restrictive nature poses a major obstacle for CNS drug development as approximately 98% of potential small-molecule therapeutics and nearly all macromolecular drug candidates fail to cross the BBB.^[232,233]

Under natural physiological conditions, four main pathways allow molecules to traverse the BBB as illustrated in Figure 13b.^[234]

- I. *Paracellular diffusion* is available to very small hydrophilic molecules via an aqueous route.
- II. *Transcellular diffusion* enables small lipophilic molecules that meet Lipinski's rules to cross the BBB.
- III. *Carrier-mediated transport* uses carrier proteins like glucose or amino acid transporters to deliver most small molecules.

* This subsection is based on a first-author paper published in *Inorganic Chemistry Frontiers*.^[73]

IV. Macromolecules are transported via vesicular pathways, consisting of endocytosis, cellular trafficking and exocytosis. Within this route, *receptor-mediated transcytosis*, is activated by the binding of a ligand to a complementary receptor, e.g. insulin or transferrin receptors. *Adsorptive-mediated transcytosis* on the other hand is a carrier-independent pathway and instead triggered by electrostatic interactions between polycationic macromolecules and negative charges on the cell surface.

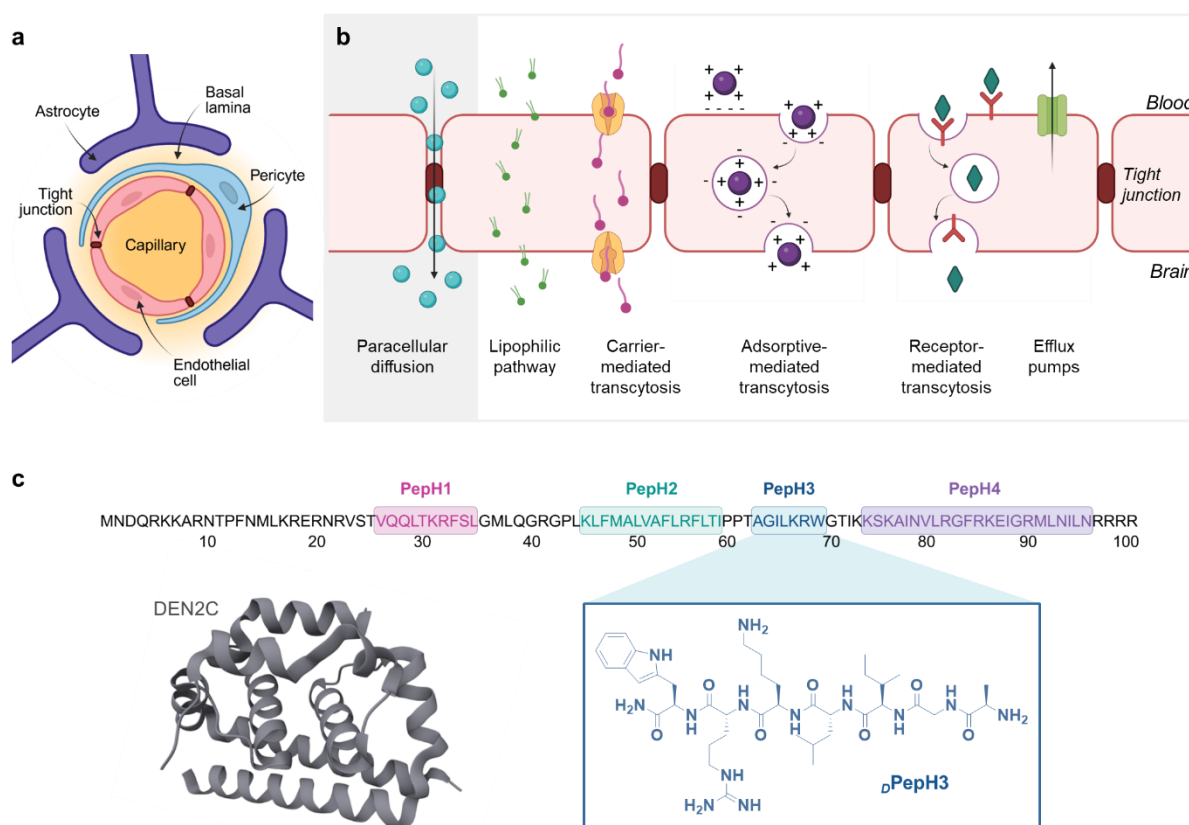


Figure 13: Crossing the blood-brain barrier. a) Physiology of the BBB.^[230] b) Transportation mechanisms across the BBB divided in paracellular and transcellular pathways.^[234] c) Chemical structure of the BBB shuttle peptide $dPepH3$ derived from the α -helical domain of Dengue virus type-2 capsid protein (DEN2C).^[207] The figure was created in <https://BioRender.com>.

Several CNS pathologies including brain tumours, stroke, diabetes, multiple sclerosis, Alzheimer's or Parkinson's disease can compromise BBB integrity, which is often detectable by MRI using contrast agents.^[231,235] Since the degree and duration of the dysfunction are variable, it is not sufficient to solely rely on the BBB leakage for drug uptake.^[235] Strategies like ultrasound or hyperosmotic agents can enhance brain uptake by transiently disrupting the BBB and enabling paracellular diffusion (Figure 13b).^[234,236] However, careful control of these treatments is necessary to prevent irreversible damage.^[228]

Consequently, most current efforts to improve CNS drug delivery focus on non-invasive transcellular pathways (Figure 13b). As many pharmacological molecules lack the required

physicochemical properties for natural passage, innovative approaches have evolved. These include functionalization of molecules like lipidation to promote passive diffusion or conjugation of targeting moieties to address receptors, application of drug delivery vehicles such as viral vectors, exosomes, inorganic and organic nanoparticles or the development of receptor-ligand mimics.^[235,236] Another particularly promising strategy involves cell-penetrating peptides, also known as “Trojan” or “shuttle” peptides. These short amphipathic or cationic sequences (5-30 amino acids) can cross cellular membranes and deliver macromolecular cargos such as nucleic acids, nanoparticles and proteins.^[237,238] Their straightforward synthesis, superior penetration efficiency and low cytotoxicity, make them attractive tools for drug delivery.^[239] With regard to brain delivery, a wide variety of BBB shuttle peptides has been identified from endogenous neurotropic proteins, including TAT, Angiopep, ApoE, or SynB1.^[240] In 2017, the groups of Castanho and Correia introduced PepH3 (AGILKRW), a seven amino-acid long peptide derived from the α -helical domain of the Dengue virus type-2 capsid protein (Figure 13c).^[207] PepH3 demonstrated promising brain penetration both *in vivo* (0.31 ± 0.07 % injected dose per gramme (ID/g) 5 min p.i.) and *in vitro* using a transwell assay ($> 60\%$ translocation).^[207,241] The peptide was found to traverse the BBB via adsorptive-mediated transcytosis (AMT), with a preference for endothelial cells, and was capable to transport even high-molecular-weight cargos.^[207,242,243] For instance, PepH3 has been conjugated to the fragment crystallisable region of an immunoglobulin G antibody, creating a platform that can easily be adapted to deliver a therapeutic payload with fast and efficient translocation.^[243] In another study, the BBB shuttle peptide was coupled to the anticancer peptide vCPP2319 to treat brain metastases originating from triple negative breast cancer.^[244,245] The peptide-peptide conjugate displayed a 2-fold increase in brain accumulation compared to vCPP2319 alone while maintaining potent antiproliferative activity ($IC_{50} = 5 \mu M$). Going to supramolecular constructs, PepH3 was recently employed to functionalize nanovesicles encapsulating a single-domain antibody for potential Alzheimer's therapy ($A\beta$ oligomer targeting), where PepH3-coating significantly enhanced the *in vitro* BBB translocation capacity compared to the unmodified vesicle.^[246] As described in section 1.3.5, also a $[Pd_2L_4]^{4+}$ metallacage was functionalized with PepH3, which enabled *in vivo* SPECT imaging upon encapsulation of $[^{99m}Tc]TcO_4^-$.^[128] The host-guest complex had a distinct biodistribution profile and achieved a brain uptake of 0.42 ± 0.06 %ID/g 5 min p.i.. Importantly, the *D*-enantiomer $_D$ PepH3 offers improved proteolytic stability with a half-life exceeding 2 h, further underscoring its suitability for brain drug delivery applications.^[241] It should be noted that brain uptake values in healthy or subcutaneous tumour-bearing mice of other reported peptide-based radiotracers were also lower than 1%.^[247] In addition, the small molecule $[^{18}F]FET$ (O-(2- $[^{18}F]$ fluoroethyl)-L-tyrosine) which is routinely used for PET imaging of cancer patients, including affected by glioblastoma^[248], showed brain uptake of below 1% (few minutes p.i.) in mice and rat models

of glioma.^[249,250] Therefore, driven by the promising data, the shuttle peptide ρ PepH3 was used in the design of a new generation of BBB-targeted $[\text{Pd}_2\text{L}_4]^{4+}$ cage for dual modality imaging via PET and SPECT, as discussed in the following chapters.

3.1.2 Ligand design and synthesis

As depicted in Figure 14, two MCg ligands **L1** and **L2** for the assembly of brain-targeted $[\text{Pd}_2\text{L}_4]^{4+}$ cages were designed from 4 components: the BBB-penetrating peptide ρ PepH3, a SiFA synthon for radiofluorination, a PEG linker (Ebes = 4-((2-(2-(2-aminoethoxy)ethoxy)ethyl)amino)-4-oxobutanoic acid) as a spacer and a Pd(II)-coordinating bispyridinyl ligand **L0** (3,5-bis(3-ethynylpyridine)phenyl) acetic acid). The main difference between the two ligands is the incorporated silicon-based fluoride acceptor. For **L1** the standard monofunctional (SiFA)BA was selected and coupled in a branched fashion to a Dap unit (2,3-diaminopropanoic acid) that was additionally inserted between the peptide and the PEG linker. The second-generation ligand **L2** features the newly developed (SiFA)SeFe. As explained in section 1.3.1, its amino acid bifunctionality leads to a reduction in the well-known SiFA lipophilicity, which could have a positive impact on the ligand's pharmacokinetics, but also enables the direct coupling of the prosthetic group in a bridged fashion, as an amino acid building block.^[168,171] This not only shortens the ligand synthesis, but reduces the ligand's steric demand as well, which might facilitate subsequent cage assembly. As a comparison, Deiser *et al.* developed a complex TATE- and DOTA-functionalized MCg ligand that hindered coordination-driven self-assembly (CDSA) of a homoleptic MCg and only produced the metallacycle (see section 1.3.5).^[127]

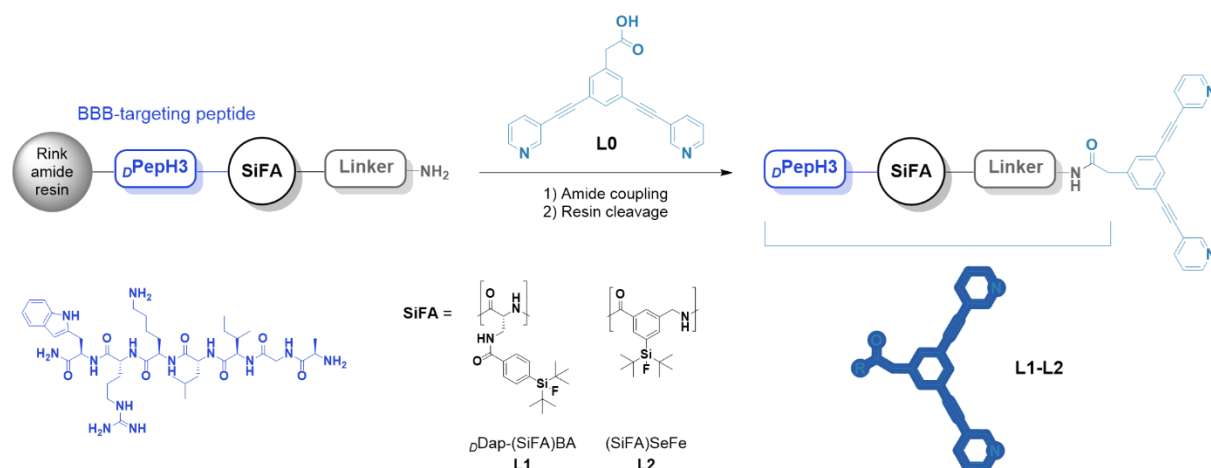


Figure 14: Design and synthesis of MCg ligands **L1** and **L2** used for the assembly of BBB-targeted $[\text{Pd}_2\text{L}_4]^{4+}$ cages. Reproduced from ref.^[73] with permission from the *Royal Society of Chemistry*.

The basic carboxylic acid ligand **L0** was synthesized via Sonogashira cross-coupling according to a literature-known procedure and obtained as a white powder in a total yield of 72% and 97% RP-HPLC purity.^[251] Synthesis of cage ligands **L1** and **L2** was then carried out via semi-automated fluorenylmethoxycarbonyl solid-phase peptide synthesis (Fmoc-SPPS) on a Rink amide resin (Figure 14). Here, *D*PepH3 was synthesized using a microwave-assisted peptide synthesizer followed by manual consecutive amide coupling of the remaining building blocks. After resin cleavage and semi-preparative RP-HPLC purification, the ligands were afforded in satisfactory yields (**L1**: 11%, **L2**: 17%) and excellent purity of 98% as evidenced by RP-HPLC. All three ligands were fully characterized by HR-ESI-MS as well as by ¹H NMR and ¹H-DOSY NMR spectroscopy (SI Figures 1-12).

3.1.3 Homoleptic metallocages

The straightforward synthesis of [Pd₂L₄]⁴⁺-type MCgs via coordination-driven self-assembly is one of their main advantages and can be achieved by mixing the ligand and metal precursor (here [Pd(MeCN)₄](BF₄)₂) in stoichiometric amounts at RT for less than one hour (Figure 15a). As the SPPS of the peptide-conjugated ligands **L1** and **L2** generally results in just a few milligrams of product, the cage CDSA was downscaled to get the most out of one ligand batch. This was accomplished by adapting the synthetic protocol to a procedure based on stock solutions yielding a 1 mM theoretical cage concentration (see Materials and Methods for details), which was used for all experiments analysed by RP-HPLC. Here, theoretical cage concentration is defined as the maximal concentration of MCg that can be obtained assuming full conversion and the formation of only one product. The synthesis of homoleptic MCgs **C1_{hom}** ([Pd₂(**L1**)₄]⁴⁺) and **C2_{hom}** ([Pd₂(**L2**)₄]⁴⁺) was first attempted in different solvents commonly used in the CDSA of MCgs, including DMSO, dimethyl formamide (DMF) and MeCN, as well as in different DMSO/H₂O mixtures (3/1, 1/1, 1/3, 1/4) relevant for later guest encapsulation studies. RP-HPLC analysis after one hour with a gradient of 40-70% MeCN in 15 min revealed that only in DMSO a clean formation of the desired cages was possible, as indicated by a shifted retention time *t_R* with respect to the ligands from 6.4 to 8.9 min for **C1_{hom}** and from 5.2 to 7.3 min for **C2_{hom}** (Figure 15b, SI Figure 13). Moreover, the presence of only minor ligand traces confirmed almost quantitative conversion at this concentration and further monitoring demonstrated a stability of at least 24 h under these conditions (SI Figure 19). While a water content of up to 50% was tolerated in the MCg assembly, DMF and MeCN were deemed unsuitable for the CDSA of these cages as the peaks of several supramolecular adducts appeared in the chromatograms (SI Figure 20). Thus, unless stated otherwise, DMSO was used as the reaction medium for the self-assembly.

NMR spectroscopy is the most widely used technique to monitor the self-assembly of MCgs as the ligand's α-pyridyl protons H_a and H_b experience a new electronic environment upon

coordination to the metal ion, which can be observed as a downfield shift in the ^1H NMR spectrum (Figure 15c).^[42] In order to obtain well-resolved peaks, cage solutions were prepared in $\text{DMSO-}d_6$ with an increased theoretical concentration of ~ 3 mM and spectra of ligands and MCgs were analysed. Here, the α -pyridyl protons of **L1** shifted from 8.78 ppm and 8.61 ppm to 9.77 ppm and 9.41 ppm, respectively, and the α -pyridyl protons of **L2** shifted from 8.79 ppm and 8.61 ppm to 9.78 ppm and 9.43 ppm, respectively, confirming the formation of supramolecular species (Figure 15c, SI Figure 14). Exemplarily, the NMR spectra of **L2** and **C2_{hom}** are compared in Figure 15c. The cage formation was further assessed via ^1H -DOSY NMR spectroscopy, a method by which the size of a molecule can be estimated by measuring its diffusion coefficient. The bigger the molecule, the slower its diffusion through the NMR solvent and the smaller the diffusion coefficient. As expected, a decrease in the diffusion coefficients was observed upon CDSA (Figure 15c, SI Figure 15). The cages' diffusion coefficients were then transformed into the corresponding hydrodynamic radii using the SEGWE calculator.^[252] While the radius of **C1_{hom}** amounts to 2.29 nm, **C2_{hom}** exhibits a smaller radius of 1.80 nm, which clearly reflects the reduced steric demand of ligand **L2**.

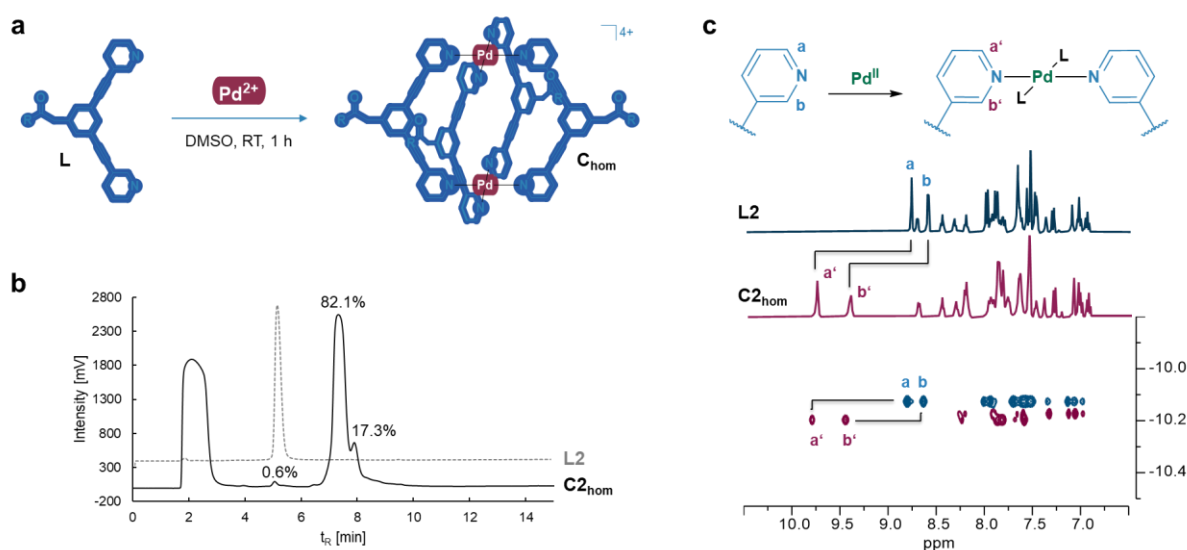


Figure 15: Self-assembly of homoleptic MCg **C2_{hom}**. a) general scheme of the homoleptic CDSA. b) RP-HPLC chromatogram (40-70% B in 15 min, C18 column) of **C2_{hom}** (1 mM) after 1 h CDSA at RT (see Materials and Methods for details). As a reference the chromatogram of the respective ligand **L2** is depicted in a grey dashed line. c) ^1H NMR (top) and ^1H -DOSY NMR (bottom) of **C2_{hom}** (purple) compared to **L2** (blue) measured at 400 MHz in $\text{DMSO-}d_6$. Characteristic proton shifts are labelled. Full NMR spectra can be found in the Supplementary Information (SI Figures 17 and 18). Adapted from ref.^[73] with permission from the *Royal Society of Chemistry*.

As NMR alone does not give information about the actual identity of the formed supramolecular species, characterization via direct injection-(DI-) and liquid chromatography-(LC-) HR-ESI-MS was performed in collaboration with Prof. Dr. Luisa Ronga and her group at the *Université de Pau et des Pays de l'Adour* (France). The difference between both techniques is the

processing of the sample: in the LC method, the components of the sample are first separated chromatographically and consecutively analysed by MS, while in the DI method, all components of the sample are analysed simultaneously. The CDSA of **C1_{hom}** was successfully evidenced in the DI mass spectrum by a peak at 661.8748 m/z corresponding to the $[\text{Pd}_2(\text{L1})_4(\text{FA})(\text{MeCN})(\text{H})_7]^{11+}$ adduct wherein formic acid (FA) and MeCN originate from the sample preparation (SI Figure 16, SI Table 1). For cage **C2_{hom}**, DI-HR-ESI-MS revealed multiple peaks that could be assigned to **C2_{hom}** adducts with FA and solvent molecules as well as to different $\text{Pd}_2(\text{L2})_3$ species as depicted in Figure 22. Moreover, an LC mass spectrum was recorded for further confirmation of **C2_{hom}**. In the total ion chromatogram (TIC), a peak at $t_R = 13.94$ min with an m/z value of 637.3228 was detected and attributed to the $[\text{Pd}_2(\text{L2})_4(\text{FA})(\text{H})_7]^{11+}$ adduct. Comparison of the experimental with the theoretical isotopic pattern indicated good agreement. Additionally, the $\text{Pd}_2(\text{L2})_3$ assembly was present in the TIC indicated by a peak at $t_R = 14.37$ min (m/z = 670.8255, $z = +8$) as well as free ligand **L2** at $t_R = 14.60$ min (Figure 16, SI Table 1). These findings suggest that chromatographic elution or the ionization process may induce partial dissociation of the MCg, particularly detachment of the fourth ligand. Nonetheless, all supramolecular species could be completely separated and fully characterized.

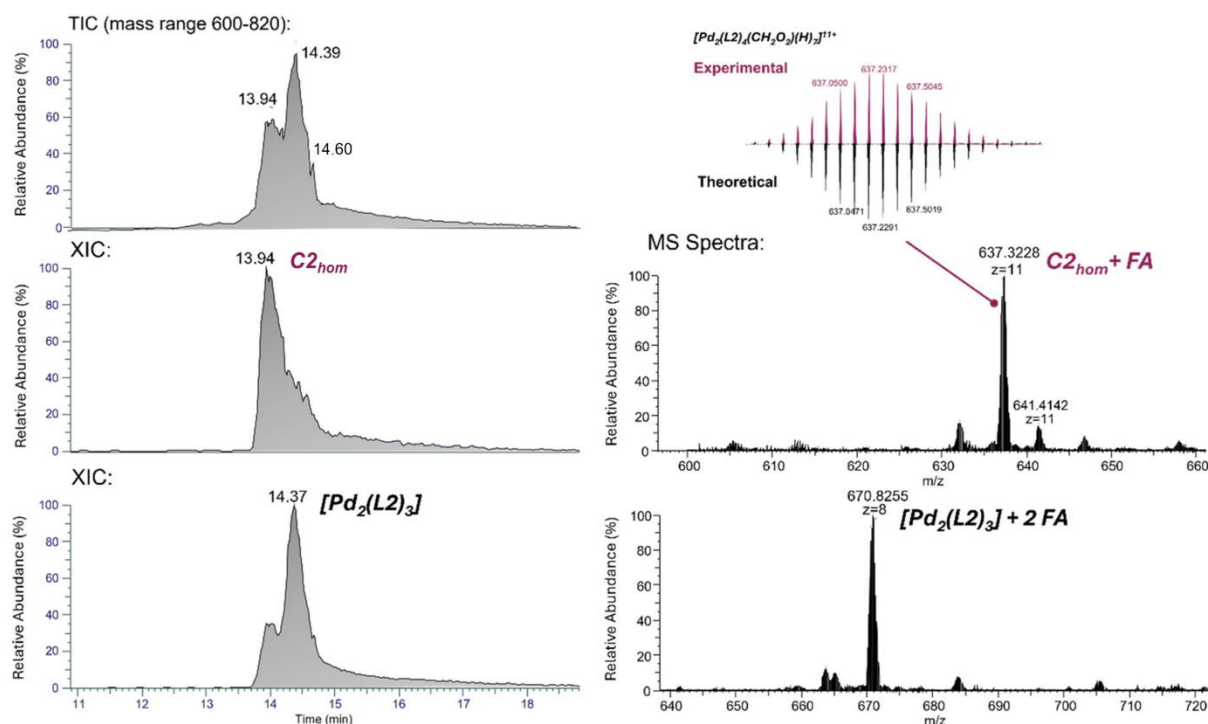


Figure 16: LC-HR-ESI-MS analysis of the homoleptic cage **C2_{hom}**. Left: Total ion chromatogram and extracted ion chromatogram (XIC) of the ions at m/z = 637.32 (**C2_{hom}**, $t_R = 13.94$ min) and at m/z = 670.83 ($[\text{Pd}_2(\text{L2})_3]$, $t_R = 14.37$ min). Right: Corresponding mass spectra of **C2_{hom}** + FA at $t_R = 13.94$ min (including isotopic pattern comparison) and $[\text{Pd}_2(\text{L2})_3] + 2$ FA at $t_R = 14.37$ min. Chromatographic details can be found in the Materials and Methods section. The figure was reproduced from ref.^[73] with permission from the *Royal Society of Chemistry*.

Subsequently, the stability of the homoleptic cages was investigated in solvents relevant to various assays, radiolabelling procedures and analytical methods. MCg solutions with 1 mM theoretical concentration were prepared and each diluted 10-fold with either DMSO, Milli-Q H₂O, MeCN, HPLC solvent (MeCN/H₂O (1/1) + 0.1% trifluoroacetic acid (TFA)), 0.9 wt% NaCl or PBS (pH 7.4). The samples were analysed by RP-HPLC with a gradient of 40-70% MeCN in 15 min (Figure 17, SI Figures 21 and 22). At this new concentration of 0.1 mM, precipitation and partial disassembly of the cages occurred in PBS and saline within one hour, however, the effect was less pronounced in the latter case. This instability is problematic for preclinical assessment, as salts are naturally present under physiological conditions, and also for encapsulation of [^{99m}Tc]TcO₄⁻, which typically is eluted from a ⁹⁹Mo/^{99m}Tc-generator in isotonic NaCl solution. In contrast, both cages showed good stability in Milli-Q H₂O, with 97% of intact **C1**_{hom} and 75% of intact **C2**_{hom} after 4 h (Figure 17a and b). Coordinating solvents such as DMSO and MeCN promoted the formation of new supramolecular assemblies with less than 50% intact cages after 24 h (Figure 17a). Notably, the HPLC conditions, in which water was added to MeCN, showed a stabilizing effect of the MCgs compared to MeCN alone (Figure 16a). Overall, **C2**_{hom} seemed to be more sensitive in the different solvents than **C1**_{hom} although the opposite was expected due to the reduced bulkiness of ligand **L2**. This might partially be a result of the lower cage concentration used in this study. For example, while **C2**_{hom} disassembled in DMSO to an extent of 50% within one hour after dilution to 0.1 mM, the cage remained intact for at least 24 h when formed with a concentration of 1 mM (SI Figure 19).

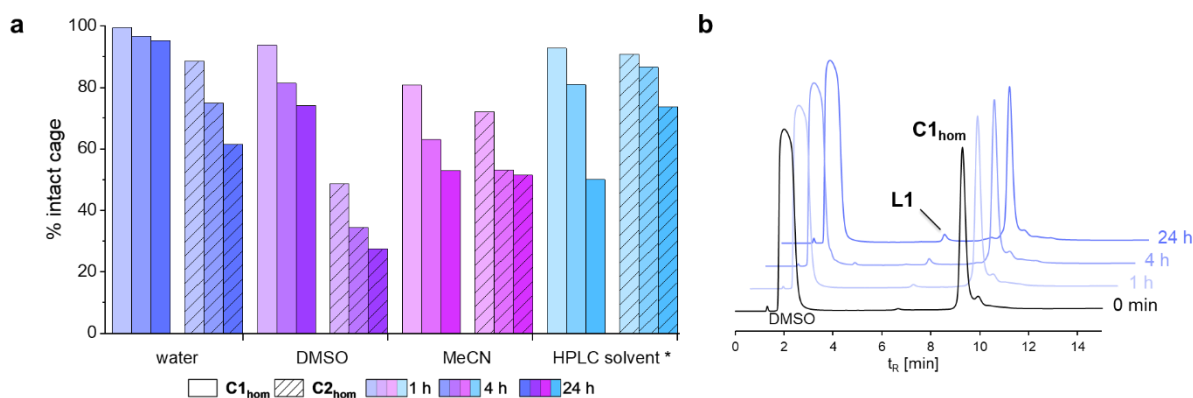


Figure 17: a) Stability of homoleptic cages **C1**_{hom} and **C2**_{hom} in various solvents analysed by RP-HPLC. After dilution with the respective solvent the theoretical cage concentration amounted to 0.1 mM. *MeCN/H₂O (1/1) + 0.1% TFA. b) Stability of **C1**_{hom} in Milli-Q water over the course of 24 h monitored by RP-HPLC (40-70% MeCN in 15 min, see Materials and Methods for details). Reprinted from ref.^[73] with permission from the *Royal Society of Chemistry*.

To further investigate the influence of the concentration on the cage formation and intactness, CDSA of **C1**_{hom} and **C2**_{hom} was directly carried out at a theoretical concentration of 0.1 mM. Under these dilute conditions, only partial cage assembly was achieved with 65% conversion

to **C1_{hom}** and 57% conversion to **C2_{hom}** (SI Figures 23 and 24), confirming that CDSA is indeed concentration dependent. This observation is particularly relevant for applications in areas like radiochemistry, where reactions are routinely carried out at the micromolar or even nanomolar level. Collectively, as demonstrated in the stability analysis, the intactness of $[\text{Pd}_2\text{L}_4]^{4+}$ cages can be affected by multiple factors, *i.e.* steric effects of *exo*-functionalities, interactions of the Pd(II) centres with nucleophilic molecules, the cage concentration as well as cage surface charges that can be sensitive to the ionic strength of the solvent. For **C1_{hom}** and **C2_{hom}**, electrostatic and nucleophilic interactions might be of particular relevance in the destabilization in solution.

In summary, CDSA of the homoleptic metallacages **C1_{hom}** and **C2_{hom}** was accomplished and the MCgs were fully characterized by three complementary analytical methods. Nonetheless, optimization of the cage stability is necessary for their application in *in vitro* and *in vivo* studies.

3.1.4 Heteroleptic metallacages

The possibility to combine ligands with different features in a single heteroleptic structure is one advantage of the modular cage design and increases the multifunctionality of these platforms. As already mentioned, the heteroleptic strategy was applied recently to introduce a complex TATE-DOTA-bearing ligand for radiolabelling with lutetium-177 resulting in a $[\text{Pd}_2\text{L}(\text{L0})_3]^{4+}$ cage that would have otherwise produced a homoleptic metallacycle $[\text{Pd}_2\text{L}_2]^{4+}$.^[127] Thus, the synthesis of analogous heteroleptic MCgs, **C_{het}** ($[\text{Pd}_2\text{L}(\text{L0})_3]^{4+}$) - with a 1:3 ligand ratio of functionalized ligand to unfunctionalized ligand **L0** - was pursued for the BBB-targeted ligands **L1** and **L2** (Figure 18a). It is hypothesized that the resulting reduction of the system's steric demand might have a positive impact on the cages' stability. Thus, statistical CDSA of **C1_{het}** ($[\text{Pd}_2(\text{L1})(\text{L0})_3]^{4+}$) was attempted mixing the ligands **L1** and **L0** in the ideal stoichiometric ratios (**L1/L0** = 1 eq./3 eq.), and reacted with 2 eq. of $[\text{Pd}(\text{MeCN})_4](\text{BF}_4)_2$ in DMSO for 1 h at RT. Of note, for NMR analysis total ligand concentrations of ~ 12 mM and Pd(II) concentrations of ~ 6 mM were used, while CDSA of HPLC samples was performed with 3-fold reduced concentrations (~ 4 mM ligand and ~ 2 mM Pd(II)). As depicted in the corresponding ¹H NMR spectra in Figure 18b, the α-pyridyl protons H_a and H_b underwent a downfield shift to 9.76 ppm and 9.40 ppm compared to the reference spectra of ligands **L0** and **L1**, which confirms the assembly of a supramolecular species. However, ¹H-DOSY NMR spectroscopy revealed peaks at different diffusion coefficients indicating the formation of multiple supramolecular assemblies upon Pd²⁺ addition. This became more evident by RP-HPLC analysis with a gradient of 30-60% MeCN in 15 min, wherein the single species could be separated and identified by ESI-MS (Figure 18c). Comparison with chromatograms of the free ligands and homoleptic cages **C0_{hom}** ($[\text{Pd}_2(\text{L0})_4]^{4+}$) and **C1_{hom}** already verified several peaks:

$t_R(\mathbf{L0}) = 3.2$ min, $t_R(\mathbf{L1}) = 11.5$ min, and $t_R(\mathbf{C0_{hom}}) = 8.3$ min (SI Figure 27). While the peak at $t_R = 6.6$ min originated from the metallacycle $[\text{Pd}_2(\mathbf{L0})_2]^{4+}$, the peaks at 9.4 min and 12.7 min could not be clearly identified by MS but were likely products of heteroleptic CDSA with varying $\mathbf{L1/L0}$ ratios. Lastly, the peak at $t_R = 11.1$ min with a percentage integral of 25% could be assigned to the desired cage $\mathbf{C1_{het}}$ and could be isolated in excellent purity (SI Figure 28). In an attempt to improve the yield of $\mathbf{C1_{het}}$, CDSA was performed with different ligand stoichiometries (2 eq. $\mathbf{L1}$ and 2 eq. $\mathbf{L0}$, 3 eq. $\mathbf{L1}$ and 1 eq. $\mathbf{L0}$). Unfortunately, this rather led to an enhanced assembly of heteroleptic species overlapping with the $\mathbf{C1_{het}}$ peak, highly unfavourable for isolation (SI Figure 29).

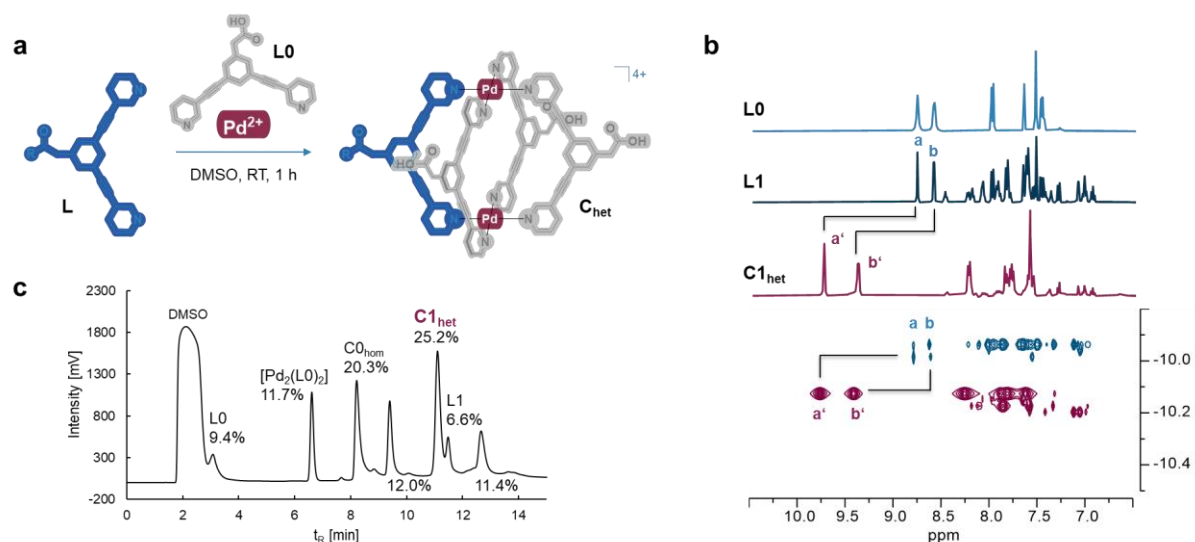


Figure 18: Self-assembly of heteroleptic MCg $\mathbf{C1_{het}}$. a) general scheme of the heteroleptic CDSA. b) ^1H NMR (top) and ^1H -DOSY NMR (bottom) of $\mathbf{C1_{het}}$ (purple) compared to the ligands $\mathbf{L1}$ (blue) and $\mathbf{L0}$ (light blue) measured at 400 MHz in $\text{DMSO-}d_6$. Characteristic proton shifts are labelled. Full NMR spectra are shown in the Supplementary Information (SI Figures 25 and 26) c) RP-HPLC chromatogram of the $\mathbf{C1_{het}}$ mixture (1 eq. $\mathbf{L1}$, 3 eq. $\mathbf{L0}$ and 2 eq. Pd(II) precursor) measured with a gradient of 30-60% MeCN in 15 min (see Materials and Methods for details).

Further, the $\mathbf{C1_{het}}$ mixture obtained in the first set of experimental conditions was then studied by LC-HR-ESI-MS in cooperation with Prof. Dr. Luisa Ronga's group. Therefore, the chromatographic method was first adapted to the other instrumental setup (UHPLC and C4 column, see Materials and Methods for details) and optimized by changing the gradient from the original 30-60% MeCN in 15 min (1 mL/min) to 20-40% MeCN in 3 min followed by 40-80% MeCN in 15 min (0.5 mL/min). This resulted in a different elution order, and in one dominant peak at $t_R = 6.7$ min (62.4%), which corresponded to $\mathbf{C1_{het}}$ (SI Figure 30). The optimized conditions were then applied in the LC-HR-ESI-MS analysis wherein three main peaks could be identified in the obtained TIC (Figure 19). The peak at $t_R = 7.35$ min could be assigned to the 5^+ and 4^+ charge states of $\mathbf{C1_{het}}$ with m/z of 594.2142 and 742.7651, respectively, and additionally confirmed by isotopic pattern comparison. Furthermore, free ligands $\mathbf{L0}$ and $\mathbf{L1}$

were detected at $t_R = 8.38$ min and $t_R = 9.31$ min. Extra DI-HR-ESI-MS analysis of the heteroleptic mixture validated again formation of **C1_{het}** by detection of the 5⁺ charge state ($m/z = 594.2141$). Moreover, a second heteroleptic species was observed in the DI spectrum, namely the 7⁺ charge state of $[\text{Pd}_2(\text{L1})_2(\text{L0})_2]^{4+}$ ($m/z = 625.4161$) as depicted in SI Figure 31. Isotopic pattern comparison of both assemblies with the respective calculated patterns showed good agreement as well.

