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METAL CARBONYLS FOR BIO-IMAGING AND -SENSING

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Abstract

Metal carbonyls, a class of organometallic complexes that consist of carbon monoxide (CO) ligands coordinated to a metal center, show intense CO vibrational frequencies in the mid-infrared (IR) 2200-1800 cm^{-1} . This region is unique to the metal carbonyl species and is typically free of interference by other biomolecules. This property can be put to good use for mid-IR bio-sensing, which was first demonstrated by Jaouen and co-workers in the development of IR-based quantitative assays for detecting various biomolecules.

In this thesis, the concept is extended to surface-enhanced Raman spectroscopy (SERS) through the development of metal carbonyl-based Raman reporters. The conjugation of two classes of metal carbonyls, namely, metal carbonyl clusters and mononuclear half-sandwich metal carbonyl complexes, onto colloidal gold nanospheres gave good CO vibrational stretches in the 1800-2200 cm^{-1} region. Alterations to the ligand sphere and the charge (for cluster complexes) were found to be effective in tuning the CO vibrational stretches over a wide frequency range. It was also observed that the SERS enhancement was highly dependent on the extent of aggregation of the colloid.

In another extension, a SERS-active planar substrate was utilized in tandem with a triosmium carbonyl cluster-boronic acid conjugate acting as a secondary saccharide probe, in a sensitive and selective SERS-based glucose assay. While investigating extension of this idea to the detection of cancer cells via their surface glycans, thiolato triosmium carbonyl clusters of the type $\text{Os}_3(\mu\text{-H})(\mu\text{-SAryl})(\text{CO})_{10}$ were found to be cytotoxic. Cell death was found to be via the induction of apoptosis.

Metal carbonyl clusters were also demonstrated to be good contrast agents for photoacoustic (PA) imaging. The cluster nuclearity was shown to be a useful parameter for fine-tuning the absorption wavelength, as increasing the cluster nuclearity shifted the absorption into the near-IR region. The high nuclearity carbonyl cluster $[\text{Os}_{10}(\mu_6\text{-C})(\text{CO})_{24}]^{2-}$ was shown to exhibit good PA properties in the NIR region, and its sodium salt was applied successfully for the whole body PA imaging of live mice.

Abbreviations and Conventions

ACN	Acetonitrile
AuNS	Gold nanospheres
BA	Boronic acid
BMFON	Bimetallic film over nanospheres
Cp	Cyclopentadienyl (C ₅ H ₅)
Cp*	Pentamethylcyclopentadienyl (C ₅ Me ₅)
CV	Cyclic voltammetry
Cy	Cyclohexanyl
DCM	Dichloromethane
DMF	Dimethylformamide
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
ESI	Electrospray ionization
EtOAc	Ethyl acetate
Fc	Ferrocene
FITC	Fluorescein isothiocyanate
HNCC	High nuclearity carbonyl cluster
HOMO	Highest occupied molecular orbital
HRMS	High resolution mass spectroscopy
IC ₅₀	50% Inhibitory concentration
IR	Infrared
LCMS	Liquid chromatography-mass spectrometry
LUMO	Lowest unoccupied molecular orbital
MSOT	Multi-spectral optoacoustic tomography
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
<i>m/z</i>	Mass-to-charge ratio
NMR	Nuclear magnetic resonance
PA	Photoacoustic
PEG	Polyethylene glycol
Ph	Phenyl
PPN	Bis(triphenylphosphine)iminium [(PPh ₃) ₂ N] ⁺
PS- <i>b</i> -PAA	Polystyrene- <i>block</i> -poly(acrylic acid)
R _f	Retention factor
RT	Room temperature
SERS	Surface-enhanced Raman spectroscopy
TEM	Transmission electron microscopy
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMNO	Trimethylamine N-oxide
μ	Bridging

Vibrational spectroscopy (IR and SERS)

ν_{CO}	Stretching frequency in the carbonyl region (2200-1800 cm^{-1})
vw	Very weak
w	Weak
m	Medium
s	Strong
vs	Very strong
br	Broad
sh	Shoulder

Nuclear magnetic resonance (NMR) spectroscopy

δ	Chemical shift
ppm	Parts per million
$\{^1\text{H}\}$	Protons decoupled
s	Singlet
d	Doublet
dt	Doublet of triplet
t	Triplet
m	Multiplet
COSY	Correlation spectroscopy

Molecule Numbering Scheme

The metallic frameworks of all molecular structures are drawn in outline only. The short line extending from the metal atom (in the molecular structure diagrams) represents a coordinate bond from carbon monoxide to the metal center (M-CO). This is illustrated in the example below:

Molecule	Molecular Formula	Molecular Structure
1	$\text{Os}_3(\text{CO})_{10}(\text{NCCH}_3)_2$	
2	a: $\text{Os}_3(\text{CO})_{10}(\mu\text{-H})(\mu\text{-S}(\text{CH}_2)_2\text{SH})$ $\text{Os}_3(\text{CO})_{10}(\mu\text{-H})(\mu\text{-SC}_6\text{H}_4\text{X})$ b: X = B(OH) ₂ = H = OH = COOH	2a 2b X = B(OH) ₂ = H = COOH = OH
3	$[\text{Os}_3(\text{CO})_{10}(\mu\text{-H})(\mu\text{-COOC}_2\text{H}_4\text{S})]_2$	
4	$\text{Os}_3(\text{CO})_9(\mu\text{-H})_2(\mu_3\text{-S})$	

5	$\text{Ph}_2\text{P}(\text{CH}_2)_2\text{CO}_2\text{N}(\text{COCH}_2)_2$
6	$\text{Os}_3(\text{CO})_{10}(\text{PPh}_2(\text{CH}_2)_2\text{CONH}(\text{CH}_2)_2\text{SH})_2$
$6.\text{H}^+$	$[\text{Os}_3(\text{CO})_{10}(\mu\text{-H})\text{PPh}_2(\text{CH}_2)_2\text{CO NH}(\text{CH}_2)_2\text{SH})_2] \text{PF}_6^-$
7	$\text{Re}_2(\text{CO})_8(\mu\text{-H})(\mu\text{-SR})$ a: R = H b: R = $(\text{CH}_2)_2\text{SH}$	<p>7a 7b</p>
8a-9a	$\text{CpM}(\text{CO})_3\text{I}$ 8a: M = Cr 9a: M = Mo	<p>8a: M = Cr 9a: M = Mo</p>
10	$\text{CpW}(\text{CO})_2\text{LI}$ a: L = CO b: L = PPh_3 c: L = PCy_3	<p>11a: L = CO 11b: L = PPh_3 11c: L = PCy_3</p>

11a	$\text{CpFe(CO)}_2\text{I}$
12a	$\text{CpRu(CO)}_2\text{I}$
12'a	$\text{Cp}^*\text{Ru(CO)}_2\text{I}$
13a	$\text{CpOs(CO)}_2\text{I}$
20	$\text{Ru}_3(\text{CO})_{10}(\mu\text{-H})(\mu\text{-NC}_5\text{H}_4)$
21	$[\text{Ru}_6(\mu_6\text{-H})(\text{CO})_{18}]^-$
22	$[\text{Ru}_2(\mu\text{-H})(\mu\text{-NC}_5\text{H}_4)_2(\text{CO})_4(\text{NC}_5\text{H}_5)_2][\text{Ru}_{10}(\mu\text{-H})(\mu_6\text{-C})(\text{CO})_{23}(\text{PPh}_3)]$

23	$[\text{Ru}_2(\mu\text{-H})(\mu\text{-NC}_5\text{H}_4)_2(\text{CO})_4(\text{PPh}_3)_2][\text{Ru}_{10}(\mu\text{-H})(\mu_6\text{-C})(\text{CO})_{20}(\text{PPh}_3)_4]$
24	$[\text{Ru}_{10}(\mu_6\text{-C})_2(\text{CO})_{24}]^{2-}$	<p>CO ligands are omitted for clarity.</p>
25	$[\text{Ru}_{11}\text{H}(\text{CO})_{27}]^{3-}$	<p>CO ligands are omitted for clarity.</p>
26	$[\text{Os}_{10}(\mu_6\text{-C})(\text{CO})_{24}]^{2-}$

List of Schemes

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Chapter 1: Organometallic Complexes for Bio-Imaging and -Sensing

1.1 Bioorganometallic chemistry

Organometallic chemistry, which lies at the intersection between organic and inorganic chemistry, involves the study of compounds with metal-carbon bond(s). Early interests were driven by its relevance to catalysis, and their industrial applications. Some important examples of industrial catalyst systems are those for the Monsanto and Cativa processes for the production of acetic acid, and Ziegler-Natta catalyst for the synthesis of polymers.¹⁻³ Generally perceived as air- and –moisture sensitive as a consequence of research in the early days, the relevance of organometallic compounds to biology was largely neglected. The term “bioorganometallics”, first coined in 1985, refers to the study and synthesis of organometallic species of biological and medical interest and has attracted much attention since then.^{4, 5} This is especially so after the first International Symposium on Bioorganometallic Chemistry (ISBOMC) which was held in 2002. Current research interest is wide-ranging and includes applications in pharmaceuticals, bio-catalysis, bio-imaging and –sensing.

The interest in organometallic complexes as biosensors is of great relevance, especially in an era with higher demand for better healthcare and disease diagnosis than before. Biosensors can be defined as analytical devices incorporated with a biological sensing element which allows them to have specificity and sensitivity for the identification of targets of interest in biological systems; they are usually coupled with physiochemical transducers to deliver complex bioanalytical measurements for quantitative or qualitative detection.^{6, 7} In bio-imaging, a visual output or quantitative measurement is produced which can be processed to give a visual image or map.

The interest in organometallic complexes for bio-sensing mainly lies in the unique properties (electrochemical, radiological or optical) that arise from the metal centers, as well as the ligand-metal interactions, which are not present in organic compounds. The metal centers exhibit diverse coordination numbers and geometry and hence can provide multiple conjugation sites for the development of multi-functional probes. The

latter have enabled various sensing modalities, in the form of electrochemical, radioisotopic and optical-based probes. A brief survey of the first two modalities will be followed by a more in-depth survey of optical modality; the latter can be described as the study of light and its interaction with matter, and includes fluorescence, vibrational spectroscopy and photoacoustic imaging.

1.2 Electrochemical-based probes

Ferrocene (Fc), which consists of a Fe^{2+} bound between two cyclopentadienyl rings in a sandwich manner, is the classic representative organometallic compound. Since its discovery more than 60 years ago,^{8,9} Fc has contributed to the development of bioorganometallic chemistry tremendously, especially in the area of pharmaceutical and electrochemical sensing.¹⁰⁻¹² An important example is its incorporation into Fc-mediated amperometric glucose sensors, which has been made available commercially since 1987, allowing diabetic patients to monitor their conditions with ease.^{6, 13} The application of Fc in electrochemical sensing has been largely driven by its intrinsic robust and fully reversible reduction/oxidation properties, as well as good aqueous stability. The ease with which Fc can be functionalized has also allowed it to be utilized as a building scaffold for bioconjugation to a wide range of biomolecules, thus allowing it to be developed into an efficient and selective electrochemical sensor.¹⁴

In one example, a helical peptide with the sequence Arg-Pro-Leu-Ala-Leu-Trp-Arg-Ser-Cys conjugated to Fc at the N-terminal was used to detect the activity of matrix metalloproteinase-7 (MMP-7) (figure 1.1).¹⁵ MMPs are a class of extracellular, zinc-dependent proteinases known to be involved in many important biological processes, and they have been identified as important biomarkers of diseases, inflammation and infection.^{16, 17} The Fc-peptide conjugate was self-assembled onto a gold electrode surface, followed by the incubation with MMP-7. The conjugate was anchored onto the gold surface via the thiol moiety at the Cys residue and, in the presence of MMP-7, the peptide is cleaved leading to loss of the Fc moiety. The substantial decrease in the redox current, detected by cyclic voltammetry (CV) and square wave voltammetry (SWV), was used for quantification. The specificity of the cleavage by MMP-7 was achieved at a much faster rate, in comparison to other subtypes of the enzyme (MMP-2 and MMP-3), with a low detection limit of 3.4 pM.

Figure 1.1. a) Schematic representation for the detection of matrix metalloproteinase: self-assembly of electrochemical Fc-peptide conjugate on the gold substrate, followed by cleavage of the peptide in the presence of MMP-7. b) CV of the Fc-peptide-Au electrode in 0.6 M NaClO₄ solution before (red curve) and after (blue curve) cleavage with 2 ng mL⁻¹ MMP-7. c) Corresponding SWV of Fc-peptide-Au electrode. All potentials are referred to an Ag/AgCl reference electrode. Adapted from ref. 15 with permission from the American Chemical Society.

