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EXPLORING THE CHELATION OF THERANOSTIC RADIOMETALS WITH SULFUR-BEARING POLYAZAMACROCYCLES

When properly harnessed, the radiation emitted by a metallic radioisotope can be employed to diagnose, monitor and treat cancer. To securely deliver the radiation to the disease site, the stable coordination of the radiometal to a bifunctional chelator, covalently coupled to a disease targeting molecule, is imperative. Herein we describe our effort to develop a class of sulfur bearing macrocycles as potential platforms for the chelation of non-standard theranostic borderline/soft radiometals.

Introduction

Radiometals have provided a breakthrough in cancer imaging and treatment due to their variety of emission profiles and half-life. Short-range and highly cytotoxic α, β^- and Meitner-Auger electrons emitters can be properly harnessed to destroy small or diffuse tumours. Exploiting either γ rays or annihilation photons, produced by β^+-emitters, the therapeutic efficacy and the disease progression can be monitored using imaging techniques such as single photon emission tomography (SPECT) and positron emission tomography (PET) **[1]**.

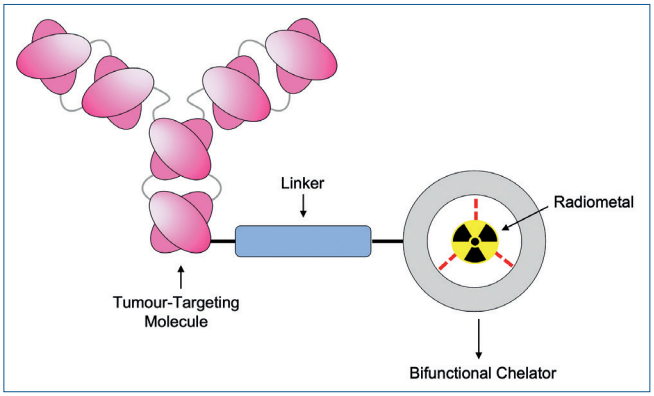

Fig. 1 - Depiction of radiometal-based radiopharmaceutical

A pillar component of radiometal-based radiopharmaceuticals is a bifunctional chelator (BFC) covalently appended to a tumor-targeting biomolecule that selectively accumulates into specific disease sites (Fig. 1) **[1, 2]**. The BFC is used to securely bind the radiometal and ensures proper delivery of the emitted radiation solely to the area of interest. The *in vivo* released radionuclide would be rapidly taken up the non-target organs (*e.g.*, kidney, liver) resulting in an unwanted radiation burden to healthy sites **[1, 2]**. Hence, it is imperative that the BFCs form thermodynamically stable and kinetically inert complexes with the radioisotope of interest to prevent *in vivo* dissociation and/or competition reactions (transmetallation and/or transchelation) **[1-3]**. BFCs not only coordinate the metal radionuclide, but they offer an anchoring point for the incorporation of the disease-targeting moiety. Moreover, they can be exploited to modulate the pharmacokinetic profile

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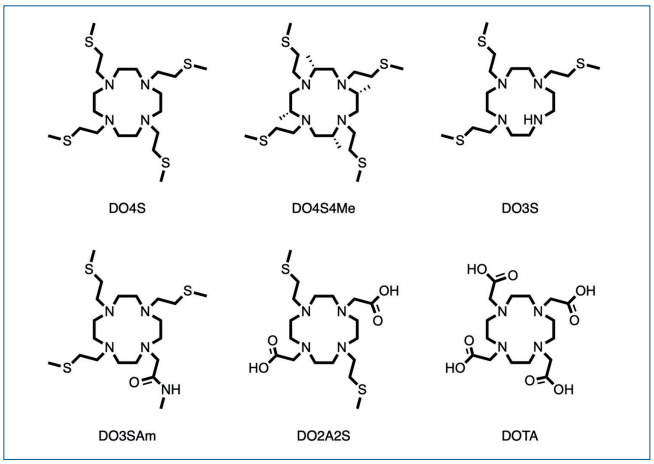