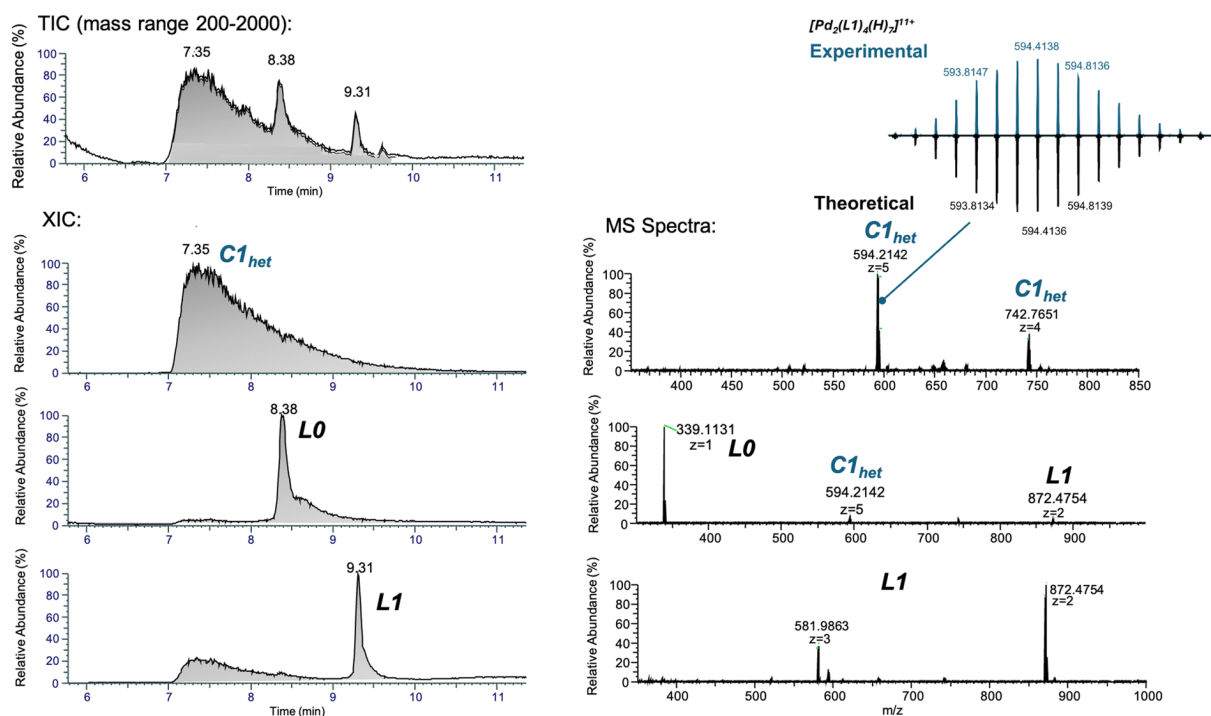


Figure 19: LC-HR-ESI-MS analysis of the heteroleptic cage **C1_{het}**. Left: TIC and extracted ion chromatogram (XIC) of the ions at $m/z = 594.21$ (**C1_{het}**, $t_R = 7.35$ min), $m/z = 399.11$ (**L0**, $t_R = 8.38$ min) and $m/z = 872.48$ (**L1**, $t_R = 9.31$ min). Right: Corresponding mass spectra of **C1_{het}** at $t_R = 7.35$ min (including isotopic pattern comparison), **L0** at $t_R = 8.38$ min and **L1** at $t_R = 9.31$ min. Chromatographic details can be found in the Materials and Methods section. The figure was reproduced from ref.^[73] with permission from the *Royal Society of Chemistry*.

Next, the self-assembly of heteroleptic cage **C2_{het}** ($[\text{Pd}_2(\text{L2})(\text{L0})_3]^{4+}$) was pursued in a similar way. Here, CDSA was carried out by combining 2 eq. of $[\text{Pd}(\text{MeCN})_4](\text{BF}_4)_2$ with either 1 eq. **L2** and 3 eq. **L0**, 2 eq. **L2** and 2 eq. **L0**, 3 eq. **L2** and 1 eq. **L0** or 2.7 eq. **L2** and 1.3 eq. **L0**. After reacting in DMSO for 1 h at RT and RP-HPLC analysis with a gradient of 30-60% MeCN in 15 min, the latter ligand stoichiometry (**L2**:**L0** = 2.7:1.3) was found to yield the most satisfactory mixture of heteroleptic species (SI Figure 32). Additionally, ^1H and ^1H -DOSY NMR spectra were recorded applying the optimal conditions, which confirmed formation of multiple supramolecular species (SI Figures 33 and 34). Again, the components of the mixture were elucidated by comparing the chromatogram with reference chromatograms, wherein **C2_{het}** was found at $t_R = 10.5$ min, but also ($[\text{Pd}_2(\text{L2})_2(\text{L0})_2]^{4+}$) could be identified at $t_R = 11.5$ min by ESI-MS of the isolated peaks (SI Figure 35). By applying the optimized HPLC gradient (20-40%

MeCN in 3 min followed by 40-80% MeCN in 15 min (0.5 mL/min)), the elution order was changed as expected and a dominant peak at $t_R = 6.8$ min (58.7%) was observed that belonged to **C2_{het}** (SI Figure 36). Due to limited time LC-HR-ESI-MS analysis was abstained from and instead the faster DI method was performed to further examine the heteroleptic mixture. Here, the desired species **C2_{het}** was found at $m/z = 583.0103$ in a 5⁺ charge state, while $\text{Pd}_2(\text{L2})_2(\text{L0})_2^{4+}$ species appeared at $m/z = 609.2678$ in a 7⁺ charge state (Figure 20). Isotopic pattern comparison verified both species.

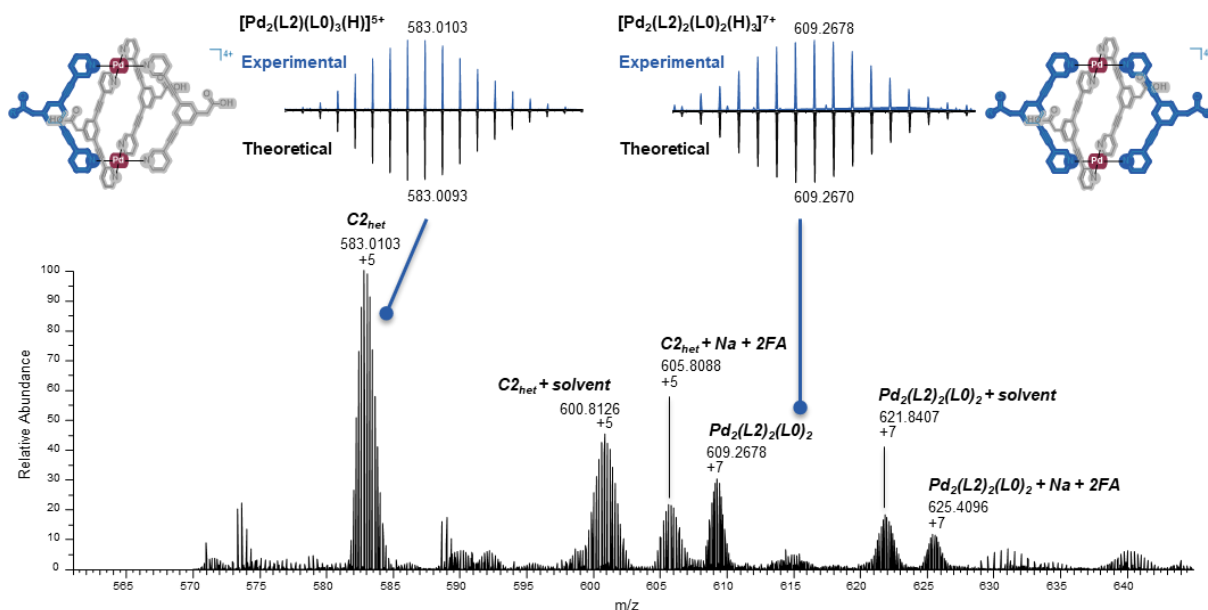


Figure 20: DI-HR-ESI-MS spectrum of the heteroleptic **C2_{het}** mixture obtained by the CDSA of **L2** (2.7 eq.) and **L0** (1.3 eq.) with $[\text{Pd}(\text{MeCN})_4](\text{BF}_4)_2$ (2.0 eq.) in DMSO. Adapted from ref.^[73] with permission from the *Royal Society of Chemistry*.

To conclude, the heteroleptic MCgs **C1_{het}** and **C2_{het}** with the general formula $[\text{Pd}_2\text{L}(\text{L0})_3]^{4+}$ were successfully synthesized and analysed by three different analytical techniques. MS analysis further revealed the existence of $[\text{Pd}_2\text{L}_2(\text{L0})_2]^{4+}$ species. The CDSA resulted in mixtures with other supramolecular species, whose distribution depended on the ligand stoichiometry used in the synthesis, but clean isolation of the desired heteroleptic cages was possible.

3.1.5 Encapsulation studies

Due to their porous nature, MCgs are well suited as drug delivery vehicles. As illustrated in Figure 21, the preparation of the host-guest complex can either be achieved after the CDSA (post-assembly) or during CDSA with the cage assembly being guided by the guest molecule (one-pot). In the following, encapsulation studies of the chemotherapeutic drug cisplatin, the SPECT tracer $^{99\text{m}}\text{Tc}$ pertechnetate and its non-radioactive surrogate perhenate will be discussed. Here, the focus was set on post-assembly encapsulation, but the one-pot approach

was tested as well in the case of the guest anion [^{99m}Tc]pertechnetate. Noteworthy, the ligand scaffold used in this work (3,5-bis(3-ethynylpyridine)phenyl) produces a hydrophobic cage cavity that does not favour hydrogen bonding interactions neither with the drug cargo nor with water molecules or H-bonding solvents, meaning that the driving force behind encapsulation is based on the hydrophobic effect. Furthermore, special attention must be paid to the selection of the MCg counterion as it can compete with the guest molecule for the internal cavity and thereby interfere with encapsulation. In the case of post-assembly encapsulation, the counterion might already occupy the cavity before being replaced by the guest. Often this results in a 'masked' guest encapsulation process that is not observable by ^1H NMR as the ligand H-atoms lining the cavity already produce shifted signals due to interactions with the counterion.^[251] Consequently, a counterion should be chosen that prefers not to enter the cage cavity. For instance, Crowley and co-workers conducted crystallographic and NMR analyses to study the counterion interactions with a $[\text{Pd}_2\text{L}_4]^{4+}$ cage featuring tripyridyl ligands similar to the ligand scaffold used in this work.^[253] While smaller anions like tosylate and NO_3^- were present in the cavity, encapsulation of fluorine-containing counterions such as SbF_6^- , BF_4^- and triflate was not observed likely due to size mismatch or electrostatic repulsions.^[118,253] As all the cages of this doctoral project are synthesized with the BF_4^- counterion using the Pd(II)-precursor $[\text{Pd}(\text{MeCN})_4](\text{BF}_4)_2$, interference with the encapsulation of guest molecules should be prevented.

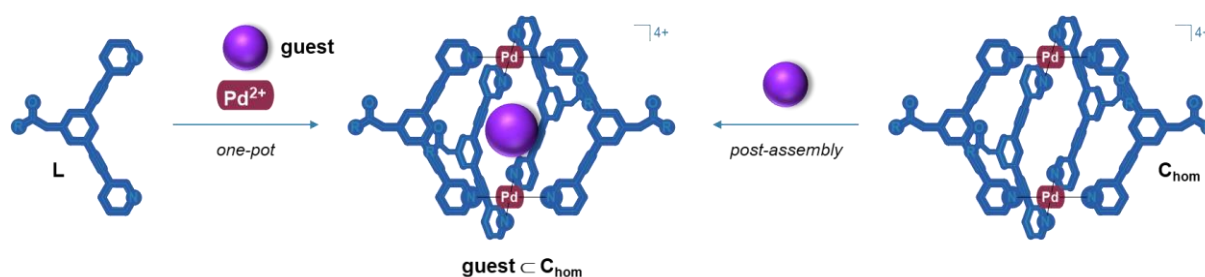


Figure 21: Schematic illustration of guest encapsulation into $[\text{Pd}_2\text{L}_4]^{4+}$ MCgs.

Cisplatin encapsulation

Encouraged by the encapsulation of cisplatin in peptide functionalized integrin-targeted $[\text{Pd}_2\text{L}_4]^{4+}$ cages, host-guest complex formation with this chemotherapeutic drug was pursued as well using the sterically less hindered homoleptic cage **C2_{hom}**.^[84]

It has been reported that cisplatin undergoes ligand exchange with DMSO, which may disturb host-guest adduct formation.^[254] Instead, the encapsulation is usually performed in DMF as it builds hydrogen bond networks and therefore, is not likely to compete with the desired cisplatin guest for encapsulation into the hydrophobic cavity.^[253] Although the CDSA of **C2_{hom}** was unfavourable in DMF as a solvent due to the appearance of various supramolecular species

(see section 3.1.3), encapsulation was still attempted under these conditions. Furthermore, the cavity of $[\text{Pd}_2\text{L}_4]^{4+}$ cages featuring 3,5-bis(3-ethynylpyridine)phenyl ligands usually holds space for two molecules of cisplatin, and the encapsulation process can be followed by ^1H NMR spectroscopy.^[119]

Thus, post-assembly encapsulation was carried out by first assembling **C2_{hom}** in DMF-*d*₇ (~ 3 mM) and then adding 2 eq. of cisplatin with respect to the cage. ^1H NMR spectra were recorded 1 h after CDSA and 30 min after encapsulation, but only negligible downfield shifts of 0.004 ppm for both α -pyridyl protons H_a and H_b were observed (Table 2, SI Figure 37). In contrast, the literature values reported for $[\text{cisplatin}]_2 \subset \text{C0}_{\text{hom}}$ were ten times higher than the shifts observed for **C2_{hom}**, so that the verification of cisplatin internalisation using ^1H NMR was considered unsuccessful (Table 2). It is likely that the long and flexible structure of ligand **L2** impeded encapsulation either through steric hindrance or by interacting directly with the cisplatin guest. Further insights could be provided by complementary analytical tools such as ^{195}Pt NMR spectroscopy or HR-ESI-MS analysis.

Table 2: Shift difference of the α -pyridyl protons H_a and H_b in Δppm before and after encapsulation of cisplatin or perrhenate into **C1_{hom}** and **C2_{hom}** compared to literature values.^[128,251]

Desired host-guest adduct	$\Delta\text{ppm}(\text{H}_a)$	$\Delta\text{ppm}(\text{H}_b)$	Literature $\Delta\text{ppm}(\text{H}_a)$	Literature $\Delta\text{ppm}(\text{H}_b)$
$[\text{cisplatin}]_2 \subset \text{C1}_{\text{hom}}$	n.d.	n.d.	~ + 0.043	~ + 0.033
$[\text{cisplatin}]_2 \subset \text{C2}_{\text{hom}}$	+ 0.004	+ 0.004		
$[\text{ReO}_4^-] \subset \text{C1}_{\text{hom}}$	- 0.011	- 0.004	~ - 0.080	~ - 0.040
$[\text{ReO}_4^-] \subset \text{C2}_{\text{hom}}$	- 0.030	+ 0.001		

Downfield shift is indicated by a positive sign and upfield shift is indicated by a negative sign.

n.d. = not determined

Perrhenate encapsulation

One established approach for radiolabelling MCgs involves the encapsulation of a radioactive guest molecule. Here, the groups of Lusby and Archibald, and Correia and Casini have demonstrated the successful encapsulation of $[\text{}^{99\text{m}}\text{Tc}]\text{TcO}_4^-$ as well as the *in vivo* stability of its radioactive host-guest complexes with different MCgs (see section 1.3.5).^[96,128] To probe the host-guest interactions by NMR spectroscopy, $[\text{}^{99\text{m}}\text{Tc}]\text{perrhenate}$ was exchanged with its non-radioactive ('cold') analogue perrhenate, and the experimental results supported by *in silico* modelling.

Building on these promising findings, the encapsulation of ReO_4^- was examined in the homoleptic cages **C1_{hom}** and **C2_{hom}**. As determined in section 3.1.3, a water content of 50% was tolerated in the CDSA of the MCgs. This becomes relevant now as water is known to support the internalization of oxo-anions as the host-guest complex is formed via electrostatic interactions and hydrogen bonding between the guest and the cavity.^[255] Therefore, **C1_{hom}** and

C2_{hom} solutions (~ 3 mM) were prepared in D₂O/DMSO-*d*₆ (1/1). 2 eq. of NaReO₄ were added and ¹H NMR spectra were measured after 30 min (SI Figure 38). While the H_a protons exhibited an upfield shift of 0.01 ppm in the case of **C1_{hom}** and 0.03 ppm in the case of **C2_{hom}** upon perrhenate addition, no significant changes were observed for the H_b protons in either case (Table 2). Compared to the larger upfield shifts reported for a similar host-guest system (H_a: ~ 0.08 ppm, H_b: ~ 0.04 ppm), only weak perrhenate encapsulation can be assumed under these conditions, probably due to a still too high DMSO content.

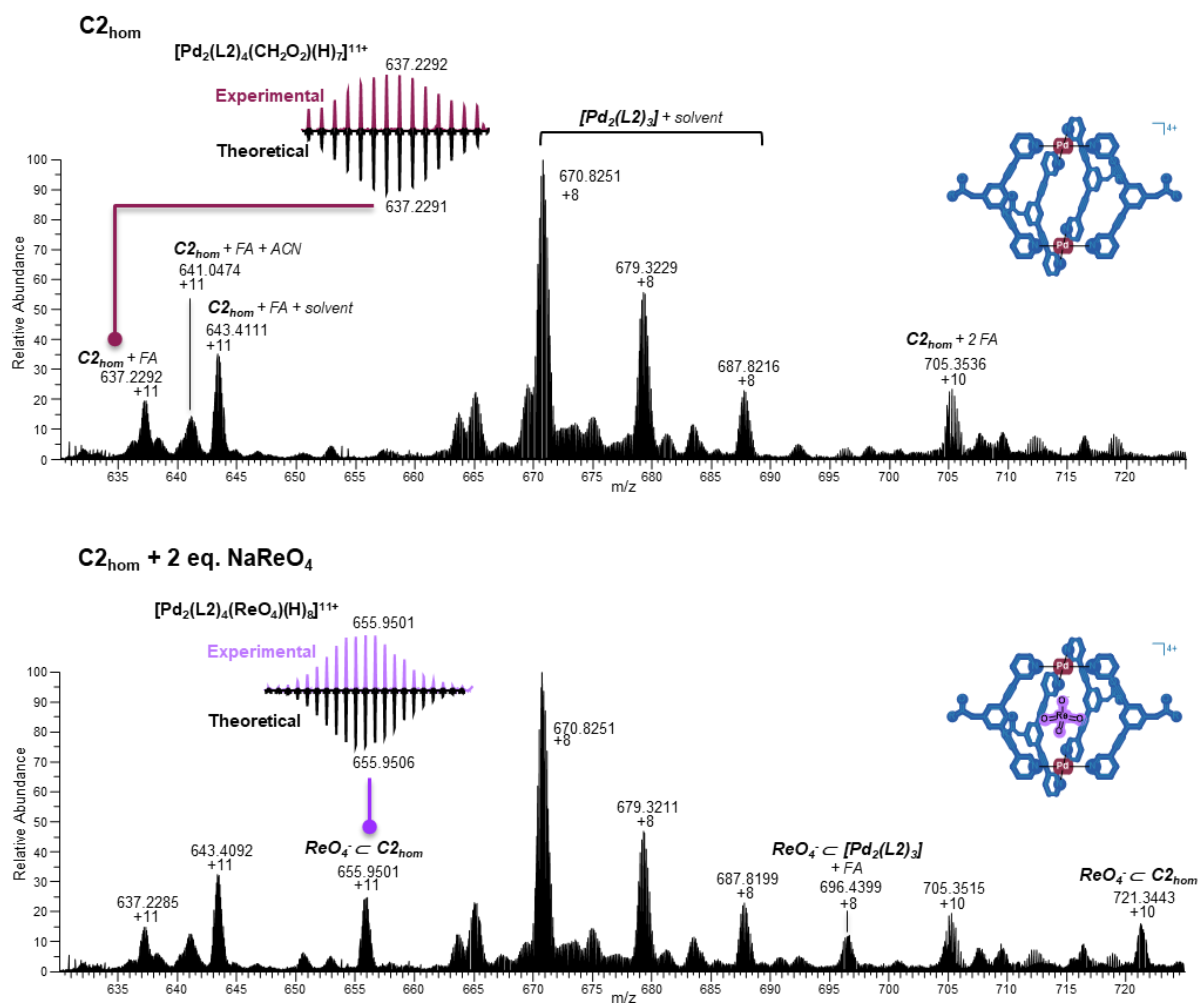


Figure 22: DI-HR-ESI-MS spectra of **C2_{hom}** (top) and **C2_{hom}** + 2 eq. of NaReO₄ (bottom). Isotopic pattern comparisons of relevant peaks are included as insets. Adapted from ref.^[73] with permission from the Royal Society of Chemistry.

Hence, the encapsulation of perrhenate by **C2_{hom}** was further investigated in media with higher percentage of water and in MCg concentrations comparable to the physiologically relevant one, by DI-HR-ESI-MS in collaboration with Prof. Luisa Ronga and co-workers. Therefore, a ~1 mM cage solution containing 2 eq. of NaReO₄ was prepared and further diluted for MS injection. As depicted in Figure 22, peaks of the $[\text{ReO}_4]^- \subset \text{C2}_{\text{hom}}$ adduct were observed at $m/z = 655.9501$ and 721.3443 , corresponding to the 11⁺ and 10⁺ charge states, and

experimental and theoretical isotopic pattern were in good accordance. Although encapsulation was not quantitative as evidenced by the present peaks of **C2_{hom}**, some affinity of ReO_4^- for the cage cavity can be confirmed. This result could also be more representative as the lower concentrations (μM) used in the MS analysis better reflect the clinical scenario. However, unlike in previous reports on related MCgs, isolation of the host-guest complex by LC separation was not possible.^[128]

Additionally, formation of heteroleptic host-guest complexes with **C1_{het}** and **C2_{het}** was attempted by adding 2 eq. of NaReO_4 to the heteroleptic mixtures. DI-HR-ESI-MS analysis of the **C1_{het}** perrhenate mixture revealed the presence of $\text{ReO}_4^- \subset \text{C1}_{\text{het}}$ by peaks at $m/z = 644.6024$ ($z = +5$) and 805.0005 ($z = +4$). Furthermore, the $\text{ReO}_4^- \subset [\text{Pd}_2(\text{L1})_2(\text{L0})_2]^{4+}$ were observed at $m/z = 661.4080$ and 771.6426 , which could be assigned to the 7^+ and 6^+ charge states, respectively (SI Figure 39, SI Table 1). As previously determined, also the homoleptic cage **C0_{hom}** is present in the **C1_{het}** mixture and therefore, the $\text{ReO}_4^- \subset \text{C0}_{\text{hom}}$ adduct ($m/z = 605.7229$, $z = +3$) did form as well upon perrhenate addition. In the DI spectrum of the **C2_{het}** perrhenate mixture both $\text{ReO}_4^- \subset \text{C2}_{\text{het}}$ ($m/z = 633.1988$, $z = +5$; $m/z = 790.9967$, $z = +4$) and $\text{ReO}_4^- \subset [\text{Pd}_2(\text{L2})_2(\text{L0})_2]^{4+}$ ($m/z = 752.4680$, $z = +6$) inclusion complexes could be observed (SI Figure 39, SI Table 1). However, at variance with **C1_{het}**, the peaks are very weak in intensity, and the most dominant signal at $m/z = 655.5997$ ($z = +7$) could not be identified yet. Nonetheless, detection of the heteroleptic host-guest adducts in this preliminary setting is already an important step in the characterization. In case of a continued investigation of the heteroleptic perrhenate encapsulation, repetition of the DI-HR-ESI-MS measurements, as well as LC separation of the host-guest adducts should be conducted.

[^{99m}Tc]pertechnetate encapsulation

As the host-guest complexes of **C2_{hom}** with perrhenate could be unambiguously characterized, the next step was to repeat the encapsulation with the “hot” counterpart [^{99m}Tc]TcO₄⁻ to obtain a radioactive host-guest system for SPECT imaging. Therefore, the procedure for a similar [^{99m}Tc]TcO₄⁻ $\subset$ $[\text{Pd}_2\text{L}_4]^{4+}$ complex, in which the encapsulation proceeded as a one-pot reaction with the cage assembly within 1 h at 37 °C, was adapted:^[128] [^{99m}Tc]TcO₄⁻ was eluted from a ⁹⁹Mo/^{99m}Tc-generator in saline, concentrated to reach an activity concentration of 0.8 MBq/ μL , of which 80 MBq were mixed with DMSO solutions of the ligand **L2** and the Pd-precursor $[\text{Pd}(\text{MeCN})_4](\text{BF}_4)_2$. Analogously, the encapsulation was performed post-assembly as well with 100 μL of a 1 mM **C2_{hom}** solution. In both approaches, formation of a cloudy precipitate could be observed within minutes upon addition of the [^{99m}Tc]pertechnetate solution, likely due to the cage's instability in the presence of saline.

To investigate the reaction mixture, the suspensions were centrifuged and the supernatant analysed by radio RP-HPLC with a gradient of 40-70% MeCN in 15 min. As shown in

Figure 23a, the radio chromatograms of the one-pot and the post-assembly encapsulation only contain a peak of free $[^{99m}\text{Tc}]\text{TcO}_4^-$ eluted at the solvent front, which is confirmed by comparison with a reference chromatogram of $[^{99m}\text{Tc}]\text{pertechnetate}$. Successful encapsulation instead would have been evidenced by a peak at the cage's usual retention time ($t_R = 7.3$ min, C18 column).

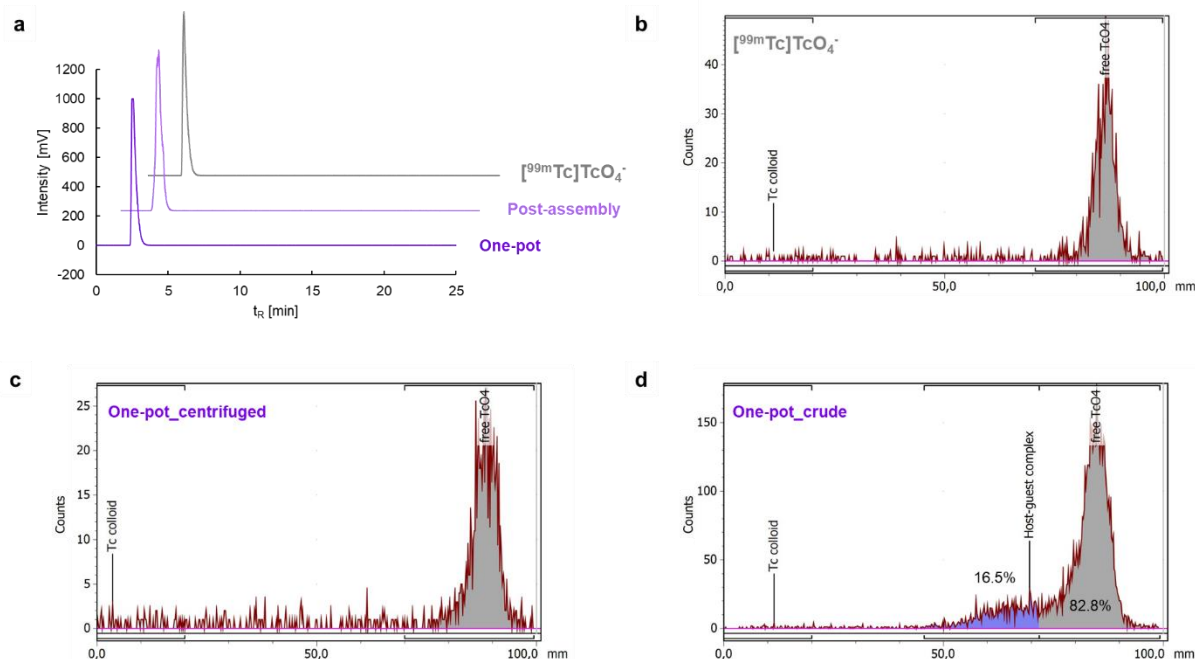


Figure 23: Chromatographic analysis of $[^{99m}\text{Tc}]\text{TcO}_4^-$ encapsulation in **C2_{hom}**. a) Comparison of RP-HPLC chromatograms (40-70% MeCN in 15 min) of one-pot encapsulation, post-assembly encapsulation and free $[^{99m}\text{Tc}]\text{pertechnetate}$. b) radio TLC chromatogram in MEK of $[^{99m}\text{Tc}]\text{TcO}_4^-$. c) radio TLC chromatogram in MEK of the one-pot encapsulation mixture after centrifugation. d) radio TLC chromatogram in MEK of the one-pot encapsulation mixture before centrifugation. Chromatographic details can be found in the Material and Methods section.

Additional analysis was conducted by radio TLC in saline and in methylethylketone (MEK), respectively (Figure 23b-d, SI Figure 40). Here, samples of both reaction mixtures were taken before and after centrifugation and compared to free $[^{99m}\text{Tc}]\text{TcO}_4^-$. For interpretation, it has to be noted that $[^{99m}\text{Tc}]\text{pertechnetate}$ always runs at the solvent front and Tc-colloid impurities stay at the baseline in both TLC conditions. Furthermore, the ^{99m}Tc -labelled compound should run at the solvent front in saline and stay at the baseline with MEK in theory, but usually optimization with additives is necessary to reach this separation. Exemplarily, the radio TLC chromatograms in MEK of the one-pot encapsulation are analysed (Figure 23b-d). For completion, similar findings were made in the case of the post-assembly encapsulation as well as in radio TLC with saline (SI Figure 40). As expected, the chromatogram of the centrifuged sample only contained a peak at the solvent front corresponding to free $[^{99m}\text{Tc}]\text{TcO}_4^-$, which agrees with the radio RP-HPLC analysis. In contrast, in the TLC chromatogram of the crude

sample (before centrifuging) a shoulder (16.5%) appeared close to the [^{99m}Tc]pertechnetate peak that might correspond to the [^{99m}Tc]TcO $_4^- \subset \mathbf{C2}_{\text{hom}}$ complex. This observation could indicate an instability of the host-guest adduct due to centrifugation, beside the already known instability of the cage in certain solvents. In any case, without further characterization of the shoulder peak to identify the actual species, e.g. by radio MS, [^{99m}Tc]pertechnetate encapsulation in these conditions must be excluded. Hence, encapsulation was not attempted using $\mathbf{C1}_{\text{hom}}$ or the heteroleptic cage mixtures as first optimization is necessary. This includes, switching the reaction medium from saline, in which [^{99m}Tc]TcO $_4^-$ is eluted, to a solvent that guarantees intactness of the Pd(II)-Cg. Furthermore, exchanging the MCg's metal centre from Pd $^{2+}$ to Pt $^{2+}$ should be considered to improve the overall kinetic inertness of the system.

To conclude, of the three attempted host-guest complexes, only the encapsulation of ReO $_4^-$ in the homoleptic and heteroleptic metallacages was partly evidenced by DI-HR-ESI-MS. The formation of cisplatin-cage adducts could not be verified by ^1H NMR and should therefore also be examined by MS, even though the used solvent DMF is less favourable for CDSA. On the other hand, while encapsulation of [^{99m}Tc]TcO $_4^-$ could have been possible due to the confirmed formation of ReO $_4^- \subset \text{MCg}$ adducts, cage and host-guest instability under the radiolabelling conditions prevented the successful outcome. Optimization of the procedure as well as enhancement of stability of the MCg structure would therefore be necessary.

3.1.6 ^{18}F -radiolabeling of ligands and metallacages

Another possibility to obtain radiolabelled MCgs is the integration of prosthetic groups or chelators in the ligand. The groups of Casini, Cuenca and Llop achieved *in vivo* PET imaging with a [Pd_2L_4] $^{4+}$ MCg *exo*-functionalized with AMBF $_3$ for ^{18}F -labelling (see section 1.3.5).^[202] In this work, the δPepH3 -conjugated ligands **L1** and **L2** contain SiFA moieties instead of trifluoroborates, allowing for radiofluorination under very mild conditions, which might be beneficial when handling peptide-based ligands. The synthesis of radiolabelled ligands and CDSA of the corresponding MCgs is discussed in the following.

^{18}F -radiolabelling of the ligands

First, radiolabelling of ligands **L1** and **L2** was via ^{19}F -to- ^{18}F IE was performed and optimized. Therefore, a Kryptofix-free procedure based on cartridges was selected wherein the nucleophilic [^{18}F]fluoride was prepared by solvent exchange from target water to NH $_4\text{HCO}_2$ (80 mg/mL, DMSO) as shown in Figure 24a.^[256] [^{18}F]Fluoride was subsequently added to the ligand solution and after reaction, purification was carried out by fixating the ligand on a cartridge, washing with Ultrapure H $_2\text{O}$ and eluting in 300 μL of ethanol (EtOH). While this method afforded excellent RCP of at least 96%, radiochemical conversion (RCC) and RCY

needed improvement. Thus, molar amount of ligand, reaction time and temperature, [^{18}F]fluoride activity as well as the elution type were varied, and RCC and RCY were determined by radio RP-HPLC as well as radio TLC (Table 3). Eventually, the optimal reaction parameters were found to be 15 nmol of ligand, addition of at least 150 MBq of [^{18}F]fluoride, 10 min reaction time at RT and an inverse elution (= elution at the loading side) of the ligand from the cartridge. Ligands **^{18}F -L1** and **^{18}F -L2** could thereby be obtained in 47% RCY and 98-99% RCP as well as satisfactory RCC of 60-67% (SI Figures 41-44).

Table 3: Optimization of RCC and RCY in the ^{18}F -labelling of ligands **L1** and **L2** by varying several reaction parameters. RCY and RCP are decay-corrected values.

Ligand	n [nmol]	t [min]	T [°C]	Activity [MBq]	Elution type	RCC [%]	RCY [%]	RCP [%]
^{18}F-L1	9	5	RT	120	normal	43	12	99
	9	5	RT	120	inverse	57	21	96
	15	5	RT	150	inverse	59	32	97
	15	10	RT	150	inverse	67	47	98
	15	10	40	120	inverse	69	46	98
^{18}F-L2	9	10	RT	135	inverse	40	27	n.d.
	9	10	RT	200	inverse	62	30	n.d.
	9	10	40	190	inverse	55	31	n.d.
	15	10	RT	200	inverse	60	47	99

n.d. = not determined

^{18}F -radiolabelling of homoleptic and heteroleptic MCgs

Radiolabelling of prosthetic groups conjugated to MCgs via *exo*-functionalization can be achieved before CDSA (pre-assembly), during CDSA (one-pot) or after CDSA (post-assembly) as explained in section 1.3.5.^[5] As the metallacages in this work are prone to disassembly under the salt-containing (NH_4HCO_2) radiofluorination condition, one-pot and post-assembly radiolabelling were not an option. Thus, synthesis of the ^{18}F -labelled MCgs was carried out pre-assembly by first radiofluorinating the ligands and then performing CDSA.

In order to combine radiolabelling with supramolecular chemistry, an optimized CDSA protocol had to be established (Figure 24a): here, 30 nmol of ligand (1 eq.) were labelled with ~ 200 MBq of [^{18}F]fluoride and eluted in EtOH. The solvent was fully evaporated and 2 eq. of $[\text{Pd}(\text{MeCN})_4](\text{BF}_4)_2$ were added in 6 μL of DMSO. The drastic reduction of the reaction volume as well as quadrupling the Pd(II)-equivalents ensured ideal concentration of the reactants (mM range) as well as sufficient driving force for the cage formation.

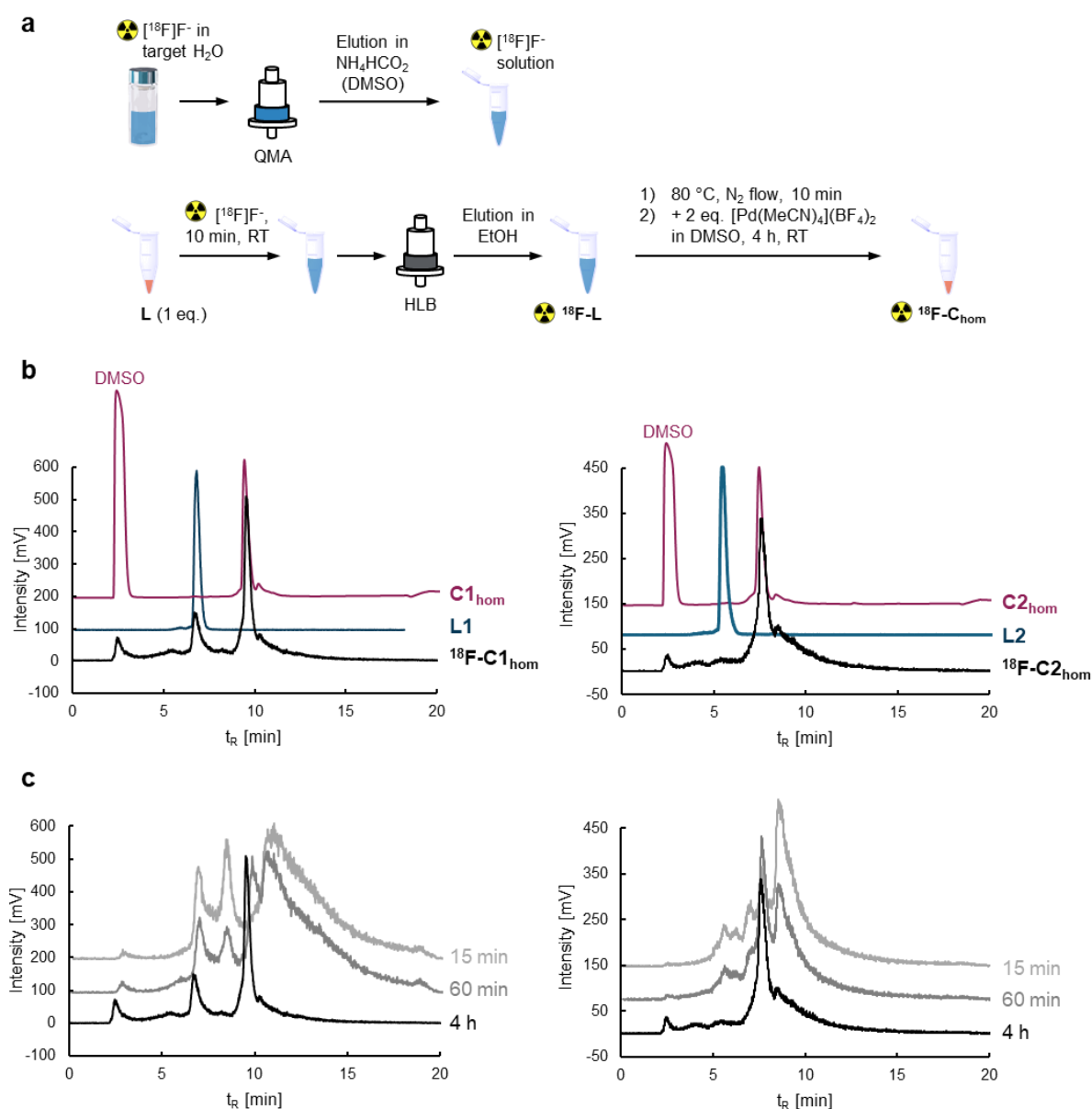


Figure 24: Synthesis of ^{18}F -labelled MCgs. a) schematic illustration of the radiofluorination procedure of the ligands and CDSA of radiolabelled cages. A silica-based ion exchange cartridge (QMA) or a copolymer-based reverse phase cartridge (HLB) were used for fixation. b) Radio chromatograms (40-70% MeCN in 15 min, C4 column) of $^{18}\text{F}\text{-C1}_{\text{hom}}$ and $^{18}\text{F}\text{-C2}_{\text{hom}}$ recorded after 4 h CDSA compared to chromatograms of the ligands and the co-injected non-radiolabelled MCgs. c) Monitoring of the CDSA of $^{18}\text{F}\text{-C1}_{\text{hom}}$ (left) and $^{18}\text{F}\text{-C2}_{\text{hom}}$ (right) over 4 h by radio RP-HPLC (40-70% MeCN in 15 min, C4 column). Chromatographic details are explained in the Materials and Methods. Reprinted from ref.^[73] with permission from the *Royal Society of Chemistry*.

The self-assembly of the homoleptic cages $^{18}\text{F}\text{-C1}_{\text{hom}}$ and $^{18}\text{F}\text{-C2}_{\text{hom}}$ was followed by radio RP-HPLC, and chromatograms were measured at 15 min, 60 min and 4 h with a gradient of 40-70% MeCN in 15 min on a C4 column (Figure 24c). To confirm successful CDSA, reference chromatograms of the unlabelled ligands and cages were recorded for comparison (Figure 24b). After 4 h reaction time, full conversion to $^{18}\text{F}\text{-C2}_{\text{hom}}$ ($t_R = 7.7$ min) was reached, whereas 18% remaining $^{18}\text{F}\text{-L1}$ ($t_R = 6.7$ min) were detected in the case of $^{18}\text{F}\text{-C1}_{\text{hom}}$

($t_R = 9.6$ min), resulting in a conversion of 73%. Furthermore, the presence of free fluoride-18 ($t_R = 2.4$ min) indicated minor defluorination in both cases ($^{18}\text{F-C1}_{\text{hom}}$: 9%, $^{18}\text{F-C2}_{\text{hom}}$: 3%). Isolation of the radiolabelled MCgs from the reaction mixtures was not possible as purification via cartridges could not separate ligands and cages, and analytical radio RP-HPLC was unsuitable due to the short-lived radioisotope ($t_{1/2} = 109.7$ min).