Other than peptides, the conjugation of Fc to peptide nucleic acid (PNA) for use as electrochemical probes has also been widely reported.¹⁸⁻²⁰ PNA, which is analogous to DNA, is a polymer consisting of a backbone formed from repeating *N*-(2-aminoethyl) glycine units linked by peptide bonds (figure 1.2a).²¹ Various purine and pyrimidine nucleobases, which are linked to the backbone via methylene carbonyl bonds, can be hydrogen-bonded to complementary bases of DNA, forming hybrid PNA/DNA, similar to that of double stranded DNA (figure 1.2b).

Aoki and co-workers reported the anchorage of Fc-PNA conjugates onto gold electrode for detection of a specific DNA strand.¹⁸ The detection mechanism is based on hybridization of the conjugate with a complementary target DNA which leads to the formation of a rigid duplex structure that restricts the Fc moiety from approaching the surface. This led to a decrease in current which can be used to quantify the concentration of the target DNA (figure 1.3).

Figure 1.2. a) Structural representation of PNA backbone and side chains (A,C,G,T = nucleobase). b) Structural representation of PNA/DNA hybrid.¹² Adapted from ref. 12 with permission from the Royal Society of Chemistry.

Figure 1.3. a) Molecular structure of the Fc-PNA conjugate. Nu represents a nucleobase (A, T, G, or C). b) Working principle of a gene sensor based on the conformational flexibility change in the probe structure induced by hybridization, where the pair of sequences in PNA and DNA is represented as an example: (i) before hybridization, and (ii) after hybridization, the formation of duplexes makes the conjugate structure more rigid, access of the Fc to the surface is restricted, and consequently the electron transfer is inhibited. Adapted from ref. 18 with permission from the Royal Society of Chemistry.

Fc-antibody conjugates have also been constructed to utilize their specific interactions with antigens to detect and study binding events. In many instances, selective binding of the conjugate to the antigen of choice is followed by separation from the free conjugate and detection of the Fc-antibody-antigen electrochemically.²²⁻²⁴ For the detection of human chorionic gonadotropin (HCG), Fc carboxylic acid conjugated to anti-HCG immunoglobulin G (Ig G) was prepared via carbodiimide coupling chemistry, and used as a detection tag in tandem with electrochemical capillary flow immunoassay (figure 1.4).²² Increasing HCG concentration leads to an increase in current which is used for quantification.

Figure 1.4. a) Immobilization of Fc on IgG using carbodiimide coupling chemistry. b) Schematic diagram of an electrochemical capillary flow immunoassay system (upper) and the working principle (lower). The reaction mixture consists of HCG, Fc-IgG and HCG-Fc-IgG complex. The mixture is passed through a capillary column that selectively traps Fc-IgG. The eluted species passes through the flow cell, and the current associated with the HCG-Fc-IgG antibody complex is monitored. Applied potential: 395 mV versus Ag/AgCl. Adapted from ref. 22 with permission from Elsevier.

1.3 Radioisotope-based probes

Positron emission tomography (PET) and single photon emission computed tomography (SPECT) represent two important diagnostic tools for the visualization of organ functions.^{25, 26} Both techniques provide good sensitivity, and utilize the detection of gamma emission produced by exogenously administered radioactive probes. The demand for more efficient and safer probes continues to drive development in radiopharmaceuticals. One radionuclide of choice for this is technetium-99m (^{99m}Tc).

The artificial element technetium (Tc) was first prepared by deuteron bombardment of molybdenum in 1937.²⁷ All the Tc isotopes are radioactive, with the metastable nuclear isomer (^{99m}Tc) being the most applicable in nuclear medicine.²⁸ The pivotal role that ^{99m}Tc plays in this field owes much to the ease of production (using the $^{99}\text{Mo}/^{99m}\text{Tc}$ generator),^{28, 29} low cost (in comparison to other radionuclides), biocompatibility and, most importantly, its ideal decay characteristics; the mono-energetic gamma ray of 140 keV is of sufficient energy for deep tissue imaging, and the short half-life of 6 h is sufficient for diagnostic procedure and minimizes radiation exposure.³⁰

There are many ^{99m}Tc -based radiopharmaceuticals available on the market, with only one (Cardiolite[®]) being an organometallic complex; the rest are coordination complexes with non-carbon atoms (figure 1.5).³¹ Nevertheless, extension of the chemistry of technetium to its organometallic compounds has revealed many exciting avenues for their development into pharmaceuticals for targeted imaging. Most of those work has been devoted to developing synthetic strategies, as $[\text{}^{99m}\text{TcO}_4]^-$ is generally the only source of ^{99m}Tc available in hospitals, in the form of an aqueous solution.

Figure 1.5. Some commercially available ^{99m}Tc -based radiopharmaceuticals.

Metal carbonyls, which consist of one or more carbon monoxide (CO) ligands bound to the metal center, are common in organometallic chemistry and more details will be described in section 1.6. To explore the use of carbon monoxide (CO) as possible ligands for coordination to metal center, $[\text{}^{99\text{m}}\text{Tc}(\text{OH}_2)_3(\text{CO})_3]^+$ was synthesized in excellent yield using CO gas and NaBH_4 (Scheme 1).³² For practical reasons, boranecarbonate became a preferred CO source as it enabled routine synthesis to be carried out clinically (scheme 1).³³ The synthesis of $[\text{}^{99\text{m}}\text{Tc}(\text{OH}_2)_3(\text{CO})_3]^+$ expanded the synthetic versatility of Tc as the aquo ligands could be easily replaced, leading to the development of many Tc-carbonyl complexes containing other ligands or biomolecules of choice.^{34, 35} Some examples include conjugation to amino acid,³⁶ annexin A5,³⁷ acridine-based nuclear targeting agent and specific cell-receptor binding peptides bombesin (BBN).³⁸

Scheme 1.1. Synthesis of $[\text{}^{99\text{m}}\text{Tc}(\text{OH}_2)_3(\text{CO})_3]^+$ and some examples of conjugates to biomolecules.

The coordination of classical organometallic ligands like cyclopentadienyl (Cp) to $^{99\text{m}}\text{Tc}$ can be achieved using water-soluble acetyl Cp derivatives.^{39, 40} An aqueous one-pot synthesis of $\text{CpTc}(\text{CO})_3$ from $[\text{}^{99\text{m}}\text{TcO}_4]^-$ was demonstrated, which likely proceeded sequentially as shown (Scheme 1.2).³⁹ More recently, facile syntheses of $^{99\text{m}}\text{Tc}(\text{arene})_2$ from $[\text{}^{99\text{m}}\text{TcO}_4]^-$ was achieved, allowing functionalized arenes to be used as building blocks.⁴¹

Scheme 1.2. Synthesis of $[^{99m}\text{Tc}(\text{OH})_2(\text{CO})_3]^+$ and some examples of conjugates to biomolecules.

1.4 Fluorescence-based probes

Optical imaging is an emerging technology for biomedical imaging. Unlike modalities such as X-ray and positron emission tomography (PET), the use of non-ionizing radiation in optical imaging is safer for both the user and the subject. Valuable physical and chemical properties, useful for diagnostics, may also be obtained when molecules absorb and scatter different wavelengths.^{42, 43} One important example is fluorescence microscopy which has become an essential tool in biology. The development of fluorescence probes from green fluorescence protein, first isolated from the *Aequorea* jellyfish,⁴⁴⁻⁴⁶ to various fluorescence small organic molecules,⁴⁷ provides a useful labelling tool for studying biological systems ranging from the cellular to single molecule level.⁴⁸⁻⁵⁰

The principle of fluorescence microscopy is dependent on light emission from the site of interest to improve contrast from the background. Fluorescence involves the emission of light upon absorption of incident light (typically of shorter wavelength) from an external source, or the natural emission from the sample.^{51, 52} The higher wavelength emitted light can be detected while the excitation light is filtered off; the difference in wavelength between the excitation and emitted light is known as Stokes shift (figure 1.6). Excellent contrast can be obtained between the area of interest and areas detected via normal methods (reflection, transmission).

Besides organic fluorophores, fluorescent organometallic complexes have also been reported. These are mainly based on three classes: cyclometalated metal

complexes, gold-based complexes and rhenium tricarbonyl polypyridyl complexes; some examples of the first two classes will be presented here.⁵²

Figure 1.6. Jablonski diagram (top), and the absorption and emission spectra (bottom) of the common fluorophore fluorescein isothiocyanate (FITC). Each vertical gray line aligns the spectra with the energy of the absorbed (arrows pointing up) or emitted (arrows pointing down) photons. The arrows are colored to represent the wavelength of the photons. For example, the purple arrow to the left represents the energy of an ultraviolet photon that can cause the molecule to transition from the ground state to the second singlet excited state. The orange arrow on the right represents the lowest energy photon that can be emitted by this molecule as it drops back from the lowest energy state S₁ to a high vibrational state of S₀. Adapted from ref. 51 with permission from Macmillan Publishers Ltd: *Nat. Methods*.

Luminescent complexes can be formed via the cyclometalation of 2-phenylpyridine with group 9 metals like iridium and rhodium. Many examples of such complexes, $[M(C-N)_2(N-N)]^+$ (where M = Ir or Rh), have been reported and they generally show good cellular uptake and photophysical properties (figure 1.7).⁵³⁻⁵⁶ For example, Ir complexes coupled with pH-responsive functionalities and photosensitizers have been employed in pH sensing and photodynamic therapy.^{57,58}

Figure 1.7. Molecular structures of selected cyclometalated $[\text{Ir}(\text{C-N})_2(\text{N-N})]$ complexes.^{53, 55, 58}

Platinum cyclometalates also possess robust luminescent properties. An example is the use of Pt(II) complexes with cyclometalated 1,3-di(2-pyridyl) benzene and derivatives in time-resolved and two-photon emission imaging microscopy of live cells (figure 1.8).⁵⁹ These complexes are highly tunable, via the substituent R, over a spectral range from blue-green to orange.⁶⁰ The complex $[\text{Pt}(\text{N-C-N})\text{Cl}]$ -a possesses high emission, good photo-stability and low toxicity. It shows good cellular accumulation, via passive uptake, within five minutes of incubation and is preferentially localized onto nuclei acids.

Figure 1.8. a) Molecular structure of the complexes $[\text{Pt}(\text{N-C-N})\text{Cl}]$. b) Confocal micrographs of human dermal fibroblasts, C8161 human melanoma, and CHO cells. Cells were incubated, washed with PBS and imaged by using an excitation wavelength of 488 nm. Paired images show grayscale and rainbow intensities respectively, identifying regions of $[\text{Pt}(\text{N-C-N})\text{Cl}]$ -a accumulation (Scale bar: 10 μm). Adapted from ref. 59.

The use of luminescent Au(I) complexes for fluorescence imaging, though rare, has also been reported. There is a report on a dinuclear Au(I) complex with a bidentate

N-heterocyclic carbene (NHC) ligand separated by methylene bridging groups; this is believed to provide a stronger support for an Au...Au interaction in comparison to the use of benzyl as the bridging group.⁶¹ This was largely motivated by the desire to tune the aurophilicity (the Au...Au interaction) and hence, the luminescent profile of the Au(I) complex (red-shifted); complexes with longer Au...Au separations are non-emissive.^{62, 63} The as-synthesized Au(I) complex was introduced into RAW264.7 cells (mouse macrophage cancer cell line) and was found to localize within the lysosomes instead of the mitochondria (figure 1.9a). In another example, Dyson and co-workers synthesized a series of Au(I) alkynyl complexes containing water-soluble 1,3,5-triaza-7-phosphaadamantane (PTA), which showed anticancer and luminescent properties (figure 1.9b).⁶⁴ These trinuclear Au(I) alkynyl complexes showed good IC₅₀ values of $3.3 \pm 0.2 \mu\text{M}$ with A2780 ovarian cancer cells, and cellular uptake was confirmed by luminescence which was detected throughout the cells.

Figure 1.9. a) Molecular structure of the dinuclear Au(I) NHC complexes and cellular distribution of the complex (200 μM) in RAW264.7 cells after incubation for 15 h at 37 $^{\circ}\text{C}$; i) brightfield image and ii) luminescence image (λ_{ex} 351 nm). The black and white arrows mark the positions of the nuclei. Adapted from ref. 61 with permission from John Wiley and Sons. b) Molecular structure of the trinuclear Au(I) alkynyl containing the PTA ligands and cellular distribution of the complex in A2780 cells after incubation for 6 h at 37 $^{\circ}\text{C}$; i) bright field image and ii) luminescence image. Adapted from ref. 64 with permission from the American Chemical Society.