Fig. 2 - Chemical structures of the ligands investigated in this work: 1,4,7,10-tetrakis(2-(methylsulfanyl)ethyl)-1,4,7,10-tetraazacyclododecane (DO4S), (2S,5S,8S,11S)-2,5,8,11-tetramethyl-1,4,7,10-tetrakis(2-(methylsulfanyl)ethyl)-1,4,7,10-tetraazacyclododecane (DO4S4Me), 1,4,7-tris(2-(methylsulfanyl) ethyl)-1,4,7,10-tetraazacyclododecane (DO3S), 1,4,7-tris(2-(-methylsulfanyl)ethyl)-10-acetamido-1,4,7,10-tetraazacyclododecane (DO3SAm), and 1,7-bis(2-(methylsulfanyl)ethyl)-4,10,diacetic acid-1,4,7,10-tetraazacyclododecane (DO2A2S) and 1,4,7,10-tetrazacyclododecane 1,4,7,10-tetracetic acid (DOTA)

Ligand	Equilibrium reaction[a]			
	$Ag^+ + L \rightleftharpoons AgL^+$	$Ag^+ + H^+ + L \rightleftharpoons AgHL^{2+}$	$Ag^+ + 2H^+ + L^{2-} \rightleftharpoons AgH_2L^+$	pAg+[b]
DO4S	16.51±0.03 16.9±0.1[c]	21.03±0.04	-	15.3
DO4S4Me	18.00±0.07 17.9±0.2[c]	20.76±0.01	-	14.5
DO3S	16.12±0.01 15.81±0.09[c]	22.09±0.04	-	13.3
DO3SAm	15.48±0.05	20.16±0.05	-	12.9
DO2A2S	13.71±0.06	19.63±0.06 19.6±0.06[c]	22.82±0.09 23.2±0.5[c]	11.2
DOTA	9.1±0.2	16.6±0.2	-	6.9
Cyclen	6.60±0.02	-	-	6

[a]L denotes the ligand in its totally deprotonated form
[b]Calculated at $C_{Ag} = 10^{-6}$ mol/L and $C_L = 10^{-5}$ mol/L, pH 7.4
[c]Obtained by UV-Vis spectrophotometric titrations
The reported uncertainty was obtained by the fitting procedure and represents one standard deviation unit

Tab. 1 - Equilibrium constants (logβ) for the Ag^+-complexes with the investigated ligands at T = 25 °C and I = 0.15 M $NaNO_3$. Unless otherwise state, logβ values were obtained by potentiometry

of the resulting molecule, especially when the latter possess low molecular weight.

Silver Complexes in Nuclear Medicine

Among the non-conventional metallic radioisotopes, ^{111}Ag ($t_{1/2}$ 7.47 days) is a promising candidate for targeted cancer therapy as well as for associated SPECT imaging due to its co-emission of medium-energy β^- particles and γ rays **[4]**. The β^+-emitters ^{103}Ag ($t_{1/2}$ 65.7 min) or ^{104}Ag ($t_{1/2}$ 69.2 min) could be used as PET imaging analogues **[3]**. The theranostic approach of using $^{103/104}Ag$ and ^{111}Ag could bring a step towards personalized medicine, where low dose scouting scans to obtain dosimetry information, followed by higher dose therapy in the same patient, could be performed.

Despite its inherent potential, no previous research has investigated $^{103/104/111}Ag$ for targeted radionuclide therapy or nuclear imaging: to boost its clinical applications, suitable ligands that can act as BFCs forming sufficiently stable Ag^+ complexes under *in vivo* conditions have still to be developed **[3]**.

For this purpose, we have designed and developed a series of *N*-functionalized 1,4,7,10-tetraazacyclododecane (cyclen) derivatives bearing soft sulfur donor arms, inserted to fulfil the binding preference dictated by the chemical softness of Ag^+ (Fig. 2) **[3, 5]**.