The CDSA of ^{18}F -labelled heteroleptic MCgs was attempted analogously by first labelling the ligands and then adding the Pd(II)-precursor and **L0**. The reaction mixtures were again analysed via radio RP-HPLC, which confirmed the formation of various radiolabelled supramolecular species (SI Figure 45). However, even though unlabelled cage mixtures were co-injected for simultaneous UV-detection, clear assignment of the peaks was impractical due to the time lag between the radio detector and the UV-detector. Furthermore, time-consuming isolation of each species by analytical radio RP-HPCL was not carried out due to the short half-life of fluorine-18.

In summary, a protocol for pre-assembly radiofluorination of SiFA-functionalized MCgs was established. Both ligands **L1** and **L2** were successfully radiolabelled and CDSA of the homoleptic cages $^{18}\text{F-C1}_{\text{hom}}$ and $^{18}\text{F-C2}_{\text{hom}}$ was complete after 4 h. The assembly of ^{18}F -labelled heteroleptic MCgs $^{18}\text{F-C1}_{\text{het}}$ and $^{18}\text{F-C2}_{\text{het}}$ resulted in a mixture of radiolabelled supramolecular coordination complexes. Unfortunately, the limited time frame makes purification of the radiofluorinated homoleptic and heteroleptic cages unsuitable. Therefore, post-assembly radiolabelling of the already purified cages would be favourable. This can be achieved by exchanging the MCgs' metal centre from Pd^{2+} to Pt^{2+} , generating cages that remain intact under the radiolabelling conditions.

3.1.7 *In vitro and in ovo evaluation*

Evaluation of biological parameters is indispensable in the optimization of pharmaceutical compounds for *in vivo* translation and is discussed in this section.

Defluorination

First, stability of the Si- ^{18}F bond was assessed in physiological conditions to determine the amount of defluorination. As free fluoride-18 is preferentially taken up by bone tissue, an inert Si-F bond is essential to minimize image background and off-target radiation.^[257] Therefore, a 3 mM K_2CO_3 solution (pH 7.4) was prepared in Ultrapure water and mixed with an EtOH solution of labelled ligand in a 9:1 ratio. Samples were taken at 0, 30, 60, 90 and 120 min and the amount of intact ligand was analysed by radio TLC (Figure 25). Overall, good stability was observed over the course of 2 h with a defluorination of only $1.7 \pm 1.1\%$ for $^{18}\text{F-L1}$ and $2.5 \pm 0.3\%$ for $^{18}\text{F-L2}$.

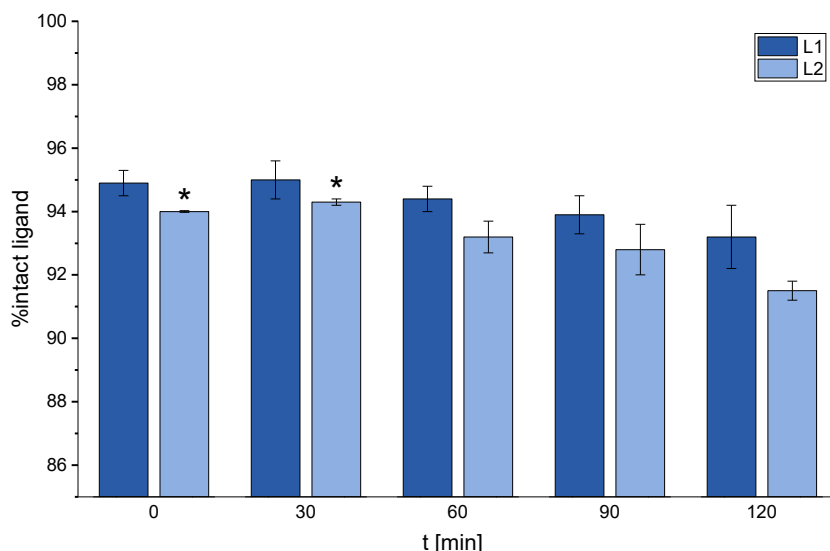


Figure 25: Si-F bond stability of ^{18}F -L1 and ^{18}F -L2 assessed in physiological conditions (K_2CO_3 , 3 mM in Ultrapure water, pH 7.4, 37 °C) and evaluated by radio TLC (see details in Materials and Methods). $n = 3$, * $n = 2$.

Lipophilicity

Lipophilicity is an important parameter that gives insights into the biodistribution and the clearance pathway of a radiopharmaceutical. While hydrophilic molecules are cleared by renal filtration, the excretion of lipophilic molecules proceeds via enterohepatic circulation.^[258,259] As clearance by the liver leads to prolonged circulation of the compound, which entails low tumour uptake as well as an increased off-target radiation level to the abdominal region, excretion via the kidneys is preferred, and radiopharmaceuticals should therefore possess the according hydrophilicity.^[259,260] Conversely, in the context of brain uptake, a study analysing the physicochemical properties of approximately 300 CNS drugs in comparison with non-CNS drugs demonstrated that BBB penetration might be favoured for lipophilic compounds ($\text{Log}D_{\text{pH}7.4} = 2\text{-}3$).^[261]

Lipophilicity can be measured by comparing the compound's distribution in a 2-phase mixture of an apolar and a polar solvent. The so-called partition coefficient can either be determined in octanol-water, referred to as $\text{Log}P$, or in octanol-PBS, known as $\text{Log}D_{\text{pH}7.4}$.^[262] However, the latter is more representative for physiological conditions due to its pH-dependence.^[263] In general, compounds with $\text{Log}P$ or $\text{Log}D_{\text{pH}7.4}$ values greater than 1 are classified as lipophilic, whereas values smaller than 1 are deemed hydrophilic.^[262]

$\text{Log}D_{\text{pH}7.4}$ determination of the radiolabelled ligands ^{18}F -L1 and ^{18}F -L2 was carried out via the "shake-flask method", in which the ligand is added to the solvents, vortexed and centrifuged. The activity of the separated solvent layers is then evaluated using a γ -counter (see Materials and Methods). While ^{18}F -L1 exhibited a $\text{Log}D_{\text{pH}7.4}$ of 2.53 ± 0.05 , a value of 2.07 ± 0.07 was calculated for ^{18}F -L2, which is a significant reduction compared to the (SiFA)BA-containing

ligand (Table 4). However, set in relation to ^{99m}Tc -labelled PepH3 ($\text{Log}P = 0.11 \pm 0.06$), the impact of the lipophilic SiFA synthons on the lipophilicity of the MCg ligands becomes evident.^[207] Even though the positive effect of incorporating the novel (SiFA)SeFe could be confirmed, the lipophilicity of both ligands is unfavourably high, and whether this leads to a promoted brain uptake needs to be assessed.

Due to the cages' instability in PBS, determination of the $\text{Log}P$ was better suited in this case. Exemplarily, the $\text{Log}P$ was measured for ^{18}F -**C2**_{hom} and amounted to -1.11 ± 0.09 , a rather questionable result. Thus, in order to investigate the MCg's intactness, a reference sample of ^{18}F -**C2**_{hom} in water was added to the procedure for analysis by radio RP-HPLC. The corresponding radio chromatogram indicated cage disassembly with 46% of remaining ^{18}F -**C2**_{hom} caused by vortexing and centrifuging (SI Figure 46). Moreover, the presence of free $^{18}\text{F}^-$ (36%) revealed major defluorination as a result of the experimental protocol and explains the negative $\text{Log}P$ value. Consequently, this method is not suitable to evaluate lipophilicity of the radiolabelled MCgs. Nonetheless, it can be assumed that the cages' lipophilicity is high as they consist of for highly lipophilic ligands.

Table 4: Summary of $\text{Log}D_{\text{pH}7.4}$ values and %HSA binding of ligands and homoleptic and heteroleptic MCgs.

Compound	$\text{Log}D_{\text{pH}7.4}$ ^a [-]	HSA binding by HPAC-HSA [%]	HSA binding by ultrafiltration ^b (unspecific binding) [%]
L1	2.53 ± 0.05	> 99 *	20.5 ± 2.4 (99.4 ± 0.0)
L2	2.07 ± 0.07	99	37.2 ± 2.3 (98.8 ± 0.1)
C1 _{hom}	n.d.	> 99 *	n.d.
C2 _{hom}	n.d.	99	n.d.
C1 _{het}	n.d.	98	n.d.
C2 _{het}	n.d.	98	n.d.

^a n = 8, ^b n = 6.

* no elution due to too strong interaction with the column.

n.d. = not determined.

Binding to human serum albumin

HSA is the most abundant protein in human blood where it exhibits prolonged circulation as it evades renal filtration.^[264] Due to its ability to bind exogenous ligands it can serve as a transporter and storage for radiopharmaceuticals and therefore has an impact on their pharmacokinetics.^[265] Sufficient HSA-binding is desired to make use of the transporter function and the depot effect. Too tight binding of over 95% on the other hand causes long blood retention, slow tumour uptake as well as enhanced off-target radiation, which could damage blood cells.^[266] Interestingly, a correlation between high lipophilicity and strong interaction with HSA was found and thus, even though $\text{Log}D_{\text{pH}7.4}/\text{Log}P$ could not be acquired for the cages, determining their HSA-binding might give further insight into their lipophilicity as well.^[266]

The HSA-interaction was first evaluated by a high-performance affinity chromatography (HPAC)-HSA assay. This method applies a column, on which HSA is immobilized. The compound's retention time can then directly be converted into the HSA-binding using a calibration curve of known standards. Samples of the ligands **L1** and **L2** as well as the corresponding homoleptic and heteroleptic MCgs were prepared, purified via analytical RP-HPLC, lyophilised and redissolved before injection. Unfavourably high HSA-binding of over 98% was observed for all compounds (Table 4). While in the case of **L1** and **C1_{hom}** no elution from the column occurred at all due to too strong interaction with HSA (> 99%), 99% binding was calculated for **L2** and **C2_{hom}**, and 98% binding for the heteroleptic cages **C1_{het}** and **C2_{het}**, which might correlate with a slightly reduced lipophilicity when incorporating (SiFA)SeFe and unfunctionalized ligand **L0**.

The HSA-binding was further assessed under physiological conditions by HSA ultrafiltration. Here, the radiolabelled compound is mixed with a solution of HSA in PBS, incubated at 37 °C and then filtered through a membrane via centrifugation. The HSA-ligand adduct is held back by the membrane while unbound ligand is pressed through the filter into the centrifugate. The activity of the filter and centrifugate is measured separately in the γ -counter and used to calculate the binding. Furthermore, a reference samples of the radiolabelled compound in PBS are prepared to determine the unspecific binding to the ultrafiltration vial. As the MCgs would not withstand the centrifugal forces, the assay was just carried out with the ^{18}F -labelled ligands. Here, HSA-binding amounted to $20.5 \pm 2.4\%$ for ^{18}F -**L1** and to $37.2 \pm 2.3\%$ for ^{18}F -**L2** (Table 4). However, the validity of these results is questionable as an unspecific binding of $99.4 \pm 0.0\%$ and $98.8 \pm 0.1\%$ was determined, respectively, meaning that the radiolabelled ligands are almost quantitatively withdrawn from the assay, which impedes proper evaluation.

In ovo studies

To investigate the consequences of the high lipophilicity and strong HSA-binding *in vivo*, the organ uptake was exemplarily studied for ^{18}F -**L1** in the chick chorioallantoic membrane model in collaboration with Prof. Tim Witney at *King's College London*, UK (Figure 26a). This preclinical platform enables cost- and time-efficient tumour imaging as well as high-throughput screening while adhering to the ethical principles of reduction, refinement and replacement (3Rs), and will be discussed in more detail in section 3.3.^[267] For the study, U87 glioblastoma tumours were cultivated on the CAM of fertilised chicken eggs, and a 60 min PET scan was acquired upon injection of ~ 3 MBq of ^{18}F -**L1** formulated in saline solution. Analysis of representative time frames as well as an *ex ovo* biodistribution 60 min p.i. reveal pronounced liver accumulation of 268.5 ± 61.0 %ID/g as well as prolonged blood circulation as expected with the ligand's high lipophilicity (Figure 26b and c). Furthermore, bone uptake of 3.55 ± 0.91 %ID/g was observed indicating slight defluorination of the ligand, which is in

accordance with the *in vitro* Si- ^{18}F stability assay.^[257] To compare the effect of the SiFA synthons examination of ^{18}F -L2 would be of interest but has not yet been carried out due to time constraints.

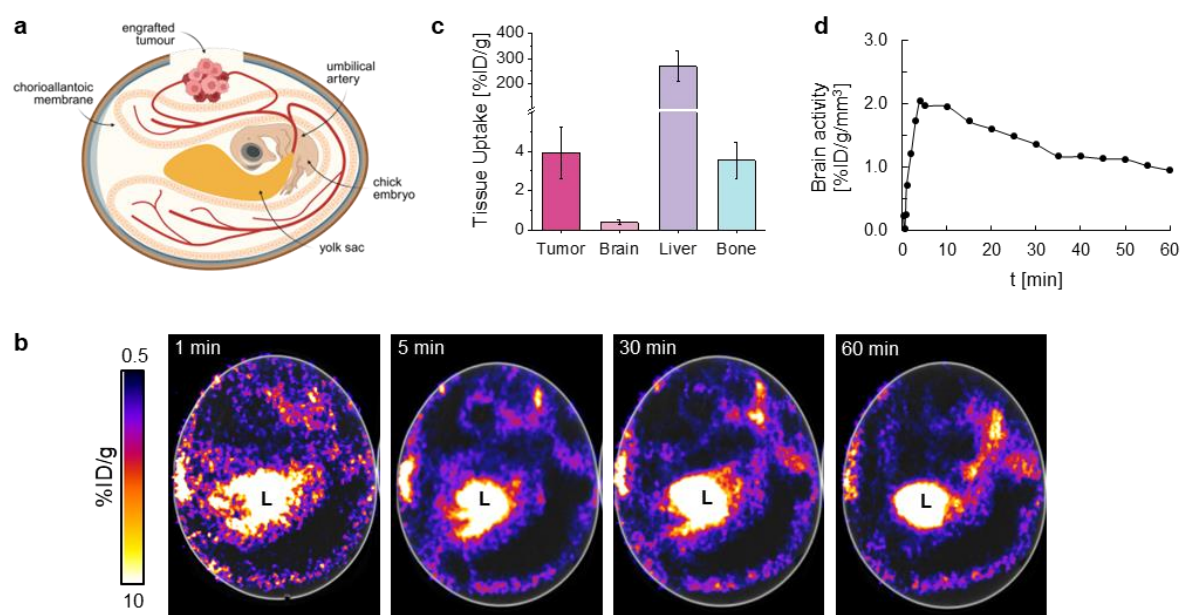


Figure 26: *In ovo* assessment of ^{18}F -L1. a) illustration of the chick CAM model. b) *in ovo* PET images at different time points from a 60 min dynamic scan ($n = 4$). The liver (L) is labelled. c) *ex ovo* biodistribution 1 h p.i. d) Time-activity curve of the brain uptake of the chick embryo. Adapted from ref.^[73] with permission from the *Royal Society of Chemistry*. Panel a was created in <https://BioRender.com>.

Although the CAM system is not primarily designed to evaluate BBB permeability, an attempt was made to quantify brain uptake of the chick embryo (Figure 26c and d). According to the time-activity curve maximal brain uptake is reached 5 min p.i. and *ex ovo* biodistribution 60 min p.i. reveals an uptake of 0.38 ± 0.13 %ID/g. Overall, the resulting values were consistent with previously reported data for the BBB shuttle peptide PepH3 (0.31 ± 0.07 %ID/g) as well as a PepH3-conjugated MCg (0.42 ± 0.06 %ID/g) assessed in mouse models.^[128,207] Nevertheless, additional studies are required to confirm these findings, ideally employing bimodal PET/MRI to improve delineation of the brain region. Moreover, a negative control with a tracer that is unable to cross the BBB should be performed to ensure that the embryonic BBB is fully developed at this stage. Complementary histology assay to analyse the BBB structure and the presence of tight junctions in particular would grant further insight into the BBB morphology of the chick embryo, provided that suitable chicken-specific antibodies for the immunohistochemical staining exist.

Despite these challenges, encouraging U87 tumour accumulation was observed *ex ovo*, with an uptake of 3.94 ± 1.31 %ID/g 60 min p.i. Moving forward, tumour targeting could be further optimized through a heteroleptic design combining the δ PepH3-conjugated ligand with a glioblastoma-targeting ligand like CPCR4, FALGEA or RAS70.^[268–270] Elaborate orthotopic *in*

vivo uptake studies could then not only assess tumour targeting but also BBB crossing of the supramolecular tracer at the same time.

To conclude, while sufficient stability of the Si- ^{18}F bond toward defluorination was observed *in vitro* and *ex ovo*, lipophilicity of the ligands and corresponding MCgs, originating from the incorporated SiFA synthons, needs to be drastically improved. This can either be achieved by optimizing the ligand via the addition of compensating amino acids like citrulline, glutamate or aspartate, or by using a more hydrophilic prosthetic group like AMBF_3 , accepting harsher radiolabelling conditions. Nonetheless, satisfactory tumour accumulation and brain uptake of the MCg ligand are promising outcomes for its potential application as BBB-penetrating radiotheranostic.

3.1.8 Conclusion on BBB-targeted $[\text{Pd}_2\text{L}_4]^{4+}$ MCgs

All in all, this project comprehensively evaluated the synthesis of brain-targeted homoleptic and heteroleptic $[\text{Pd}_2\text{L}_4]^{4+}$ MCgs and further explored their use for radiopharmaceutical applications. Two $\text{D}^{\text{D}}\text{PepH3}$ -conjugated bispyridinyl ligands featuring different SiFA synthons were successfully synthesized and radiofluorinated. The corresponding homoleptic metallocages were formed by CDSA and generation of their ^{18}F -labelled analogues was achieved by pre-assembly radiolabelling. In the context of CDSA, it was found that the reaction conditions including the solvent and concentration of building blocks had a crucial impact on the formed species as well as the conversion. In the mM range, almost full cage assembly was ensured, whereas CDSA in lower concentrations resulted in incomplete conversion and dilution led to cage disassembly. In a next step, heteroleptic MCg mixtures were synthesized by statistical self-assembly, wherein isolation of single species was possible. Assembly of ^{18}F -labelled heteroleptic mixtures could be evidenced as well, but the short fluorine-18 half-life prevented species separation. While encapsulation of cisplatin and $[\text{}^{99\text{m}}\text{Tc}]\text{TcO}_4^-$ failed due to stability issues of the cages, formation of host-guest complexes with perrhenate, the 'cold' surrogate of $[\text{}^{99\text{m}}\text{Tc}]\text{pertechnetate}$, was confirmed for both homoleptic and heteroleptic MCgs. Beside problematic instability, high lipophilicity and HSA-binding limit the potential of these systems for *in vivo* applications. Improvements could be implemented by ligand optimization and by replacing Pd^{2+} with the more kinetically inert Pt^{2+} to form $[\text{Pt}_2\text{L}_4]^{4+}$ cages, which would allow for post-assembly modification and optimized radiolabelling. In summary, these results underscore the challenges that remain in developing targeted SCC-based theranostics for potential clinical translation in nuclear medicine. Nonetheless, their structural versatility and straightforward chemical modification provide a powerful platform for creating multimodal theranostics.

3.2 Design concept for brain-targeted platinum(II) metallacages

With the progress made in the area of $[\text{Pt}_2\text{L}_4]^{4+}$ cages as discussed in section 1.2.2, structures with complex ligands for biomedical and radiopharmaceutical applications will be derived soon. Their enhanced kinetic stability not only allows for safe *in vitro* and *in vivo* testing, but also enables clean isolation, post-assembly modification (PAM) as well as post-assembly radiolabelling, procedures that were not possible for the SiFA-containing BBB-targeted $[\text{Pd}_2\text{L}_4]^{4+}$ MCgs. In fact, our group just reported on a microwave-assisted method for the assembly of *N*-hydroxysuccinimide (NHS) ester-activated $[\text{Pt}_2(\text{L}^{\text{NHS}})_4]^{4+}$ (L^{NHS} = 2,5-dioxopyrrolidin-1-yl 2-(3,5-bis(pyridin-3-ylethynyl)phenyl)acetate), featuring the same ligand backbone of the Pd(II) cages (C0_{hom}) explored in this work.^[271] CDSA of the Pt(II)-MCg was followed by PAM with *i.a.* a DOTA chelator for radiometal complexation and an integrin-targeted peptide via amide coupling.^[271] Thus, encouraged by these advancements, two synthetic approaches to generate BBB-targeted homoleptic $[\text{Pt}_2\text{L}_4]^{4+}$ and heteroleptic $[\text{Pt}_2\text{L}(\text{L}')_3]^{4+}$ cages via PAM were developed (Figure 27).

The homoleptic route starts with solid-phase synthesis of the δ PepH3-containing amine linker, followed by resin cleavage and amide coupling to the NHS-activated Pt(II)-cage (Figure 27a). Whether full conversion (PAM of all four activated ligands of the Pt(II)-MCg) can be reached in the coupling step might be dependent on the complexity and steric hindrance of the amine linker. Furthermore, as the BBB-shuttle peptide sequence (AGILKRW) contains a lysine residue, unwanted side reactions may occur between its amino group and the NHS-activated carbonic acid of the cage during amide coupling. This issue can be circumvented by the implementation of a suitable protecting group (PG) strategy. Classically, PGs like *tert*-butyloxycarbonyl or 2,2,4,6,7-pentamethyl dihydrobenzofuran-5-sulfonyl are employed during Fmoc-SPPS, as they are acid-labile and are automatically removed when cleaving the ligand off the resin with TFA.^[272] However, in this case, the lysine side-chain amino group must remain protected during resin cleavage, so it cannot interfere with cage coupling. This requires the introduction of a TFA-stable PG that can instead be removed after conjugating the ligand to the MCg. Here, the 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl PG, which can be cleaved with 2% hydrazine, or the allyloxycarbonyl PG, removable in the presence of $[\text{Pd}(\text{PPh}_3)_4]$, would be convenient candidates.^[272] A more expensive photochemical alternative would be the application of a photolabile PG that can be cleaved by UV irradiation.^[273] To identify the most appropriate option, stability studies of the basic Pt(II)-cage under the respective deprotection conditions are essential to ensure MCg integrity upon PG removal. Once purified and characterized, the resulting *exo*-functionalized homoleptic $[\text{Pt}_2\text{L}_4]^{4+}$ cage could then be evaluated for $^{99\text{m}}\text{Tc}[\text{TcO}_4]^-$ encapsulation and subsequently applied in brain SPECT imaging studies.

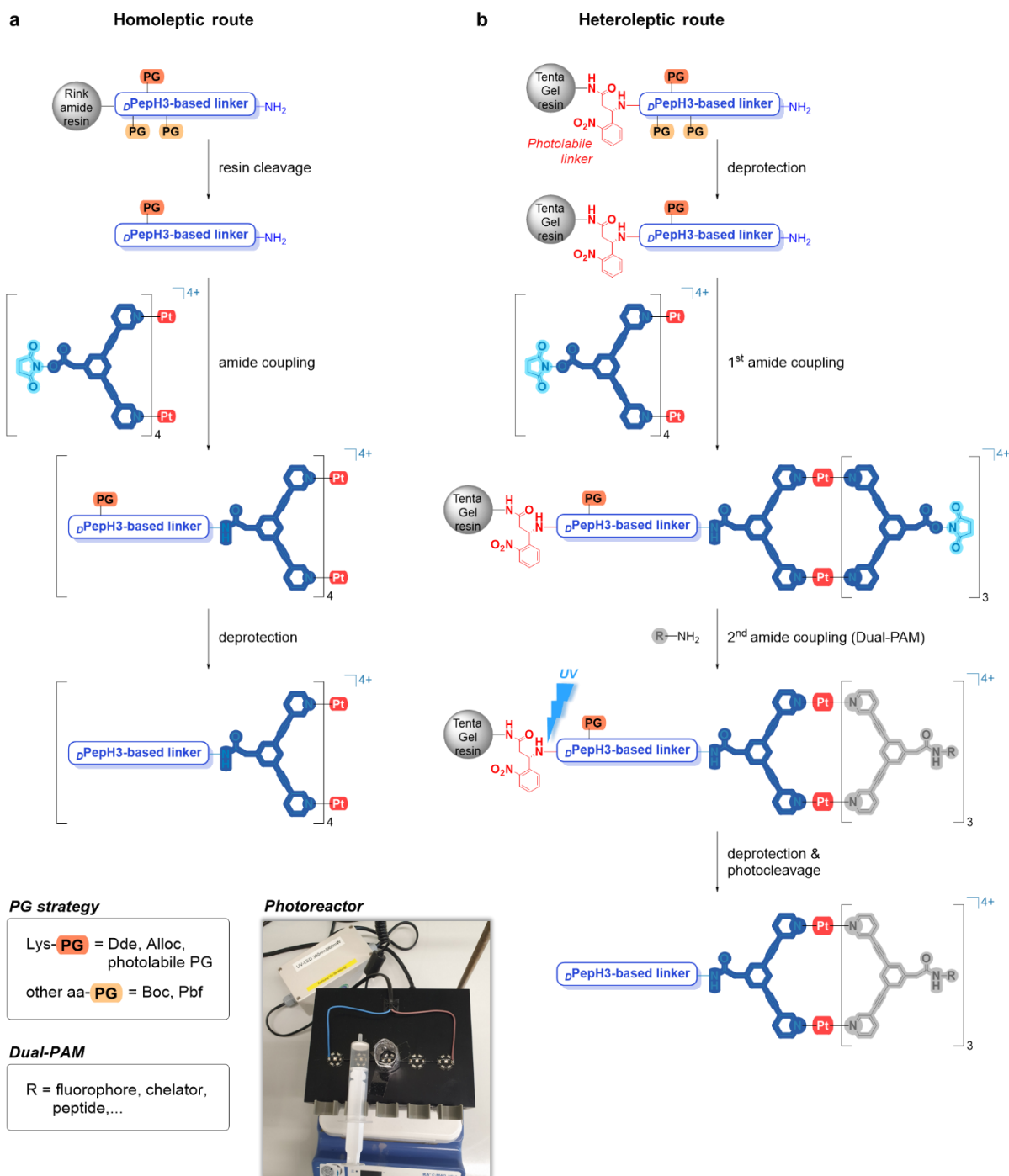


Figure 27: Proposed synthetic routes for the assembly of BBB-targeted a) homoleptic $[\text{Pt}_2\text{L}_4]^{4+}$ and b) heteroleptic $[\text{Pt}_2\text{L}(\text{L}')_3]^{4+}$ MCg via PAM. The photoreactor for resin cleavage was developed together with the TUM chemistry central electronics shop (ZBE).

Abbreviations: Alloc = allyloxycarbonyl, Boc = tert-butyloxycarbonyl, Dde = 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl, Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl.

A heteroleptic route to obtain Pt(II)-cages featuring only one BBB-penetrating ligand could be envisaged based on a solid-phase PAM approach wherein the NHS-activated Pt(II)-MCg is conjugated to the dPepH3 -containing linker while still on the resin to generate the desired monofunctionalized $[\text{Pt}_2\text{L}(\text{L}^{\text{NHS}})_3]^{4+}$ species (Figure 27b). Further, the three remaining NHS-

activated ligands can be conjugated on resin to other functional groups (e.g. fluorophores, chelators, etc.) to achieve $[Pt_2L(L')_3]^{4+}$ systems for multimodality imaging or targeting. For this synthetic strategy to be successful, there are two main considerations: first, a thoughtful PG strategy must be applied to prevent side reactions between the cage and the Δ PepH3 lysine sidechain during amide coupling just as for the homoleptic approach. Second, conventional resin cleavage in TFA needs to be replaced as the acidic conditions could induce disassembly of the *exo*-functionalized heteroleptic cage. In this context, mild removal from the solid support is achievable with photochemical methods. This was demonstrated by Boros and co-workers who reported on the solid-phase synthesis of ready-to-inject radiopharmaceuticals.^[274] Their peptide-based ligands were conjugated to a photolabile linker (Anp = (S)-3-amino-3-(2-nitrophenyl)propanoic acid), radiolabelled and released from the resin upon UV irradiation (λ = 365 nm) in a simple 3D-printed photoreactor setup. TentaGelTM S-NH₂ resin was selected as the photostable solid support due to its high photochemical cleavage yield. Moreover, on-resin washing allowed the removal of excess reagents and resulted in a clean radiopharmaceutical formulation for *in vivo* administration.^[274] Consequently, the TentaGel resin and the photosensitive Anp linker were implemented in the synthetic protocol of solid-phase PAM for heteroleptic Pt(II)-cages as well, which now consists of 6 steps, namely Fmoc-SPPS of the Δ PepH3-linker, a first amino acid deprotection, amide coupling of the NHS-activated $[Pt_2L_4]^{4+}$, second amide coupling of an additional functionality, lysine deprotection and photocleavage (Figure 27b). The realization of this approach has already been started with the construction of a photoreactor containing 5 LED lamps (365 nm, 960 mW), a cooling body and syringe holders. Furthermore, reproducible synthesis and photocleavage of a short model peptide (WRK-Fmoc) was achieved (data not shown). As a next step, on-resin amide coupling of the Pt(II)-cage to a simple PEG linker and subsequent photocleavage is planned before repeating the solid-phase PAM trial with the more complex Δ PepH3-based linker. Nonetheless, it is difficult to predict whether this pathway solely produces the desired monofunctionalized species.

To conclude, PAM is a powerful tool to decorate $[Pt_2L_4]^{4+}$ -MCgs with a plethora of functional linkers, including targeting peptides, radionuclide chelators and fluorescent tags. It also facilitates functionalization with challenging modalities that, when incorporated pre-assembly, could decompose under the harsh CDSA conditions or sterically hinder cage formation. While the proposed homoleptic route for BBB-targeted Pt(II)-cages is based on PAM in solution, the heteroleptic route suggests solid-phase PAM followed by photocleavage, which includes on-resin washing, simplifying subsequent purification protocols. Moreover, the possibility to add a second functional group by dual-PAM on the resin further increases the multimodality of the heteroleptic system. In both the homo- and the heteroleptic route straightforward amide coupling was selected for the PAM step, employing an NHS-ester activated Pt(II)-cage. As this

MCg is slightly sensitive under ambient conditions, immediate use without purification and a dry atmosphere is required. For prospective PAM protocols, alternative conjugation methods, such as cycloaddition reactions, that apply less sensitive Pt(II)-MCg intermediates, should therefore be considered.

3.3 Methodology: the chick chorioallantoic membrane model*

With growing emphasis on animal welfare and in accordance with the 3R principles (reduction, refinement, replacement), the development of alternative preclinical models is of increasing importance. One such model is the chick chorioallantoic membrane (CAM), an avian system mentioned in section 3.1.7, which represents a valuable alternative to mouse xenografts in cancer research (Figure 28).^[275] The CAM is a highly vascularized double membrane that forms in fertilized chicken eggs around the embryo through the fusion of the chorion and allantois mesodermal layers six to seven days post-fertilization (embryonic day E6-7).^[276,277] Functionally, it mediates Ca^{2+} transport from the eggshell to the embryo to support bone mineralization and provides an oxygen-rich environment through gas exchange.^[278,279] Moreover, the chick embryo remains naturally immunodeficient until day E18, which facilitates rapid growth of tumours and other tissues engrafted on the CAM as well as an enhanced take rate.^[280] For this reason, the model has already been employed in studies of tumour growth, metastasis, angiogenesis as well as drug delivery.^[281–284] It allows for high-throughput screening of therapeutic agents and is compatible with a variety of imaging techniques.^[275,285] Moreover, as tumours are grown externally on the CAM rather than within animals, background signals from overlapping tissues can be minimized allowing clearer evaluation of the tumour uptake. Notably, under European law (Directive 2010/63/EU), the CAM model is considered in line with the 3R until day E14, meaning that animal protocols and ethical approvals are not required for its use.^[276] Furthermore, the ease of handling the eggs combined with the time- and space-efficiency makes the avian model a highly accessible and available model compared to mouse xenografts, which require substantial housing, handling and husbandry as well as administrative responsibilities, especially when working with costly immunodeficient strains.^[267,285,286]

* Parts of this subchapter are based on a work published in the *Journal of Medicinal Chemistry*.^[171]

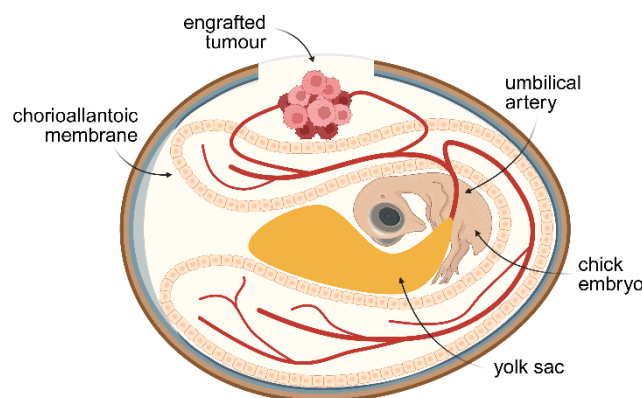


Figure 28: Schematic illustration of the chick CAM model. The figure was created in <https://BioRender.com>.

Nonetheless, the CAM model has limitation. Being non-mammalian, it differs from murine models in metabolism and pharmacokinetics, reducing its translational relevance.^[275] The fast tumour growth on the CAM also restricts the experimental time window and variations in tumour vasculature can influence drug uptake.^[282] In addition, the early embryonic developmental stage results in poor CT contrast, complicating organ localization in image analysis, and the incomplete BBB might be unsuitable for brain uptake studies.^[73] Despite these drawbacks, the CAM is a promising high-throughput and low-cost preclinical tool that can complement murine models and contribute to reducing the number of animals in *in vivo* studies.

Because of these attractive features, the chick CAM model has gained attention also in the radiopharmaceutical field. So far, it has been employed to evaluate the biodistribution and stability of radiotracers labelled with fluorine-18, technetium-99m, iodine-125 and lutetium-177, providing valuable data for validation and comparability with mouse models.^[287,288] More recently, tumour imaging applications have also been reported. For instance, uptake of [¹⁸F]F-siPSMA-14 into PSMA-positive tumours was demonstrated both *in ovo* and *in vivo* by PET/MRI.^[289] Similarly, a ⁶⁸Ga-labelled HER2 affibody enabled visualization of breast cancer tumours in the CAM model, yielding results consistent with those obtained in mouse xenografts.^[290] In another study, Smith *et al.* compared *in ovo* and *in vivo* uptake of [¹⁸F]FDG and (4S)-4-(3-[¹⁸F]fluoropropyl)-L-glutamate in non-small cell lung cancer tumours and successfully used the latter to monitor response to external beam radiotherapy via PET imaging.^[267]

To broaden the range of tumours and radiotracers studied, the sstR2-specificity of a SiFA-containing radiohybrid (¹⁸F][^{nat}Ga]Ga-(SiFA)SeFe-rhTATE2) was investigated in the chick CAM model in collaboration with the group of Prof. Tim Witney at *King's College London*, UK (Figure 29a).^[171] The tumour uptake was determined in two different cancer cell lines: in addition to sstR2-expressing rat-derived AR42J pancreatic cells, sstR2-negative U87 human

glioblastoma cells were employed as a negative control. The differential sstR2-expression of the two cell lines was confirmed by Western Blotting as depicted in Figure 29b, with α -tubulin being visualized to confirm sufficient gel loading.

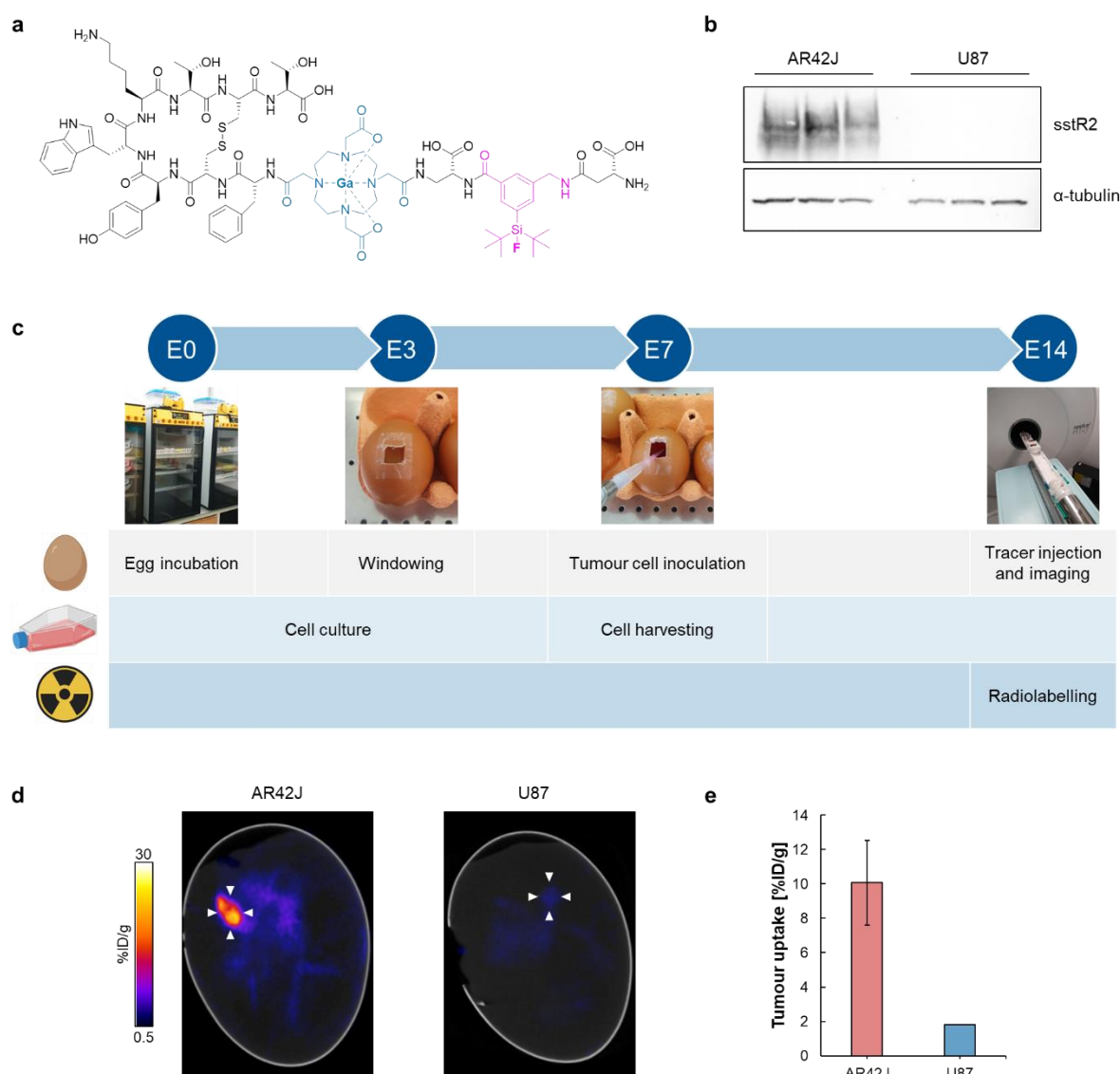


Figure 29: Investigation of the sstR2-addressing radiohybrid tracer $[^{18}\text{F}][^{\text{nat}}\text{Ga}]\text{Ga}-(\text{SiFA})\text{SeFe-rhTATE2}$ in AR42J tumours grown on the chick CAM. a) chemical structure of $[^{\text{nat}}\text{F}][^{\text{nat}}\text{Ga}]\text{Ga}-(\text{SiFA})\text{SeFe-rhTATE2}$. b) expression of sstR2 in AR42J and U87 cell lysates (n=3). Uncropped images of the gels are shown in SI Figure 47. c) timeline of *in ovo* imaging studies with radiotracers. d) Representative *in ovo* PET/CT images of $[^{18}\text{F}][^{\text{nat}}\text{Ga}]\text{Ga}-(\text{SiFA})\text{SeFe-rhTATE2}$ in sstR2-positive AR42J (n=4) and sstR2-negative U87 tumours (n=1) on the chick CAM 40-60 min p.i. Tumours are indicated by white arrows. e) AR42J and U87 tumour uptake of the tracer as determined by *ex ovo* biodistribution 1 h p.i. Panels c, d and e were adapted from ref.^[171] with permission from *American Chemical Society*.