1.5 Transition metal carbonyls as mid-infrared (IR) probes

As mentioned earlier, a class of organometallic compounds which has found wide applications in biology is the transition metal carbonyl complexes, i.e., complexes with CO ligand(s) coordinated to the metal center. The earliest example of a metal carbonyl, Ni(CO)_4 , was discovered by Ludwig Mond in 1890, which subsequently led to the Mond process which uses CO gas to purify and extract nickel from its ores in the form of Ni(CO)_4 .⁶⁵

1.5.1 Bonding in transition metal carbonyls

CO is an unsaturated ligand due to the C-O multiple (triple) bond. Hence, it can be regarded as a soft ligand with π -accepting property. Its interaction with a metal center involves the donation of a lone pair of electrons from the carbon atom to the metal, and back donation from the metal to an anti-bonding orbital of the C-O bond (figure 1.10a). The extent of π -back donation is usually the main factor affecting the C-O bond order, which can be affected indirectly by the effective charge on, and ligands coordinated to, the metal. This in turn affects the vibrational frequency. Bonding to a metal center generally reduces the C-O bond order, and the different bonding modes that a CO ligand can adopt, ranging from triply-bridging to terminal, leads to characteristic vibrational frequencies in the range of $1650\text{--}2200\text{ cm}^{-1}$ (figure 1.10b-c).⁶⁶ Hence, the CO vibrational frequencies of metal carbonyls, situated in a region which is relatively devoid of interference from other functional groups, are ideal for sensing applications.

Figure 1.10. a) Orbital diagram showing the bonding between carbon monoxide with a transition metal centre. b) Different bonding modes of CO with different number of metal centers, forming the triply-bridge to terminal CO with vibrational frequencies in the range of 1650-2200 cm^{-1} . c) IR spectra recorded in absorbance mode of untreated mucosa cells (bottom), cells treated with a triosmium carbonyl cluster (middle and top). Boxed region shows carbonyl (CO) stretching vibrations of the organometallic fragment. Adapted from ref. ⁶⁷ with permission from the American Chemical Society.

1.5.2 Applications based on CO vibrational intensities

The absorption intensity of metal carbonyls can be applied for quantitative sensing. Similar to fluorescence spectroscopy, the absorbance of the CO vibrational frequencies follows the Beer-Lambert law: $A = \epsilon bc$, where A is the absorbance, ϵ is the molar absorptivity coefficient ($\text{M}^{-1} \text{cm}^{-1}$), b is the path length (cm) and c is the analyte concentration (M). The viability of such quantification has allowed the development of mid-IR assays based on metal carbonyl, and was first realized by the group of Jaouen in a steroid hormonal assay to study the hormonal receptors on breast cancer cells.^{5, 68}

- Steroidal hormonal assay

This assay was driven by the need for an alternative to the hazardous and costly radio-isotopic method. The concept was demonstrated with the use of an $\eta^6\text{-Cr(CO)}_3$ -estradiol derivative which binds onto estrogen receptors in lamb uterus cytosol.^{5, 68} The precipitated protein, incubated with the α -derivative, was then prepared in the form of a CsI pellet and quantified by FT-IR spectroscopy; the area of the $A_1(\nu_{\text{CO}})$ peak to the mass of the pellet gave a linear plot (figure 1.11).

Figure 1.11. a) FT-IR (νCO) spectrum of precipitated lamb uterine cytosol incubated with $\eta^6\text{-Cr}(\text{CO})_3\text{-estradiol } \alpha\text{-derivative}$, prepared in CsI pellet. b) Correlation between the weight of the pellet and the area of the A_1 (νCO) peak at 1957 cm^{-1} . Adapted from ref. 5.

The conjugation to estradiol was subsequently extended to a derivative with an alkyne functionality at the $\text{C17}\alpha\text{-}$ position. This allowed for complexation with di- and tri-nuclear metal carbonyl clusters like $[\text{CpMo}(\text{CO})_2]_2$, $[\text{Co}_2(\text{CO})_6]$ and $[\text{Os}_3(\text{CO})_{10}]$ respectively (figure 1.12).^{69, 70} Conjugation of metal carbonyls to other receptor binding molecules have also been reported, such as, mifepristone and hexestrol, with the former used for progesterone and glucocorticoid receptor assays, and the latter for estrogen receptor assays.^{71, 72}

Figure 1.12. Molecular structures of metal carbonyl-steroid derivatives used for steroidal hormonal assay.

- Carbonylmetalimmunoassay (CMIA)

Another quantitative assay subsequently developed was the carbonylmetalimmuno-assay (CMIA) which utilized analytes labelled with metal carbonyls for mid-IR quantification down to femtomolar concentrations.⁷³ The analyte to be detected was first conjugated to a stable metal carbonyl fragment; this conjugate can be referred to as the tracer. In this competition-type immunoassay, a known amount of the tracer was incubated with the antibodies (the binding proteins) and unknown amounts of the analyte to be detected, leading to the competition between the tracer and the analyte for binding to the limited amount of antibody. The unbound tracer was then extracted with organic solvents and quantified by FT-IR spectroscopy, allowing indirect determination of the amount of the analyte present (figure 1.13).^{74, 75}

Figure 1.13. Schematic representation of the working principles of CMIA. Adapted from ref. 74 with permission from the Royal Society of Chemistry.

This assay has been utilized for the detection of anti-epileptic drugs like diphenylhydantoin, carbamazepine, phenobarbital, diphenylhydantoin, the anti-depressant nortriptyline and cortisol, with the use of the corresponding metal carbonyl conjugates as tracers (figure 1.14).^{73, 76-79} The simultaneous detection of two or more targets, also known as multiplex detection, can be incorporated into CMIA. FT-IR spectroscopy is suitable for multiplex detection owing to the narrow bandwidths of the vibrational bands, and is advantageous for achieving minimal or non-overlapping of the signals. Duplex and triplex detection of anti-epileptic drugs like carbamazepine, phenobarbital and diphenylhydantoin were achieved, using a different metal carbonyl for each target.^{80, 81}

Figure 1.14. Molecular structures of metal carbonyl derivatives used as tracers in CMIA.

Enhancements to the CMIA sensing platform have also been made over the years. This includes the development of solid-phase CMIA, which improves on the purification step and minimizes the interference from water. For example, streptavidin-coated microtiter plates and nitrocellulose membrane allow for immobilization of metal carbonyl dendrimer conjugates.⁸²⁻⁸⁴ Nanoparticle-based platforms have also been demonstrated with the use of gold nanoparticles to anchor disulfide-linked metal carbonyls onto the surface via strong Au-S interactions (figure 1.15a). Further functionalization with antibodies using carbodiimide coupling chemistry turns it into a secondary label to detect mouse monoclonal anti-staphylococcal enterotoxin A (SEA) antibody via polarization modulation infrared reflection-absorption spectroscopy (PM-IRAAS) and atomic force microscopy (AFM).^{85, 86}

More recently, $\text{CpFe(CO)}_2\text{(N-maleimidato)}$ was immobilized onto amine or thiol-functionalized magnetic nanoparticles (MNP) followed by conjugation to antibodies (figure 1.15b).⁸⁷ The nanoparticles-based platform is attractive as it allows the metal carbonyl-ligand ratio to be increased, thereby enhancing the CO vibrational signal, as well as providing a mean to easily modify the support without having to functionalize the metal carbonyl directly.

Figure 1.15. a) Synthesis of CpFe(CO)₂-gold nanoparticle conjugates and immobilization of goat anti-mouse IgG antibody. Adapted from ref. 85 with permission from Elsevier. b) Synthesis of CpFe(CO)₂-MNP conjugates and immobilization of IgG antibody. Adapted from ref. 87 with permission from Elsevier.

- IR probes for cellular imaging

The CO vibrational bands of metal carbonyls can also be utilized as a spectroscopic handle for cellular imaging. Such applications rest on qualitative rather than quantitative measurements, largely due to the uneven topology of the cells. Accurate quantification can be complicated and requires determination of the optical path length which, in one case, was measured via 3D reconstructed IR images, with the absorption coefficient pre-determined from a similar setup of the IR microscope.⁸⁸ Quantification has also been carried out indirectly by measuring the absorbance from a large number of cells incubated with the complex deposited on a nitrocellulose disk and air-dried, to obtain a calibration curve.⁸⁹

The first application of metal carbonyls for mid-IR cellular imaging was reported by Leong and co-workers, using the water-soluble and stable [Os₃(CO)₁₀(μ-H)(μ-S(CH₂)₁₀COO)]Na, an inorganic salt of a fatty acid

conjugated to a metal carbonyl cluster, and its phosphatidylcholine analogue, as the probe (figure 1.16).⁶⁷ Chemical mapping at 2013 cm^{-1} , which corresponded to one of the CO vibration bands of the cluster, of oral mucosa cells was carried out with an IR microscope fitted with a 64×64 pixel image plate detector. The IR images correlated well to the optical image, indicating internalization of the compound into the cells.

Figure 1.16. a) Molecular structure of $[\text{Os}_3(\text{CO})_{10}(\mu\text{-H})(\mu\text{-S}(\text{CH}_2)_{10}\text{COO})]\text{Na}$ and the phosphatidylcholine analogue. The short lines extending from Os represent CO ligands. b) Normal optical image and c) false-color IR image of oral mucosa cells treated with $[\text{Os}_3(\text{CO})_{10}(\mu\text{-H})(\mu\text{-S}(\text{CH}_2)_{10}\text{COO})]\text{Na}$ ($32\text{ }\mu\text{M}$), taken at 2013 cm^{-1} , one of the carbonyl vibration band. Adapted from ref. 67 with permission from the American Chemical Society.

The interest in metal carbonyl compounds for biological applications has partly been spurred on by the discovery that carbon monoxide (CO) is produced endogenously through heme catabolism and is a vital cell signaling mediator and regulator,^{90, 91} and reports that it induces vasorelaxation and exhibits anti-inflammatory and cytoprotective effects against disease pathology.⁹¹⁻⁹⁴ As carriers for carbon monoxide, metal carbonyls are potential CO-releasing molecules (CORMs) for controlled and site-specific delivery.^{95, 96} Release of CO from the complexes can be triggered by various external factors which include light; the term “photoCORMs” is used to describe such complexes.^{97, 98} Zobi and Quaroni, *et. al.*, reported the synthesis of a manganese photoCORM conjugated to the 5'-OH of the ribose sugar of vitamin B₁₂ and its delivery into live 3T3 fibroblasts.⁹⁹ The cellular uptake and distribution of the B₁₂-MnCORM

was monitored by synchrotron FTIR spectromicroscopy in the live cells (figure 1.17). The moderate spatial resolution, however, only allowed the nucleus and cytoplasm to be distinguished and accumulation of the compound in the nucleus was supported by atomic absorption spectrophotometric measurements on the cell fractions.

Figure 1.17. a) Molecular structure of the vitamin B₁₂-Mn CORM conjugate and optical images of a 3T3 fibroblast after incubation with the conjugate (1 mM). The top and bottom inserts show images reconstructed from integration of the intensity of the carbonyl stretching vibrations at 2018 and 1900 cm⁻¹, respectively. The scale represents units of integrated band area in an absorbance vs wavenumber spectrum. Nc = cell nuclear area. Adapted from ref. 99 with permission from the American Chemical Society.

Another growing interest is the development of multimodal imaging probes for correlative and more precise detection. There have been a number of reports on rhenium tris-carbonyl derivatives, which display IR and luminescent properties, as single-core, multimodal probes to image breast cancer cells.¹⁰⁰⁻¹⁰² In one example, MDA-MB-231 cells incubated with the rhenium tris-carbonyl derivative, ReCl(CO)₃-pyta-C₁₂N₃ (pyta = 2-pyridyl-1,2,3-triazole), was imaged by synchrotron radiation FT-IR spectromicroscopy (SR-FTIR-SM), to produce chemical mapping of the complexes within the cell (figure 1.18).¹⁰⁰ The IR images were correlated with the fluorescent image of the cells, which showed

luminescence due to the metal-to-ligand charge transfer (MLCT) of the complex.

Although the IR and fluorescence bimodal probes can facilitate correlation studies between IR and fluorescence imaging, the former technique remains the more reliable for the quantification of cellular uptake.¹⁰² This is because the quantum yield of a fluorescent probe is highly susceptible to degradation through photo-bleaching and quenching, and it is also highly dependent on the environment, including the temperature and solvent.^{102, 103} On the other hand, IR vibrations tend to be more stable and easily quantified via a calibration curve. Although the probe environment can induce frequency shifts and/or broadening, the peak area remains relatively consistent, making quantification more reliable.