To evaluate the potential of the proposed ligands as BFCs for silver-based radiopharmaceuticals, we have explored the thermodynamic and the structural properties of their Ag^+ complexes. The bare macrocycle, cyclen, and DOTA, a commonly employed chelator in nuclear medicine applications, were also considered in our study for comparison purposes.

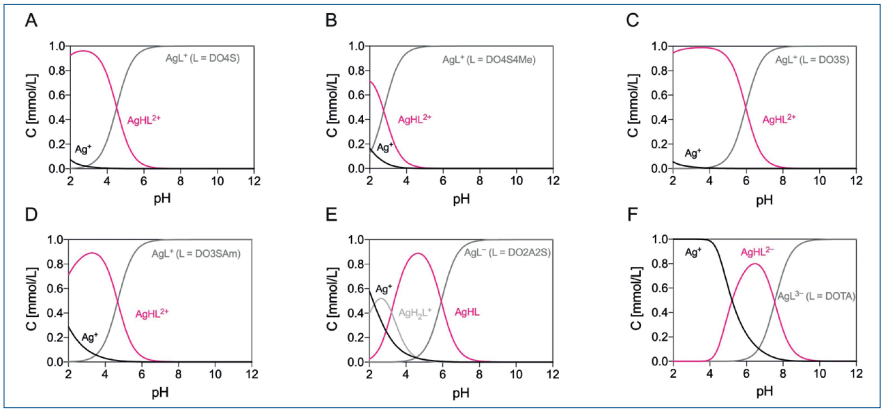

Fig. 3 - Distribution diagrams of the Ag^+ complexes formed by (A) DO4S, (B) DO4S4Me, (C) DO3S, (D) DO3SAm, (E) DO2A2S and (F) DOTA ($C_{Ag} = C_{ligand} = 1 \cdot 10^{-3}$ M)

Combined pH- and pAg-potentiometric and UV-Vis spectrophotometric experiments demonstrated that these ligands form highly stable 1:1 metal-to-ligand complex, AgL (L denotes the completely deprotonated ligand), at physiological pH **[3]**. The complex stability was remarkably high also in acidic solutions where protonated species (*e.g.,* AgHL and/or AgH_2L) predominate **[3]**. The determined equilibrium constants are summarized in Tab. 1 while the distribution diagrams are shown in Fig. 3.

As already discussed, the formation of highly stable complexes with the radionuclide of interest is of utmost importance for medical application. In order to compare the Ag^+ complex stability of the examined ligands, our thermodynamic data were used to compute the pAg^+ values ($pAg^+ = -\log[Ag^+]$) as this parameter takes into account the influence of ligand basicity (not described herein; the interested reader is referred to **[5]**) and metal-ion hydrolysis: the higher the pAg^+, the stronger the complex (Tab. 1).

Ligands bearing four sulfide arms, DO4S and DO4S4Me, demonstrated to form the most stable complexes. A relatively small pAg^+ difference was observed between DO4S and DO3S/DO3SAm: this could be explained by structural factors as only two among the four sulfur atoms can simultaneously coordinate Ag^+ in DO4S. The highest stability displayed by the compounds bearing four sulfide arms can thus be attributed to statistical effects taking place in the presence of the fourth thioether chain which promotes the complexation. The molecule with only two sulfanyl arms (DO2A2S) forms Ag^+ complexes which are less stable than those bearing three arms, but its pAg^+ values at physiological pH are still higher than those of DOTA and cyclen, pointing out the utmost impact of S on Ag^+ stabilization **[3]**.

NMR measurements and Density Functional Theory (DFT) calculations gave structural insight into the Ag^+ complexes structure (the interested reader is referred to **[3]**).

The ability of the ligands forming the most stable complexes with Ag^+ (DO4S and DO4S4Me) to label $[^{111}Ag]Ag^+$ was assessed. Both showed high specificity for the radiometal, gave quantitative incorporation at mild reaction conditions and the resulting radio-complexes possessed high stability and inertness as well **[3]**.

The obtained results indicate that the proposed series of ligands are promising candidates for the chelation of silver radioisotopes: these premises could open the way toward preclinical application of silver as a theranostic radionuclide when bound to biological vectors.