In ovo tumour PET imaging studies generally follow four key stages, described in detail in the Supplementary Information (Figure 29c): Embryo development is initiated by incubating the fertilized chicken eggs in a warm and humidified atmosphere, which is defined as day E0. On day E3, the eggs are checked for viability and windows are cut in the eggshell. Tumour cells

are harvested on day E7 and engrafted onto the CAM through the prepared window. Here, introduction of micro-bleeding on the CAM prior to cell inoculation promoted initiation of tumour formation and angiogenesis. Tumours are then allowed to grow until day E14, when the radiotracer is labelled and administered into a CAM vein for PET imaging.

Ahead of the imaging study, essential parameters such as egg viability and tumour growth were monitored to help estimating the number of eggs required per experiment. First, the egg fertilization was tracked and a rate of $72.5 \pm 27.4\%$ was observed (SI Figure 48a). The relatively high deviation might be the result of a disrupted cold chain during egg delivery. Following tumour cell inoculation, embryo viability and tumour take rates were assessed (SI Figure 48a). Growth of U87 tumours on the CAM is well-established as the human-derived cells are tolerated by the chick embryo, resulting in favourable embryo viability of $87.3 \pm 13.9\%$ and a take rate of $75.0 \pm 18.5\%$. By contrast, engraftment of rat-derived AR42J cells markedly reduced embryo viability ($16.7 \pm 39.6\%$) and produced a less consistent take rate of $56.4 \pm 33.1\%$. Additionally, tumour growth was optimized by testing different Matrigel volumes (10 μL and 20 μL) and cell numbers (1, 2 and 3×10^6) (SI Figure 48b). Ultimately, suspensions of 3×10^6 cells in 20 μL of Matrigel produced tumours of appropriate size by day E14, with mean weights of 122.4 ± 43.2 mg for U87 and 169.6 ± 41.8 mg for AR42J. Nevertheless, AR42J tumours often appeared flat and slight necrosis could occasionally be observed, which is consistent with the reduced tolerance of rat-derived cells. Postponing the day of tumour cell engraftment from day E7 to day E9, allowing for prolonged undisturbed embryo development, might increase the tolerability, but switching to human-derived sstR2-positive cell lines such as MiaPaCa-2 or QGP-1 may prove more suitable for future applications.^[291,292]

On day E14, $[\text{natF}][\text{natGa}]\text{Ga}-(\text{SiFA})\text{SeFe-rhTATE2}$ was labelled via ^{19}F -to- ^{18}F IE according to an established protocol, followed by *in ovo* PET imaging.^[256] The PET/CT images acquired 1 h p.i. shown in Figure 29d as well as *ex ovo* biodistribution (Figure 29e) revealed strong tracer accumulation in AR42J tumours (10.1 ± 2.5 %ID/g), whereas uptake in U87 tumours was minimal (~ 1.8 %ID/g), confirming the high sstR2 specificity of $[\text{natF}][\text{natGa}]\text{Ga}-(\text{SiFA})\text{SeFe-rhTATE2}$. To obtain further insight into the comparability of *in ovo* and *in vivo* models, additional tumour uptake studies of this tracer in AR42J-tumour bearing mice would be advisable.

All in all, this proof-of-concept study highlights the feasibility of the chick CAM model in assessing specific tumour uptake and quantification. While not every cell line is equally compatible with the model, it supports reproducible growth of human-derived cancer cell lines. The straightforward set-up, short experimental timeline and capacity for high-throughput screening make the CAM platform a valuable preclinical tool for evaluating radiopharmaceutical candidates before advancing to mouse studies and might therefore be able to fill the gap between *in vitro* and *in vivo* research.

4 Conclusions and Outlook

This work set out to design and investigate brain-targeted $[\text{Pd}_2\text{L}_4]^{4+}$ cages for dual-modality nuclear imaging. Therefore, two generations of metallacage (MCg) ligands, **L1** and **L2**, incorporating the blood-brain barrier (BBB)-penetrating peptide D^{D} PepH3 as well as two different silicon-based fluoride acceptor (SiFA) synthons ((SiFA)BA and (SiFA)SeFe) for radiolabelling with the PET isotope F-18, were successfully synthesized via Fmoc-SPPS in good yields and excellent purity, as confirmed by RP-HPLC, NMR spectroscopy and HR-ESI-MS. Both ligands were radiofluorinated via ^{18}F -to- ^{19}F isotopic exchange (IE) employing a Kryptofix-free method. Satisfactory RCY and RCP was achieved after optimization and good stability against defluorination was observed *in vitro* and *in ovo*. While SiFAs offer mild radiolabelling conditions, their intrinsic high lipophilicity can impair pharmacokinetics of a radiotracer. Therefore, the optimized amino acid-like (SiFA)SeFe was introduced in the design of the second-generation ligand **L2**, markedly reducing the $\text{Log}D_{\text{pH}7.4}$ value compared with the (SiFA)BA-bearing ligand **L1**. The unfavourable lipophilicity of **L1** was additionally showcased in the chick chorioallantoic membrane (CAM) model by PET imaging as well as *ex ovo* biodistribution, resulting in pronounced liver uptake and prolonged blood circulation. The latter might also be enhanced by strong HSA binding of the ligand, which directly correlates with high lipophilicity. To validate the positive effect of (SiFA)SeFe, CAM PET imaging with ^{18}F -**L2** should be performed. Additionally, ligand optimization would be advisable to further improve the lipophilicity and could be achieved by incorporating hydrophilic moieties or replacing the SiFA synthon with other ^{18}F -labelling moieties. Nevertheless, ^{18}F -**L1** demonstrated *in ovo* brain uptake in comparable amounts to a similar host-guest system and free PepH3 as well as promising U87 tumour uptake, encouraging its potential for brain cancer imaging, though the intactness of the chick BBB at this early developmental stage requires confirmation. In addition, the low bone CT contrast of the chick embryo complicates localization of the brain and thus, monitoring tracer uptake *in ovo*. By switching the complementary morphological imaging from CT to MRI, this bottleneck could be optimized.

As a next step, homoleptic cages **C1_{hom}** and **C2_{hom}**, as well as heteroleptic cages **C1_{het}** and **C2_{het}** (with unfunctionalized **L0**) were successfully assembled by coordination-driven self-assembly (CDSA) within one hour at RT, as evidenced by RP-HPLC, NMR spectroscopy and HR-ESI-MS. While homoleptic CDSA yielded a single cage species, heteroleptic CDSA produced statistical mixtures of supramolecular adducts separable by analytical RP-HPLC. Formation of $[\text{Pd}_2\text{L}_4]^{4+}$ cages proved strongly dependent on the reaction conditions including the reaction medium and the concentrations of reactants. In the case of the cages synthesized in this work, DMSO/H₂O ($\leq 50\%$ H₂O) and 1 mM theoretical cage concentration provided optimal conversion. Stability studies revealed sufficient integrity in organic solvents and water,

but rapid disassembly in the presence of salts, limiting applicability under physiological conditions. Host-guest chemistry of the MCgs was investigated by following the encapsulation of cisplatin, perrhenate, as well as [^{99m}Tc]pertechnetate. While the formation of a cisplatin-MCg adduct could not be confirmed, encapsulation of ReO_4^- into homoleptic and heteroleptic cages was evidenced by HR-ESI-MS. Even though partial inclusion of the non-radioactive analogue was achieved, encapsulation of the SPECT tracer [^{99m}Tc] TcO_4^- was unsuccessful, both post-assembly and in a one-pot reaction with CDSA due to cage instability. Following the successful ^{18}F -labelling of the ligands, synthesis of ^{18}F -labelled homoleptic cages was pursued via pre-assembly radiolabelling where the cage is assembled after radiofluorination of the ligand. Careful matching of the IE and CDSA protocols by reducing the reaction volume and increasing the Pd(II) equivalents enabled complete formation of $^{18}\text{F}\text{-C1}_{\text{hom}}$ and $^{18}\text{F}\text{-C2}_{\text{hom}}$ within 4 h. This optimization represented an important advancement toward establishing the clinical applicability of MCgs for PET imaging, which requires simple, reproducible and kit-like radiolabelling procedures. Although heteroleptic ^{18}F -labelled mixtures were obtained as well, time-consuming purification by analytical radio RP-HPLC would be necessary to isolate one species, which is not suitable for a short-lived isotope like fluorine-18. Post-assembly radiolabelling, where labelling is performed after CDSA and isolation of the cage, would therefore, be the more favourable approach, but is currently not feasible due to MCg instability.

Overall, the lability of $[\text{Pd}_2\text{L}_4]^{4+}$ and the formation of mixtures of SCCs during self-assembly remain the main bottleneck, complicating characterization, radiolabelling and *in vivo* application. Transitioning to Pt(II)-cages would be an elegant solution as they are more kinetically inert and with the recent advances made in their synthesis incorporation of complex and sterically hindering ligands might soon be achievable. Herein, first synthetic routes toward BBB-targeted homo- and heteroleptic $[\text{Pt}_2\text{L}_4]^{4+}$ cages was developed that included MCg functionalization via post-assembly modification, tailored protecting group strategy and photocleavage. An alternative approach to increase cage stability that has also been investigated in this work, is the assembly of heteroleptic MCgs replacing *exo*-functionalized ligands with less sterically demanding ones, which in our case resulted in statistical CDSA. One solution to achieve formation of solely one defined cage species is the use of asymmetrical ligands that support the square planar coordination geometry of the metal centre only by one specific ligand combination, as demonstrated by Severin *et al.* for Pd(II)-Cgs featuring bispyridyl ligands.^[69] Another possible way to form heteroleptic Pd(II)-MCgs, which is currently under investigation in our group for the assembly of $[\text{Pd}_2\text{L}(\text{L0})_3]^{4+}$ cages, relies on solid-phase synthesis of the functionalized ligand L and subsequent *in situ* CDSA. To ensure mild MCg cleavage from the solid support, an established photochemical method is applied wherein a photolabile linker ((*S*)-3-amino-3-(2-nitrophenyl)propanoic acid) is installed on a

photostable resin (TentaGel™ S-NH₂) ahead of ligand synthesis (similar to the PAM approach of heteroleptic Pt(II)-MCgs).^[274] After cage assembly, introducing a second functionalized ligand L', and washing, the MCg can then be cleaved off the resin upon UV irradiation ($\lambda = 365$ nm) in a simple photoreactor setup (Figure 30). While solid-phase cage assembly and cleavage still need to be assessed, this approach could be an interesting tool for the design of peptide-based heteroleptic Pd(II)-MCgs.

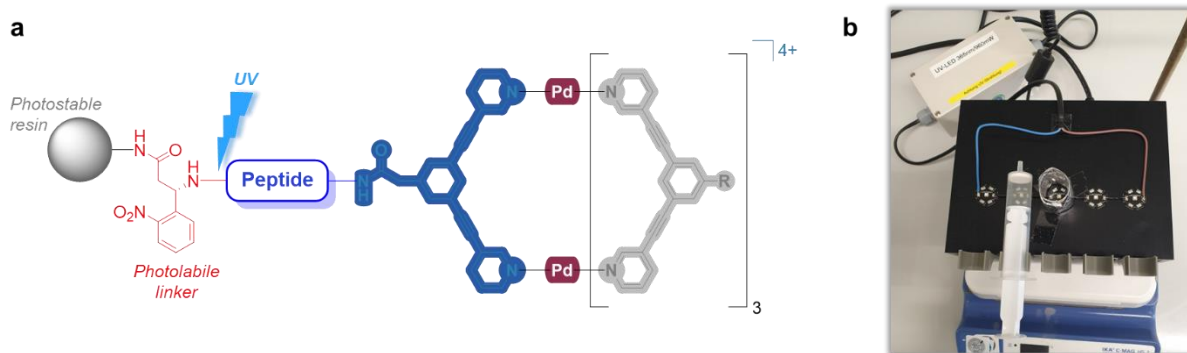


Figure 30: a) Schematic illustration of a $[\text{Pd}_2\text{L}(\text{L}')_3]^{4+}$ MCg formed by solid-phase ligand synthesis and *in situ* CDSA. Incorporation of a photolabile linker allows for mild resin cleavage upon UV irradiation. R = moiety bearing a fluorophore, chelator, peptide etc. b) Photoreactor setup suitable for photocleavage in peptide synthesis syringes consisting of 5 LED lamps (365 nm, 960 mW), a cooling body (black) and syringe holders. The reactor was developed together with the TUM chemistry central electronics shop (ZBE).

Finally, the applied chick CAM model is a complementary preclinical tool bridging the gap between *in vitro* and *in vivo* studies, which allows growing tumours on a highly vascularized membrane. Being cost-, time- and space-effective as well as in line with the 3R principles, it has gained attention for high-throughput screening of drug candidates. To further validate its applicability, the sstR2-addressing radiohybrid tracer $[\text{}^{18}\text{F}][\text{}^{\text{nat}}\text{Ga}]\text{Ga}(\text{SiFA})\text{SeFe-rhTATE2}$ was investigated in the *in ovo* setup. Clear accumulation in AR42J tumours and negligible uptake in sstR2-negative U87 controls was observed, confirming tracer specificity.

To conclude, 3D SCCs offer a promising platform for biomedical applications in imaging, therapy and drug delivery, enabled by their versatile, modular assembly including the heteroleptic approach, their porous nature and their limitless functionalization. This work highlights both the challenges and opportunities of $[\text{Pd}_2\text{L}_4]^{4+}$ MCgs for radiopharmaceutical development including preclinical studies in the chick CAM model, which was found suitable for screening of specific tumour uptake and provides a future outlook on the design of functionalized $[\text{Pt}_2\text{L}_4]^{4+}$ cages.

5 Materials and Methods

5.1 General Methods

Chemicals and Radionuclides

All reagents and solvents were obtained from commercial suppliers and used without further purification. The synthesis of SiFA building blocks followed previously reported procedures, and product purity (> 95%) was confirmed by RP-HPLC.^[163,171]

[¹⁸F]fluoride in [¹⁸O]H₂O was provided by *Klinikum rechts der Isar* (Munich, Germany) and *Guy's and St Thomas' Hospital* (London, UK). The ⁹⁹Mo/^{99m}Tc generator was delivered by *Klinikum rechts der Isar* (Munich, Germany).

NMR spectroscopy

¹H NMR and ¹H-DOSY NMR spectra were collected on a Bruker Avance III HD 400 MHz instrument (*Bruker Cooperations*, Billerica, USA). Chemical shifts (δ) are reported in parts per million (ppm) relative to the residual solvent peak and coupling constants (*J*) are given in Hertz (Hz). Multiplicities are denoted as singlet (s), doublet (d), doublet of doublets (dd), triplet (t), doublet of triplets (dt), doublet of quartets (dq) and multiplet (m). Hydrodynamic radii were calculated using the SEGWE calculator by *Manchester NMR Methodology Group*.

Chromatographic methods

Flash column chromatography of ligand **L0** was carried out on a Biotage® Isolera One purification system (*Biotage*, Uppsala, Sweden) employing Sfär Silica D columns (*Biotage*, Uppsala, Sweden). The separation was performed using a gradient of *n*-hexane/EtOAc (100:0 → 0:100) over 30 column volumes, followed by a second gradient of EtOAc/MeOH (100:0 → 90:10) over 10 column volumes.

Analytical and semi-preparative RP-HPLC analyses were performed on gradient systems from *Shimadzu Corp.* (Kyoto, Japan) equipped with an SPD-20A dual-wavelength UV/Vis detector (λ = 220 nm, 254 nm), two LC-20AD pumps, a CBM-20A communication module and a CTO-20A column oven. Analytical separations were carried out at 1 mL/min while semi-preparative runs were performed at 10 mL/min flow rate. Ligand quality control and cage formation analyses were conducted using a MultoKrom® 100-5 C18 column (125×4.6 mm, 5 μm; *Chromatographie Service GmbH*, Langerwehe, Germany). Analytical HPLC employed gradients of H₂O (0.1% TFA) (A) and MeCN (2% H₂O, 0.1% TFA) (B) while semi-preparative purification used H₂O (0.1% TFA) (A) and MeCN (5% H₂O, 0.1% TFA) (B).

Radiolabelled products were analysed on a *Shimadzu* gradient HPLC system equipped with an SPD-20A UV/Vis detector (λ = 220 nm, 254 nm), two LC-20AD pumps, a CBM-20A unit, a HERM LB500 NaI scintillation detector and a FlowStar² LB514 radio detector (*Berthold*

Technologies GmbH, Bad Wildbad, Germany). Radiolabelled ligands were loaded on a MultoKrom® 100-5 C18 column (125×4.6 mm, 5 µm) at 1 mL/min while radiolabelled cages were analysed on a MultoHigh® Bio 300-5 C4 column (150×4.6 mm, 5 µm) at the same flow rate.

Radio TLC was performed using a ScanRAM detector (*LabLogic*, Sheffield, UK) and analysed with Laura software. ¹⁸F-labelled compounds were analysed on silica gel coated aluminium plates F254 in MeCN/H₂O (8/2, 10% NaOAc (2 M), 1% TFA) while ^{99m}Tc-labelled compounds were analysed on Whatman™ 1 CHR chromatography (*cytiva*) in either MEK or saline as the mobile phases.

Mass spectrometry

HR-ESI-MS of ligands and cages was performed on an Orbitrap Q-Exactive Plus mass spectrometer (*Thermo Fisher Scientific*) using both direct-infusion and liquid chromatography methods.

For LC-MS, the mass spectrometer was coupled to a Dionex Ultimate 3000 UHPLC system (*Thermo Fisher Scientific*). For analysis of homoleptic cages **C1_{hom}** and **C2_{hom}** (10 µL injection volume), a SUPELCO BIOshell A400 Protein C4 column (150×1 mm, 3.4 µm, 400 Å; *Sigma Aldrich*) was used with mobile phases A: H₂O + 0.1% FA and B: MeCN + 0.1% FA. The flow rate was 0.05 mL/min with gradients from 20-40% B over 3 min followed by 40-80% B over 15 min. For analysis of heteroleptic cages **C1_{het}** and **C2_{het}**, an Acclaim™ 120 C18 column (100×4.6 mm, 5 µm, 120 Å; *Thermo Fisher Scientific*) was employed under the same gradient conditions at a flow rate of 0.5 mL/min. Electrospray ionization was carried out in positive mode with a capillary voltage of 3.50 kV. The capillary temperature was 320 °C, with sheath gas, auxiliary gas and sweep gas set at 20, 12 and 1 (arbitrary units), respectively, and auxiliary gas temperature at 100 °C.

For DI-MS, samples were infused at 0.01 mL/min with ionization in positive mode and 3.50 kV capillary voltage. The capillary temperature was set to 300 °C, with sheath/auxiliary/sweep gases at 20, 12 and 0 (arbitrary units), respectively, and auxiliary gas temperature at 50 °C.

5.2 Software and AI-assisting tools

Mass spectra were processed via the FreeStyle software provided by *Thermo Fisher Scientific*. Crystal structures were visualized using the *CCDC Mercury* software. Chemical structures were generated in ChemDraw. OriginLab Data Analysis software and Excel were used as plotting tools. Figures were composed with PowerPoint and BioRender.

In accordance with ethical recommendations on the use of AI tools, ChatGPT was applied to refine already written paragraphs of this thesis in order to improve clarity and readability of the content.^[293]

5.3 Ligand synthesis

Synthesis of BBB-shuttle peptide ***d***PepH3

dPepH3 (AGILKRW) was synthesized using microwave-assisted SPPS on a Liberty Blue™ synthesizer (CEM, Matthews, USA). The peptide was assembled on ProTide Rink Amide resin (loading capacity: 0.65 mmol/g) with Fmoc-protected *D*-amino acids. The final Fmoc group was not removed and the peptide was stored on the resin for subsequent use.

Synthesis of 2-(3,5-bis(pyridine-3-ylethynyl)phenyl)acetic acid (**L0**)

Ligand **L0** was prepared according to a literature-reported protocol in a three-step synthesis consisting of a protection, a Sonogashira coupling and a deprotection.^[251]

3,5-dibromobenzoic acid (1.15 g, 3.91 mmol, 1.0 eq.) was dissolved in acetone (40 mL). After addition of benzyl bromide (0.54 mL, 769 mg, 4.50 mmol, 1.2 eq.) and K₂CO₃ (811 mg, 5.87 mmol, 1.5 eq.) the suspension was heated to 56 °C for 3 h and then cooled to RT. The reaction mixture was diluted with ethyl acetate (100 mL) and washed with water (3×80 mL) and brine (1×80 mL). The organic phase was dried over Mg₂SO₄, filtered and the solvents were removed under reduced pressure. The protected intermediate benzyl 3,5-dibromobenzoate was obtained quantitatively in form of yellow oil and then mixed with 3-ethynylpyridine (1.208 g, 11.73 mmol, 3.0 eq.) and triethylamine (80 mL) in a flame dried Schlenk flask under N₂-atmosphere for subsequent Sonogashira coupling. After addition of Pd(PPh₃)₂Cl₂ (274 mg, 0.391 mmol, 0.1 eq.) and CuI (74 mg, 0.391 mmol, 0.1 eq.) the reaction mixture was stirred at 90 °C for 24 h. The suspension was cooled to RT, diluted in ethyl acetate (120 mL) and filtered. The solvent was removed from the filtrate under reduced pressure, and the remaining brown oil was purified by flash column chromatography to yield benzyl 3,5-bis(pyridin-3-ylethynyl)benzoate as an orange crystalline solid. This was then dissolved in acetonitrile (50 mL) and a solution of NaOH (391 mg, 9.78 mmol, 2.5 eq.) in water (20 mL) was added dropwise to the first solution for benzyl deprotection. The reaction mixture was stirred at reflux for 4.5 h. After cooling down, the solution was concentrated to 15 mL and the product was precipitated by addition of HCl_(aq) (6 M). The precipitate was filtered and washed twice with cold ethyl acetate and diethyl ether. The precipitation was repeated with the filtrate and the combined precipitates were dried under reduced pressure, affording **L0** as a white solid in 72% overall yield and 97% purity.

RP-HPLC (10-90% B in 15 min): *t_R* = 6.2 min.

¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) = 8.79 (d, *J* = 2.3 Hz, 2 H, H_a), 8.61 (dd, *J* = 4.8, 1.8 Hz, 2 H, H_b), 8.01 (dt, *J* = 7.9, 2.0 Hz, 2 H, H_d), 7.70 (s, 1 H, H_e), 7.57 (d, *J* = 1.7 Hz, 2 H, H_f), 7.49 (dd, *J* = 7.9, 4.8 Hz, 2 H, H_c), 3.66 (s, 2 H, H_g).

MS (ESI positive): calculated monoisotopic mass for $C_{22}H_{14}N_2O_2$: 338.11; found by ESI-MS: $m/z = 339.11 [M+H]^+$.

Synthesis of δ PepH3- δ Dap((SiFA)BA)-Ebes-L0 (L1)

Fmoc deprotection: Resin-bound, N-terminally Fmoc-protected δ PepH3 (60.0 μ mol, 1.0 eq.) was treated with 20% piperidine in DMF (5 mL) at RT for two cycles (15 min + 5 min). The resin was washed with DMF (6 $\times$ 5 mL).

Fmoc- δ Dap(Dde)-OH (*N*-alpha-(9-fluorenylmethyloxycarbonyl)-*N*-beta-[(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl]-*D*-2,3-diaminopropionic acid) (90.0 μ mol, 1.5 eq.), TBTU (*N,N,N',N'*-tetramethyluronium-tetrafluoroborate) (90.0 μ mol, 1.5 eq.), HOAt (1-hydroxy-7-azabenzotriazole) (90.0 μ mol, 1.5 eq.) and 2,4,6-trimethylpyridine (300 μ mol, 5.0 eq.) were dissolved in DMF (5 mL). After 2 min pre-incubation, the solution was added to the resin and shaken for 2 h at RT. The resin was washed with DMF (6 $\times$ 5 mL) and *N*-methyl-2-pyrrolidone (NMP, 6 $\times$ 5 mL). Four coupling in branched position, the Dde-group was removed by treating with $NH_2OH \cdot HCl$ (6.0 mmol, 100 eq.) and imidazole (4.5 mmol, 75 eq.) in NMP/DCM (5/2, 7 mL) for 2 h at RT. The resin was then washed with NMP (6 $\times$ 5 mL) and DMF (6 $\times$ 5 mL).

Amide coupling: Fmoc-(SiFA)BA (72.0 μ mol, 1.2 eq.), TBTU (90.0 μ mol, 1.5 eq.) and HOAt (90.0 μ mol, 1.5 eq.) were dissolved in DMF (5 mL), followed by *N,N*-diisopropylethylamine (210 μ mol, 3.5 eq.). After pre-incubation for 30 min, the mixture was added to the deprotected resin and shaken for 2.5 h at RT. The resin was subsequently washed with DMF (6 $\times$ 5 mL).

Fmoc-Ebes (*N*-[8-(9-fluorenylmethyloxycarbonyl)amino-3,6-dioxaoctyl]succinamic acid) (90.0 μ mol, 1.5 eq.) and **L0** (90.0 μ mol, 1.5 eq.) were sequentially coupled to the resin-bound intermediate. Each coupling step was preceded by *Fmoc-deprotection* followed by *amide coupling*.

Resin cleavage and purification: The final ligand was released from the resin by treatment with TFA/triisopropyl silane/ H_2O (95/2.5/2.5, 2 $\times$ 5 mL) for 2 $\times$ 1 h at RT. The combined fractions were stirred for 1 h and concentrated under an N_2 stream. Additional treatment with TFA (5 mL, 2 h at RT) was performed followed by solvent removal under N_2 . The crude product was purified by semi-preparative RP-HPLC and lyophilized, yielding **L1** as a white solid (11.5 mg, 11% yield, 98% purity).

RP-HPLC (10-90% B in 15 min): $t_R = 9.7$ min.

1H NMR (400 MHz, $DMSO-d_6$) δ (ppm) = 10.78 (s), 8.78 (d, $J = 3.1$ Hz, 2H), 8.61 (d, $J = 6.5$ Hz, 2H), 8.49 (d, $J = 4.4$ Hz), 8.26 (d, $J = 6.6$ Hz), 8.21 (d, $J = 5.9$ Hz), 8.14 – 8.08 (m), 8.04 – 7.97 (m), 7.98 – 7.92 (m), 7.89 – 7.79 (m), 7.68 (s), 7.67 – 7.61 (m), 7.61 – 7.53 (m), 7.52 – 7.43 (m), 7.39 (s), 7.32 (d, $J = 7.1$ Hz), 7.11 (d, $J = 2.6$ Hz), 7.08 – 7.01 (m), 7.00 – 6.92 (m), 4.43 (dq, $J = 13.6, 6.7$ Hz), 4.33 – 3.92 (m), 3.81 – 3.58 (m), 3.53 – 3.46

(m), 3.43 (t, $J = 5.8$ Hz), 3.36 (t, $J = 5.9$ Hz), 3.20 (dt, $J = 30.4, 5.7$ Hz), 3.12 – 2.92 (m), 2.72 (d, $J = 7.5$ Hz), 2.36 (s), 1.85 – 1.36 (m), 1.25 (d, $J = 7.4$ Hz), 1.11 (s), 1.01 (d, $J = 1.1$ Hz), 0.85 (d, $J = 6.4$ Hz), 0.83 – 0.73 (m).

MS (ESI positive): calculated monoisotopic mass for $C_{90}H_{124}FN_{19}O_{14}Si$: 1741.93; found by ESI-MS: $m/z = 582 [M+3H]^{3+}$, 872 $[M+2H]^{2+}$.

Synthesis of δ PepH3-(SiFA)SeFe-Ebes-L0 (L2)

Fmoc-(SiFA)SeFe (72.0 μ mol, 1.2 eq.), Fmoc-Ebes (90.0 μ mol, 1.5 eq.) and L0 (90.0 μ mol, 1.5 eq.) were sequentially coupled to the resin-bound peptide. Each step involved standard *Fmoc-deprotection* followed by *amide coupling*, as outlined above. Afterwards, *resin cleavage and purification* were performed, affording **L2** as a white solid (17.6 mg, 17% yield, 98% purity).

RP-HPLC (10-90% B in 15 min): $t_R = 9.5$ min.

1H NMR (400 MHz, DMSO- d_6) δ (ppm) = 10.79 (s), 8.79 (s, 2H), 8.73 (d, $J = 6.5$ Hz), 8.61 (dd, $J = 4.9, 1.7$ Hz, 2H), 8.52 – 8.43 (m), 8.39 – 8.30 (m), 8.28 – 8.17 (m), 8.00 (dt, $J = 7.9, 1.8$ Hz), 7.98 – 7.78 (m), 7.75 – 7.62 (m), 7.61 – 7.53 (m), 7.53 – 7.45 (m), 7.39 (s), 7.32 (d, $J = 7.9$ Hz), 7.12 (d, $J = 2.4$ Hz), 7.09 – 7.01 (m), 7.00 – 6.92 (m), 4.44 (dt, $J = 19.4, 6.9$ Hz), 4.33 (d, $J = 5.9$ Hz), 4.30 – 3.93 (m), 3.74 (d, $J = 5.8$ Hz), 3.50 (dd, $J = 4.3, 2.6$ Hz), 3.43 (t, $J = 5.7$ Hz), 3.37 (t, $J = 5.9$ Hz), 3.29 – 3.14 (m), 3.14 – 2.94 (m), 2.73 (d, $J = 7.5$ Hz), 2.46 – 2.29 (m), 2.24 (d, $J = 0.6$ Hz), 2.08 (s), 1.97 – 1.90 (m), 1.71 – 1.58 (m), 1.56 – 1.42 (m), 1.37 (d, $J = 7.1$ Hz), 1.23 (s), 1.11 (s), 1.03 – 0.97 (m), 0.88 – 0.70 (m).

MS (ESI positive): calculated monoisotopic mass for $C_{88}H_{121}FN_{18}O_{13}Si$: 1684.91; found by ESI-MS: $m/z = 562 [M+3H]^{3+}$, 844 $[M+2H]^{2+}$, 1686 $[M+H]^+$.

5.4 CDSA and stability of MCgs

CDSA of metallacages for RP-HPLC analysis

For the preparation of homoleptic MCgs, ligand **L1** or **L2** (2.0 eq., 5 mM in DMSO) was combined with $[Pd(MeCN)_4](BF_4)_2$ (1.0 eq., 20 mM in DMSO) and DMSO was added to reach a theoretical cage concentration of 1 mM. Heteroleptic cage assemblies were obtained by mixing either 1.0 eq. of **L1** (5 mM in DMSO) with 3.0 eq. of **L0** (5 mM in DMSO) for **C1_{het}**, or 2.7 eq. of **L2** (5 mM in DMSO) with 1.3 eq. of **L0** (5 mM in DMSO) for **C2_{het}**, in the presence of 2.0 eq. of $[Pd(MeCN)_4](BF_4)_2$ (20 mM in DMSO). DMSO was added to adjust the final theoretical cage concentration to 1 mM.

All reactions proceeded to completion within 1 h at RT and were analysed by analytical RP-HPLC. For homoleptic assemblies, separation was performed using a gradient of 40-70% B over 15 min. In contrast, heteroleptic assemblies yielded mixtures of coordination species with

varying ligand ratios. Therefore, two alternative separation gradients were tested: (i) 30-60% B over 15 min at a flow rate of 1 mL/min with 0.1% TFA, and (ii) 20-40% B over 3 min, followed by 40-80% B over 15 min at a flow rate of 0.5 mL/min with 0.1% FA as additive.

[Pd₂(L1)₄](BF₄)₄ (C1_{hom})

RP-HPLC (40-70% B in 15 min): *t_R* = 8.9 min. Yield 88.7%.

[Pd₂(L2)₄](BF₄)₄ (C2_{hom})

RP-HPLC (40-70% B in 15 min): *t_R* = 7.3 min. Yield 82.1%

C1_{het} (mixture)

RP-HPLC (30-60% B in 15 min): *t_R* = 3.2 (L0), 6.6 ([Pd₂(L0)₂]), 8.3 (C0_{hom}), 9.4, 11.1 (C1_{het}), 11.5 (L1), 12.7 min ([Pd₂(L1)₂(L0)₂]).

RP-HPLC (20-40% B in 3 min, 40-80% B in 15 min): *t_R* = 6.7 (C1_{het}), 7.5, 9.9 (L1), 11.4, 13.1, 15.4 min.

C2_{het} (mixture)

RP-HPLC (30-60% B in 15 min): *t_R* = 10.2, 10.5 (C2_{het}), 11.5 ([Pd₂(L2)₂(L0)₂]), 12.1, 12.6, 13.0 min.

RP-HPLC (20-40% B in 3 min, 40-80% B in 15 min): *t_R* = 6.8 (C2_{het}), 7.2, 9.2 min (L2).

Stability studies of homoleptic MCgs

For stability assessment, **C1_{hom}** and **C2_{hom}** (20 µL, 1 mM) were diluted with 180 µL of various solvents – DMSO, H₂O, MeCN, MeCN/H₂O (1/1, 0.1% TFA), PBS (pH 7.4) or saline solution (0.9 wt% NaCl) – to obtain a final cage concentration of 0.1 mM. Samples were incubated for different time intervals (0 min, 30 min, 60 min, 4 h and 24 h) at RT and stability was analysed by RP-HPLC.

CDSA of metallocages for NMR analysis

NMR samples were prepared by dissolving ~ 7 mg of ligand **L1/L2** (2.0 eq.) in 400 µL DMSO-d₆, followed by recording ¹H and ¹H-DOSY NMR spectra. To this solution, [Pd(MeCN)₄](BF₄)₂ (1.0 eq.) dissolved in 25 µL DMSO-d₆ was added. After 1 h at RT, ¹H and ¹H-DOSY NMR spectra were measured to confirm homoleptic cage formation.

For monitoring CDSA of heteroleptic cages, ~ 2 mg of **L1** (1.0 eq.) with 3.0 eq. of **L0** for **C1_{het}**, or ~ 6 mg of **L2** (2.7 eq.) with 1.3 eq. of **L0** for **C2_{het}**, were dissolved in 400 µL DMSO-d₆. ¹H and ¹H-DOSY NMR spectra were acquired before addition of [Pd(MeCN)₄](BF₄)₂ (2.0 eq. in 25 µL DMSO-d₆). After 1 h at RT, spectra of the heteroleptic cages were collected. It has to be noted that the reaction produced a mixture of supramolecular assemblies, which is why peak assignment was omitted.

[Pd₂(L1)₄](BF₄)₄ (C1_{hom})

¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) = 10.78 (s), 9.77 (s, 2H), 9.41 (s, 2H), 8.52 – 8.44 (m), 8.27 – 8.17 (m), 8.13 – 8.04 (m), 8.04 – 7.91 (m), 7.90 – 7.75 (m), 7.73 – 7.60 (m), 7.58 (d, *J* = 4.8 Hz), 7.47 (t, *J* = 5.4 Hz), 7.40 (s), 7.32 (d, *J* = 8.1 Hz), 7.11 (s), 7.09 – 7.01 (m), 7.00 – 6.92 (m), 4.50 – 4.37 (m), 4.33 – 3.84 (m), 3.46 (s), 3.42 – 3.28 (m), 3.19 – 2.92 (m), 2.75 – 2.69 (m), 2.34 (s), 2.07 (s), 1.75 (s), 1.46 (dt, *J* = 20.2, 7.5 Hz), 1.25 (t, *J* = 7.1 Hz), 1.11 (s), 1.06 – 0.93 (m), 0.88 – 0.72 (m).

[Pd₂(L2)₄](BF₄)₄ (C2_{hom})

¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) = 10.80 (s), 9.78 (s, 2H), 9.43 (s, 2H), 8.72 (d, *J* = 6.2 Hz), 8.48 (t, *J* = 5.8 Hz), 8.33 (d, *J* = 5.6 Hz), 8.24 (d, *J* = 7.8 Hz), 8.06 – 7.77 (m), 7.68 (d, *J* = 5.6 Hz), 7.58 (d, *J* = 7.6 Hz), 7.51 (d, *J* = 5.4 Hz), 7.42 (s), 7.32 (d, *J* = 8.1 Hz), 7.11 (d, *J* = 2.3 Hz), 7.10 – 7.01 (m), 7.01 – 6.92 (m), 4.51 – 4.38 (m), 4.32 (d, *J* = 5.7 Hz), 4.29 – 4.11 (m), 3.76 (s), 3.46 (s), 3.41 – 3.29 (m), 3.24 – 2.92 (m), 2.72 (d, *J* = 7.2 Hz), 2.42 – 2.27 (m), 2.24 (s), 2.08 (s), 1.92 (s), 1.75 (s), 1.71 – 1.56 (m), 1.57 – 1.41 (m), 1.36 (d, *J* = 7.0 Hz), 1.21 (d, *J* = 3.0 Hz), 1.11 (s), 0.99 (s), 0.88 – 0.70 (m).

CDSA of metallacages for MS analysis

Stock solutions (20 mM in DMSO) of **L0**, **L1**, **L2** and [Pd(MeCN)₄](BF₄)₂ were prepared prior to assembly. Homoleptic cages **C1_{hom}** and **C2_{hom}** were obtained by mixing ligands **L1** or **L2** with the Pd(II) precursor in a 2:1 ratio in DMSO, yielding the respective coordination complexes after 1 h at RT. For **C1_{het}**, stock solutions of **L1** and **L0** were combined in a 1:3 ratio (final concentrations 3 and 9 mM, respectively) in DMSO. For **C2_{het}**, **L2** and **L0** were mixed in a 1:2 ratio (final concentrations 4 and 8 mM, respectively). In both cases, the ligand mixtures were stirred with 0.5 eq. (6 mM) of [Pd(MeCN)₄](BF₄)₂ for 1 h at RT to reach a theoretical concentration of 3 mM. The resulting solutions of homoleptic and heteroleptic cages were diluted with H₂O containing 5% (v/v) MeCN and 0.1% (v/v) FA to a final cage concentration of 2 μ M and subsequently analysed by DI- and LC-HR-ESI-MS. Assigned mass peaks are listed in SI Table 1 in the Supplementary Information.

5.5 Encapsulation studies**Encapsulation of cisplatin**

For analysis of cisplatin encapsulation by ¹H NMR spectroscopy, a **C2_{hom}** solution (1.0 eq.) was prepared in DMF-*d*₇ as explained previously. 2.0 eq. of cisplatin (in 25 μ L DMF-*d*₇) was added and after 30 min at RT ¹H NMR spectra were measured. No significant shift differences compared to the cage spectra could be observed.

Encapsulation of perrhenate

Encapsulation of ReO_4^- was analysed by ^1H NMR spectroscopy and HR-ESI-MS.

NMR encapsulation studies of homoleptic MCgs **C1_{hom}** and **C2_{hom}** was carried out in $\text{DMSO-d}_6/\text{D}_2\text{O}$ (1/1). To the cage solutions (1.0 eq.), prepared as described previously, was added NaReO_4 (2.0 eq. in 25 μL $\text{DMSO-d}_6/\text{D}_2\text{O}$ (1/1)) and after 30 min at RT ^1H NMR spectra were recorded. No significant shift differences compared to the cage spectra could be observed.

For MS analysis of the ReO_4^- encapsulation, homoleptic cage **C2_{hom}** as well as heteroleptic cages **C1_{het}** and **C2_{het}** (3 mM in DMSO each) were diluted with Milli-Q water to reach a percentage of 67% H_2O . A freshly prepared stock solution of NaReO_4 (10 mM in Milli-Q water) was added to each cage solution to obtain a cage-to- NaReO_4 ratio of 1:2 (0.9 mM:1.8 mM) in 72% H_2O . Following 30 min incubation at RT, the mixtures were further diluted to a theoretical cage concentration of 4 μM in H_2O containing 5% (v/v) MeCN and 0.1% (v/v) FA, and subsequently analysed by DI- or LC-HR-ESI-MS. Assigned mass peaks are listed in SI Table 1 in the Supplementary Information.