Figure 1.18. a) Molecular structure of $\text{ReCl}(\text{CO})_3\text{-pyta-C}_{12}\text{N}_3$. b) MDA-MB-231 cell incubated with $\text{ReCl}(\text{CO})_3\text{-pyta-C}_{12}\text{N}_3$ (10 μM , 1 h). (i) Bright field image (scale bar 10 μm). (ii–iv) SR-FTIR mappings, pixel size: 3 x 3 μm^2 : (ii) phosphate asymmetric stretching (green), (iii) E-band (red), (iv) A₁-band (cyan). (v) Epifluorescence image, localization of the complex (green). (vi–vii) Overlays of SR-FTIR hot spots: (vi) overlay (yellow) of ii (green) and iii (red), (vii) overlay (white) of iii (red) and iv (cyan). Adapted from ref. 100 with permission from the Royal Society of Chemistry.

Figure 1.20. Molecular structure of CpRe(CO)_3 -hydroxytamoxifen conjugate and images of MDA-MB-231 cells. Top row: control cells; bottom row: cells treated with a $10\ \mu\text{M}$ solution of the conjugate for 1 h at $37\ ^\circ\text{C}$. a) AFM topography; b–f) PTIR mappings at different wavelengths, with the AFM contours superimposed as black lines; the wavelengths correspond to the following bands: phosphate (b), amide (c), tricarbonyl (d and e), and out of band (f). Adapted from ref. 89 with permission from John Wiley and Sons.

1.5.3 Sensing based on frequency shifts in CO vibrations

Besides the use of intensity for quantitation, qualitative shift in frequency of the CO vibrations has also proven to be useful for sensing applications. The frequency of the CO vibration is largely governed by the extent of π -back donation from the metal to the CO ligand. Hence, ligands in the coordination sphere of the metal can easily affect the electron density of the metal, giving rise to changes in the CO vibrational frequency. The design and application of metal carbonyl “bioprobes” that give a spectroscopic response upon interaction with the target was first discussed by Stephenson.¹⁰⁶ The term “bioprobes” refers to molecular structures that can provide information about biological systems and are required to possess functions such as recognition, transduction and response. Much work has been carried out, and these are summarized in table 1.1. Detailed discussions will be made on the applications in sensing of pH, protein interaction and solvent environment.

Table 1.1. Use of various metal carbonyls for sensing applications via frequency shifts in CO vibrations.

Entry	Applications	Compounds	Ref.	Sensing mechanism
1	pH sensing		107	Metal carbonyls with pH sensitive groups like –COOH and –NH ₂ can be used for pH sensing.
2	Metal cation sensing (e.g. alkali metal ions)		108, 109	The interaction of crown ethers with metal cations can effect a change to the metal carbonyls electronically and/or structurally, resulting in shifts in CO frequencies
		109
		110
		111	

3	Anion sensing (e.g. carboxylate and fluoride)	<p style="text-align: center;">$R = C_6H_5 \text{ or } CH_3$</p>	112	Metal carbonyls coordinated to ligands with free – NH or –OH groups can act as hydrogen bond donors for interaction with anions via hydrogen bonding
		113	
4	Hydrogen bond sensing (e.g. indole, diphenylamine and 2, 4, 6- trimethylphenol)	<p style="text-align: center;">$M = Cr \text{ or } W$</p>	114	Sensing of hydrogen bond donors was achieved via the nitrogen lone pair of dipyridylethene ligand coordinated to $M(CO)_5$
5	π -interaction sensing (e.g. 1, 3, 5-trimethoxy- benzene and 1, 2- dimethoxy- benzene)		115	π -stacking interaction between the arene ring of Cr carbonyl with aromatic analytes can lead to changes in the CO frequencies.

6	Protein interaction sensing (e.g. α -chymotrypsin)		116	The generation of $(\eta^5\text{-dienyl})\text{-Fe(CO)}_3$ moiety from $(\eta^4\text{-diene})\text{-Fe(CO)}_3$, allows the reaction with an amine functionality which produce a distinct spectroscopic response.
7	Solvent environment sensing (e.g. various organic solvents like DCM, ether, acetone <i>et. al.</i>)	<p> a: $R^1 = \text{OMe}, R^2 = R^3 = \text{H}$ b: $R^1 = R^2 = R^3 = \text{H}$ c: $R^1 = R^3 = \text{H}, R^2 = \text{OMe}$ d: $R^1 = R^3 = \text{H}, R^2 = \text{Ph}$ e: $R^1 = R^3 = \text{OMe}, R^2 = \text{H}$ a: $R^4 = \text{Cl}, R^5 = \text{H}$ b: $R^4 = R^5 = \text{OMe}$ </p>	117	Solvent-induced frequency shifts in different organic solvents were quantified and can be distinguished in binary or ternary solvent systems.
		<p> a: $R = \text{OMe}, L = \text{CO}$ b: $R = \text{H}, L = \text{CO}$ c: $R = \text{H}, L = \text{PPh}_3$ </p>	118, 119	

- pH sensing

Metal carbonyl π -complexes containing pH-sensitive groups like carboxylic acids and amines, can be utilized as pH-sensitive IR probes (Figure 1.21).¹⁰⁷ Examples include η^6 -(1-carboxybenzene)Cr(CO)₃ and η^4 -1-carboxy-1,3-pentadiene)Fe(CO)₃. Protonation/ deprotonation of these groups affect the $\nu_{\text{sym}}(\text{CO})$ and $\nu_{\text{asym}}(\text{CO})$ vibrational modes; deprotonation of the carboxylic acid group increases the electron density of the π -conjugated system, thereby increasing π -back donation from the metal to C-O π^* anti-bonding orbital, which is translated into a shift to lower wavenumber of the CO vibrations.

Figure 1.21. a) Molecular structures of η^6 -(1-carboxybenzene)Cr(CO)₃ and η^4 -1-carboxy-1,3-pentadiene)Fe(CO)₃ and their pH sensing capability. b) FTIR spectra in the 2050-1800 cm^{-1} region of η^6 -(1-carboxybenzene)Cr(CO)₃ in buffer solutions over the pH range 2.1-6.0. Adapted from ref. 107 with permission from the American Chemical Society.

The response to pH can also be adjusted through the use of other pH-sensitive groups like hydroxyl or amine groups, or through different substituents on the π -system. A combination of the two above-mentioned pH-active probes with non-overlapping CO frequencies can act effectively as a “dual-sensor” which allows simultaneous but independent measurements to be made. Since the probes have different pKa (2.9 and 4.2 for the Cr(CO)₃ and Fe(CO)₃ derivatives, respectively), they respond to different pH and hence, allow a wider pH range that can be probed.

- Protein interaction sensing

A responsive metal carbonyl which gives a spectral change upon reaction with a functional group may be advantageous for reaction monitoring, especially in cases where excess probes are difficult to remove. The complex $(\eta^4\text{-diene})\text{-Fe(CO)}_3$, which bears a labile pyridinium group, can be deprotonated to form the $(\eta^5\text{-dienyl})\text{-Fe(CO)}_3$ moiety, which can then react readily with an amine functionality (figure 1.22).¹¹⁶

Figure 1.22. a) Reaction of $(\eta^4\text{-diene})\text{-Fe(CO)}_3$ with amine functionality in proteins. b) (a-f) FTIR spectra of $(\eta^4\text{-diene})\text{-Fe(CO)}_3$ in pH 7.5 buffer solution containing α -chymotrypsin recorded at intervals over 3 h. (g) Component bands resulting from decomposition of spectrum (c) using a scale expansion of 2. (h) FTIR spectrum of $[\text{Fe(CO)}_3(\text{C}_6\text{H}_7\text{NH}_2\text{Pr}^i)](\text{PF}_6)$ in buffer. Adapted from ref. 116 with permission from the Royal Society of Chemistry.

Incubation of the protein α -chymotrypsin with excess of the complex led to the emergence of high frequency shoulders on the ν_{sym} and ν_{asymm} stretches. Curve-fitting analysis resolved the spectrum of the bound complex from that of the excess complex, and identified to be the isolated species $[\text{Fe(CO)}_3(\text{C}_6\text{H}_7\text{NH}_2\text{Pr}^i)](\text{PF}_6)$. The interaction of $(\eta^4\text{-diene})\text{-Fe(CO)}_3$ with various amino acids and poly(lysine) also afforded species exhibiting similar spectra, and the study was extended to other enzymes like ribonuclease A, alkaline phosphatase and triacylglycerol lipase.¹²⁰ With a change in pH, which results in deprotonation/protonation of the amine group, the spectrum of the

enzyme-bound complex undergo further shifts, thus demonstrating its dual-function as a tag and pH sensor on the protein surface.

- Solvent environment

The observation of solvent-induced frequency shifts in metal carbonyls was made as early as in the 1960s by R. S. Nyholm and co-workers.¹²¹ Typically, CO vibrational frequencies are shifted and broadened in the presence of polar solvents. Quantitative interpretation of the shifts was first carried out by Stephenson et. al. using a series of (η^6 -arene)-Cr(CO)₃ and (η^4 -cyclohexadiene)-Fe(CO)₃ complexes (figure 1.23).¹¹⁷

Figure 1.23. a) Molecular structures of various (η^4 -cyclohexadiene)-Fe(CO)₃ complexes used for solvent environment sensing. b) Symmetric and antisymmetric stretching frequencies of Fe(CO)₃-(a) in various solvents: 1, hexane; 2, cyclohexane; 3, toluene; 4, dichloromethane; 5, diethyl ether; 6, tetrahydrofuran; 7, acetonitrile; 8, ethyl acetate; 9, acetone; 10, 1-octanol; 11, dimethyl formamide; 12, dimethyl acetamide; 13, acetic anhydride. c) Variation of ν_{sym} and ν_{asym} of Fe(CO)₃-(a) in binary mixtures of ethyl acetate and dimethyl acetamide. Reproduced from ref. 117 with permission from Elsevier.

Bellamy-type plots of ν_{sym} against ν_{asym} stretches of Fe(CO)₃-(a) in various solvents showed that the frequency shifts gave a better correlation with the solvent environment than the band broadening (figure 1.23b). The vibrational frequencies of Fe(CO)₃-(a) in a binary solvent mixture of ethyl acetate and

dimethyl acetamide showed good quantitative correlation with the composition, and the correlation for a ternary solvent mixture could be resolved with principal components analysis. Extension of the idea to metal carbonyl grafted onto a solid support for sensing binary to ternary solvent mixtures was demonstrated with $(\eta^4\text{-cyclohexadiene})\text{-Fe(CO)}_2(\text{L})$, where $\text{L} = \text{CO}$ or PPh_3 , covalently attached onto silica particles via a 3-(diethoxymethylsilyl)propyl linker.^{118, 119}

1.5.4 Applications based on CO vibrational lifetime

The vibrational lifetime of metal carbonyls is another feature which can be used for sensing applications. The vibrational lifetime of metal carbonyls in organic solvents was first reported in 1987.¹²² Applications of this for sensing only surfaced recently and are generally used in protein and membrane studies.

- Ultrafast vibrational lifetime of metal carbonyls in proteins and membrane studies

The vibrational lifetime of metal carbonyl in water was first measured by Kubarych and co-workers in their study on the water-soluble $[\text{RuCl}_2(\text{CO})_2]_2$, also known as CORM-2.¹²³ The vibrational relaxation was probed by two-dimensional (2D) IR spectroscopy, and showed that there was a great contrast between the relaxation time in water (3.12 ± 0.29 ps) compared to that in a polar organic solvent like methanol (42.25 ± 3 ps). This property was subsequently utilized in a study to probe the hydration environment at the protein-water interface (figure 1.24).¹²⁴ The study employed metal carbonyls as a site-specific IR label on globular proteins, such as hen egg white lysozyme (HEWL) and human lysozyme (HuLys). The proteins were labelled by incubation with tricarbonylchloro(glycinato) ruthenium(II) (CORM-3); while HEWL was labelled at the His15 residue to form $[(\text{His15})\text{Ru(CO)}_2(\text{H}_2\text{O})_3]^{2+}$, HuLys was labelled at the His78 residue.¹²⁵ Vibrational relaxation times of the ruthenium carbonyl residue measured in H_2O , D_2O , and in an amphiphilic alcohol co-solvent of D_2O -trifluoroethanol to mimic a hydrophobic environment, suggested that the residue on HEWL experienced dynamically constrained water in a largely hydrophobic surface of the protein, while that on HuLys was more exposed to solvent and experienced bulk-like hydration dynamics. The

ruthenium carbonyl-labelled HEWL was also used to investigate hydration and protein dynamics using a glycerol/D₂O mixture.¹²⁶

Figure 1.24. a) Molecular structure of ruthenium carbonyl (RC) protein label on HEWL and HuLys. b) Crystal structures of HEWL-RC (PDB code 2XJW) overlaid with the crystal structure of native HuLys (PDB code 2ZIJ). While no crystallographic data are available for the HuLys-RC complex, the binding location is proposed by comparison with the HEWL-RC complex. c) Linear FTIR spectra of HEWL-RC (i) and HuLys-RC (iv) in D₂O and H₂O. The 2D-IR rephasing spectra for HEWL-RC (ii) and HuLys-RC (v) in D₂O are shown for a waiting time of t₂ = 500 fs. Monitoring the amplitude of the 2004 cm⁻¹ peak as a function of waiting time, t₂, provides the vibrational lifetime of the mode. For HEWL-RC there is no observable isotope effect in the vibrational relaxation between D₂O and H₂O (iii), whereas HuLys-RC shows a very clear isotope effect (vi). Adapted from ref. 124 with permission from the American Chemical Society.