Copper in Nuclear Medicine

Copper is another element that has attracted great interest in recent years, because of its several radioisotopes with half-life and emission properties suitable for diagnostic and therapeutic applications **[6, 7]**. ^{64}Cu ($t_{1/2}$ 12.7 h) is suitable for both PET imaging and cancer therapy due to its unique decay profile, which combines β^+, β^- and electron capture emissions. Furthermore, ^{64}Cu can provide a matched PET imaging pair with the pure β^- emitter ^{67}Cu ($t_{1/2}$ 61.9 h) **[4]**. However, the inertness of $^{64/67}Cu^{2+}$-complexes *in vivo* can be thwarted by the biologically triggered redox switching between Cu^{2+} and Cu^+ that may bring to demetallation processes **[4]**.

As the ligands proposed herein incorporate both N and S donors, they can potentially stabilize the two oxidation states of copper, thus preventing the reductive-induced demetallation pathway. To assess this strategy, we have investigated the kinetic, thermodynamic and structural properties of the Cu^{2+} and Cu^+ complexes of the ligands shown in Fig. 2. The slow equilibration at acidic pH combined with the high stability of the Cu^{2+} complexes hampered the determination of the equilibrium constants by conventional pH-potentiometry. Therefore, UV-Vis

Ligand	Cu^{2+}			Cu^+		
	Equilibrium reaction[a]	logβ	pCu^{2+d}	Equilibrium reaction[a]	logβ	pCu^{+d}
DO4S	$Cu^{2+} + L \rightleftharpoons CuL^{2+}$	19.8±0.1[b] 19.6±0.4[c]	17.7	$Cu^{+} + L \rightleftharpoons CuL^{+}$	19.8±0.2	17.2
DO3S	$Cu^{2+} + L \rightleftharpoons CuL^{2+}$	20.34±0.06[b] 20.10±0.08[c]	17.5	$Cu^{+} + L \rightleftharpoons CuL^{+}$	17.2±0.2	14.5
DO3SAm	$Cu^{2+} + L \rightleftharpoons CuL^{2+}$	19.8±0.2[b] 19.7±0.2[c]	17.2	-	-	-
DO2A2S	$Cu^{2+} + H+ + L^{2-} \rightleftharpoons CuHL^{+}$ $Cu^{2+} + L^{2-} \rightleftharpoons CuL$	24.22±0.09[b] 22.05±0.3[c] 21.9±0.2[b]	19.4	$Cu^{+} + L^{2-} \rightleftharpoons CuL^{-}$	16.7±0.1	14.1

[a]L denotes the ligand in its totally deprotonated form
[b]Obtained by UV-Vis spectrophotometric titrations
[c]Obtained by Ag^+-Cu^{2+} competition (no ionic strength control)
[d]Calculated at $C_{Cu} = 10^{-6}$ mol/L and $C_L = 10^{-5}$ mol/L and pH 7.4
The reported uncertainty was obtained by the fitting procedure and represents one standard deviation unit

Tab. 2 - Overall stability constants (logβ) of the $Cu^{2+/+}$ complexes with the investigated ligands at *I* = 0.15 mol/L NaCl and T = 25 °C **[4]**

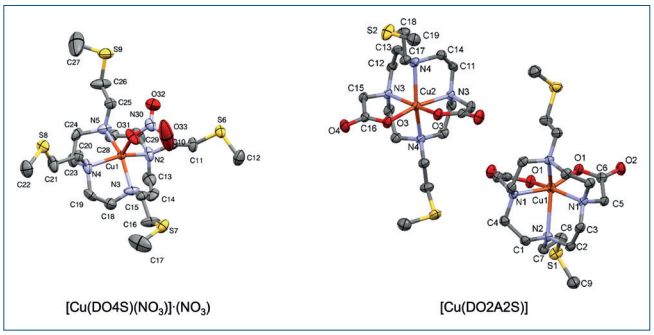

Fig. 4 - X-Ray crystal structure of [Cu(DO4S)(NO_3)]·(NO_3) and [Cu(DO2A2S)] [4]