Encapsulation of [$^{99\text{m}}\text{Tc}$]pertechnetate

Encapsulation of [$^{99\text{m}}\text{Tc}$] TcO_4^- into **C2_{hom}** was carried out analogous to a previously reported procedure.^[128] [$^{99\text{m}}\text{Tc}$] TcO_4^- was eluted from a $^{99}\text{Mo}/^{99\text{m}}\text{Tc}$ -generator in saline and concentrated to reach an activity concentration of 0.8 MBq/ μL . For the one-pot encapsulation, 100 μL (80 MBq) of the [$^{99\text{m}}\text{Tc}$]pertechnetate solution was mixed with 10 μL of $[\text{Pd}(\text{MeCN})_4](\text{BF}_4)_2$ (2.0 eq., 20 mM, DMSO) and added to 80 μL of **L2** (4.0 eq., 5 mM, DMSO). For the post-assembly encapsulation, 100 μL (80 MBq) of the [$^{99\text{m}}\text{Tc}$]pertechnetate solution was mixed with 100 μL of **C2_{hom}** (1 mM, DMSO). After 1 h at 37 °C white precipitates formed in both reaction mixtures due to instability of the cage, which were analysed by radio TLC and, after centrifugation, by radio RP-HPLC.

5.6 ^{18}F -radiolabelling of ligands and MCgs

^{18}F -radiolabelled ligands

Radiolabelling of the SiFA moiety was performed via an IE reaction analogous to a literature-known procedure.^[256] Approximately 800 MBq of [^{18}F]fluoride in [^{18}O] H_2O was loaded onto a SEP-Pak® Light (46 mg) Accell™ Plus QMA cartridge from Waters (Milford, USA) (preconditioned with 10 mL Ultrapure water) and subsequently dried with 8 mL anhydrous DMSO. The trapped [^{18}F]fluoride was eluted into a LoBind Eppendorf tube using 40 mg NH_4HCO_2 dissolved in 500 μL anhydrous DMSO. An aliquot of the eluate containing ~ 250 MBq [^{18}F]fluoride was then added to 15 nmol of ligand (1 mM in DMSO), and the labelling reaction was allowed to proceed for 10 min at RT. The reaction was quenched with

10 mL Ultrapure water, after which the labelled ligand was retained on a *Waters Oasis®* HLB (30 mg) Light cartridge (preconditioned with 10 mL EtOH and 10 mL Ultrapure water). The cartridge was washed with 10 mL Ultrapure water, and the product was eluted with 300 μ L EtOH. Radiochemical analysis was carried out by radio RP-HPLC and radio TLC.

^{18}F -*D*PepH3-*D*Dap((SiFA)BA)-Ebes-L0 (^{18}F -L1)

RCY: 44%, RCP: 98%.

Radio RP-HPLC (10-80% B in 15 min): t_R = 12.4 min.

^{18}F -*D*PepH3-(SiFA)SeFe-Ebes-L0 (^{18}F -L2)

RCY: 47%, RCP: 99%.

Radio RP-HPLC (30-60% B in 15 min): t_R = 10.4 min.

Self-assembly of ^{18}F -labelled cages

For synthesis of homoleptic radiolabelled MCgs ^{18}F -**C1**_{hom} and ^{18}F -**C2**_{hom}, 30 nmol of **L1** or **L2** (1.0 eq., 5 mM in DMSO) were radiofluorinated as described above. The obtained solutions of ^{18}F -**L1** and ^{18}F -**L2** were evaporated at 80 °C under a gentle N₂ stream. Subsequently, 3 μ L of [Pd(MeCN)₄](BF₄)₂ (2.0 eq., 20 mM in DMSO) and 3 μ L of DMSO were added to the dried labelled ligands. The CDSA proceeded for 4 h, after which completion was confirmed by radio RP-HPLC.

The synthesis of heteroleptic radiolabelled cages was performed similarly. For ^{18}F -**C1**_{het}, 15 nmol of **L1** (1.0 eq., 5 mM in DMSO) were radiofluorinated and the elution solvent was removed. To the dried labelled ligand ^{18}F -**L1** was added 9 μ L of **L0** (3.0 eq., 5 mM in DMSO) and 6 μ L of [Pd(MeCN)₄](BF₄)₂ (8.0 eq., 20 mM in DMSO). For ^{18}F -**C2**_{het}, 20 nmol of **L2** (2.7 eq., 5 mM in DMSO) were radiofluorinated and after solvent removal, 2 μ L of **L0** (1.3 eq., 5 mM in DMSO), 1 μ L of [Pd(MeCN)₄](BF₄)₂ (8.0 eq., 20 mM in DMSO) and 3 μ L of DMSO were added. In both cases, CDSA resulted in a mixture of supramolecular species that could not be identified.

^{18}F -[Pd₂(L1**)₄](BF₄)₄ (^{18}F -**C1**_{hom})**

Radio RP-HPLC (40-70% B in 15 min): t_R = 9.6 min.

^{18}F -[Pd₂(L2**)₄](BF₄)₄ (^{18}F -**C2**_{hom})**

Radio RP-HPLC (40-70% B in 15 min): t_R = 7.7 min.

5.7 Evaluation of biological parameters

Stability of the Si- ^{18}F bond

To simulate physiological conditions, 90 μ L of 3 mM aqueous K₂CO₃ buffer (pH 7.4) was placed in a LoBind Eppendorf tube, followed by the addition of 10 μ L of radiolabelled ligand

^{18}F -L1/ ^{18}F -L2. The mixture was incubated at 37 °C and 10 μL aliquots were withdrawn at 0, 30, 60, 90 and 120 min for analysis of intactness of the Si- ^{18}F bond by radio TLC. Three replicates were performed for each ligand.

Lipophilicity determination ($\text{Log}D_{\text{pH}7.4}$)

The octanol-PBS partition coefficients ($\text{Log}D_{\text{pH}7.4}$) of ^{18}F -L1 and ^{18}F -L2 were determined by adding 0.5 MBq of the respective radiolabelled ligand to 1.5 mL LoBind Eppendorf tubes containing 500 μL of octanol and 500 μL of PBS ($n=8$). Samples were vortexed for 3 min at RT and centrifuged at 9000 rpm for 5 min at RT using a Heraeus Pico 17 centrifuge (*Thermo Scientific*, Waltham, USA; 24 $\times$ 1,5/2,0 mL rotor with ClickSeal lid). From each phase, 200 μL were collected and the radioactivity was measured separately using a 2480 Wizard γ -counter (*PerkinElmer Inc.*, Langerwehe, Germany). Due to instability during vortexing, the lipophilicity of the corresponding cages could not be determined.

$$\text{Log}D_{\text{pH}7.4} (^{18}\text{F}\text{-L1}) = 2.53 \pm 0.05.$$

$$\text{Log}D_{\text{pH}7.4} (^{18}\text{F}\text{-L2}) = 2.07 \pm 0.07.$$

Binding to human serum albumin

HSA binding was assessed by two different methods: HPAC-HSA and HSA ultrafiltration. Chromatographic HSA binding assays were performed using a CHIRALPAK immobilized HSA column (50 $\times$ 3.0 mm, 5 μm ; *Daicel Chiral Technologies*, Illkirch, France) at a constant flow rate of 0.5 mL/min. The mobile phases consisted of 50 mM NH_4OAc buffer (pH6.6) (A) and isopropanol (B). Elution was carried out with a linear gradient of 0-20% B over 3 min, followed by isocratic elution at 20% B for 57 min. The column oven was maintained at 25 °C throughout the assay. Prior to analysis, 20 μL aliquots (1 mM in DMSO) of ligands L1 and L2, homoleptic cages C1_{hom} and C2_{hom} and heteroleptic cages C1_{het} and C2_{het} were purified by analytical RP-HPLC, with the entire peak collected, lyophilized and resuspended in 20 μL A/B (1/1). To quantify the HSA binding based on the determined retention times of the compounds, reference samples with known HSA binding affinities (benzyl alcohol, aniline, phenol, benzoic acid, carbamazepine, 4-nitrophenol, estradiol, probenecid, glibenclamide) were analysed, and a calibration curve was generated using a sigmoidal fit calculated in OriginLab Data Analysis software. The HSA binding affinity of the tested ligands and cages amounted to at least 98% as listed in Table 4.

Dynamic HSA binding determination was carried out by HSA ultrafiltration ($n=6$). Therefore, 225 μL of HSA solution (777.8 μM in PBS) was combined with 25 μL (0.5 MBq) of radiolabelled ligand ^{18}F -L1 and ^{18}F -L2, respectively, resulting in a final volume of 250 μL and an HSA concentration of 700 μM . Reference samples of ^{18}F -L1 and ^{18}F -L2 in PBS only were prepared alongside to determine unspecific binding. After mixing and centrifugation, samples were

incubated at 37 °C for 30 min. Subsequently, samples were transferred into Millipore Centrifree® ultrafiltration devices (30 kDa nominal molecular weight cutoff, methylcellulose micro-partition membranes; *Merck KGaA*) and subjected to ultracentrifugation at 3200 rpm for 40 min using a *Thermo Scientific* Heraeus Megafuge 16R with a BIOShield 720 rotor. Finally, the membranes and filtrates were measured separately in a 2480 Wizard γ -counter (*PerkinElmer Inc.*, Langerwehe, Germany).

Uncorrected HSA binding and unspecific binding B was calculated according to following equation with A being the determined activity in counts per minute:

$$B [\%] = \frac{A_{\text{membrane}}}{A_{\text{membrane}} + A_{\text{filtrate}}} \cdot 100$$

Specific HSA binding B_{spec} was determined by correcting the uncorrected value with the unspecific binding using the respective mean values and standard deviations:

$$B_{\text{spec}} [\%] = \frac{B_{\text{HSA}} - B_{\text{unspecific}}}{100 - B_{\text{unspecific}}} \cdot 100$$

Due to extremely high unspecific binding to the ultrafiltration vials of over 98%, the determined specific HSA binding values shown in Table 4 were deemed insignificant. Furthermore, instability toward centrifugation impeded the evaluation of the radiolabelled homoleptic cages.

5.8 Cell culture and sstR2 expression

Cell culture

AR42J cells (*Sigma-Aldrich*, Gillingham, UK) were maintained in RPMI medium (*Thermo Fisher*) supplemented with 10% foetal bovine serum (*Thermo Fisher*), 2.5 vol% L-glutamine solution (200 mM, *Sigma Life Sciences*) and 1 vol% MEM nonessential amino acids (v/v) at 37 °C in a humidified 5% CO₂ incubator. Cells were subcultured every 2-4 days at ~ 80% confluence. For passaging, the medium was removed, the cell lawn rinsed with PBS (6 mL, 37 °C) and cells detached with PBS-based Enzyme-Free Cell Dissociation Buffer (*Thermo Fisher*; 5 mL, 5 min, 37 °C). The resulting suspension was collected in RPMI medium (5 mL, same supplements), centrifuged (1300 rpm, 3 min, RT) and the cell pellet resuspended in fresh medium. A fraction (1/2-1/3) of the suspension was transferred to new culture flasks and the total volume adjusted to 25 mL with supplemented RPMI medium.

U87 cells (*ATCC*, Teddington, UK) were cultured in MEME (*Sigma Life Sciences*) supplemented with 10% fetal bovine serum (*Thermo Fisher*), 1 vol% L-glutamine (*Sigma Life Sciences*) and 1 vol% penicillin-streptomycin (*Sigma Aldrich*) at 37 °C in an incubator with humidified atmosphere (5% CO₂). Cells were passaged every 3-4 days at ~ 80% confluence. For subculturing, spent medium was discarded, the cell lawn rinsed with PBS (10 mL, 37 °C) and cells detached using trypsin/ethylenediaminetetraacetic acid (5 mL, 5 min, 37 °C). The detached cells were collected in MEME (20 mL, same supplements), centrifuged (1300 rpm,

3 min, RT) and the pellet resuspended in fresh MEME (6 mL). A portion (1/6) of the suspension was seeded into new flasks, with volume adjusted to 25 mL with supplemented MEME.

In all cases, cell density was routinely monitored by inverted microscopy and cell lines were tested Mycoplasma-free every month.

Cell lysates

To determine the sstR2 expression in the cell lines, AR42J cells (6.05×10^5 cells/mL, 10 mL) in RPMI medium (*Thermo Fisher*) and U87 cells (1.21×10^6 cells/mL, 10 mL) in MEME (*Sigma Life Sciences*) were seeded into 10 cm dishes and incubated at 37°C in a humidified 5% CO₂ atmosphere. During lysate preparation, both dishes and buffers were kept on ice. One day after seeding culture medium was removed. The cells were washed with PBS (3 × 5 mL, *Sigma Life Sciences*) and subsequently, 400 µL of PierceTM RIPA buffer (*Thermo Scientific*) supplemented with 4 µL of HaltTM Protease and Phosphatase Inhibitor Cocktail (100×) (*Thermo Scientific*) was added. The cells were thoroughly detached from the dish surface using a cell scraper, and the suspension transferred to an Eppendorf tube and centrifuged at 21130 × g at 4°C for 10 min (*eppendorf* Centrifuge 5424 R). The cell lysates were collected and stored at -80°C until further use. Three biological replicates were performed for each cell line.

Bicinchoninic Acid Assay

Protein concentrations in the cell lysates were quantified with a PierceTM BCA Protein Assay Kit (*Thermo Scientific*). After 30min incubation at 37°C (+5% CO₂), absorption was measured on a VarioskanTM LUX microplate reader (*Thermo Scientific*) and protein concentrations determined using a calibration curve. Duplicates were performed.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Samples were prepared with 15 µg protein (cell lysates), 10% NuPAGETM Sample Reducing Agent (10×) (*invitrogen*), 25% NuPAGETM LDS Sample Buffer (4×) (*invitrogen*) and filled up with water. Prior to loading, the samples were heated to 70°C for 10 min in a block heater SBH130D (*Stuart*). A Midi 12% CriterionTM TGX Stain-FreeTM Precast Gel (*Bio-Rad*) was placed in a CriterionTM Cell (*Bio-Rad*) and filled with ten-fold diluted 10× Tris/Glycine/SDS Buffer (*Bio-Rad*). The gel was loaded with 30 µL of each protein sample, 10 µL of 1/1 protein ladder mixture of SeeBlueTM Plus2 Pre-stained Protein Standard (*invitrogen*) and MagicMarkTM XP Western Protein Standard (*invitrogen*), and a blank to separate samples and ladder. The proteins were separated at 140 V for 1 h using a PowerPacTM Basic Power Supply (*Bio-Rad*).

Western Blot

The separated proteins on the SDS gels were transferred to a Western membrane using a Trans-Blot[®] TurboTM Transfer Pack (*Bio-Rad*) and a Trans-Blot[®] TurboTM Transfer System (*Bio-*

Rad). The loaded membrane was incubated at RT in an iBind™ Flex device (*invitrogen*) with 4 mL of primary rabbit sstR2 antibody [UMB1] (*abcam*, AB134152) and 4 mL of secondary anti-rabbit IgG HRP-linked antibody (*Cell Signaling Technology*, 7074) for 4 h. Both antibodies were diluted with iBind™ Solution Kit (*invitrogen*), and washes with 4 mL and 12 mL of iBind™ solution, respectively were carried out after each incubation step. A control was performed with alpha-Tubulin Rabbit monoclonal antibody (*Cell Signaling Technology*, 2125) as the primary antibody. Afterwards, the membrane was washed with TBST Buffer (5 × 5 min) and visualised on an iBright™ FL1000 (*invitrogen*) with Amersham ECL Western Blotting Detection Reagents (*GE Healthcare*).

5.9 CAM studies

All *in ovo* experiments were conducted using fertilized Dekalb white or brown eggs (*Henry Stewart & Co. Ltd*, UK). To comply with regulatory guidelines, experiments were performed prior to day E14, at which stage a Home Office license is not required.

***In ovo* tumour inoculations**

The procedure was adapted from the literature.^[267] Before use, eggs were stored for up to 14 days at 12-14 °C in a humidified wine cooler (*Haller*). For tumour engraftment onto the chick CAM, eggs were disinfected with *Brinsea* disinfectant (100×) and transferred to an incubator (*Brinsea*) maintained at 38.7 °C and 48% humidity. The first day of incubation at this temperature was considered embryonic day 0. Until E3, the incubator trays were gently tilted to loosen the CAM from the shell. On E3, eggs were removed for window cutting. After rolling the eggs to prevent the CAM from adhering to the shell, a hole was pierced at the wide base above the air cell, and approximately 5 mL of albumin was withdrawn using a 19G needle and syringe. The puncture was resealed with Scotch Magic tape. A square of tape was then applied to the shell surface, and four small holes were made through the tape in a rectangular arrangement. Using these as guides, a 1×2 cm rectangular window was cut with fine dissection scissors, avoiding damage to the inner membrane. The window was resealed with tape, and the eggs were returned to the incubator until E7. AR42J and U87 cells were cultured as previously described and supplied with fresh medium 24 h before harvesting. On E7, cells were collected, resuspended in culture medium, and aliquots containing 3×10⁶ cells were prepared. These were centrifuged at 500×g for 3 min at 4 °C (Centrifuge 5424 R, *Eppendorf*) and kept on ice. In parallel, Matrigel® Basement Membrane Matrix (*Corning*) was thawed on ice. Eggs were then removed from the incubator, placed on cushioned holders and windows were reopened to expose the CAM. After gently drying the membrane with sterile lens tissue, 20 µL of Matrigel containing the cell pellet was applied directly onto the CAM surface. Windows were resealed, eggs were labelled and incubation continued until E14. On E14, eggs were placed

on cushioned holders and the windows enlarged to allow direct injection of radiotracers into CAM blood vessels for tumour imaging. Embryos were humanely discarded after completion of the experiments.

Preparation of ^{18}F -labelled radiotracers

The radiolabelled ligands ^{18}F -L1 (15 nmol) and $[^{18}\text{F}][^{\text{nat}}\text{Ga}]\text{Ga}-(\text{SiFA})\text{SeFe-rhTATE2}$ (9 nmol) were synthesized according to the previously described procedure. The resulting ethanolic solutions were concentrated to 100 μL under a N_2 stream at 60 $^\circ\text{C}$ and subsequently diluted with PBS until the ethanol content was below 25%. An aliquot was withdrawn for quality control (data not shown) by radio RP-HPLC using a 1260 Infinity II LC system (*Agilent Technologies*) equipped with a GABI Nova detector (*Elysia-Raytest*). The separation was carried out on a ZORBAX Eclipse XDB-C18 analytical column (250 $\times$ 4.6 mm, 5 μm ; *Agilent Technologies*) with a mobile phase consisting of MeCN (5% H_2O , 0.1% TFA) and H_2O (0.1% TFA), applying a gradient from 10% to 80% MeCN over 15 min at a flow rate of 1.5 mL/min. For *in ovo* administration, 150 μL samples containing approximately 5 MBq of activity was prepared by further dilution with 0.9 wt% saline solution.

In ovo PET imaging

On E14, a CAM vein was cannulated and 90 μL of medetomidine solution (1 mg/mL, *Virbac*) was applied directly onto the CAM surface. Eggs were left at RT for 15 min prior to receiving an intravenous bolus injection of approximately 3 MBq of the radiolabelled tracer (< 150 μL) on the imaging bed, followed by a 50 μL PBS flush (*Sigma Life Science*). In the case of ^{18}F -L1 a 60 min dynamic PET scan was conducted while for $[^{18}\text{F}][^{\text{nat}}\text{Ga}]\text{Ga}-(\text{SiFA})\text{SeFe-rhTATE2}$ a static PET scan after 60 min was acquired on a *Mediso* NanoScan PET/CT system (1-5 coincidence mode; 3D reconstruction; CT-based attenuation and scatter correction). Throughout imaging, eggs were maintained at 37 $^\circ\text{C}$. CT scans were performed for attenuation correction under the following parameters: 180 projections, semi-circular acquisition, 50 kVp, 300 ms exposure time. PET data were reconstructed using the Tera-Tomo 3D algorithm (4 iterations; 6 subsets; 400-600 keV; 0.3 mm³ voxel size). Dynamic PET data were reconstructed into 19 time bins (4 $\times$ 15 s, 4 $\times$ 60 s, 11 $\times$ 300 s). Image analysis was carried out with VivoQuant software (v2.5, *Invicro Ltd.*). Regions of interest were manually defined based on the PET signal and summed dynamic PET (5-30 min) was used to identify the brain region. From these regions of interest, time-activity curves were generated as % injected dose per mL, and the area under the curve was subsequently calculated.

Ex ovo biodistribution

Biodistribution analysis was performed immediately following the PET scans. Embryos were sacrificed by cervical dislocation, and samples of tumour, brain, liver and bone were excised.

Each organ was transferred into pre-weighed scintillation vials, weighed, and measured for radioactivity using a γ -counter (LKG 1282 CompuGamma, *LKB-Wallac*). Uptake values were expressed as %ID/g. To enable quantification, a dilution series of the respective radiolabelled tracer in PBS was prepared, the activity determined with a Carpentec system, and the samples measured on the γ -counter alongside the organs to generate a calibration curve.

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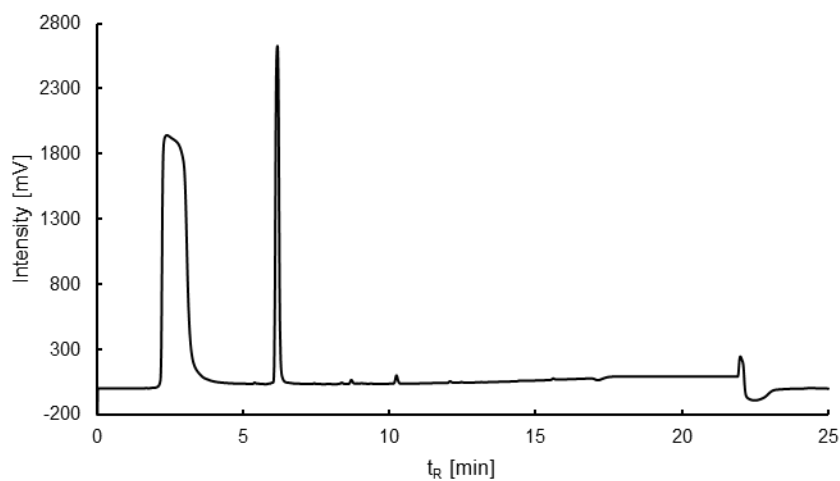
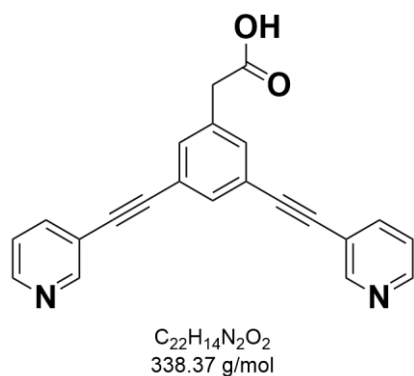
Supplementary Information

SI Table 1: Calculated and experimental m/z values (most abundant isotope) for MCg species and host-guest adducts with perrhenate. Table was adapted from ref.^[73] with permission from the *Royal Society of Chemistry*.

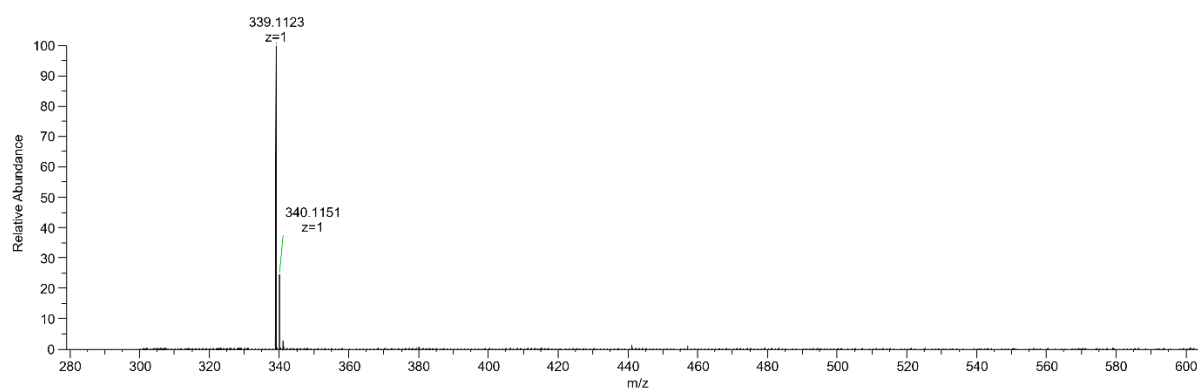
Compound	Formula	m/z		Observed Adducts
		Theoretical	Measured	
C1_{hom}	C ₃₆₀ H ₄₉₆ F ₄ N ₇₆ O ₅₆ Pd ₂ Si ₄	661.8758 (z=11)	661.8747 664.2367	[M ⁴⁺ +FA+ACN+7H ⁺] ¹¹⁺ [M ⁴⁺ +2FA+Na ⁺ +7H ⁺] ¹¹⁺
[Pd ₂ (L1) ₃]	C ₂₇₀ H ₃₇₂ F ₃ N ₅₇ O ₄₂ Pd ₂ Si ₃	686.4560 (z=8)	686.4570 692.3329 695.2059 700.5811 704.9545 709.2046	[M ⁴⁺ +FA+4H ⁺] ⁸⁺ [M ⁴⁺ +2FA+4H ⁺] ⁸⁺ [M ⁴⁺ +2FA+Na ⁺ +4H ⁺] ⁸⁺ [M ⁴⁺ +2DMSO+4H ⁺] ⁸⁺ [M ⁴⁺ +Cl ⁻ +2DMSO+5H ⁺] ⁸⁺ [M ⁴⁺ +2Cl ⁻ +2DMSO+6H ⁺] ⁸⁺
C2_{hom}	C ₃₅₂ H ₄₈₄ F ₄ N ₇₂ O ₅₂ Pd ₂ Si ₄	637.3200 (z=11) 705.5519 (z=10)	637.3203 641.0483 643.5020 705.5536	[M ⁴⁺ +FA+7H ⁺] ¹¹⁺ [M ⁴⁺ +FA+ACN+7H ⁺] ¹¹⁺ [M ⁴⁺ +2FA+Na ⁺ +7H ⁺] ¹¹⁺ [M ⁴⁺ +2FA+6H ⁺] ¹⁰⁺
ReO ₄ ⊂ C2 _{hom}	C ₃₅₂ H ₄₈₄ F ₄ N ₇₂ O ₅₆ Pd ₂ ReSi ₄	655.9506 (z=11)	655.9501	[M ₃ ⁺ +8H ⁺] ¹¹⁺
[Pd ₂ (L2) ₃]	C ₂₆₄ H ₃₆₃ F ₃ N ₅₄ O ₃₉ Pd ₂ Si ₃	670.8237 (z=8)	670.8237 683.4458 687.8202	[M ⁴⁺ +2FA+4H ⁺] ⁸⁺ [M ⁴⁺ +2DMSO+Cl ⁻ +5H ⁺] ⁸⁺ [M ⁴⁺ +2DMSO+2Cl ⁻ +6H ⁺] ⁸⁺
ReO ₄ ⊂ [Pd ₂ (L2) ₃]	C ₂₆₄ H ₃₆₃ F ₃ N ₅₄ O ₄₃ Pd ₂ ReSi ₃	696.4408 (z=8)	696.4399	[M ³⁺ +FA+5H ⁺] ⁸⁺
C1_{het}	C ₁₅₆ H ₁₆₆ FN ₂₅ O ₂₀ Pd ₂ Si	594.2134 (z=5) 742.7652 (z=4)	594.2142 742.7651	[M ⁴⁺ +H ⁺] ⁵⁺ [M ⁴⁺] ⁴⁺
ReO ₄ ⊂ C1 _{het}	C ₁₅₆ H ₁₆₆ FN ₂₅ O ₂₄ Pd ₂ ReSi	644.6020 (z=5)	644.6024	[M ³⁺ +2H ⁺] ⁵⁺
[Pd ₂ (L1) ₂ (L0) ₂]	C ₂₂₄ H ₂₇₆ F ₂ N ₄₂ O ₃₂ Pd ₂ Si ₂	625.4160 (z=7)	625.4161	[M ⁴⁺ +3H ⁺] ⁷⁺
ReO ₄ ⊂ [Pd ₂ (L1) ₂ (L0) ₂]	C ₂₂₄ H ₂₇₆ F ₂ N ₄₂ O ₃₆ Pd ₂ ReSi ₂	661.4078 (z=7)	661.4080	[M ³⁺ +4H ⁺] ⁷⁺
C2_{het}	C ₁₅₄ H ₁₆₃ FN ₂₄ O ₁₉ Pd ₂ Si	582.8092 (z=5)	582.8102 605.8088	[M ⁴⁺ +H ⁺] ⁵⁺ [M ⁴⁺ +2FA+Na ⁺] ⁵⁺
ReO ₄ ⊂ C2 _{het}	C ₁₅₄ H ₁₆₃ FN ₂₄ O ₂₃ Pd ₂ ReSi	633.1977 (z=5)	633.1988	[M ³⁺ +2H ⁺] ⁵⁺
[Pd ₂ (L2) ₂ (L0) ₂]	C ₂₂₀ H ₂₇₀ F ₂ N ₄₀ O ₃₀ Pd ₂ Si ₂	609.2671 (z=7)	609.2677 625.4096	[M ⁴⁺ +3H ⁺] ⁷⁺ [M ⁴⁺ +2FA+Na ⁺ +2H ⁺] ⁷⁺
ReO ₄ ⊂ [Pd ₂ (L2) ₂ (L0) ₂]	C ₂₂₀ H ₂₇₀ F ₂ N ₄₀ O ₃₄ Pd ₂ ReSi ₂	752.4674 (z=6)	752.4680	[M ³⁺ +3H ⁺] ⁶⁺

Characterization of the ligands

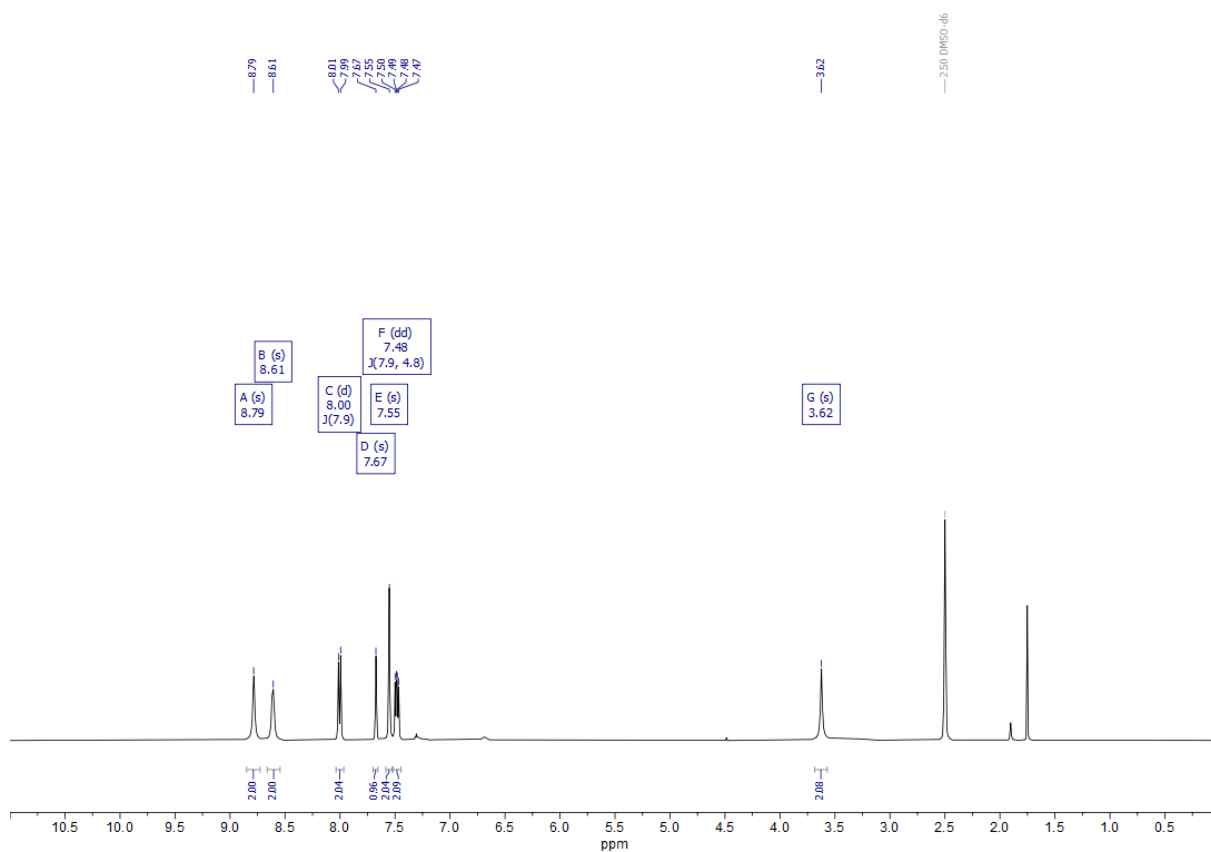
2-(3,5-bis(pyridin-3-ylethynyl)phenyl)acetic acid (**L0**)



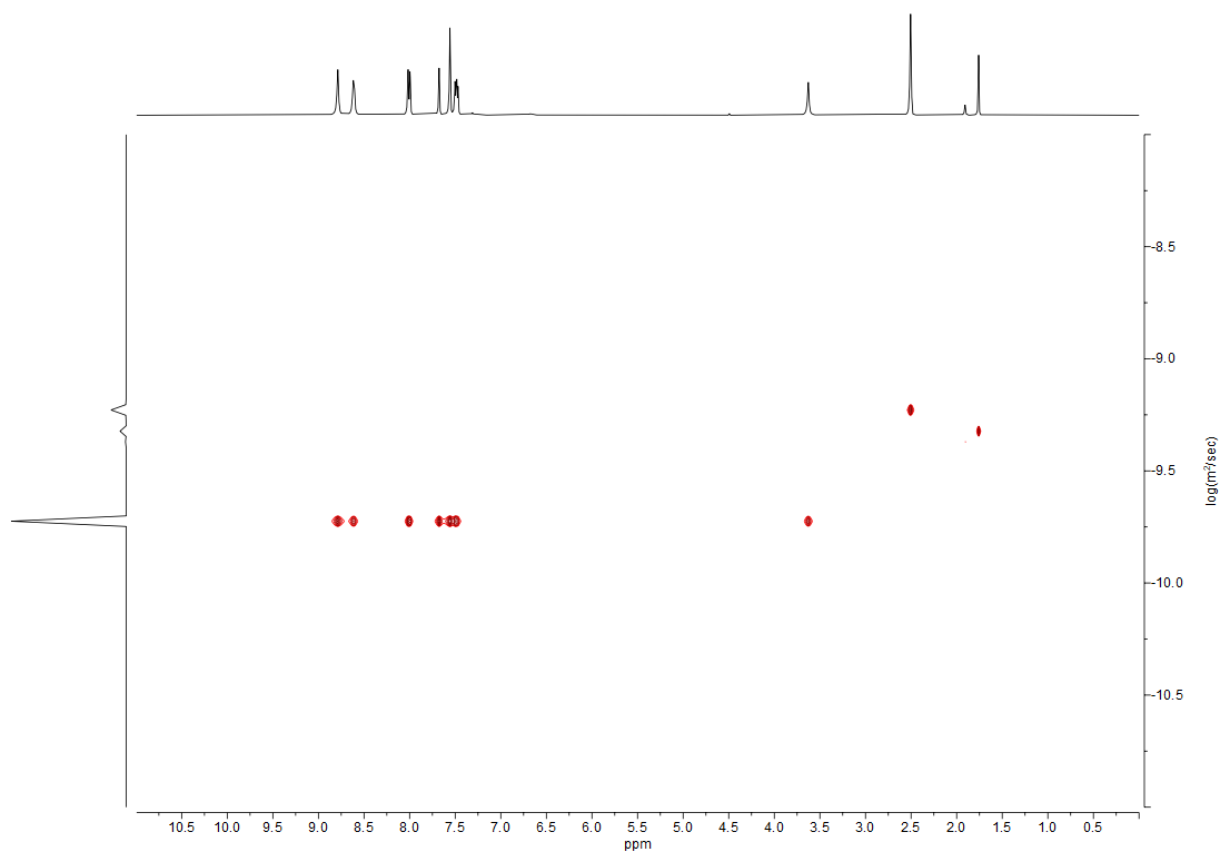
SI Figure 1: RP-HPLC chromatogram of **L0** measured with a gradient of 10-90% B in 15 min.



SI Figure 2: HR-ESI-MS spectrum of **L0**.

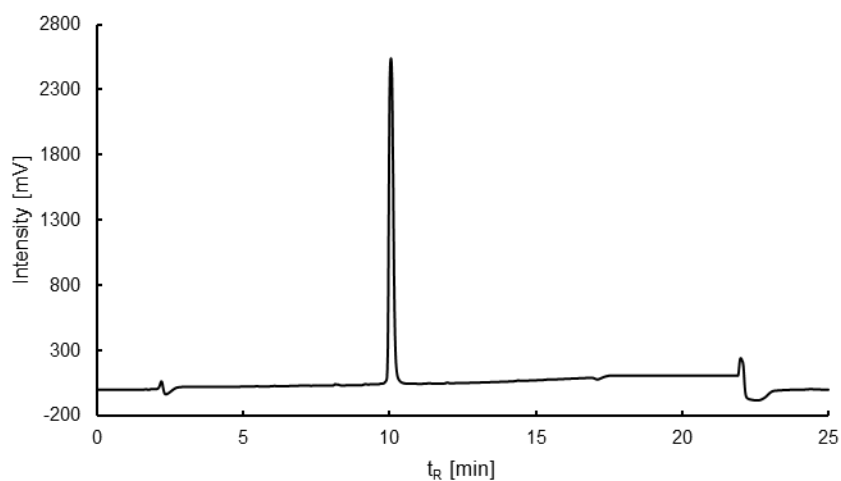
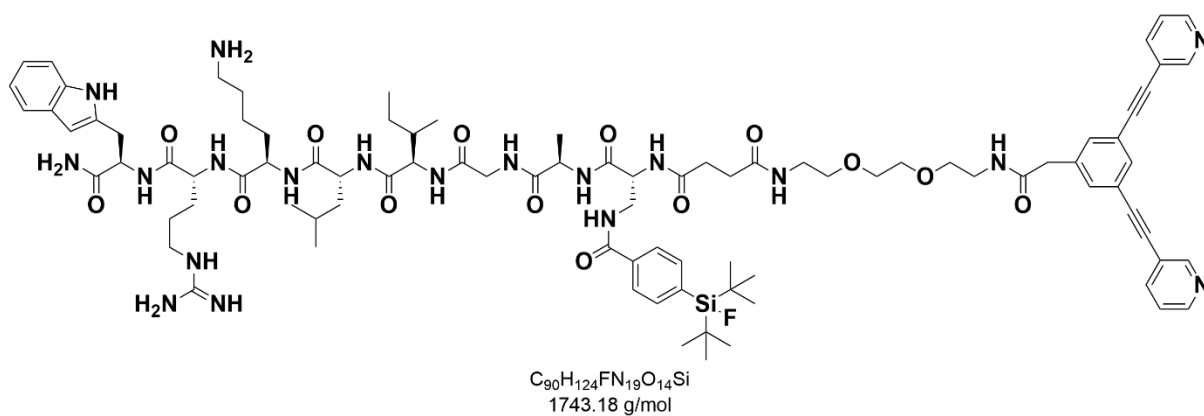


SI Figure 3: ¹H NMR spectrum of **L0** measured in DMSO-*d*₆ at 400 MHz.