A similar dependence of the CO frequencies and lifetime on the solvent environment was also demonstrated with a series of CpRe(CO)₃ with linkers to methanethiosulfonate to tag cysteine residues (figure 1.25a).¹²⁷ The CpRe(CO)₃ moiety was also bioconjugated to the ribosomal protein CTL9 through an alkynyl linkage which was then coupled to an azidohomoalanine residue on the C-terminal domain of the protein via a “click” reaction (figure 1.25b).¹²⁸ The vibrational lifetimes of the metal carbonyl probe in H₂O and D₂O were found to be different, showing accessibility to the solvent.

Figure 1.25. a) Incorporation of $\text{CpRe}(\text{CO})_3$ with an alkylated methanethiosulfonate onto thiol functionality on proteins. b) Bioorthogonal incorporation of a $\text{CpRe}(\text{CO})_3$ alkynyl derivative onto residue 109 of the protein CTL9, located in a loop, using the “click” reaction. Adapted from ref. 128 with permission from the American Chemical Society.

The water-membrane interface in a model lipid membrane has also been probed with a chromium tricarbonyl-labelled cholesterol (figure 1.26).¹²⁹ The frequency-frequency correlation function (FFCF) at the water-membrane interface showed a distinct three-fold slowdown compared to that in bulk water. A similar incorporation of $\text{W}(\text{CO})_6$ into the alkane interior of the membrane bilayer allowed sensing of its environment through the CO vibrations, and probing of the internal structural dynamics via 2D-IR measurements.¹³⁰ It was found that the addition of cholesterol induced a slowdown in the structural dynamics, and an abrupt change occurred when the concentration reached 35%; this change was also observed in the FT-IR absorption spectra and ascribed to a phase change.

Figure 1.26. a) Incorporation of $Cr(CO)_3$ labelled cholesterol into membrane constructs such as bicelles or unilamellar vesicles. bi) Rapidly acquired spectral diffusion (RASD) measures the FFCF of the conjugate in bulk water and at the bicelle–water interface. The faint lines are the full data set; the thick colored lines are the result of a 100 fs windowed moving average. The fits are shown as black lines. (ii) Rephasing decay for the bicelle sample showing the full data set (blue) and the biexponential fit. The sub-5-ps lifetime is indicative of water exposure. Adapted from ref. 129 with permission from the American Chemical Society.

1.6 Transition metal carbonyls as Raman probes

Despite the demonstrated success of metal carbonyls for mid-IR sensing and imaging, there are also several limitations associated with this technique. First, the strong water absorption in living cells or biological media can overlap and interfere with the CO stretches. Secondly, IR microscopy has a spatial resolution (for CO vibrations) of about 6 μm which is generally inadequate for sub-cellular imaging. Though this can be circumvented by techniques such as PTIR and SR-FTIR, sophisticated instrumental set-ups are required for these.

- IR vs Raman characteristics of CO

The inelastic scattering of a photon was first observed experimentally by C.V. Raman in 1928.¹³¹ It involves a change in the polarizability of the molecule (with respect to the vibrational motion) induced by the oscillating electric field of the incident light. Light scattered from a molecule contains both elastic Rayleigh and inelastic Raman scattering. Rayleigh scattering corresponds to the scattering at the same frequency of the incident light, while Raman scattering involves a shift in the

frequency of the incident light, which can be to a lower frequency (Stokes, $\nu_0 - \nu_j$) or to a higher frequency (anti-Stokes, $\nu_0 + \nu_j$); the shifts are associated with vibrational modes of the molecule (figure 1.27).¹³²

Figure 1.27. a) Jablonski diagram representing the energy transition for Rayleigh, Raman scattering and IR absorption. b) A schematic Raman spectrum showing the placement of the Stokes and anti-Stokes shift in relation to the Rayleigh line. Adapted from ref. 74 with permission from the Royal Society of Chemistry.

Rayleigh scattering is of much greater intensity than Raman scattering (a factor of $\sim 10^6 - 10^8$) and by the Boltzmann distribution, more molecules will be in the vibrational ground state than the excited state at ambient conditions and hence, Stokes scattering is of higher intensity than anti-Stokes scattering. The Stokes scattering is what is usually recorded as the Raman shift with respect to the Rayleigh scattering. The difference between IR and Raman spectroscopy are summarized in table 1.2.

Table 1.2. Summary and comparison between IR and Raman spectroscopy

	IR spectroscopy	Raman spectroscopy
Excitation wavelength	Mid-IR region from 2500 nm to 25000 nm (4000 to 400 cm ⁻¹)	Visible-near IR region from 532 to 1064 nm
Nature of techniques	Light absorption	Inelastic light scattering
Mechanism	Requires a dipole moment change	Requires a change in polarizability
Spatial resolution at the diffraction limit	~ 1.3 to 12.5 μ M	~ 266 nm to 532 nm
Limitations	Low spatial resolution and prone to interference by water	Weak signals for CO vibrational frequencies.

The intensity of a Raman signal is inherently weak, and its molecular determinant is termed the Raman cross-section (β); it is analogous to molar extinction in absorption spectroscopy. In general, β is a function of the chemical bonds and other electronic factors.¹³³ For instance, molecules with extended π systems such as benzene, naphthalene and anthracene, in which the electrons are easily polarized, have large β . In contrast, small molecules without electron-rich atoms, like H₂ and CO, have small β and hence produce weak Raman scattering. The CO ligand in a complex will likewise have a small Raman cross-section. For applications in biological systems,¹³⁴⁻¹³⁷ Raman spectroscopy has two advantages over IR spectroscopy – the relatively weak Raman scattering of water, and sub-cellular resolution with simple instrumentation. The latter derives from the fact that the incident light for Raman excitation is in the visible to near-IR range and since spatial resolution scales as $\sim\lambda/2$, it provides nm as opposed to μ m resolution for IR spectroscopy.

- **Metal carbonyls as Raman probes for cellular imaging**

Despite the fundamental limitation of weak Raman scattering in metal carbonyls, uptake of the manganese complex [Mn(tpm)(CO)₃]Cl (tpm = tris(1-pyrazolyl)methane) in HT29 human colon cancer cells was demonstrated with 3D Raman imaging; the complex was determined to be localized in the nuclear membrane and nucleolus (figure 1.28).^{138, 139}

Figure 1.28. i) Molecular structure of $[\text{Mn}(\text{tpm})(\text{CO})_3]\text{Cl}$. ii) (A) Optical image of a HT29 human colon cancer cell incubated with an aqueous solution (2 mM) of $[\text{Mn}(\text{tpm})(\text{CO})_3]\text{Cl}$ for 3 h. (B, C) Raman images reconstructed from integrating the intensities of the C-H and C=O stretching peaks. The integration range was $2800\text{--}3050(\pm 2)\text{ cm}^{-1}$ for (B) and $1945\text{--}1965(\pm 2)\text{ cm}^{-1}$ for (C). (D) Overlaid image of panels (B) and (C). (E–G) Cross-section Raman images along the x,z-direction of the same cell. Scanning positions are indicated by the white bar in the optical image. The scale bar for the Raman images is 6 μm . Reproduced from ref. 138 with permission from John Wiley and Sons.

- Detection through surface-enhanced Raman spectroscopy (SERS)

A paradigm shift occurred with the discovery of Raman intensity enhancement by Fleischmann and co-workers, and the subsequent investigation by Jeanmaire and Van Duyne.^{140, 141} The phenomenon refers to the increase in the Raman intensity of a molecule on the surface of a metallic nanostructure; enhancement factors of 10^6 to 10^9 have been reported. It led to the development of surface enhanced Raman scattering (SERS), and widespread applications followed.^{137, 142–145} The mechanism of SERS has been studied over the last few decades,^{146–148} and it is now widely accepted that the enhancement is the product of two mechanisms: electromagnetic and chemical enhancement. Electromagnetic enhancement refers to the resonance interaction of light with the localized surface plasmon of the metallic surface, generating enhanced local electric field, which is responsible for the increase in Raman signals; the surface plasmon is the collective electron cloud at the surface of the metallic nanostructure.^{147, 149} Chemical enhancement refers to a metal-to-adsorbate charge-transfer that can occur with chemisorbed species.

SERS application requires the use of highly reproducible substrates to enhance the CO vibrations and is generally based on two types of substrate – colloidal and planar substrates. Colloidal substrates, which include plasmonic nanoparticles suspended in liquid phase, are frequently used in the formulation of SERS tags for used in biological systems. These tags typically consist of Raman-active molecules that act as reporters adsorbed onto the surface of plasmon-resonant gold or silver nanoparticles protected

with a coating, and bio-functionalized with targeting biomolecules.¹⁵⁰ Even though silver gives a stronger SERS enhancement than gold, the latter is preferred due to their better biocompatibility, size distribution and long-term stability.^{151, 152}

A number of works, including a review, on SERS tags that comprise organic molecules as reporters for SERS imaging have appeared,^{150, 153, 154} but the first report on the use of metal carbonyls as Raman reporters in a SERS tag for live cell imaging was more recent, and comprised the triosmium carbonyl cluster $\text{Os}_3(\mu\text{-H})_2(\text{CO})_{10}$ conjugated onto gold nanoparticles.¹⁵⁵ This organometallic-nanoparticle (OM-NP) conjugate showed strong Raman signals in the CO vibration region ($1800\text{--}2200\text{ cm}^{-1}$), with an enhancement factor estimated at $\sim 15,000$. The OM-NP conjugates were non-toxic, showed good aqueous dispersibility and stability, and could be easily functionalized with the desired biomolecule (L) to give OM-NP-L conjugate for targeted delivery; in this case, they were biofunctionalized with an antibody against epidermal growth factor receptors (EGFR), which are over-expressed in oral squamous cell carcinoma (OSCC) (figure 1.29).

Figure 1.29. i) Preparation of OM-NP-L conjugates using the strong interactions of $\text{Os}_3(\text{CO})_{10}(\mu\text{-H})_2$ with gold nanoparticles. OM-NP conjugates are then functionalized with PEG and antibodies to give conjugate OM-NP-L conjugates. ii) Bright-field, dark-field, and SERS mapping images of (a–e) OSCC cells, (f–j) SKOV cells treated with OM-NP-L, and (k–p) OSCC cells treated with anti-EGFR prior to incubation with OM-NP-L. All SERS mapping images of CO (2030 cm^{-1}) and protein (1600 cm^{-1}) were taken at an interval of $1\text{ }\mu\text{m}$ (633 nm excitation). Adapted from ref. 155 with permission from John Wiley and Sons.

The essential components of these tags are the metal carbonyl and the SERS-active substrate. Furthering their application will require examination of these two

components. For example, it will be of interest to explore the tunability of the CO vibrational frequencies of such conjugates. This will enable a new class of Raman reporters that can provide non-overlapping signals in the 2200-1800 cm^{-1} region, thus allowing the use of more than one conjugates in multiplex applications. To extend the practicality of this system, it will also be desirable to examine SERS-active substrates in other forms apart from the colloidal form. One is the SERS planar substrate, which comprises a plasmonic nanomaterial immobilized onto a flat surface. Such planar substrates are known to give excellent reproducibility and SERS performance, and can potentially be used to develop chip-based sensors.