spectrophotometric out-of-cell and in-cell titrations, potentiometric measurements (at pH>4), and spectrophotometric Ag^{+}-Cu^{2+} competition experiments were performed [4]. According to both spectrophotometric and potentiometric data, the formation of the CuL complexes in the entire pH range was evidenced with the pure sulfur bearing ligands. For Cu^{2+}-DO2A2S, the formation of this species was also confirmed, but also the monoprotonated complex, $CuLH^{+}$, was detected at pH below 4 [4]. The overall stability constants determined are given in Tab. 2.

To compare the stability of the Cu^{2+} complexes formed by different ligands, the pCu^{2+} ($pCu^{2+} = -\log[Cu^{2+}]_{free}$) at the biologically relevant pH of 7.4 was computed (Tab. 2). The obtained results revealed that the investigated ligands form highly stable Cu^{2+} complexes, with a pCu^{2+} value higher or comparable to those of the well-known $^{64/67}Cu^{2+}$ chelators NOTA, DOTA, and TETA (*e.g.*, $pCu^{2+}_{NOTA} = 18.2$; $pCu^{2+}_{DOTA} = 17.7$; $pCu^{2+}_{TETA} = 16.2$) [4].

The solution structure of the Cu^{2+} complexes was investigated using a combination of UV-Vis and Electron Paramagnetic Resonance (EPR) spectroscopies and supported by DFT calculations [4]. For Cu^{2+}-DO4S, Cu^{2+}-DO3S, Cu^{2+}-DO3SAm, the copresence of isomers having either no ([4N]) or one coordinated sulfide atom ([4N]S_{ax}) was found [4]. With DO4S, a crystal suitable for X-Ray diffraction was obtained. In the solid-state, each Cu^{2+} ion is surrounded by four N of the macrocyclic ring and a nitrate anion in a square pyramidal geometry in a structure that resembles that of the [4N] isomer detected in solution (Fig. 4) [4]. As for Cu^{2+}-DO2A2S, the same coordination as for Cu^{2+}-DOTA was found at pH values above 4 ([2N,2O]$2N_{ax}$) which is maintained in the solid state (Fig. 4). The Cu^{2+}-DO2A2S structure changed at acidic pH, when the carboxylic arms are protonated, as one sulfur atom replace all carboxylates in the metal ion binding [4].

To gain insight into the ability of the Cu^{2+} complexes to withstand bioreductive-induced decomplexation *in vivo*, cyclic voltammetry (CV) studies were perf ormed. The CV analyses indicated that no demetallation occurs when Cu^{2+} is reduced to Cu^{+}, with all ligands being able to accommodate both copper oxidation states [4]. This study has also allowed to calculate the stability constants of the Cu^{+} complexes (Tab. 2). The long-term stability was evaluated by generating *in situ* the Cu^{+} complexes by electrolysis. The NMR characterization of the latter, combined with DFT calculations, indicated that all ring nitrogens and one rapidly exchanging sulfur are present in the metal coordination sphere of both Cu^{+}-DO4S and Cu^{+}-DO2A2S, thus pointing out that for the latter a coordination sphere switching occurred when Cu^{2+} was reduced to Cu^{+} [4]. The ability to stabilize cupric as well as cuprous ions makes these chelators a promising scaffold for $^{64}Cu/^{67}Cu$ complexation.

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Esplorando la chelazione di radiometalli teranostici con poliazamacrocicli solforati

Se adeguatamente sfruttate, le radiazioni emesse da un radioisotopo metallico possono essere impiegate per diagnosticare, monitorare e curare il cancro. Per fornire in modo mirato la radiazione al sito della malattia, è necessaria la coordinazione stabile del radiometallo con un chelante bifunzionale, accoppiato covalentemente ad una molecola direzionante. Qui descriviamo il nostro tentativo di sviluppare una classe di macrocicli contenenti zolfo come potenziali sistemi per la chelazione di radiometalli *borderline/soft* non convenzionali.