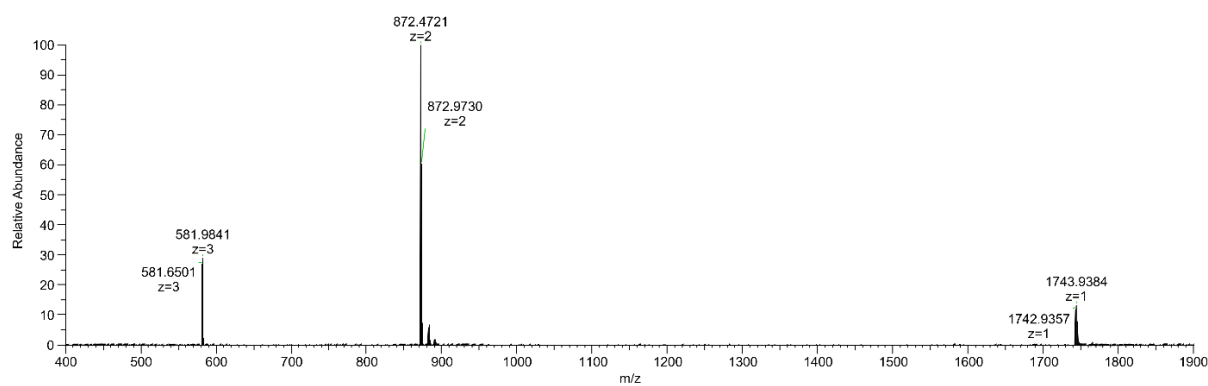


SI Figure 4: ¹H-DOSY NMR spectrum of **L0** measured in DMSO-*d*₆ at 400 MHz.

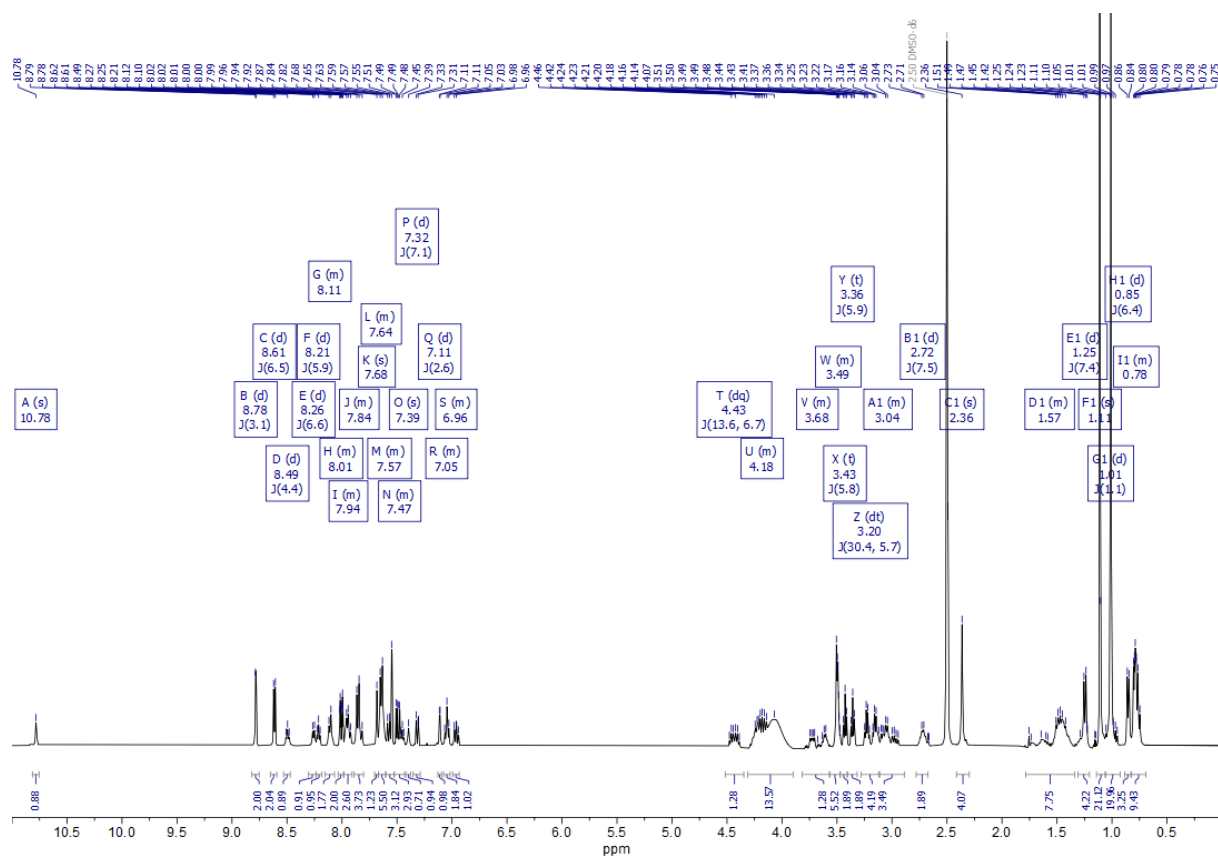
***D*PepH3-*D*Dap((SiFA)BA)-Ebes-L0 (L1)**



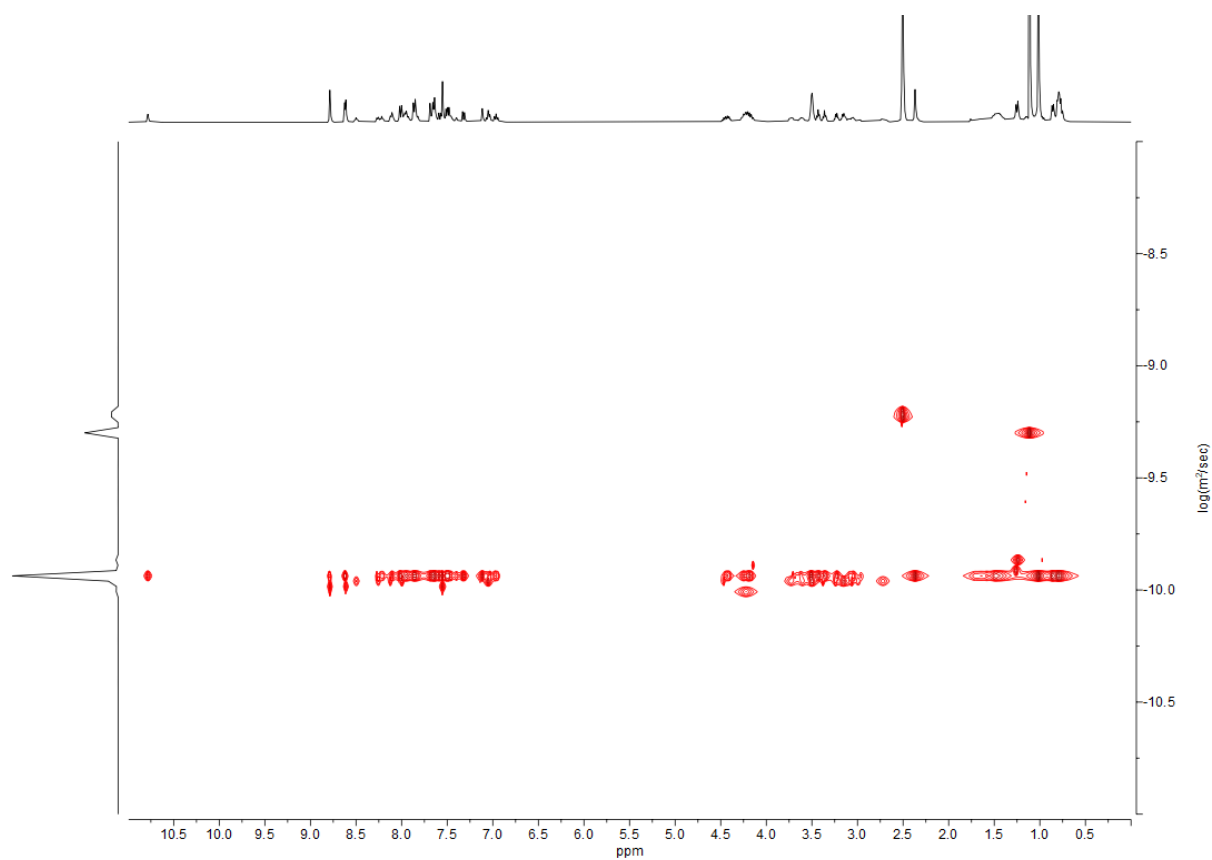
SI Figure 5: RP-HPLC chromatogram of **L1** measured with a gradient of 10-90% B in 15 min on a C18 column.



SI Figure 6: HR-ESI-MS spectrum of **L1**.

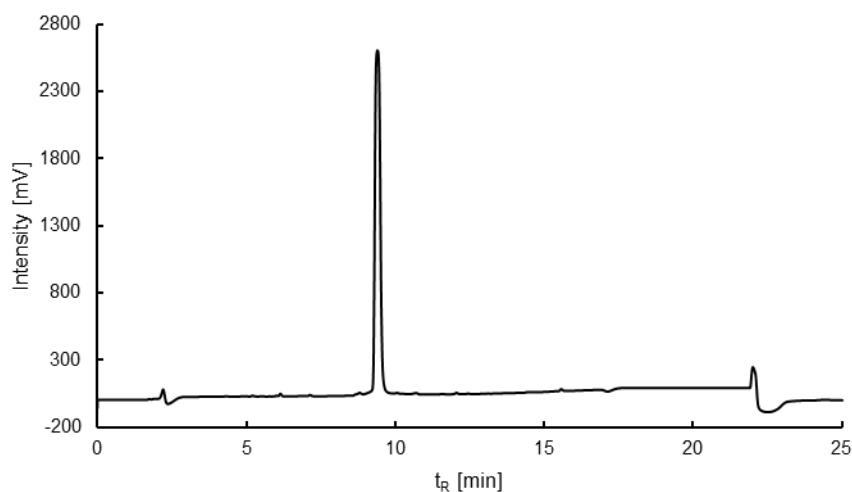
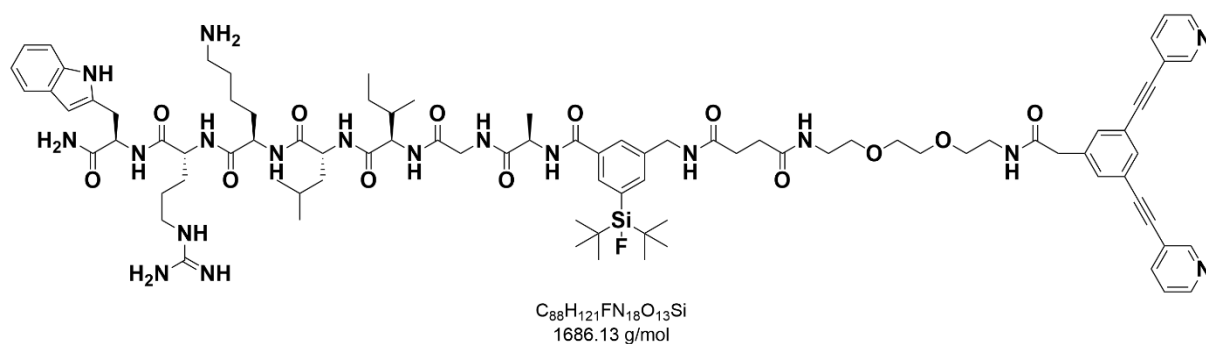


SI Figure 7: ^1H NMR spectrum of L1 measured in DMSO- d_6 at 400 MHz.

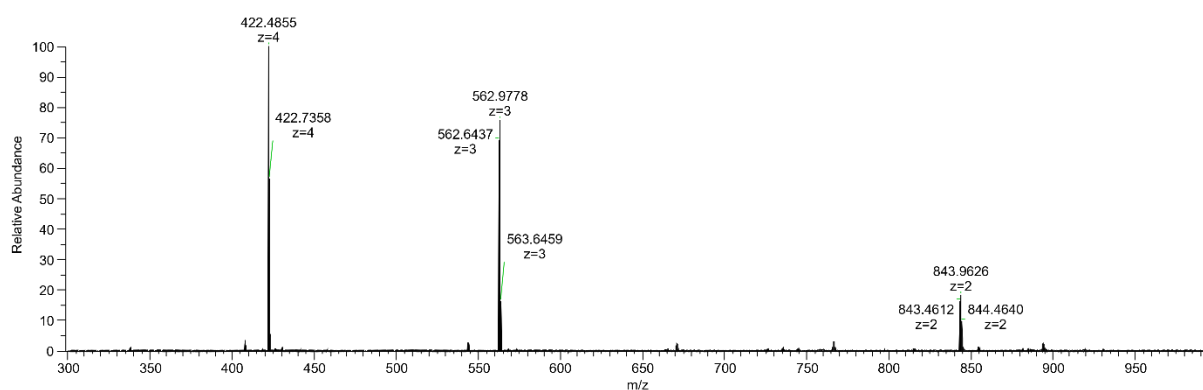


SI Figure 8: ^1H -DOSY NMR spectrum of L1 measured in DMSO- d_6 at 400 MHz.

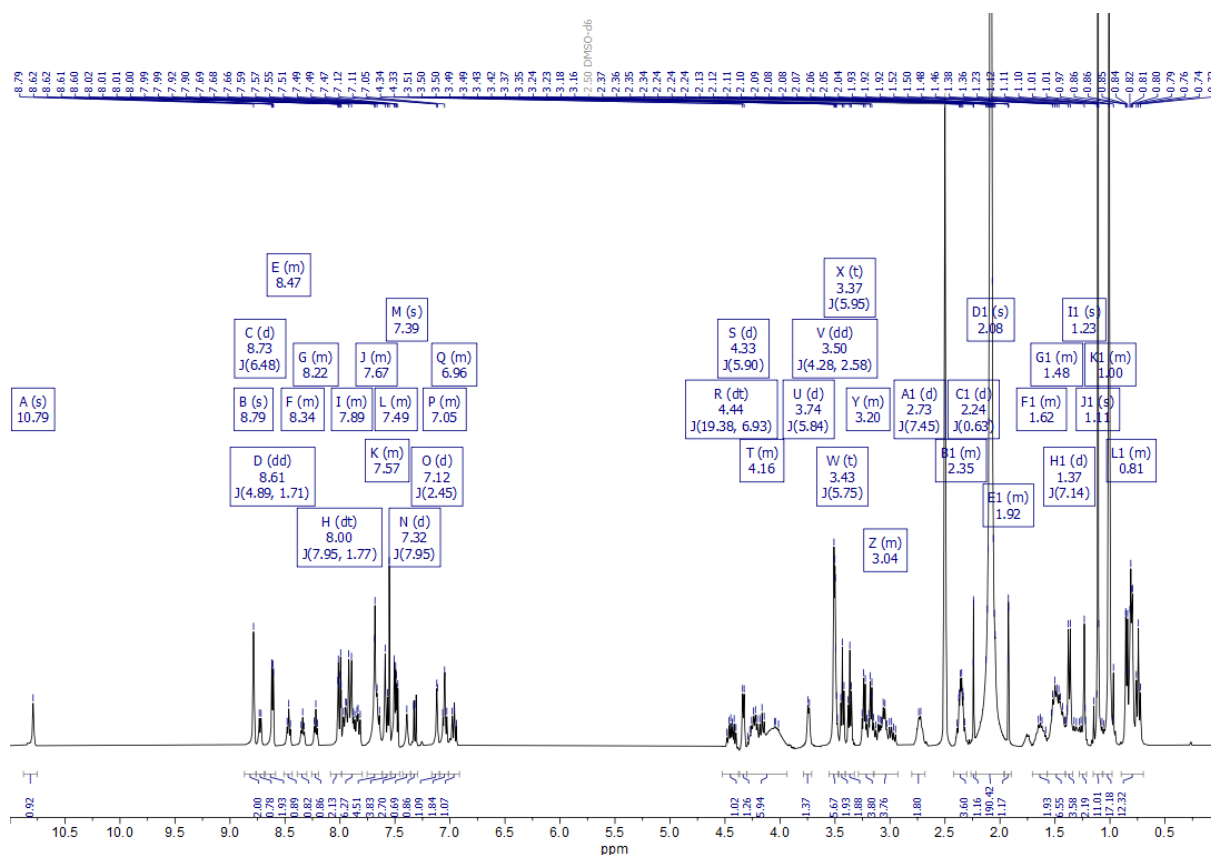
***d*PepH3-(SiFA)SeFe-Ebes-L0 (L2)**



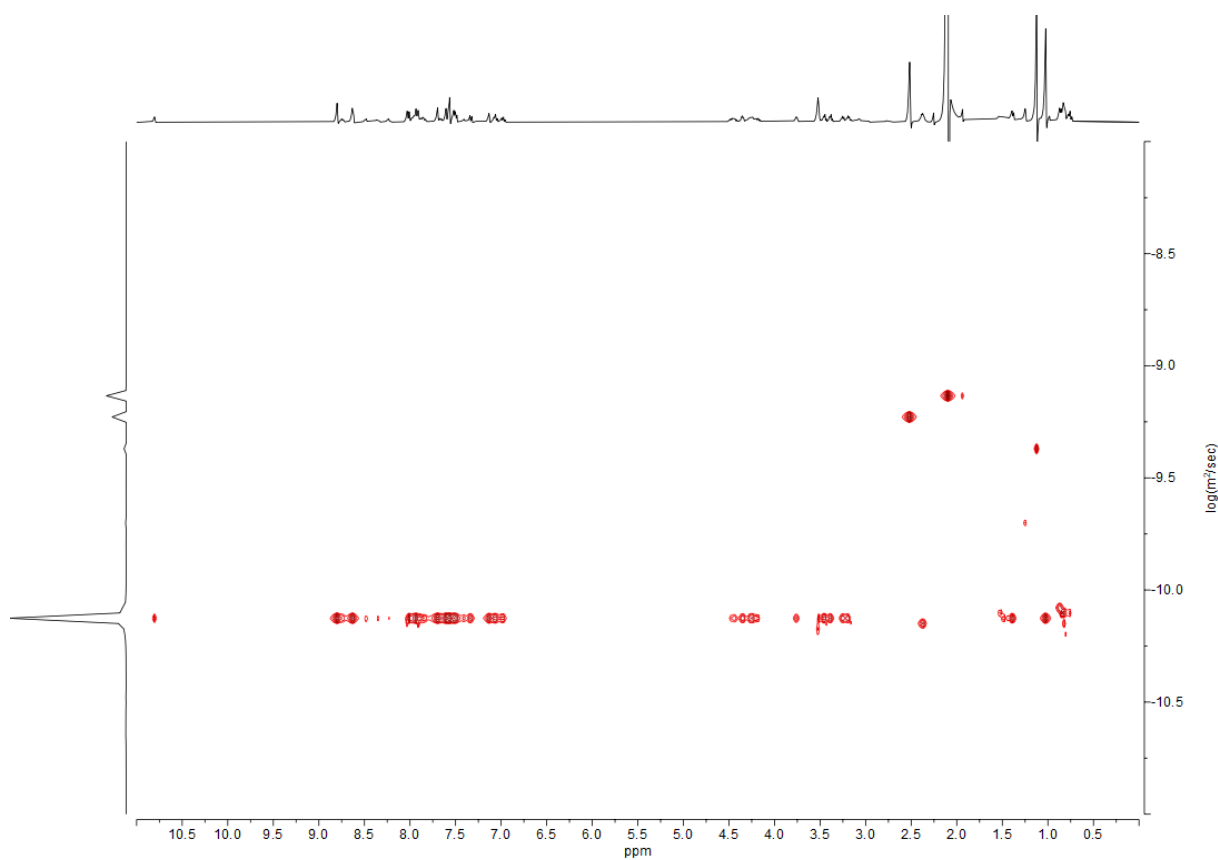
SI Figure 9: RP-HPLC chromatogram of **L2** measured with a gradient of 10-90% B in 15 min on a C18 column.



SI Figure 10: HR-ESI-MS spectrum of **L2**.



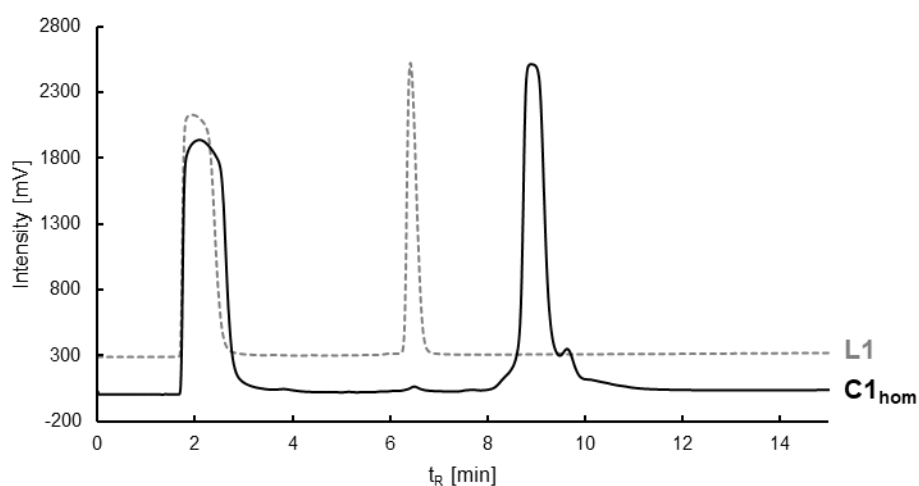
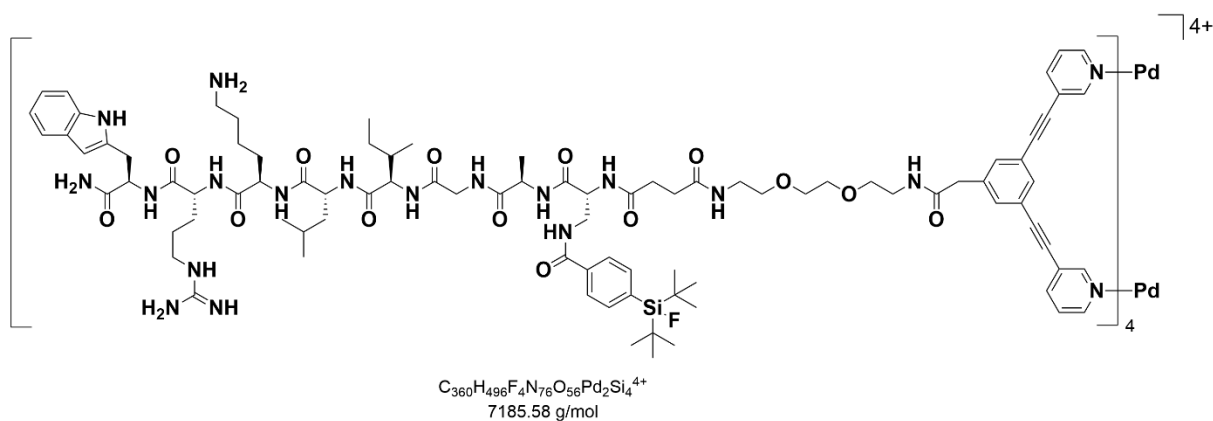
SI Figure 11: ^1H NMR spectrum of **L2** measured in $\text{DMSO}-d_6$ at 400 MHz.



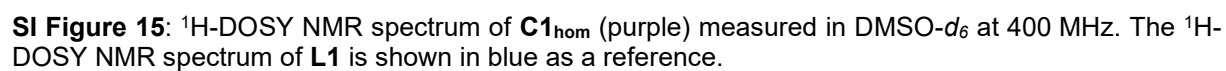
SI Figure 12: ^1H -DOSY NMR spectrum of **L2** measured in $\text{DMSO}-d_6$ at 400 MHz.

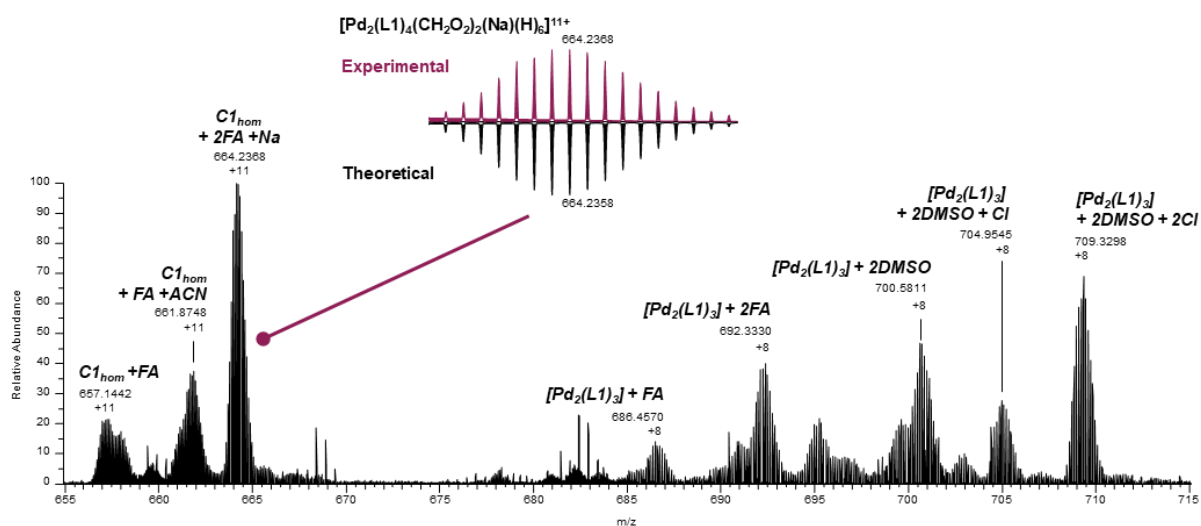
Homoleptic metallacages **C1_{hom}** and **C2_{hom}**

Characterization of $[Pd_2(L1)_4](BF_4)_4$ (**C1_{hom}**)



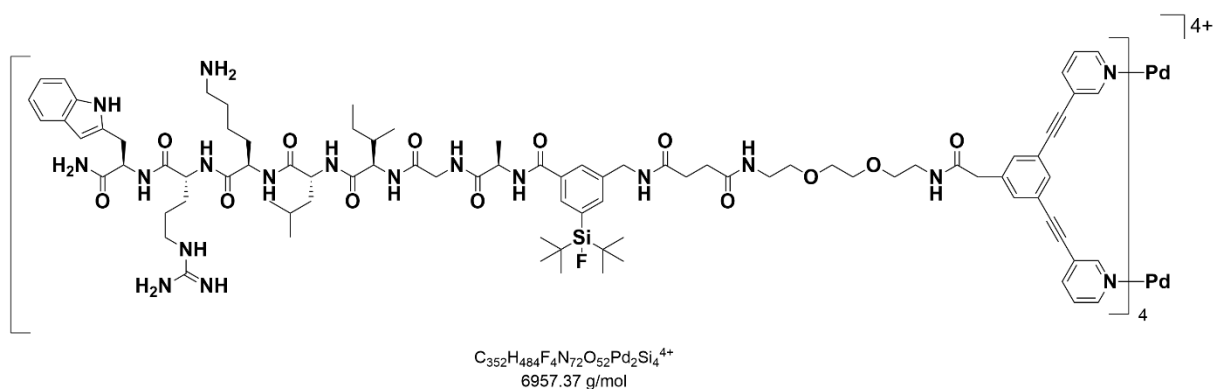
SI Figure 13: RP-HPLC chromatogram (40-70% B in 15 min, C18 column) of **C1_{hom}** (1 mM) after 1 h CDSA at RT. As a reference the chromatogram of the respective ligand **L1** is depicted in a grey dashed line.

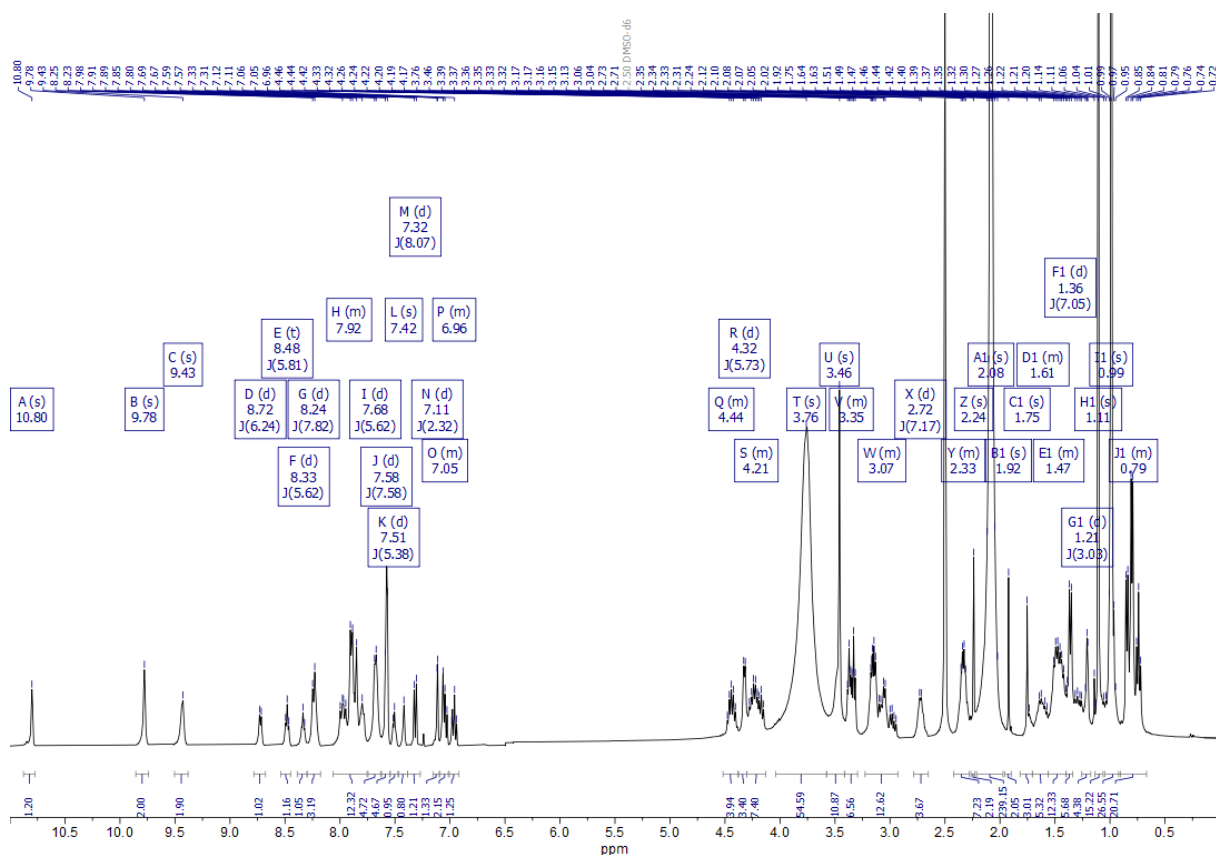




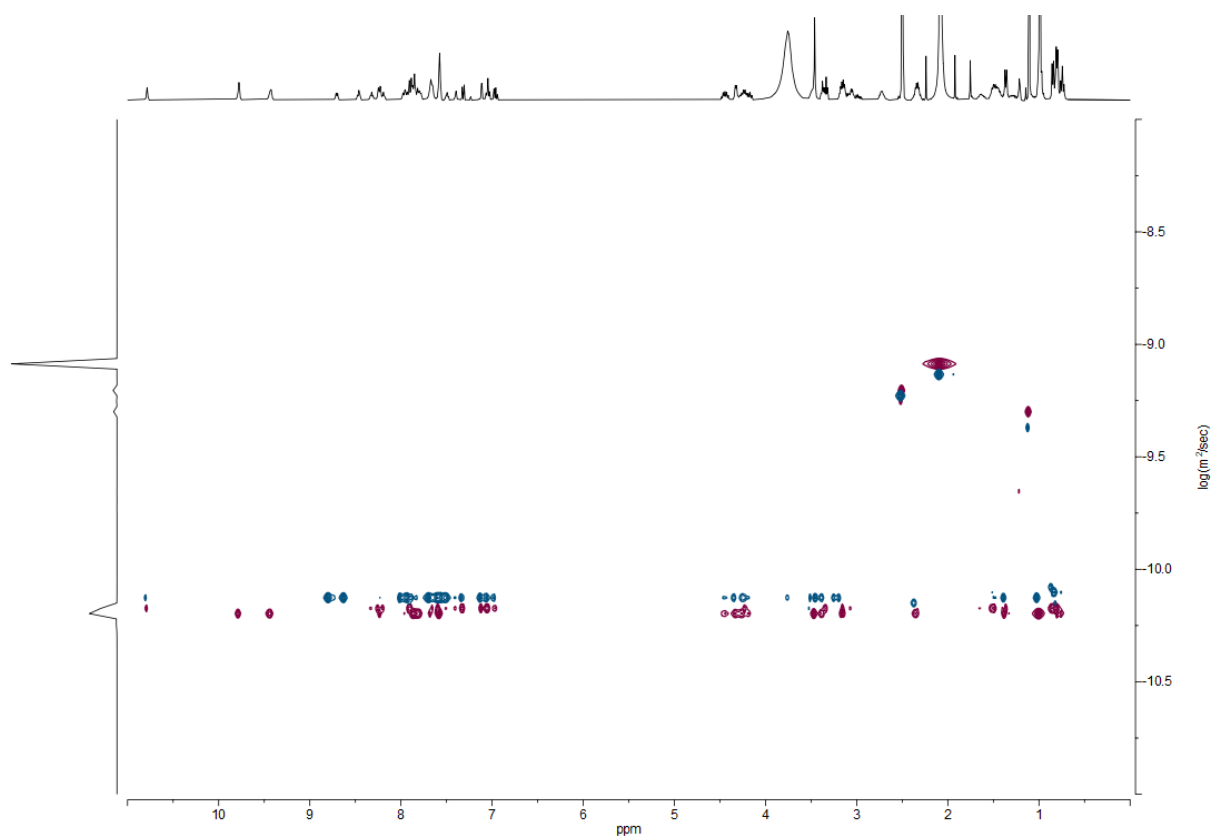
SI Figure 16: DI-HR-ESI-MS of $C1_{hom}$.

Characterization of $[Pd_2(L2)_4](BF_4)_4$ ($C2_{hom}$)



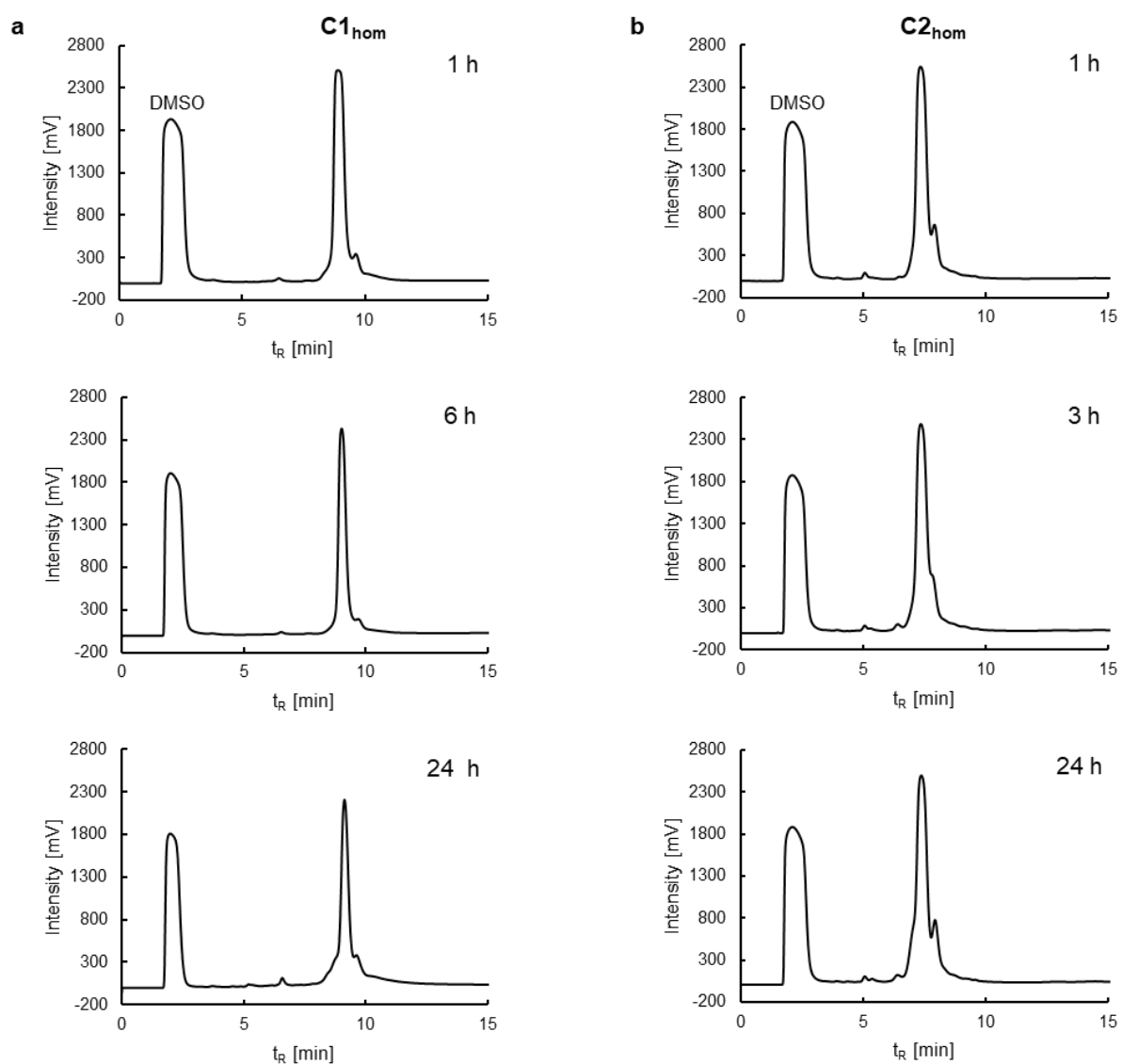


SI Figure 17: ¹H NMR spectrum of **C2_{hom}** measured in DMSO-*d*₆ at 400 MHz.



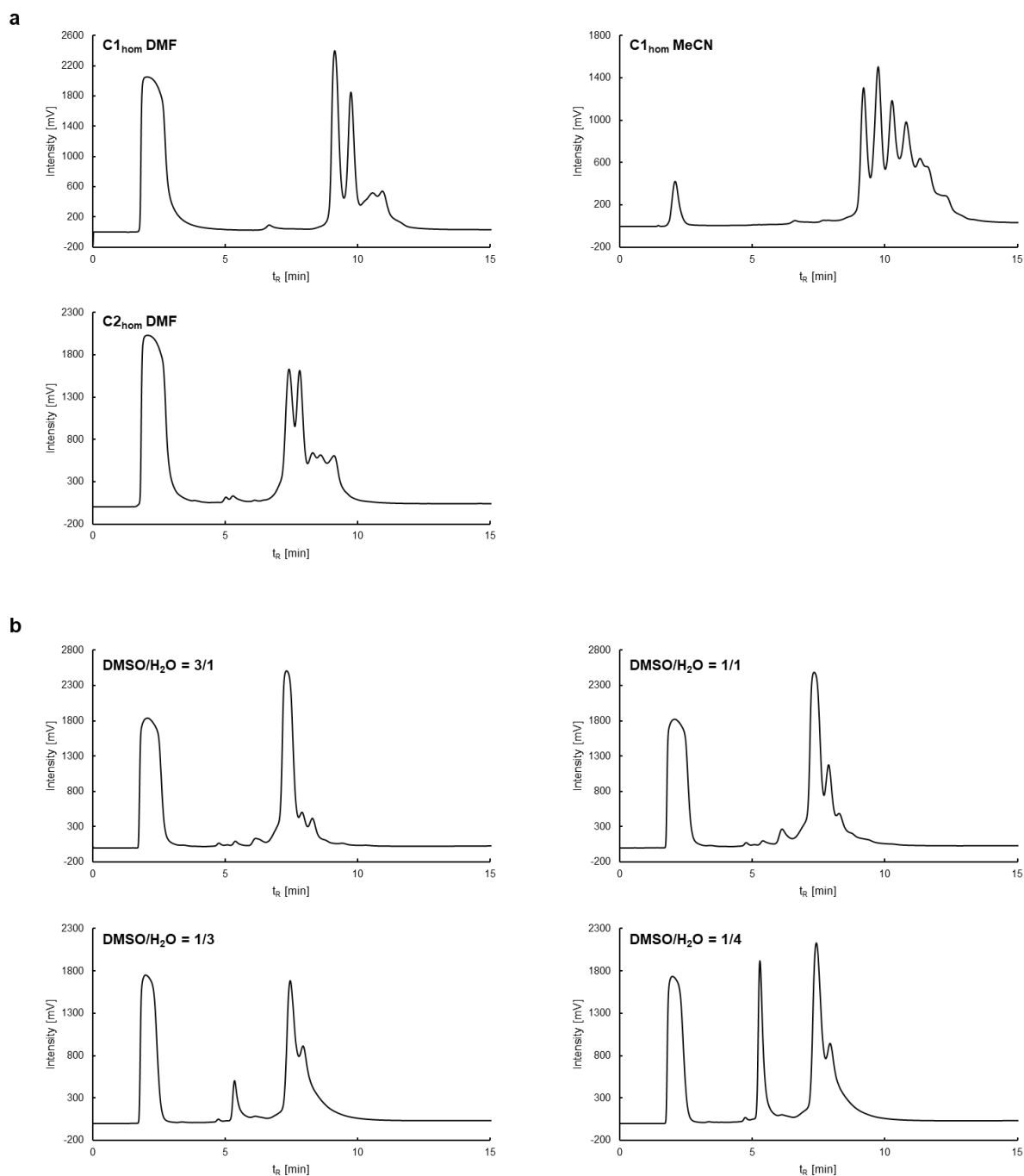
SI Figure 18: ¹H-DOSY NMR spectrum of **C2_{hom}** (purple) measured in DMSO-*d*₆ at 400 MHz. The ¹H-DOSY NMR spectrum of **L2** is shown in blue as a reference.