1.7 Transition metal carbonyl as photoacoustic probe

Pure optical imaging modalities like multiphoton microscopy, confocal microscopy and optical coherence tomography often suffer from poor depth penetration (restricted to the optical transport mean free path in biological tissue) as a result of the strong scattering by soft tissues.¹⁵⁶⁻¹⁵⁸ To circumvent the fundamental depth limitation, a hybrid modality, photoacoustic imaging, was developed and its first functional imaging application was demonstrated in 2003.¹⁵⁹ This emerging imaging modality effectively combines the strengths of both optical and acoustic imaging, i.e., strong optical contrast with high spatial resolution, and is thus being recognized as an attractive, non-invasive method for *in vivo* imaging.¹⁶⁰⁻¹⁶²

Photoacoustic imaging is based on the photoacoustic effect, first described by Alexander Graham Bell in 1880.¹⁶³ The effect refers to the generation of acoustic waves resulting from thermoelastic expansion following the absorption of light (figure 1.30).^{162, 164, 165} The ultrasound waves as detected by the transducers can be reconstructed to generate an image based on algorithms in photoacoustic computed tomography or the detection in point-by-point raster scanning in the case of photoacoustic microscopy.¹⁶⁰ The optical contrast is determined by the absorption of light by the object of interest, while the resolution is derived from the detected ultrasonic emission. Since biological tissue scattered ultrasound wave less as compared to light, reconstructed images of higher spatial resolution can be obtained. Photoacoustic imaging has allowed a wide range of *in vivo* studies, including probing dynamic changes in the hemoglobin oxygen saturation of skin, brain hemodynamic changes and tumor angiogenesis.^{159, 166, 167}

Figure 1.30. Illustration of the photoacoustic (PA) mechanism. When a target absorbs light, photoacoustic waves are generated via thermo-elastic expansion. The photoacoustic waves propagate and are detected by an ultrasonic transducer. Adapted from ref. 160 with permission from the American Chemical Society.

Although photoacoustic imaging can be used to visualize the distribution of endogenous molecules like oxy- and deoxy-hemoglobin, exogenous contrast agents are, nevertheless, often introduced into biological systems for site-specific imaging. Various contrast agents have been developed, including single-walled carbon nanotubes,¹⁶⁸ gold nanostructures,^{169, 170} fluorescent proteins,¹⁷¹ and cyanine dyes.¹⁷² More recently, Leong and Olivo *et. al.* have demonstrated the use of a water-soluble triosmium carbonyl cluster for *in vivo* imaging of the rat cerebral cortex vasculature (figure 1.31).¹⁷³ This highly stable contrast agent allows the brain vasculature, especially the superior sagittal sinus (SSS), to exhibit higher photoacoustic contrast as compared to their intrinsic contrast and the use of carbon nanotubes. Although the photoacoustic contrast of the triosmium carbonyl cluster is high, a major drawback lies in the incident wavelength of 410 nm; tissue penetration is low at this wavelength and thus an invasive procedure is required to carry out the imaging. It is therefore of interest to identify metal carbonyls that absorb in the near-IR (NIR) range (680 to 1000 nm) which can allow the use of NIR light for deeper tissue penetration and less scattering; such metal carbonyls can potentially be used for whole body *in vivo* imaging.

Figure 1.31. a) Molecular structure of the water-soluble triosmium carbonyl cluster. a) Photograph of the rat cortical blood vessels, with the superior sagittal sinus (SSS) indicated. The ROI cross-section location is indicated by a blue arrow. b) Photoacoustic B-scan images acquired before, and 20 min after, the administration of a 30 μ M aqueous solution of the cluster or carbon nanotubes. The SSS is highlighted with a red oval. d) The brain vasculature shows up clearly in the photoacoustic C-scan. Adapted from ref. 173 with permission from the Royal Society of Chemistry.

1.8 Aim and objectives

From the foregoing survey and analysis, it is clear that the application of metal carbonyls in bioimaging has come some way with current development lies with SERS and photoacoustic imaging. The aim of this project is therefore to explore the use of metal carbonyls as SERS- and photoacoustic-based reporters for various bio-imaging and-sensing applications.

Specific objectives:

1. Investigate different classes of metal carbonyls as Raman reporters with colloidal gold nanospheres in SERS-based applications.
2. Demonstrate the use of metal carbonyl clusters with SERS planar substrates in SERS-based bioassays.
3. Develop metal carbonyls as near-IR reporters for whole body *in vivo* photoacoustic imaging.

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Chapter 2: Surface-Enhanced Raman Spectroscopy (SERS) of Metal Carbonyls on Gold Nanospheres

Surface-enhanced Raman spectroscopy (SERS) has garnered much attention as an important and robust optical technique for bio-sensing. Most SERS applications involve the sensing of various molecules or analytes that are close to the surface of the plasmonic metallic substrate, giving valuable chemical information for qualitative or quantitative analysis. This technique has been utilized to develop optical probes, commonly known as “SERS tags”, which are essentially plasmonic nanoparticles with Raman reporters attached onto the surface. Such SERS tags, akin to fluorescent probes, can be functionalized and applied for sensitive SERS-based sensing and imaging.¹⁻⁵ The components of SERS tags, the plasmonic nanoparticle and the Raman reporter, are two important considerations for the development of effective and functional tags. Over the past few decades, most efforts have been dedicated to the design of novel metallic nanoparticles with high plasmonic activity for better SERS enhancement.² Some examples include gold nanostars,⁶ gold nanoflowers,⁷ gold-silver bimetallic particles,⁸ and gold aggregates.⁹ On the other hand, the development of highly sensitive Raman reporters with unique spectral characteristics remains less explored. An example is the work by Olivo and Chang who reported the screening of an 80-member tricyanocyanine library for the selection of lead compounds with strong signals for *in vitro* and *in vivo* SERS detection.^{10, 11} The study and design of Raman reporters to allow for a library of compound with non-overlapping signals remain important as it will allow the use of multiple SERS tags for multiplex detection – the simultaneous detection of more than one target.^{11, 12}

The Raman reporters used thus far are all organic-based, with the exception of an organometallic-based reporter, a triosmium carbonyl cluster, conjugated onto gold nanoparticles for the SERS detection of cancer cells.¹³ The advantage of using a metal carbonyl as the Raman reporter lies in the CO vibrational stretch in the region of 2200-1800 cm^{-1} , which is free from interference by other biomolecules, thereby offering a

clear spectral window for sensing applications. This chapter details the investigation into the SERS effect of various metal carbonyls on gold nanospheres (AuNS), in an attempt to identify compounds with unique spectral characteristics that can potentially be used for multiplex applications.

2.1 Metal carbonyl clusters as Raman reporters

2.1.1 Synthesis and design of metal carbonyl clusters

The first example of metal carbonyl-gold nanoparticle conjugates involved the use of an electron-deficient triosmium carbonyl cluster, $\text{Os}_3(\text{CO})_{10}(\mu\text{-H})_2$, as the Raman reporter.¹⁴ The interaction between the gold nanoparticle with the cluster was deduced to be direct bonding between the gold atoms and the three-center two-electron $\text{Os}(\mu\text{-H})\text{Os}$ moiety. In order to have tunability of the CO vibrational frequencies and yet have strong binding to the gold surface, however, a general design comprising a metal carbonyl cluster, a linker and a sulfur-containing functionality was envisaged (figure 2.1).

Figure 2.1. a) Components of a metal carbonyl cluster as Raman reporters. b) Binding of the metal carbonyl clusters onto AuNS via the sulfur groups.

The cluster-binding functionality, ligand and charge form an integral part of the cluster and have direct effect in tuning the CO vibrational frequencies. The linker modulates the distance between the cluster and the sulfur functionality, thereby affecting the AuNS-cluster distance. Lastly, the sulfur functionality is needed to anchor the metal carbonyl clusters onto the AuNS surface, as sulfur compounds can bind covalently with gold nanoparticles via an Au-S bond. This is not limited to the

more commonly used thiols, but can include disulfides and thioethers as well.^{10, 15-19} In this study, 60 nm AuNS was chosen as the substrate for investigating the SERS activity of the metal carbonyls as it has better biocompatibility, a narrow size distribution, and is less prone to oxidation compared to the silver counterpart. The size of around 60 nm is also regarded as a good size for both *in vitro* applications and for SERS enhancement.^{2, 10, 20-22}

A simple model that fits the requirements is **2a**. It is essentially a thiolato-bridged (μ -S) triosmium carbonyl cluster, with a two-carbon linker and a free thiol functionality on the other end. Given the high reactivity of the precursor, cluster **1**, towards thiols to form stable μ -S clusters, **2a** was synthesized from the reaction of **1** with 1,2-ethanedithiol according to a reported method (scheme 2.1).²³⁻²⁵

Scheme 2.1. Synthesis of clusters **2a**, **3b** and **4**.

The cluster-binding μ -S functionality could also be replaced by a μ, κ^2 -O,O' functionality, *viz.*, cluster **3a**, which can be synthesized from the same precursor **1**; this would modulate the CO vibrational frequencies. However, the higher reactivity of thiol

over carboxylic acid with triosmium clusters made it difficult to obtain **3a** from the reaction of **1** with 3-mercaptopropionic acid as the soft SH group reacted more readily and strongly with the soft Lewis acidic metal center than the hard COOH group.^{26, 27} Instead, cluster **1** was reacted with 3,3'-dithiodipropionic acid to give the carboxylate-bridged cluster **3b** as the disulfide was much less reactive than free thiol. Cluster **3b** is expected to react with AuNS via cleavage of the S-S bond to form similar species as that of **3a**.¹⁷ Another cluster which was synthesized was the sulfur-capped (μ_3 -S) cluster **4**.²⁸ The sulfur atom in **4** could act as the cluster-binding functionality, linking group, as well as the anchor onto the AuNS.

The IR spectra of **2a**, **3b** and **4** showed distinct CO vibrational frequencies (figure 2.2); their symmetric stretch are at 2113, 2109 and 2121 cm^{-1} , respectively.²⁹⁻³¹ The intensity of this stretch is usually the weakest in the IR spectrum but shows up strongly in the Raman spectrum.

Figure 2.2. Overlay of IR spectra (ν_{CO}) of cluster **2a**, **3b** and **4** in DCM.

The CO vibrational frequency can also be tuned through modification of the ligand sphere or by introducing a charge on the cluster. As mentioned in section 1.5.1, an electron-donating phosphine can increase the electron density on the metal center, thereby increasing π -back donation to the π^* orbitals of C-O, and shifting the CO vibrations to lower frequencies. On the other hand, the CO vibrations for a cationic

cluster are shifted to higher frequencies because of the decrease in π -back donation to the π^* orbitals of C-O.

Tuning the CO vibrational frequency through these two modifications was made with cluster **6**, which was obtained from the reaction of **5** with **1**, followed by cysteamine (scheme 2.2).

Scheme 2.2. Synthesis of clusters **6** and **6.H⁺**.

The phosphine **5**, with an N-hydroxy succinide (NHS) ester group, was synthesized according to a reported method.³² The orthogonality of **5** allowed the phosphine to coordinate to the osmium, while the N-hydroxy succinimide (NHS) ester was selective towards the amine functionality of cysteamine. Cluster **6** has both di-phosphine substitution and a thiol functionality, with the phosphines acting as both the ligand and cluster-binding functionality. The cluster was purified and characterized by IR, HRMS, ¹H, ³¹P{¹H} and ¹H{³¹P} NMR spectroscopy, and a COSY experiment. The ³¹P{¹H} NMR spectrum showed three signals which can be attributed to the presence of *trans*-

trans and *cis-trans* isomers, a common observation for diphosphine-substituted triosmium carbonyl clusters.³³

Protonation of **6** with CF₃COOH afforded the cationic cluster **6.H⁺**, which could be isolated in moderate yield after anion exchange using NH₄PF₆. Successful protonation of **6** is evident from the colour change from orange to yellow. Cluster **6.H⁺** was characterized fully by IR, ¹H, ¹H{³¹P}, ³¹P{¹H} NMR spectroscopy, and a COSY experiment. As expected, the CO vibrations for **6.H⁺** were shifted to higher frequency with respect to those of **6** (figure 2.3).

Figure 2.3. Overlay of IR spectra (νCO) of cluster **6** and **6.H⁺** in DCM.

The effect of changing the metal centres was also investigated with the rhenium clusters **7a** and **7b**, which contain a free SH functionality; they were synthesized following a reported method (scheme 2.3).³⁴

Scheme 2.3. Synthesis of clusters **7a** and **7b**.

2.1.2 SERS spectra of metal carbonyl clusters on AuNS

The as-synthesized clusters were incubated with 60 nm AuNS, followed by centrifugation and re-dispersion in water. The SERS spectra for **2a**, **3b** and **4** in the 1800-2200 cm^{-1} region are shown in figure 2.4.

Figure 2.4. SERS spectra (νCO) of cluster **2a**, **3b** and **4** incubated with AuNS.

The spectra for **2a** and **3b** were similar in the pattern, with a broad peak at $\sim 2000 \text{ cm}^{-1}$ and a sharp peak at higher frequency at 2110 and 2114 cm^{-1} for **2a** and **3b**, respectively, and the shift to higher wavenumber for **3b** is due to the more electron withdrawing carboxylate group. These strong signals can therefore be useful for SERS

detection. On the other hand, the SERS spectrum for **4** did not show a strong symmetric stretch (at 2121 cm^{-1}) but showed broad bands from 1962 to 2076 cm^{-1} . This could be due to the close proximity of the binding group (sulfur atom) to the osmium core, resulting in direct interaction of the gold atoms with the triosmium core; gold compounds are reported to have a high affinity for the osmium core.³⁵⁻³⁹ The simple SERS spectra of conjugates **2a** and **3b** appear to be derived from a single structural moiety of the clusters near the AuNS surface, while the broader and more complex spectra of conjugate **4** could be due to the different bonding interactions between the cluster and AuNS.

The attempts at conjugating $\mathbf{6.H}^+$ to AuNS led to irreversible aggregation; the AuNS changed colour from red to purple upon the addition of $\mathbf{6.H}^+$. The cationic nature of $\mathbf{6.H}^+$ probably disrupted the mutual repulsion of the negatively charged nanoparticles. Nevertheless, the SERS spectrum of the conjugate showed signals in the $1800\text{--}2200\text{ cm}^{-1}$ region; the symmetric stretch for the conjugates of **6** and $\mathbf{6.H}^+$ shows up strongly at 2082 and 2121 cm^{-1} , respectively, and do not overlap with that for **2a** (figure 2.5).

Figure 2.5. SERS spectra (vCO) of cluster **2a**, **6** and $\mathbf{6.H}^+$ incubated with AuNS.

The AuNS conjugates of cluster **7a** and **7b** showed drastically different SERS spectra, even though the clusters had similar CO vibrational frequencies in their IR spectra (figure 2.6). The SERS spectrum for the AuNS conjugate of **7a** showed broad bands at 1939 and 2009 cm^{-1} , with weak signals at higher wavenumber. On the other hand that for the AuNS conjugate of **7b** showed a distinct, strong peak at 2114 cm^{-1} which corresponded to the symmetric stretch. Taken together with the SERS spectra of the conjugates of **2a** and **4**, it is clear that the linker length does have an effect on the SERS spectrum. The linker modulates the distance and hence the interaction between the gold surface and the cluster core, producing SERS spectra with different features.

Figure 2.6. SERS spectra (vCO) of clusters **7a** and **7b** incubated with AuNS.

A comparison of the CO stretching frequencies for the clusters with the SERS frequencies of their AuNS conjugates is given in table 2.1. A few points can be noted:

- 1) The symmetric stretch shows up sharply in the SERS spectra.
- 2) There is good correlation between the symmetric stretch in the IR spectra of the clusters with the SERS spectra of the AuNS conjugates, with minimal frequency shifts.

- 3) Changing the ligands or charge is effective for tuning the CO vibrational frequency. Changing to a different cluster binding functionality or transition metal is less effective.
- 4) The length of the linker has a direct effect on the SERS spectra; too short a linker (one atom between the cluster and AuNS) leads to broad signals while a longer linker gives a more defined spectrum with a sharp symmetric stretch.

Table 2.1. IR frequencies of **2a** to **7b** and the respective SERS frequencies of the AuNS conjugates.

Compounds	^a IR (ν_{CO} , cm^{-1})	SERS (ν_{CO} , cm^{-1})
2a	2109(w), 2068(s), 2059(m), 2022(s), 1998(m), 1986(m, sh)	2110(s), 1996(m, br)
3b	2113(w), 2076(s), 2063(m), 2025(s), 2013(s), 1982(w)	2114(s), 2008(m, br) 1985(m, sh)
4	2121(w), 2084(s), 2057(s), 2037(m), 2007(m), 1994(m)	2120(w), 2076(w), 2020(s, br) 1962(m, br)
6	2084(w), 2025(s), 1997(vs), 1969(m, br)	2082(s), 1992(m, br) 1950(m, br)
6.H+	2121(w), 2080(w), 2067(m), 2038(vs), 2025(m)	2121(s), 2066(m)
7a	2117(w), 2093(m), 2019(s), 1999(m), 1965(m)	2150(w, br), 2116(m), 2101(w), 2009(s, br), 1939 (m, br)
7b	2115(w), 2091(m), 2017(vs), 1999(s), 1964(s)	2114(s), 2004(m, br), 1993(m, br)

^aIn DCM.

2.2 Half-sandwich metal carbonyl complexes as Raman reporters

In an attempt to expand the scope of metal carbonyl-based Raman reporters, half-sandwich carbonyl complexes of the group 6 and 8 metals were investigated. Half-sandwich or piano-stool metal carbonyl complexes are organometallic complexes with a cyclic polyhapto ligand bound to an metal center with CO ligands.⁴⁰ Arene and cyclopentadienyl groups often serve as reliable spectator ligands in forming η^6 -arene and η^5 -cyclopentadienyl (Cp) complexes, respectively. In comparison to metal carbonyl clusters, mononuclear metal carbonyl complexes have a number of advantages. Firstly, the number of CO vibrational bands is reduced, giving a simpler

spectrum; this reduces the possibility of overlapping bands when more than one tag is used. Secondly, the synthesis and chemistry of half-sandwich complexes are very well-established and can provide many synthetic routes and opportunities for derivatization.⁴¹⁻⁴⁴ Lastly, the use of less metal can reduce the cost and complexity of the system.

Similar to the metal carbonyl clusters, the CO vibrations of the half-sandwich metal carbonyls were tuned through the use of different transition metals and ligand set. To allow the anchorage of the complexes onto AuNS, complexes with a good-leaving group (iodide) were used; it is envisaged that the electron-rich gold metal atoms can react with the metal center via nucleophilic substitution of the iodide. The half-sandwich complexes **8-13** were synthesized according to reported methods (figure 2.7), with the exception of **11a** which was obtained commercially.⁴⁵⁻⁵¹

Figure 2.7. a) The CO vibrational frequency of a half-sandwich metal carbonyl complex can be tuned via the Cp ring, metal center and the ligands. b) Molecular structures of half-sandwich metal carbonyl complexes used in the study.

The complexes were conjugated to AuNS by incubation, and they generally showed good CO stretching vibrations in the 1800-2200 cm^{-1} region (figure 2.8). Attempts at conjugating the chromium (**8a**) and iron (**11a**) analogues were futile, however; no Raman signal in the 1800-2200 cm^{-1} was observed, and the AuNS showed signs of irreversible aggregation. Presumably, the first row transition metal complexes were not sufficiently stable for such conjugation. A comparison of the SERS spectra of the conjugates of **9a-10a** and **12a-13a**, which differ in their metal

center, show that the two peaks for all the four complexes fall within a narrow range (1943 to 2046 cm^{-1}). This would pose problems when the conjugates are utilized for multiplex detection and quantification. On the other hand, the SERS spectra of the conjugates of **12'a**, **10b** and **10c** are shifted to lower frequency compared to those of **12a** and **10a**, respectively. This is consistent with the presence of more electron-donating ligands in the former. The SERS spectra for all conjugates showed similar pattern as their respective IR spectra, indicating that the geometry of the complexes on the AuNS remained relatively the same as the free complexes.

Figure 2.8. SERS spectra (vCO) of **9a-13a** incubated with AuNS.

Table 2.2. IR frequencies of **9a** to **13a** and the respective SERS frequencies of the AuNS conjugates.

Compounds	^a IR (νCO , cm^{-1})	SERS (νCO , cm^{-1})
9a	2043(m), 1967(s, br)	2030(s), 1958(m, br)
10a	2037(m), 1952(s, br)	2026(s), 1946(m, br)
12a	2049(s), 1999(s)	2045(s), 1964(m, br)
13a	2034(m), 1978(s)	2024(s), 1969(m, br)
12'a	2028(s), 1977(s)	2026(s), 1975(m)
10b	^b 1955(m), 1876(s)	1953(s), 1870(m)
10c	^b 1942(s), 1859(m)	1944(s), 1852(w)

^aIn DCM, unless otherwise stated. ^bIn THF.

Unlike the case in the cluster-based metal carbonyls which showed little or no frequency shifts in the symmetric stretch on conjugation to the AuNS (section 2.2), the SERS spectra (νCO) of the AuNS conjugates of some of the half-sandwich metal carbonyl complexes showed significant shifts to lower frequency on conjugation (table 2.2). This is indicative of the increase in electron density at the metal center in the conjugates, which can be attributed to the Au-M interaction; such an interaction is lacking in the cluster conjugates where the cluster is separated from the AuNS by a linker. This is consistent with the expectation that there is direct bonding interaction between the half-sandwich complexes and the AuNS, via elimination of the iodide (figure 2.9).

Figure 2.9. Proposed interaction of the half-sandwich metal complexes with AuNS.

A separate study using the bromide and chloride analogues of **10a** incubated with AuNS did not show any CO vibrational frequency in the SERS spectra (appendix A2.27). This can probably be attributed to the lower reactivity of the bromide and chloride analogues as compared to **10a**, thus the conjugate did not form.

2.3 Effect of aggregation

During the course of the above studies, it was observed that while the AuNS played a critical role in producing detectable Raman signals (no signal was observed in the absence of AuNS), obtaining consistency in the SERS intensity was a problem. We suspected that this was due to variation in the extent of aggregation across different samples. Gold colloids are known to be very temperamental, and in our case, can be difficult to control. Aggregation can be easily induced during the washing steps where high centrifugal force is applied.⁵² A highly ionic environment and low pH can also reduce the negative charges on the particle surface. This reduces mutual repulsion, resulting in aggregation.⁵²⁻⁵⁴

To understand the origin of the SERS enhancement in the metal carbonyl-AuNS conjugates, the AuNS conjugate of cluster **2a** was prepared and subjected to centrifugation at low speed (1200g for 10 min) to minimize aggregation. Aliquots of the conjugates were scanned under the Raman microscope, and weak signals were detected (figure 2.10). The conjugates were then topped up with water and spun down at a much higher speed (10 000g for 2 min). Aggregation was observed, with some irreversible precipitation. Aliquots of these were scanned and, this time, stronger signals were observed.

Figure 2.10. SERS spectra (vCO) of **2a** incubated with AuNS with centrifugation at low and high speed respectively.

In another study, the AuNS conjugates of **8a-11a**, after centrifugation and re-dispersion in water, were characterized by UV-vis spectroscopy to assess the state of aggregation of the conjugates. The conjugates showed similar optical spectra as that for the non-conjugated 60 nm gold nanoparticles, except for a broad shoulder above 600 nm, which is indicative of aggregation (figure 2.11a).^{53, 55} Transmission electron microscopy (TEM) images of the conjugates showed spherical Au particles in contact with neighboring particles (figure 2.11b). However, this could be due to aggregation during sample preparation on the TEM grid and under the high vacuum conditions in the TEM chamber. Hence, TEM characterization is not conclusive for evaluation of the state of aggregation.

Other workers have reported that SERS enhancement is particularly strong in the gaps between plasmon-coupled metal nanoparticles, commonly referred to as “hot-spots”.^{56, 57} A recent study has shown that monomers of Ag and AuNS do not show SERS enhancement and suggested that the observed SERS signals reported in many earlier work could have originated from aggregation of the colloids; the close inter-particle distance in those aggregates can generate hot-spots for SERS enhancement.⁵² That study clarified the misrepresentation of Ag and AuNS as highly SERS-active substrates, and was well-supported by theoretical simulations.^{52, 58} In another work, Chen and co-workers described the SERS scattering in the hot-spots of colloidal nanoparticles, and reported that dimers and trimers gave 16 and 87 times enhancements, respectively, in comparison to the intensity from monomers.⁵³ Hence, a small amount of aggregation in a sample can effectively generate a strong SERS signal, but often erroneously regarded to be from the monomers. Given the low Raman cross-section of the CO ligand and the low SERS-activity of AuNS, it is likely that the observed SERS spectra for the various metal carbonyls resulted from aggregation of the colloid during sample preparation, and not from the AuNS monomer.

Figure 2.11. a) Optical absorption spectra and b) TEM images of **9a-10a** and **12a-13a** incubated with AuNS.

2.4 Conclusion

A qualitative survey of two classes of metal carbonyls, namely, metal carbonyl clusters and half-sandwich metal carbonyl complexes, as Raman reporters was carried out. The cluster design (cluster-linker-anchor) serves as a good model for the tuning of the CO vibrational frequency. Tunability over a larger frequency range was achieved with the use of ligands and charge on the cluster core, while variations in the binding functionality and the use of different transition metals were less effective. The linker, which modulates the distance, hence, the interaction between the cluster and AuNS, has little effect in tuning the CO frequency but played an important role in altering the SERS spectral feature. Lastly, the sulfur-containing anchor is important for strong interaction with AuNS.