Stability of $C1_{hom}$ and $C2_{hom}$ in DMSO (1 mM)



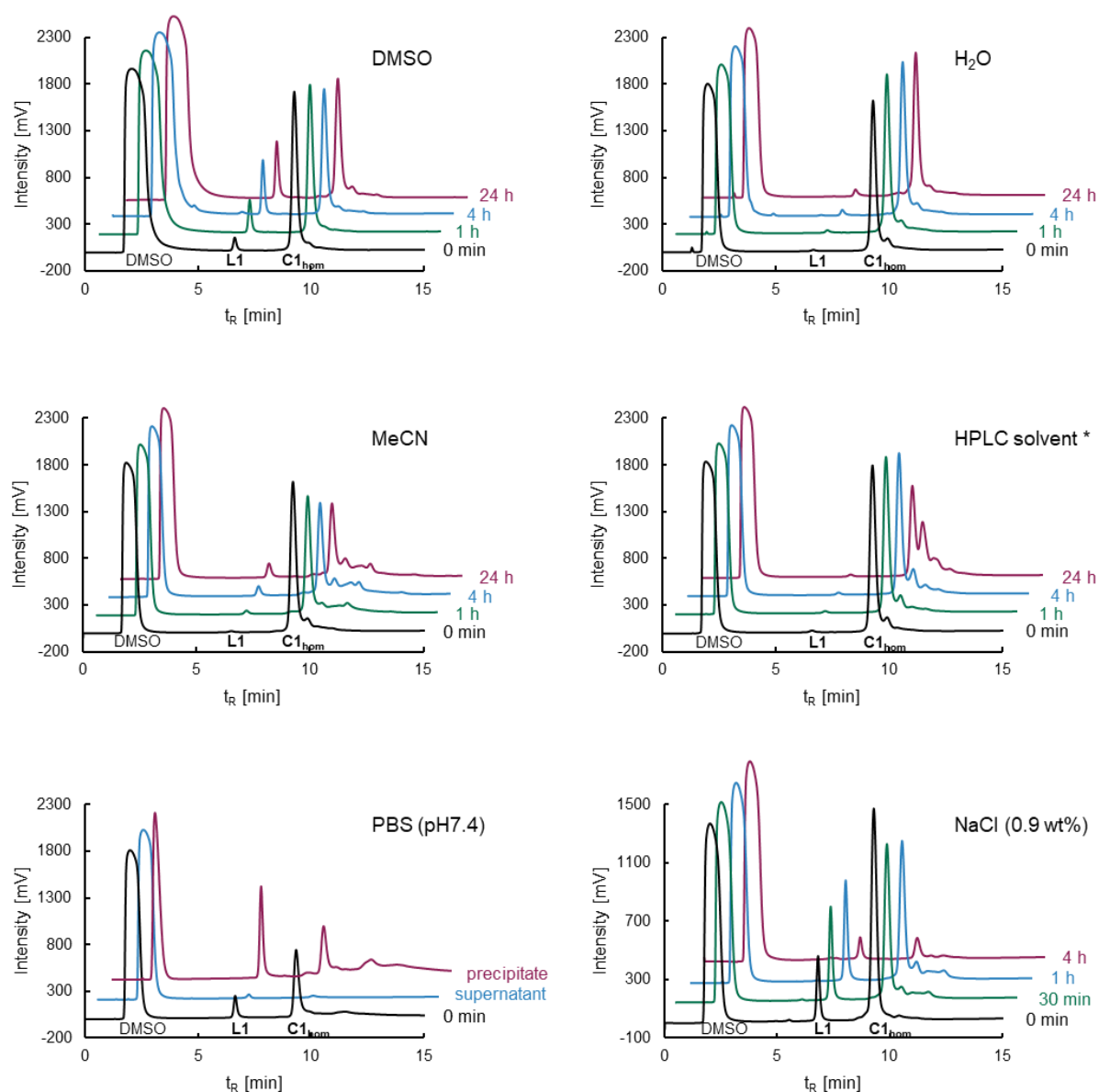
SI Figure 19: Stability of $C1_{hom}$ (a) and $C2_{hom}$ (b) after 1 h, 6 h/3 h and 24 h CDCA in DMSO at RT and 1 mM concentration evaluated by RP-HPLC (40-70% B in 15min, C18 column).

CDSA of $C1_{hom}$ and $C2_{hom}$ in different solvents (1 mM)

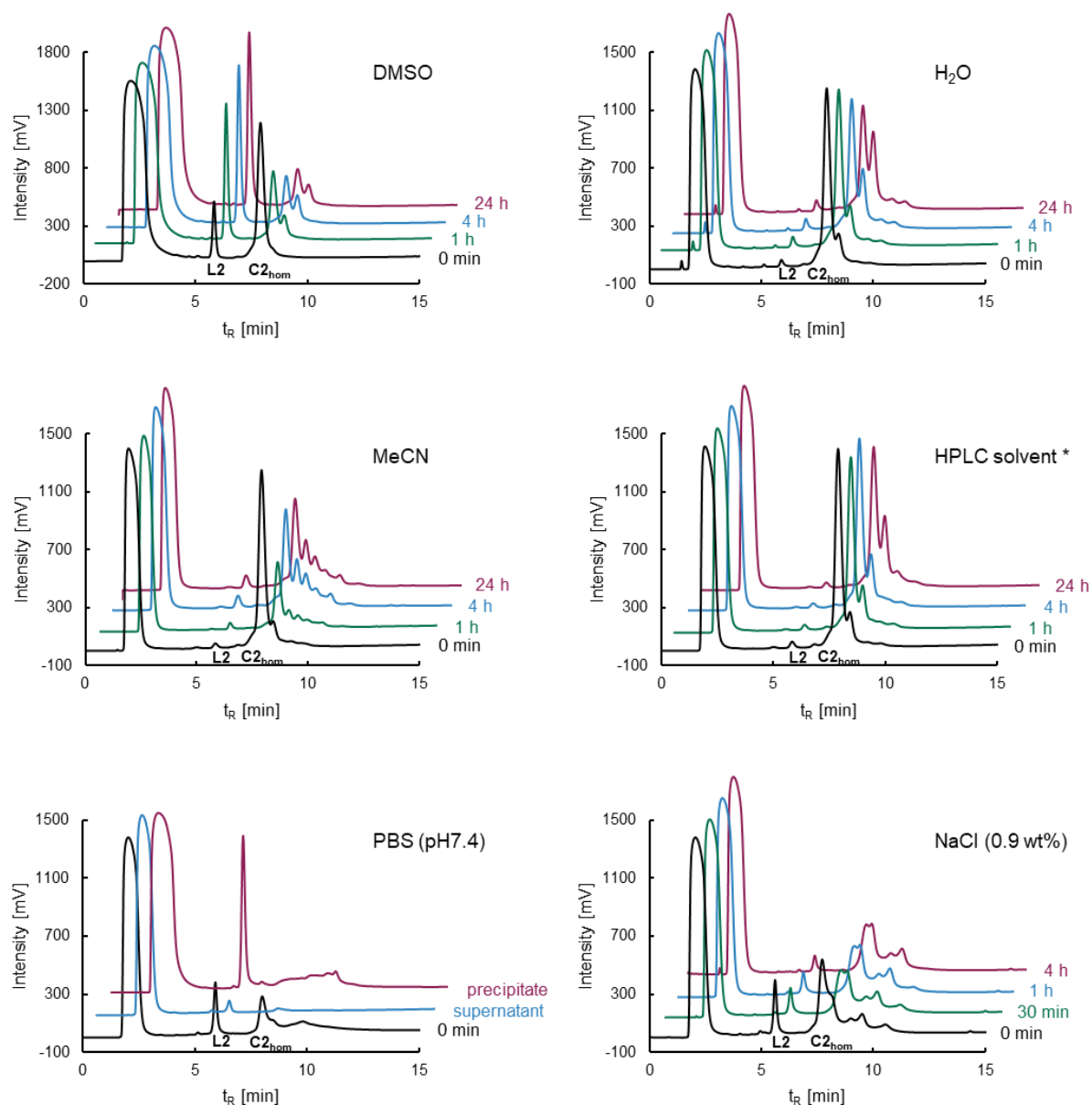


SI Figure 20: CDSA of $C1_{hom}$ and $C2_{hom}$ (1 mM, 1 h, RT) in different solvents. a) RP-HPLC chromatograms of $C1_{hom}$ (40-70% in 15 min, C18 column) and $C2_{hom}$ (40-70% B in 15 min, C18 column) formed in DMF and MeCN. b) RP-HPLC chromatograms (40-70% B in 15 min, C18 column) of $C2_{hom}$ formed in different ratios of DMSO/H₂O.

Stability of $C1_{hom}$ and $C2_{hom}$ in different solvents (0.1 mM)

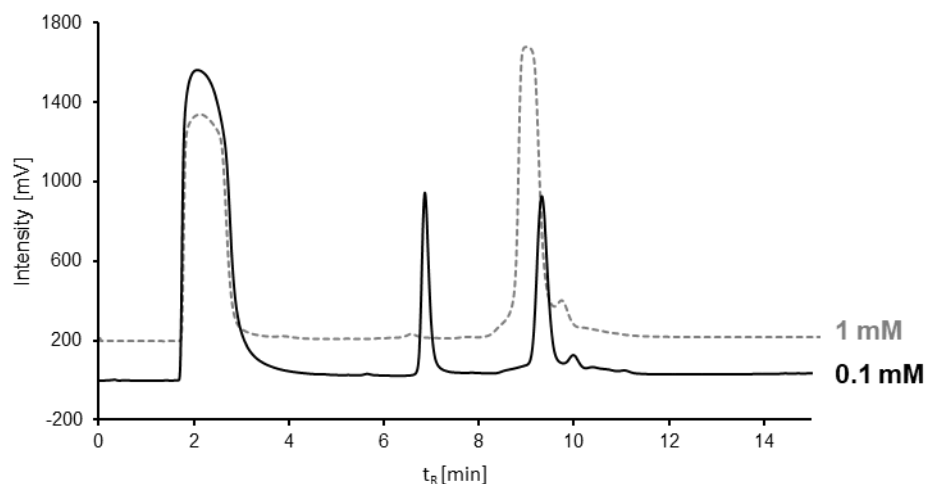


SI Figure 21: Stability of $C1_{hom}$ (0.1 mM) in different solvents at RT monitored by RP-HPLC (40-70% B in 15 min, C18 column) up to 24 h. Precipitation occurred in salt containing solutions.

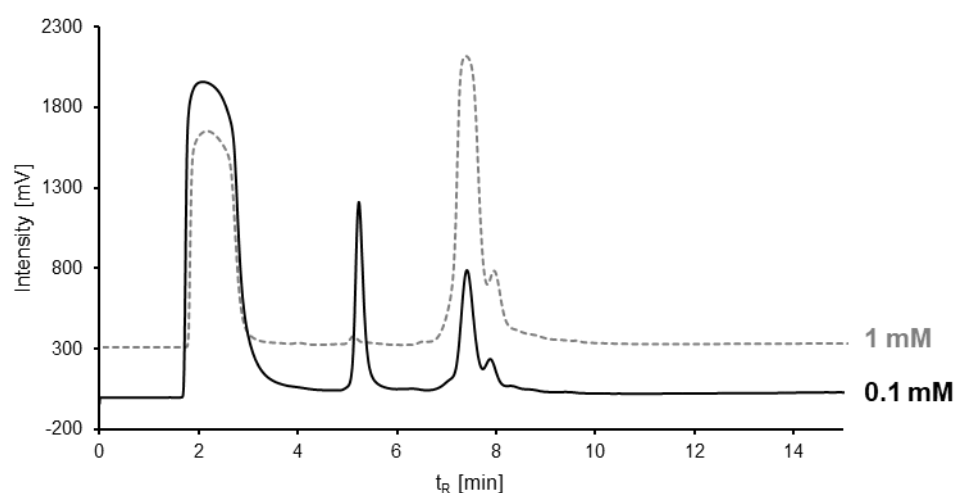


SI Figure 22: Stability of $C2_{hom}$ (0.1 mM) in different solvents at RT monitored by RP-HPLC (40-70% B in 15 min, C18 column) up to 24 h. Precipitation occurred in salt containing solutions.

Concentration-dependent formation of $C1_{hom}$ and $C2_{hom}$



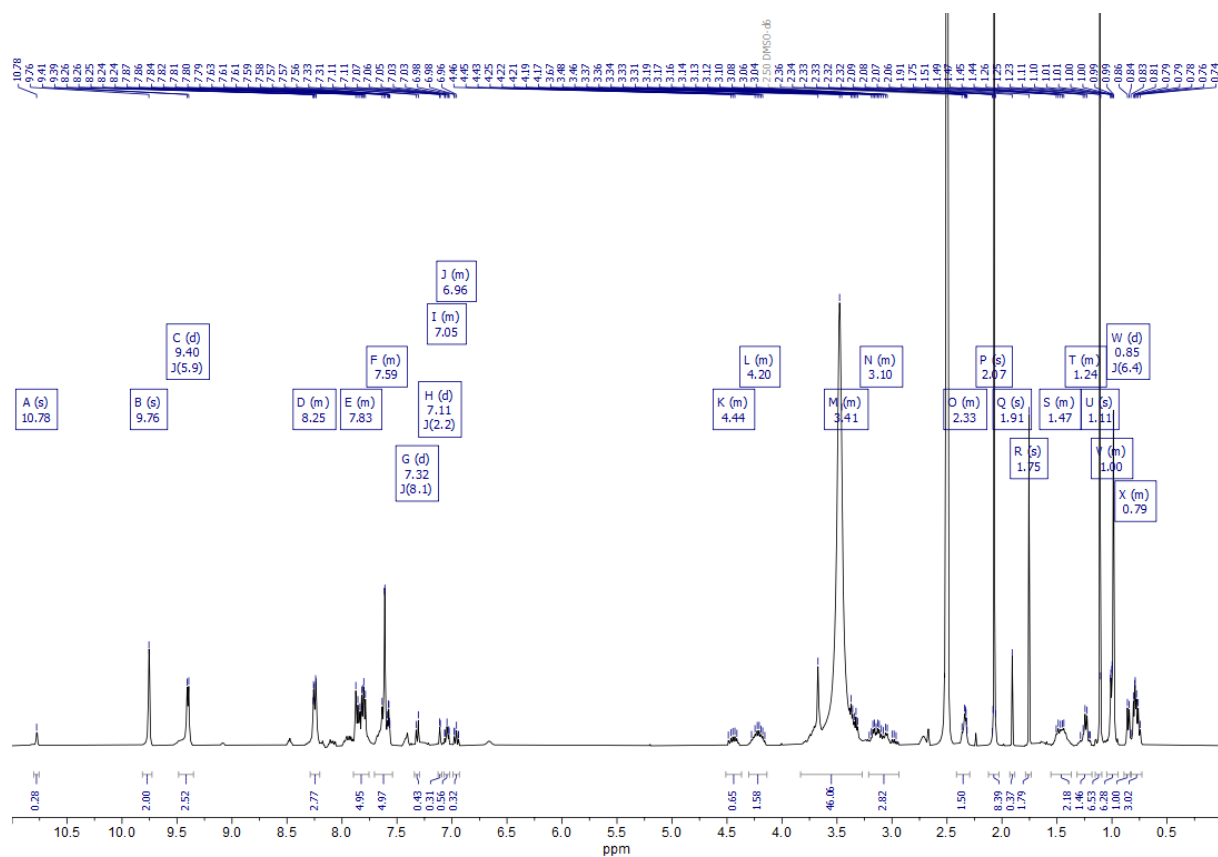
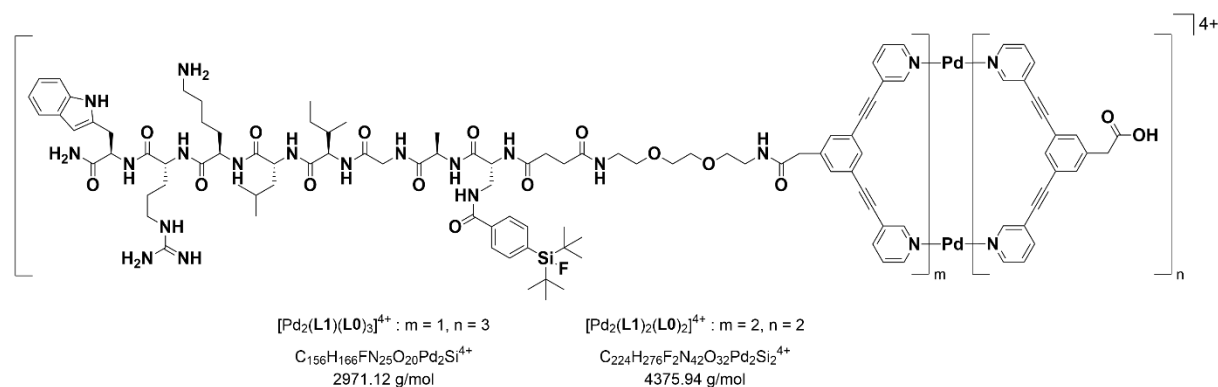
SI Figure 23: RP-HPLC chromatogram (40-70% B in 15 min, C18 column) of $C1_{hom}$ formed in a theoretical concentration of 0.1 mM in DMSO (1 h, RT). As a reference the chromatogram of $C1_{hom}$ in 1 mM concentration is depicted in a grey dashed line.



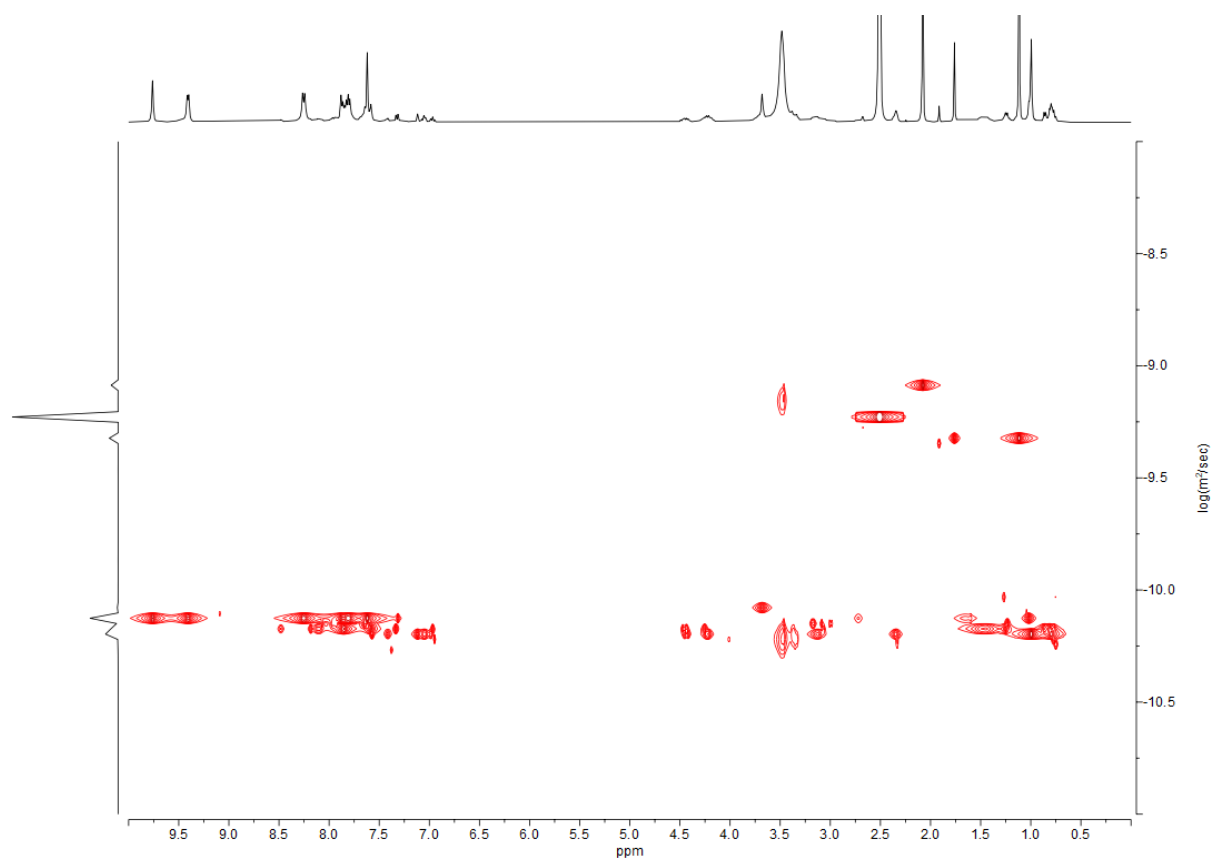
SI Figure 24: RP-HPLC chromatogram (40-70% B in 15 min, C18 column) of $C2_{hom}$ formed in a theoretical concentration of 0.1 mM in DMSO (1 h, RT). As a reference the chromatogram of $C2_{hom}$ in 1 mM concentration is depicted in a grey dashed line.

Heteroleptic metallacages **C1_{het}** and **C2_{het}**

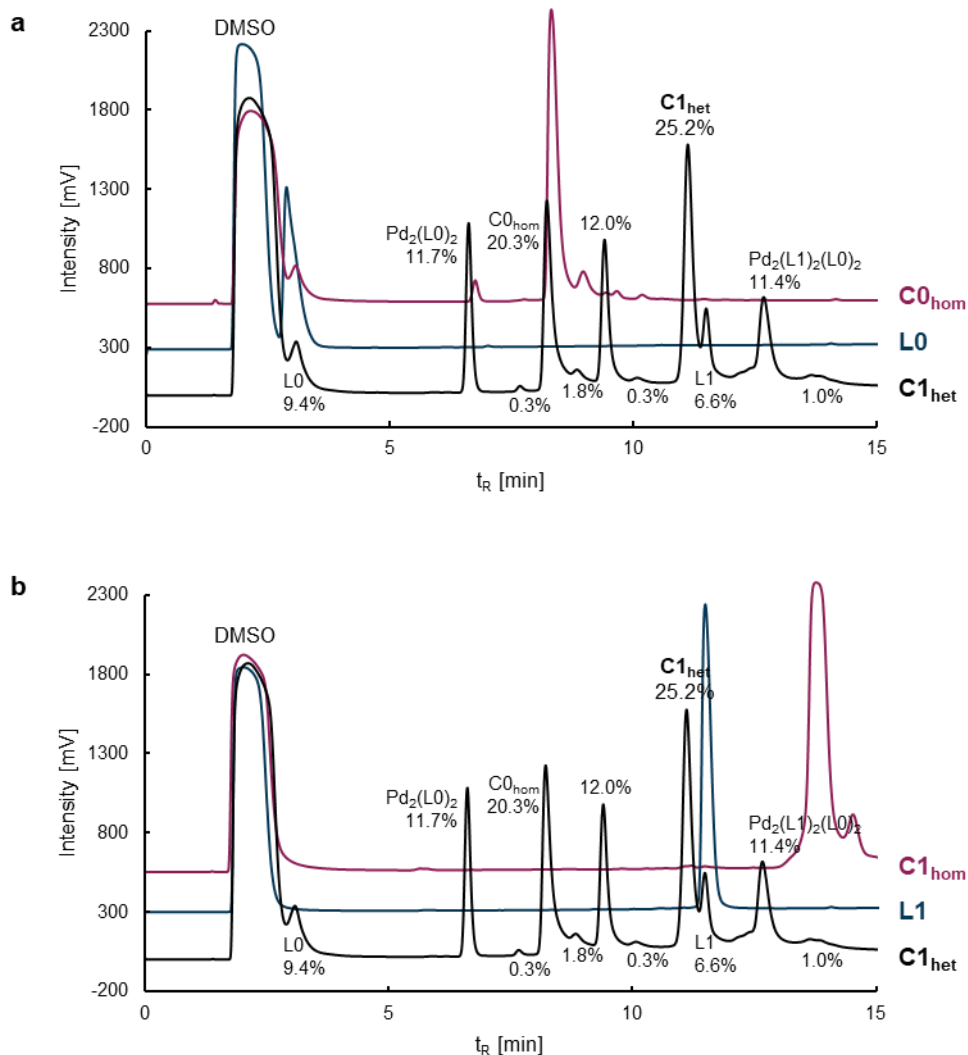
Characterization of **C1_{het}**



SI Figure 25: ^1H NMR spectrum of heteroleptic species obtained by the CDSA of **L1** (1.0 eq.) and **L0** (3.0 eq.) with $[\text{Pd}(\text{MeCN})_4](\text{BF}_4)_2$ (2.0 eq.) measured in $\text{DMSO}-d_6$ at 400 MHz.

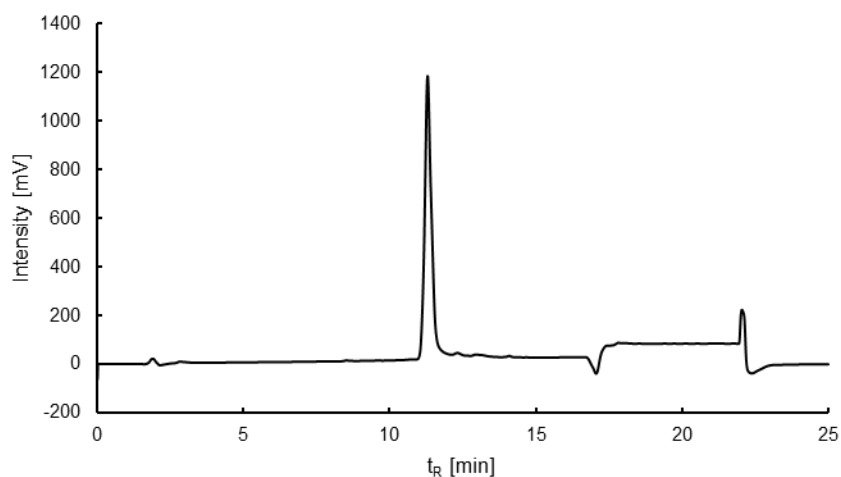


SI Figure 26: ¹H-DOSY NMR spectrum of heteroleptic species obtained by the CDSA of **L1** (1.0 eq.) and **L0** (3.0 eq.) with [Pd(MeCN)₄](BF₄)₂ (2.0 eq.) measured in DMSO-*d*₆ at 400 MHz.

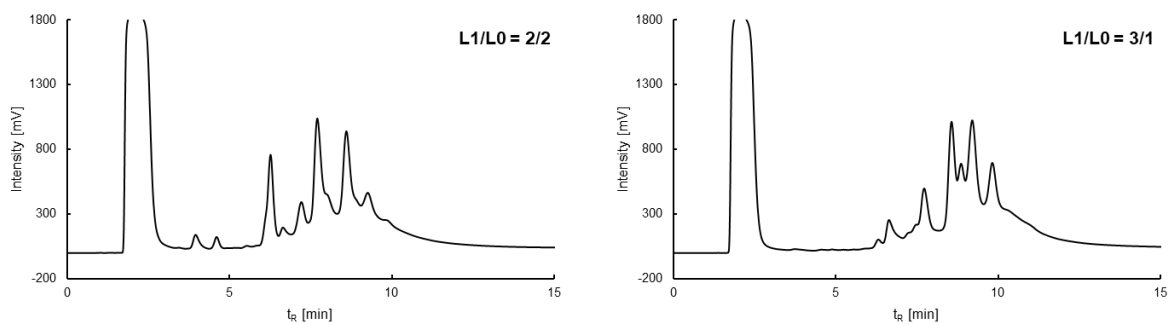


SI Figure 27: Comparison of the RP-HPLC chromatograms (30-60% B in 15 min, C18 column) of heteroleptic species obtained by the CDSA of **L1** (1.0 eq.) and **L0** (3.0 eq.) with $[\text{Pd}(\text{MeCN})_4](\text{BF}_4)_2$ (2.0 eq.) in DMSO (1 h, RT). a) Comparison of the chromatogram with the ones of **L0** and **C0_{hom}**, and b) with the ones of **L1** and **C1_{hom}** for identification of peaks.

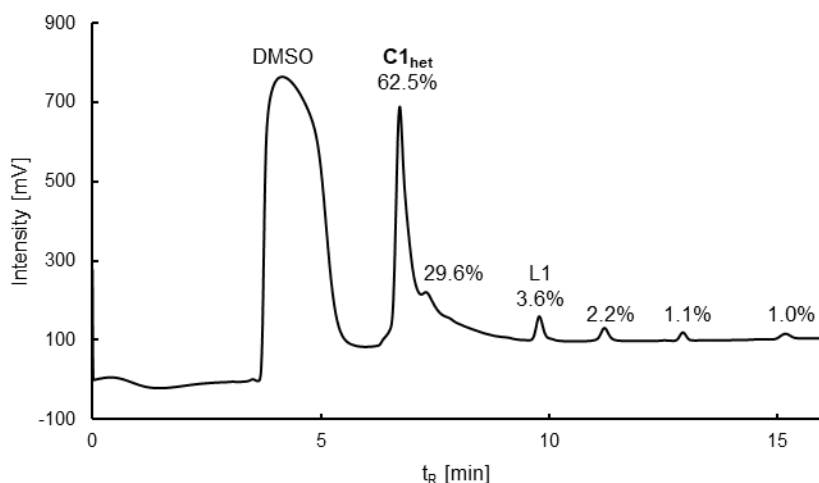
t_R = 3.2 (L0), 6.6 ($\text{Pd}_2\text{L0}_2$), 8.3 (C0_{hom}), 9.4, 11.1 (C1_{het}), 11.5 (L1), 12.7 min ($\text{Pd}_2(\text{L1})_2(\text{L0})_2$).



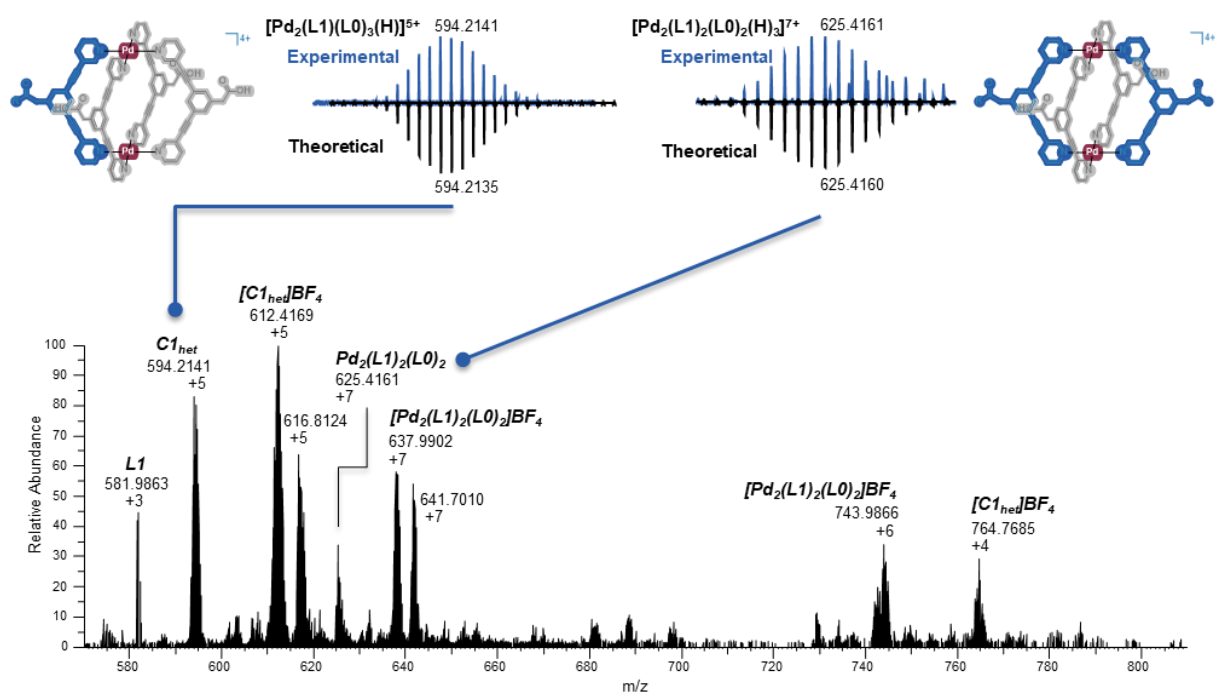
SI Figure 28: RP-HPLC chromatogram (30-60% B in 15 min, C18 column) of **C1_{net}** after isolation.



SI Figure 29: RP-HPLC chromatograms (40-70% B in 15 min, C18 column) of heteroleptic species obtained by 1 h CDSA of $[\text{Pd}(\text{MeCN})_4](\text{BF}_4)_2$ (2.0 eq.) with different ratios of **L1/L0** (2/2, 3/1) in DMSO at RT.

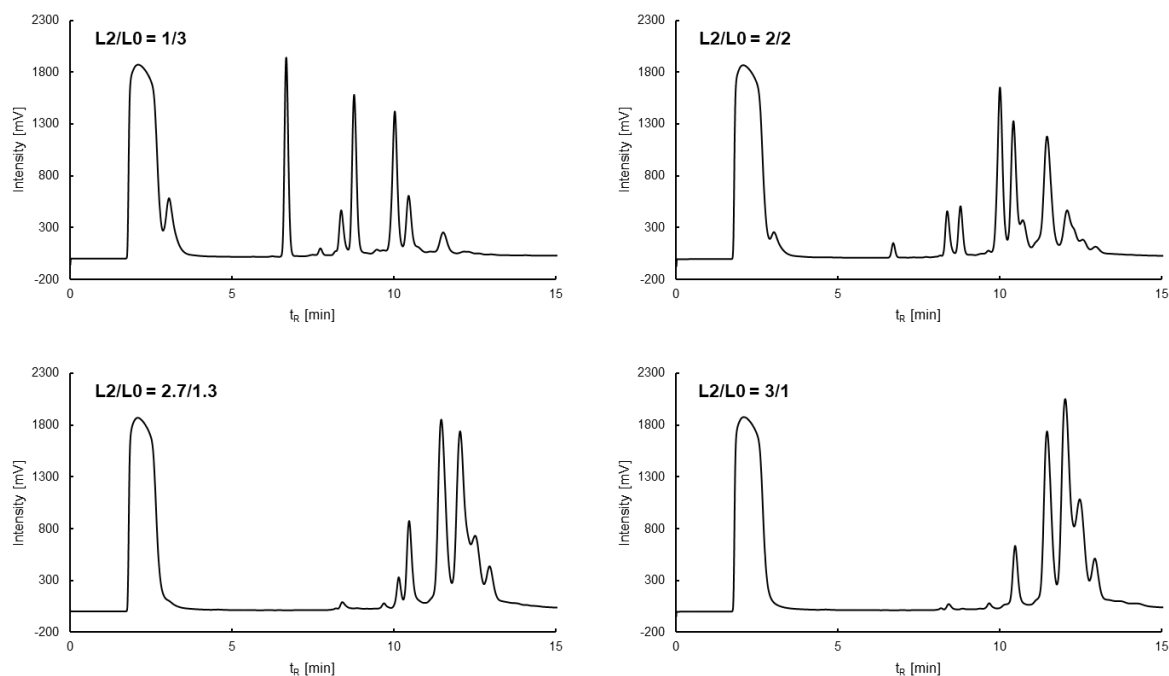
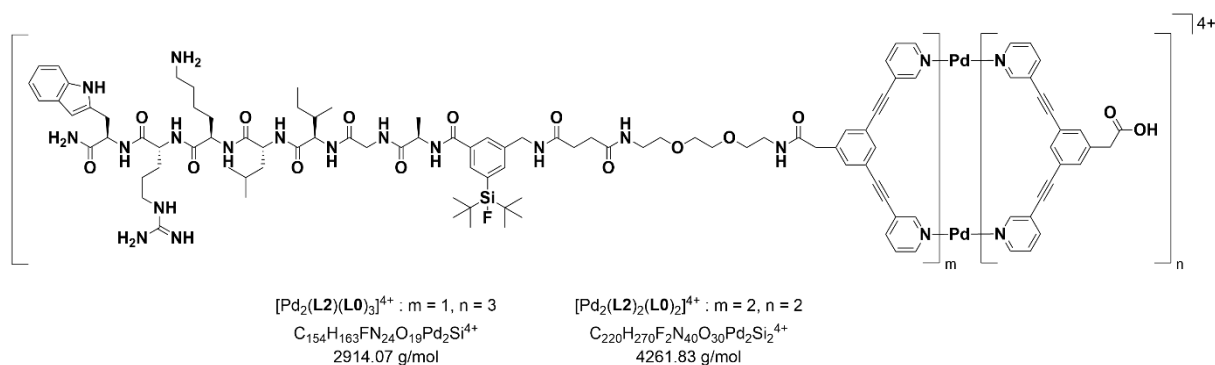


SI Figure 30: RP-HPLC chromatogram (20-40% B in 3 min, 40-80% B in 15 min, 0.5 mL/min, C18 column) of heteroleptic species obtained by the CDSA of **L1** (1.0 eq.) and **L0** (3.0 eq.) with $[\text{Pd}(\text{MeCN})_4](\text{BF}_4)_2$ (2.0 eq.) in DMSO (1 h, RT).

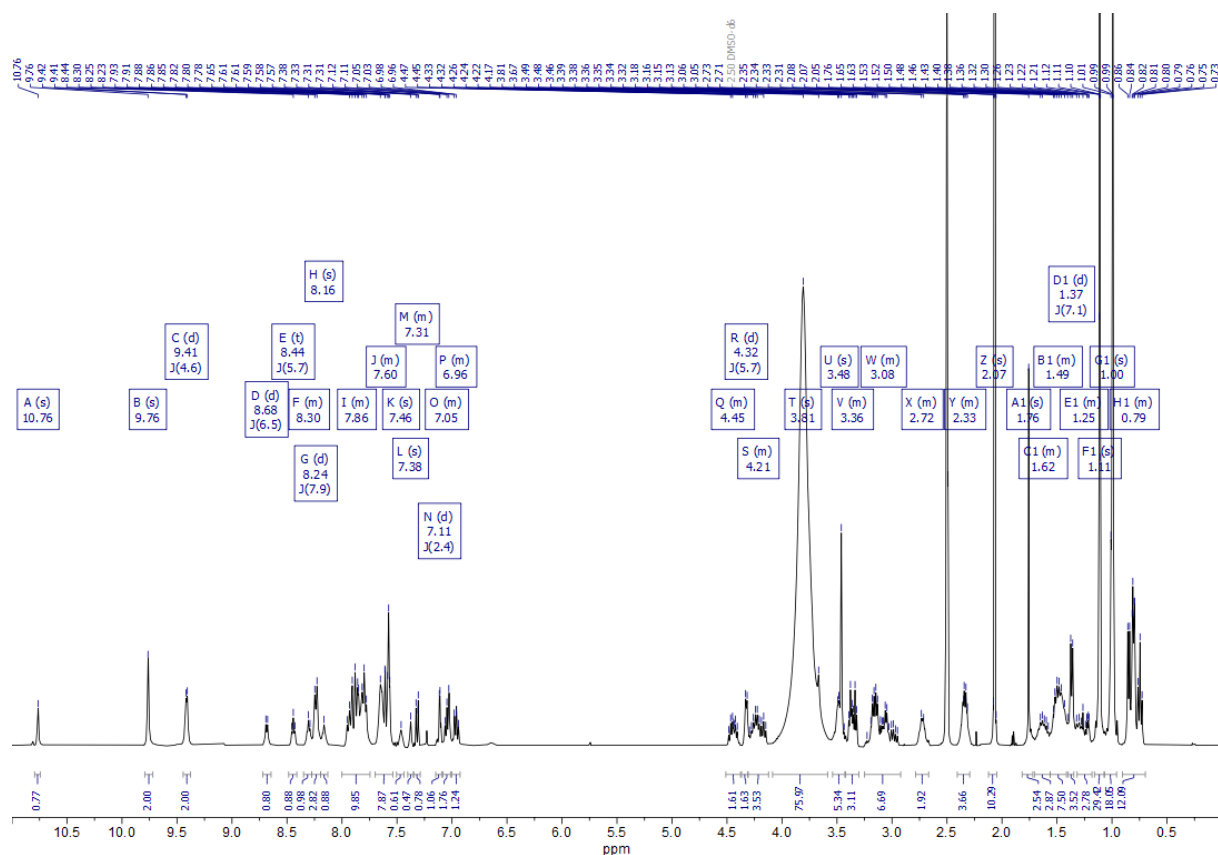


SI Figure 31: DI-HR-ESI-MS of heteroleptic species obtained by the CDSA of **L1** (1.0 eq.) and **L0** (3.0 eq.) with $[\text{Pd}(\text{MeCN})_4](\text{BF}_4)_2$ (2.0 eq.) in DMSO.

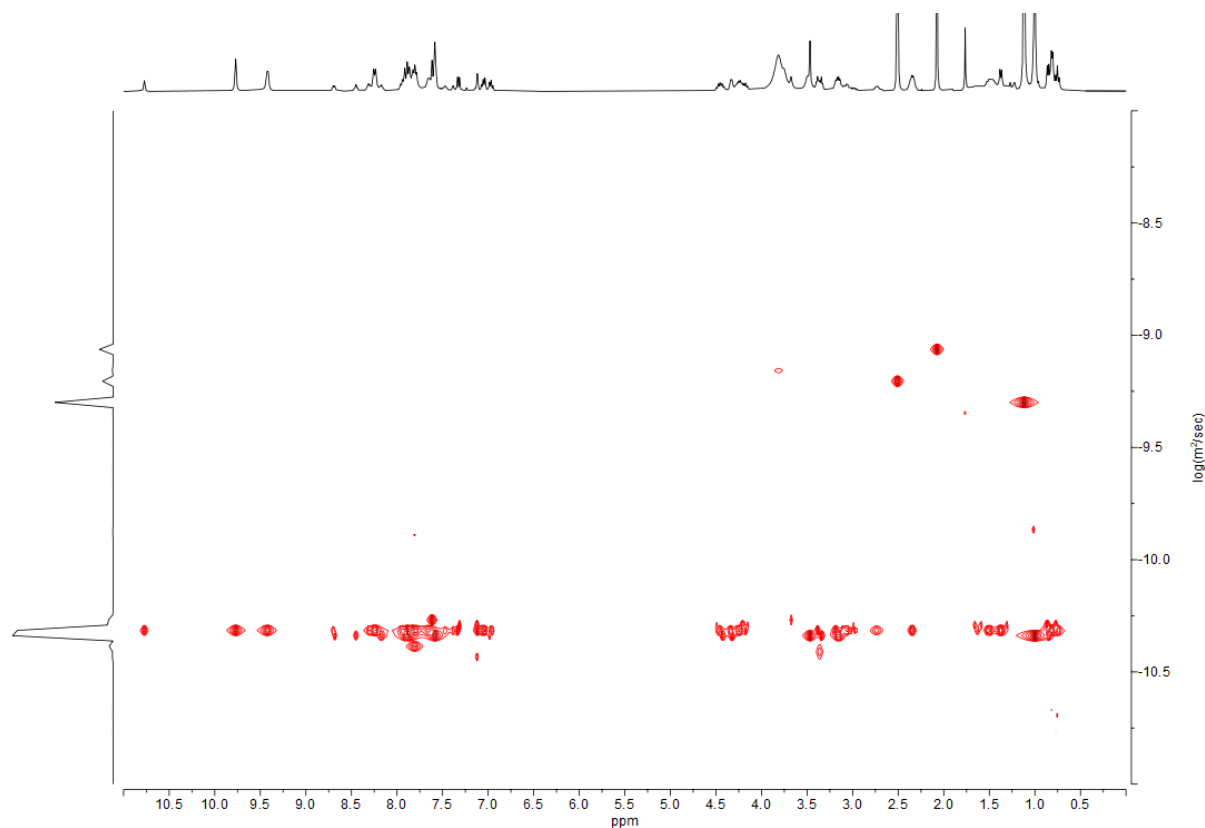
Characterization of $C2_{het}$



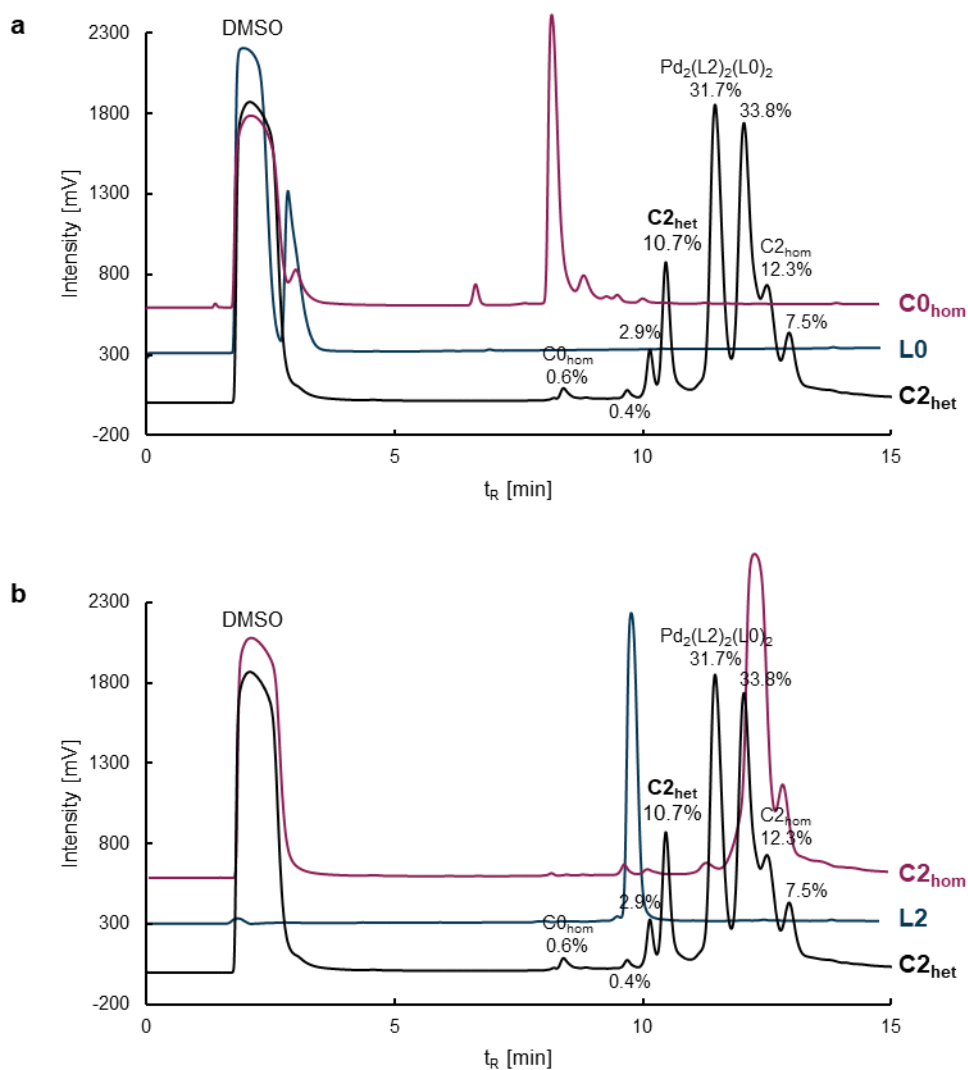
SI Figure 32: RP-HPLC chromatograms (30-60% B in 15 min, C18 column) of heteroleptic species obtained by CDSA of $[Pd(MeCN)_4](BF_4)_2$ (2.0 eq.) with different ratios of **L2/L0** (1/3, 2/2, 2.7/1.3, 3/1) in DMSO (1 h, RT).



SI Figure 33: ^1H NMR spectrum of heteroleptic species obtained by the CDSA of **L2** (2.7 eq.) and **L0** (1.3 eq.) with $[\text{Pd}(\text{MeCN})_4](\text{BF}_4)_2$ (2.0 eq.) measured in $\text{DMSO}-d_6$ at 400 MHz.

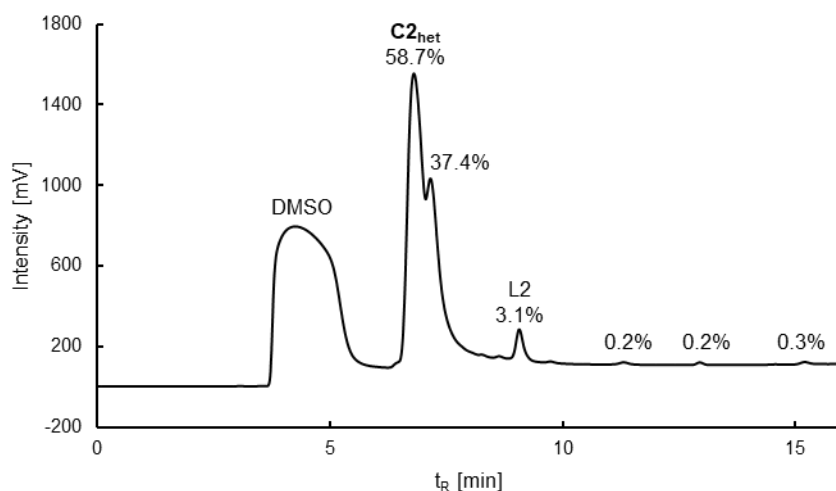


SI Figure 34: ^1H -DOSY NMR spectrum of heteroleptic species obtained by the CDSA of **L2** (2.7 eq.) and **L0** (1.3 eq.) with $[\text{Pd}(\text{MeCN})_4](\text{BF}_4)_2$ (2.0 eq.) measured in $\text{DMSO}-d_6$ at 400 MHz.



SI Figure 35: Comparison of the RP-HPLC chromatograms (30-60% B in 15 min, C18 column) of heteroleptic species obtained by the CDSA of **L2** (2.7 eq.) and **L0** (1.3 eq.) with $[\text{Pd}(\text{MeCN})_4](\text{BF}_4)_2$ (2.0 eq.) in DMSO (1 h, RT). a) Comparison of the chromatogram with the ones of **L0** and **C0_{hom}**, and b) with the ones of **L2** and **C2_{hom}** for identification of peaks.

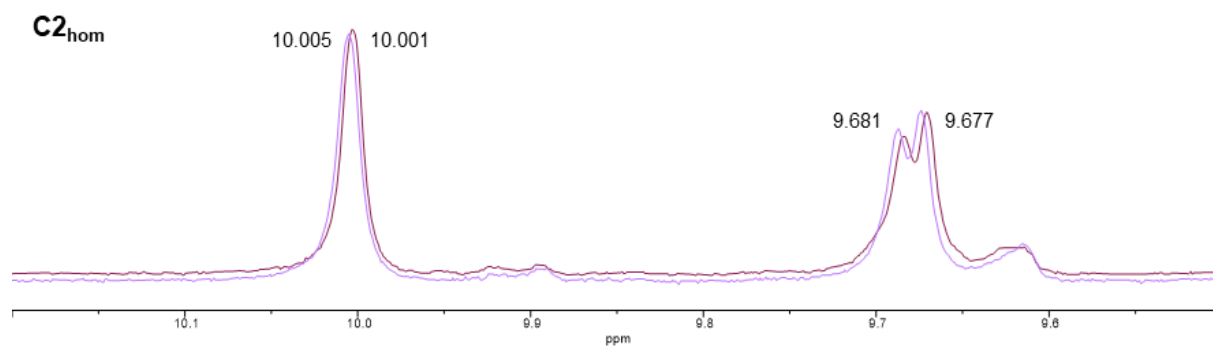
t_R = 10.2, 10.5 (**C2_{het}**), 11.5 ($\text{Pd}_2(\text{L2})_2(\text{L0})_2$), 12.1, 12.6 (**C2_{hom}**), 13.0 min.



SI Figure 36: RP-HPLC chromatogram (20-40% B in 3 min, 40-80% B in 15 min, 0.5 mL/min, C18 column) of heteroleptic species obtained by the CDSA of **L2** (2.7 eq.) and **L0** (1.3 eq.) with $[\text{Pd}(\text{MeCN})_4](\text{BF}_4)_2$ (2.0 eq.) in DMSO (1 h, RT).

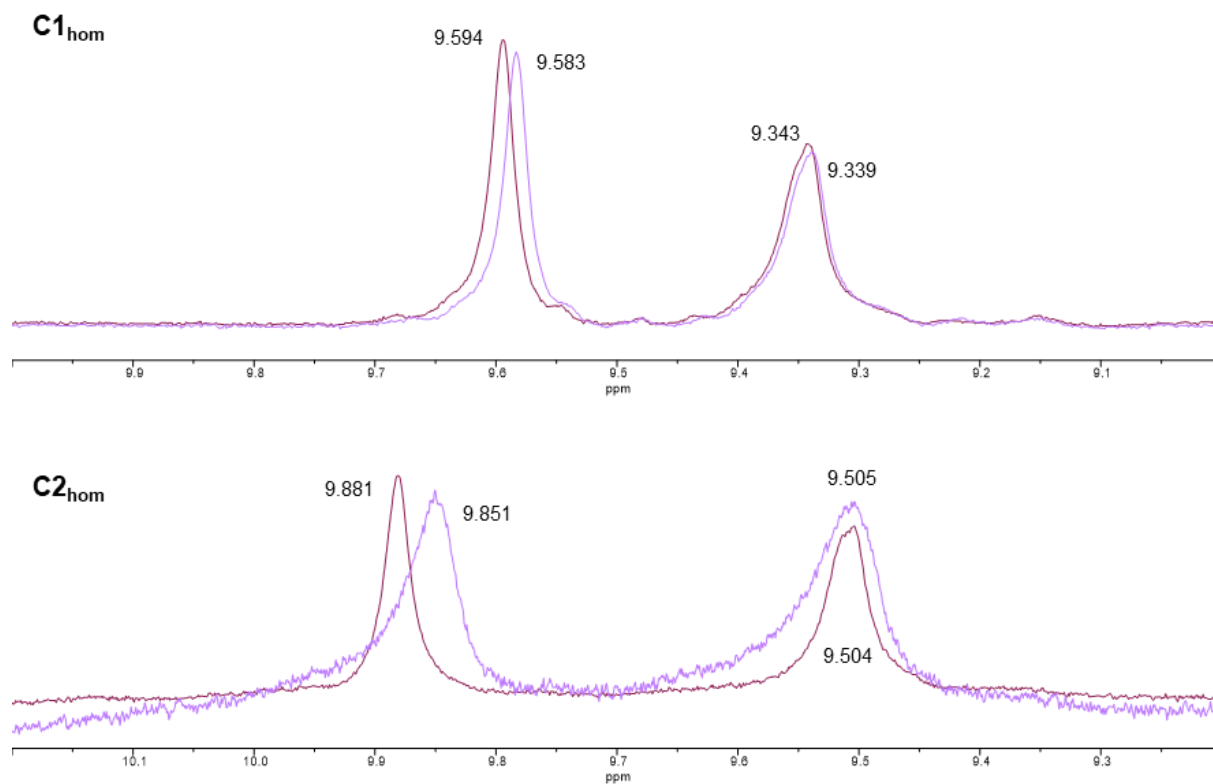
Encapsulation studies

Encapsulation of cisplatin



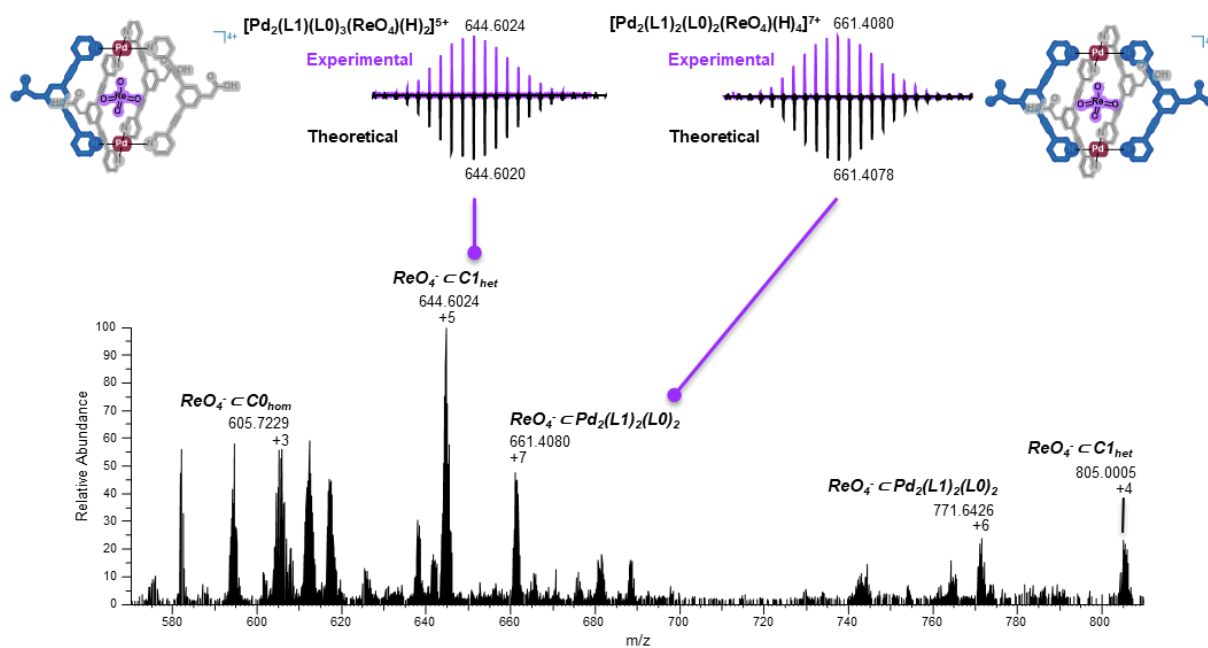
SI Figure 37: Superimposed ^1H NMR spectra of **C2_{hom}** (dark purple) and **C2_{hom}** + 2 eq. cisplatin, 30 min (light purple). The spectra were recorded in $\text{DMF-}d_7$ at 400 MHz.

Encapsulation of perrhenate

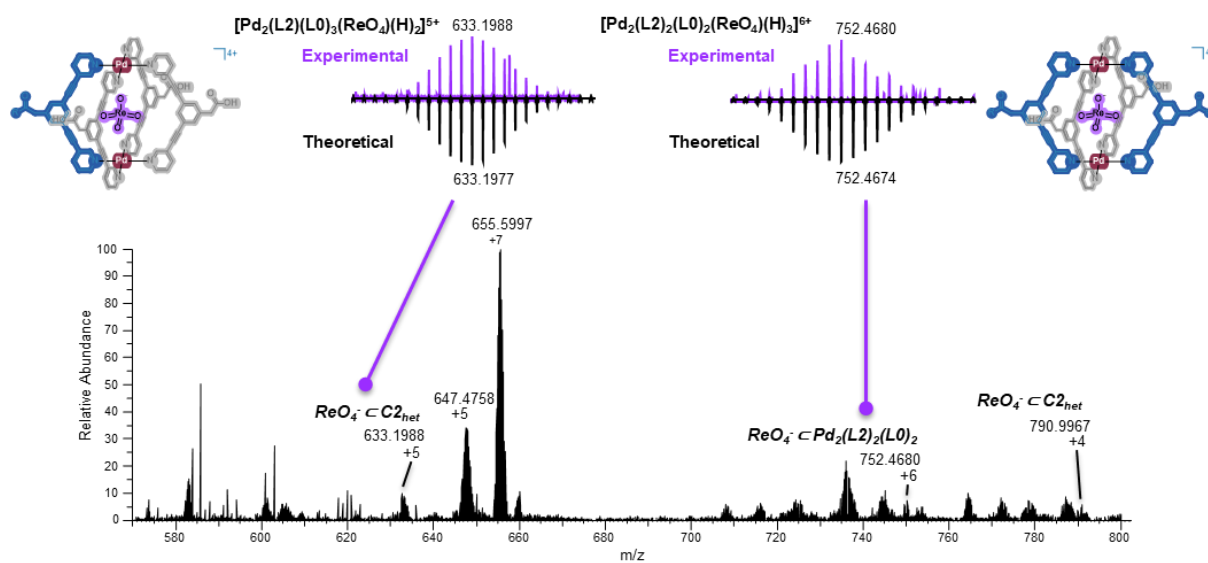


SI Figure 38: ^1H NMR spectra of the cages (dark purple) C1_{hom} (top) and C2_{hom} (bottom) superimposed with the corresponding ^1H NMR spectra of $\text{C}_{\text{hom}} + 2$ eq. NaReO_4 , 30 min (light purple). The spectra were recorded in $\text{DMSO}-d_6/\text{D}_2\text{O}$ (1/1) at 400 MHz.

C1_{het} + 2 eq. NaReO₄

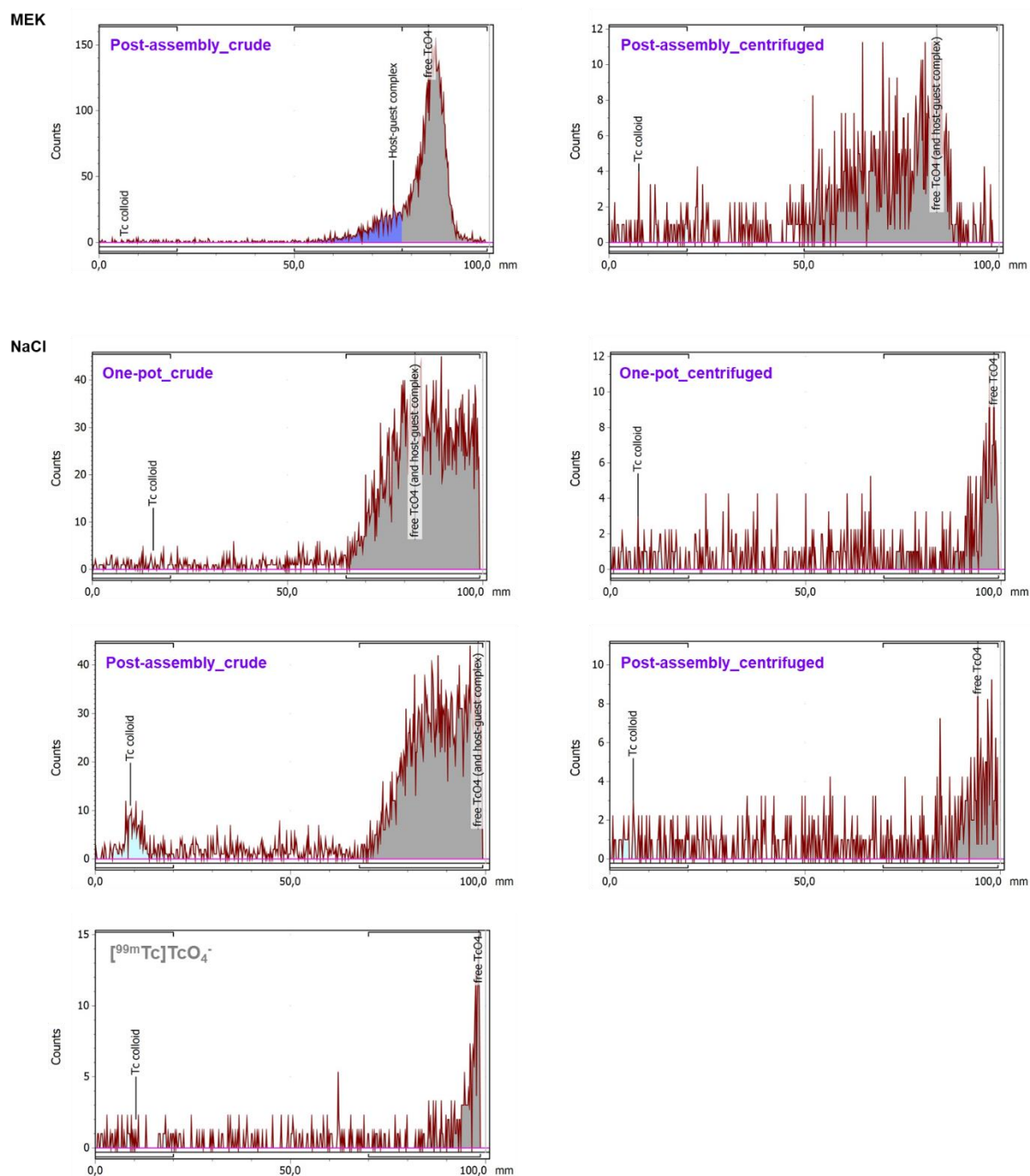


C2_{het} + 2 eq. NaReO₄



SI Figure 39: DI-HR-ESI-MS spectra of **C1_{het}** mixture + 2 eq. NaReO₄ (top) and **C2_{het}** mixture + 2 eq. NaReO₄ (bottom) in DMSO (+72% H₂O). Isotopic pattern comparisons are shown in the graph insets.

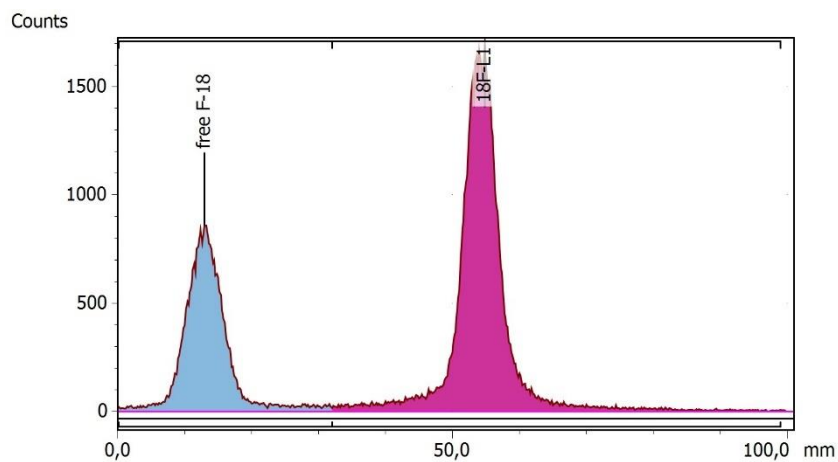
Encapsulation of [^{99m}Tc]pertechnetate



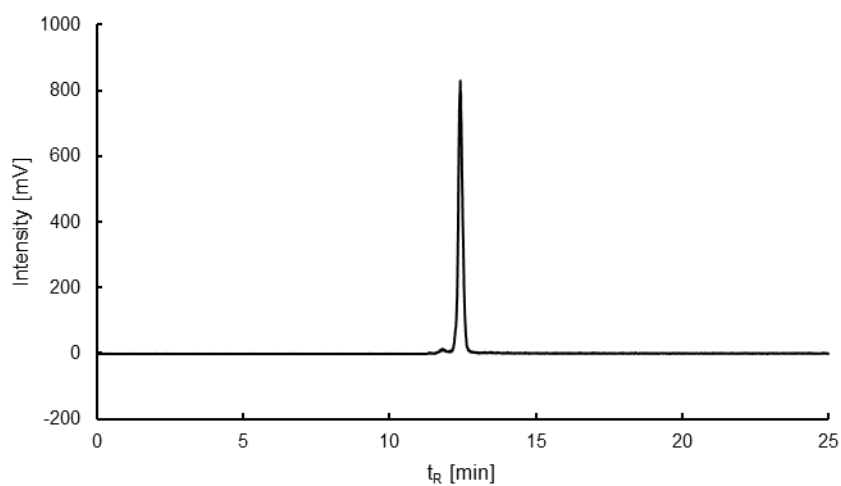
SI Figure 40: Remaining radio TLC chromatograms (run in MEK and saline) of [^{99m}Tc]TcO₄⁻ encapsulation (one-pot and post-assembly) in **C2_{hom}** before and after centrifugation.

Characterization of radiolabeled ligands

^{18}F -L1

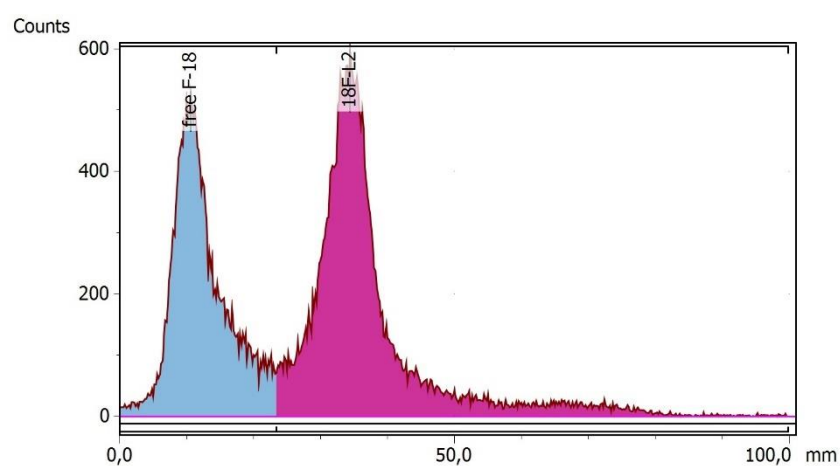


SI Figure 41: Reaction control of ^{18}F -L1 via radio TLC using MeCN/H₂O, 8/2, +10% sodium acetate (2 M), +1% TFA as the solvent system.

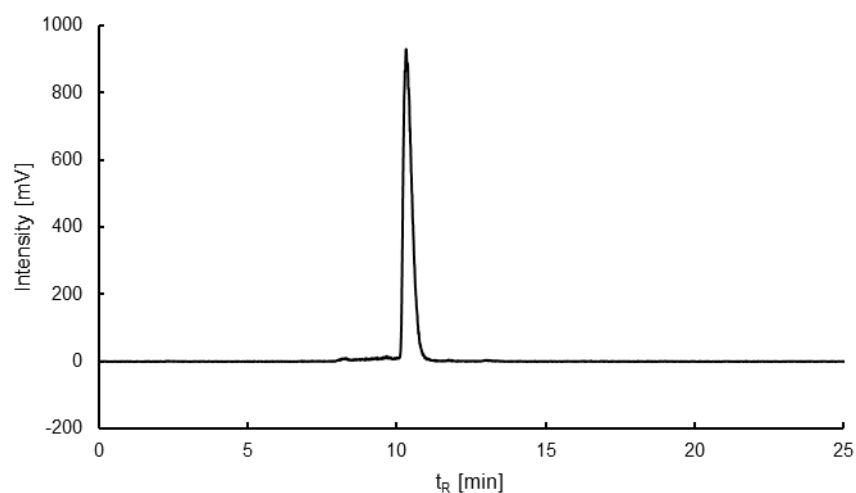


SI Figure 42: Radio RP-HPLC chromatogram of ^{18}F -L1 measured with a gradient of 10-80% B in 15 min on a C18 column.

¹⁸F-L2

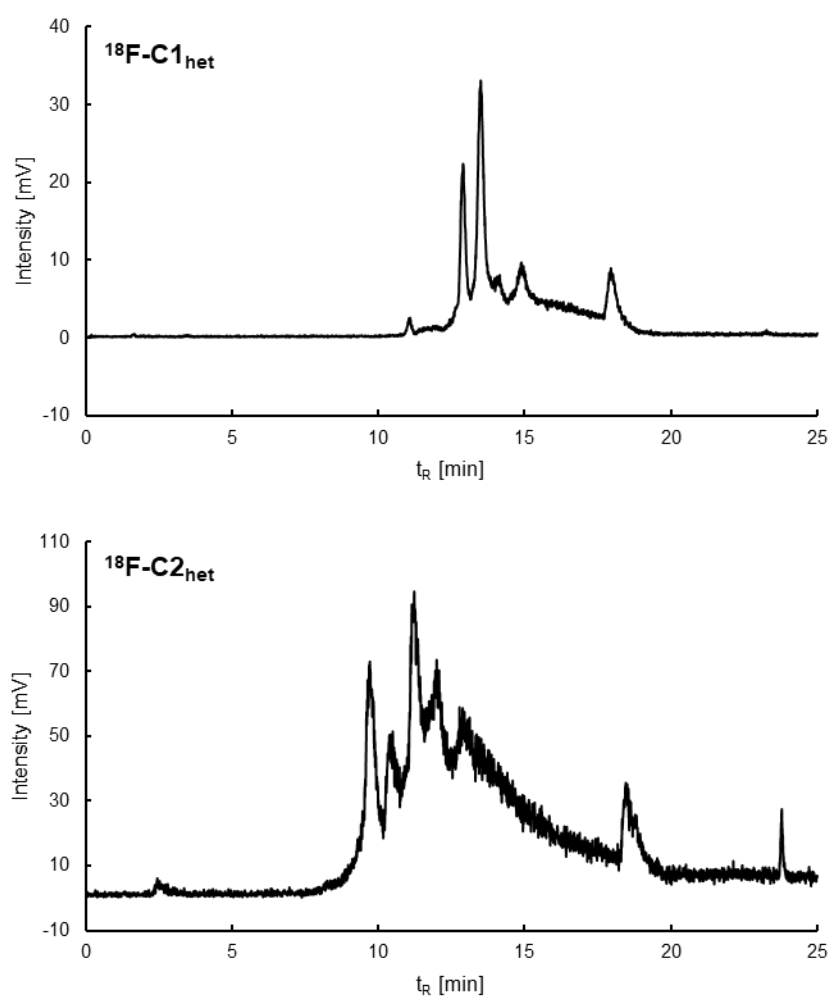


SI Figure 43: Reaction control of ¹⁸F-L2 via radio TLC using MeCN/H₂O, 8/2, +10% sodium acetate (2 M), +1% TFA as the solvent system.

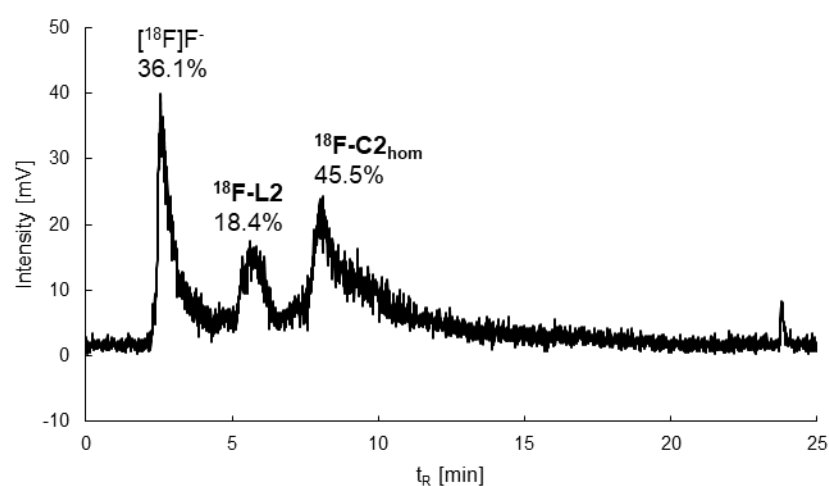


SI Figure 44: Radio RP-HPLC chromatogram of ¹⁸F-L2 measured with a gradient of 30-60% B in 15 min on a C18 column.

^{18}F -labelled MCgs

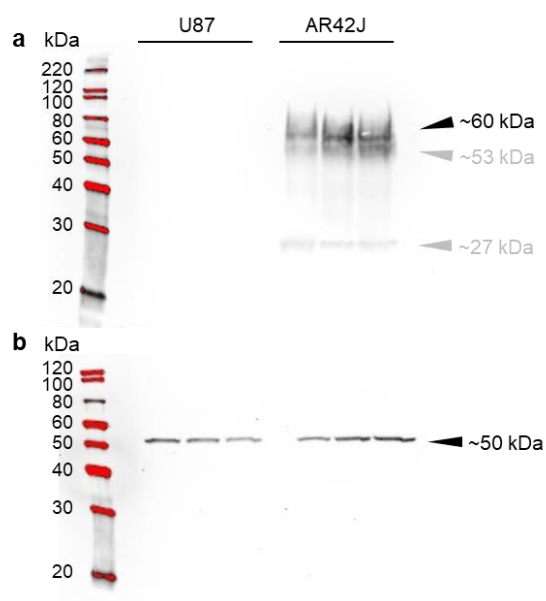


SI Figure 45: Radio RP-HPLC chromatograms (30-60% in 15 min, C4 column) of $^{18}\text{F-C1}_{\text{het}}$ and $^{18}\text{F-C2}_{\text{het}}$ mixtures formed by pre-assembly radiolabelling and 4 h CDSA at RT.

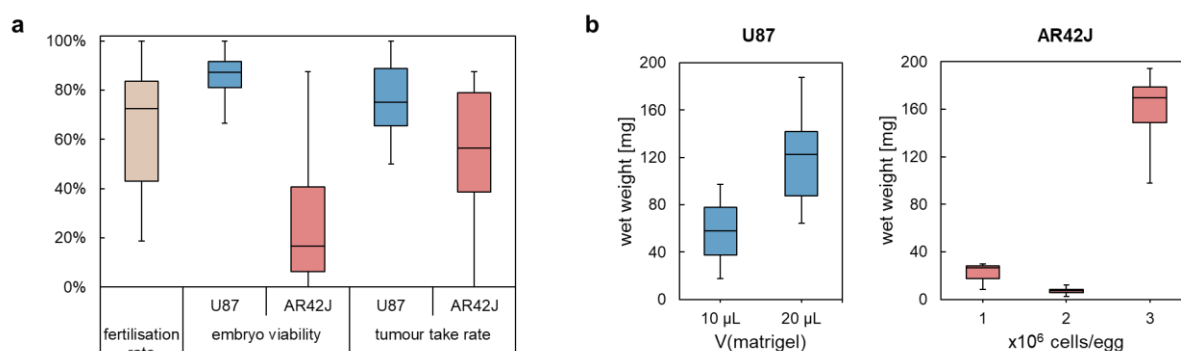


SI Figure 46: Radio RP-HPLC chromatogram (40-70% B in 15 min, C4 column) of $^{18}\text{F-C2}_{\text{hom}}$ after undergoing the logP procedure.

CAM Methodology



SI Figure 47: Full size Western blots of a) sstR2 expression in U87 and AR42J cell lysates and b) α -tubulin control for U87 and AR42J lysates. Main bands are labelled in black. As the ladder was used Magic Mark™ XP Western Protein Standard.



SI Figure 48: a) Box and whiskers plots of monitoring egg fertilization as well as embryo viability and tumour take rate upon inoculation of U87 and AR42J cells. b) Box and whiskers plots of U87 and AR42J tumour growth optimization.