The extension of the study to half-sandwich metal carbonyl complexes of group 6 and 8 metals gives the advantages of simpler spectra and easier modification with the more established chemistry. Similar to the clusters, tunability of the CO vibrational stretches can be obtained with the use of various metals and ligands (Cp ring and phosphines). Direct interaction between the AuNS and the metal centers with the elimination of iodide as the leaving group was deduced to be the mode of interaction; this can do away with the need for sulfur-containing functionality for anchorage onto AuNS. However, the use of other halides as leaving group can have different result and first row transition metal complexes (Cr and Fe) were found to be not sufficiently stable for such conjugation.

Lastly, the SERS enhancement was deduced to be from the aggregation of the AuNS during the sample preparation and not from the AuNS monomer. Rational design and tuning of the SERS signal on metal carbonyls are feasible and can potentially contribute to the current library of organic Raman reporters. Taken together, these findings serve as important considerations for design and use of metal carbonyls for SERS applications.

2.5 Experimental section

2.5.1 General methods

All reactions and manipulations were carried out under an argon atmosphere using standard Schlenk techniques. Solvents that were used for reaction were distilled over the appropriate drying agents under argon before use. Purification of compounds was generally carried out by column chromatography on silica gel, or by preparative thin-layer chromatography (TLC) using 20 cm x 20 cm plates pre-coated with silica gel 60 F₂₅₄. Infrared (IR) spectra were recorded on a Bruker Alpha FT-IR spectrometer. Solution spectra were recorded in DCM solution, unless otherwise stated, in a solution IR cell with NaCl windows and a path length of 0.1 mm, at a resolution of 2 cm⁻¹. For compounds in THF solution, a solution IR cell with CaF₂ windows and a path length of 0.1 mm was used. Optical absorption spectra were recorded using a Hitachi U-2900 double-beam spectrophotometer using quartz cuvette with path length of 10 mm. ¹H, ¹³C{¹H}, ³¹P{¹H}, ¹H{³¹P} and COSY NMR spectra were recorded on a JEOL ECA 400 or ECA 400SL (400 MHz) spectrometer, in CDCl₃. ¹H, ¹³C{¹H} chemical shifts are referenced to the residual proton resonance of the solvent. HRMS were recorded in ESI mode on a Waters UPLC-Q-ToF MS mass spectrometer. The Raman spectral measurements were carried out using a Renishaw InVia Raman (UK) microscope with a Peltier cooled CCD detector and an excitation wavelength at 785 nm, where the laser beam was directed to the sample through a 50× objective lens, which was used to excite the sample and also to collect the return Raman signal. All Raman spectra were processed with the WiRE3.4 software. The laser power at the sample was measured to be 20.8 mW and the exposure time was set at 10 s, throughout the measurements. Prior to each measurement, the instrument was calibrated against a silicon standard with a Raman peak centered at 520 cm⁻¹.

Copper specimen grids (300 mesh) with formvar/carbon support film were purchased from Beijing XXBR Technology Co. TEM images were collected from a JEM-1400 (JEOL) Transmission Electron Microscopy operated at 100 kV. TEM grids were treated with oxygen plasma in a Harrick® plasma cleaner/sterilizer for 1 min to improve the surface hydrophilicity. A sample solution (10 µL) was dropped onto the hydrophilic side of the TEM grid. Filter paper was used to wick off the excess solution on the TEM grid, which was then left to dry in air for 5 min.

Clusters **1**,⁵⁹ **2a**,²³ **4**,²⁸ **7a**,³⁴ and phosphine **5**,³² were prepared according to reported procedures with slight modifications. Half-sandwich complexes **8a-10a**,⁴⁵⁻⁴⁷ **10b-c**,⁵¹ **12a**,⁴⁸ **12'a**,⁶⁰ and **13a**,⁴⁹ were also synthesized according to reported procedures with slight modifications. Gold nanospheres (60 nm) were purchased from BBI Solutions. Os₃(CO)₁₂ was purchased from Oxkem Ltd, and all other reagents were purchased from other commercial sources and used as supplied without further purification.

2.5.2 Synthesis of cluster 3b

Cluster **1** (57 mg, 0.061 mmol) and 3,3'-dithiodipropionic acid (7.7 mg, 0.037 mmol) were dissolved in THF (8 mL) and left to stir overnight at room temperature. Solvents and volatiles were removed in vacuo and the yellow residue was purified by TLC using DCM:hexane (1:1, v/v) as eluent to yield cluster **3b**.

Yield: 9 mg (15 %)

R_f = 0.56

IR (DCM, cm⁻¹): 2113(w), 2076(s), 2063(m), 2025(s), 2013(s), 1982(w).

¹H NMR (400 MHz, CDCl₃): δ 2.65-2.58 (m, 8H, CH₂CH₂), -10.46 (s, 2H, OsHOs).

¹³C{¹H} NMR (100.46 MHz, CDCl₃): δ 189.50, 183.61, 181.67, 175.97, 174.40, 174.29, 37.09, 34.20.

2.5.3 Synthesis of cluster 6

Cluster **1** (30 mg, 0.032 mmol) was dissolved in DCM (6 mL) and stirred under argon. Phosphine **5** (25 mg, 0.071 mmol) was added and the mixture left to stir for 30 min. The solution turned from yellow to orange, and monitoring by IR spectroscopy showed the presence of the di-substituted phosphine cluster. Cysteamine (6 mg, 0.071 mmol) was then added and the reaction mixture was left to stir for 2 h. Solvents and volatiles were removed *in vacuo* and the residue was purified by flash column chromatography and TLC. However, it was noted that purification by flash column chromatography and silica TLC led to tailing and the excess phosphine could not be separated from the product. Hence, the residue was purified by reversed-phase TLC using ethyl acetate:hexane (1:1, v/v) as eluant to yield the product as a yellow-orange solid.

Yield: 35 mg (72 %)

R_f = 0.53

IR (DCM, cm⁻¹): 2084(w), 2025(s), 1997(vs), 1969(m, br).

^1H NMR (400 MHz, CDCl_3): δ 7.48-7.42 (m, 20H, Ar), 5.75 (br, 2H, NH), 3.33 (dt, 4H, $J = 6.3, 6.3$, $\text{NHCH}_2\text{CH}_2\text{SH}$), 3.03 (br, 4H, $\text{PCH}_2\text{CH}_2\text{CO}$), 2.58 (dt, 4H, $J = 6.6, 8.7$, $\text{NHCH}_2\text{CH}_2\text{SH}$), 2.12-2.06 (m, 4H, $\text{PCH}_2\text{CH}_2\text{CO}$), 1.30 (t, 2H, $J = 8.7$, SH).

$^{31}\text{P}\{^1\text{H}\}$ NMR (160.26 MHz, CDCl_3): δ -8.76, -10.45, -13.21.

HRMS-ESI m/z calculated for $\text{C}_{44}\text{H}_{41}\text{N}_2\text{O}_{12}\text{P}_2\text{S}_2^{186}\text{Os}^{190}\text{Os}^{192}\text{Os}$ $[\text{M}+\text{H}]^+$ 1483.0314, found 1483.0278.

2.5.4 Synthesis of cluster $6\cdot\text{H}^+$

Cluster **6** (40 mg, 0.027 mmol) was dissolved in DCM (2 mL), followed by the addition of CF_3COOH (60 μL , 0.78 mmol). The reaction turned from orange to yellow immediately and was left to stir for 15 min. NH_4PF_6 (2 mg, 0.21 mmol) dissolved in deionized water (2 mL) was added and the biphasic mixture was stirred vigorously for 2 h. The organic layer was extracted, washed with deionized water (3 x 2 mL) and dried over anhydrous MgSO_4 . Solvent was removed in vacuo and the yellow residue was purified by column chromatography, using $\text{MeOH}:\text{DCM}$ (1:500 to 6:500, v/v) as eluent with gradual increase of eluent strength, to yield the product as a yellow solid.

Yield: 27 mg (61 %)

IR (DCM, cm^{-1}): 2121w, 2080w, 2067m, 2038vs, 2025m.

^1H NMR (400 MHz, CDCl_3): δ 7.62-7.47 (m, 20H, Ar), 7.01 (t, 2H, $J = 5.5$, NH), 3.29 (dt, 4H, $J = 6.1, 6.1$, $\text{NHCH}_2\text{CH}_2\text{SH}$), 3.09-3.04 (m, 4H, $\text{PCH}_2\text{CH}_2\text{CO}$), 2.54 (dt, 4H, $J = 7.3, 7.3$, $\text{NHCH}_2\text{CH}_2\text{SH}$), 2.24-2.18 (m, 4H, $\text{PCH}_2\text{CH}_2\text{CO}$), 1.35 (t, 2H, $J = 8.3$, SH), -19.48 (t, 1H, $J = 7.3$, OsHOs).

$^{31}\text{P}\{^1\text{H}\}$ NMR (160.26 MHz, CDCl_3): δ -12.03 (OsP), -143.69 (PF_6).

HRMS-ESI m/z calculated for $\text{C}_{44}\text{H}_{41}\text{N}_2\text{O}_{12}\text{P}_2\text{S}_2^{187}\text{Os}^{190}\text{Os}^{192}\text{Os}$ $[\text{M}]^+$ 1484.0333, found 1484.0366.

2.5.5 Synthesis of cluster **7b**

Cluster $\text{Re}_2(\text{CO})_8(\text{NCCH}_3)_2$ (104 mg, 0.153 mmol), synthesized from a reported method,³⁴ and 1,2-ethanedithiol (129 μL , 1.53 mmol) were dissolved in toluene (5 mL) and heated at 80 $^\circ\text{C}$ for 30 min. Solvents and volatiles were removed *in vacuo* and the residue was purified by TLC using $\text{DCM}:\text{hexane}$ (1:1, v/v) as eluant to yield cluster **7b** as a colourless oil.

Yield: 3 mg (3 %)

$R_f = 0.61$

IR (DCM, cm^{-1}): 2115w, 2091m, 2017vs, 1999s, 1964s.

^1H NMR (400 MHz, CDCl_3): δ 3.01 (t, 2H, $J = 7.3$, $\text{SCH}_2\text{CH}_2\text{SH}$), 2.86 (dt, 2H, $J = 7.3, 8.3$, $\text{SCH}_2\text{CH}_2\text{SH}$), 1.79 (t, 1H, $J = 8.3$ Hz, SH), -14.07 (s, 1H, ReHRe).

LCMS-ESI m/z : 660.29 $[\text{M-H-CO}]^-$, 632.83 $[\text{M-H-2CO}]^-$.

2.5.6 Preparation of AuNS conjugates of metal carbonyl clusters 2a-7b

In a typical reaction, 60 nm AuNS (3 mL, 2.6×10^{10} particles/mL) was centrifuged and re-dispersed in deionized water till about 990 μL . Freshly prepared solutions of the clusters in DMSO (10 μL , 10 mM)* were added and allowed to incubate at room temperature for 2 h. Excess complexes were removed by centrifugation (1200g, 20 min), and the conjugate pellets were resuspended in deionised water (100 μL). An aliquot (20 μL) of the solution was dropped onto a glass slide and scanned under the Raman microscope. An excitation wavelength of 785 nm, with exposure time of 10 s and three accumulations was used throughout the measurement.

*For clusters **6** and **6.H⁺**, 10 μL of 5 mM DMSO solution was added instead.

2.5.7 Preparation of AuNS conjugates of half-sandwich metal carbonyl complexes 8a-13a

In a typical reaction, 60 nm AuNS (3 mL, 2.6×10^{10} particles/mL) was centrifuged and re-dispersed in ethanol till about 990 μL . Freshly prepared solutions of **8a-11a** in ethanol (10 μL , 10 mM) were added and allowed to incubate at room temperature for 2 h. Excess complexes were removed by centrifugation (1200g, 20 min), and the conjugate pellets were resuspended in deionised water (100 μL). An aliquot (20 μL) of the solution was dropped onto a glass slide and scanned under the Raman microscope. An excitation wavelength of 785 nm, with exposure time of 10 s and three accumulations was used throughout the measurement.

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Chapter 3: Metal Carbonyl-Boronic Acid Conjugate - a SERS Probe for Glucose Detection and its Cytotoxicity

According to a report by the World Health Organization, 422 million people worldwide have diabetes in 2014, a drastic rise from 108 million in 1980.¹ Diabetes is a chronic metabolic disease characterized by the pancreas not being able to produce enough insulin, or the body not being able to utilize the insulin produced effectively.^{2,3} Type two diabetes, which is the major contributor to the rise in this disease, is largely caused by obesity – a product of rising living standard worldwide. Over the past decade, the occurrence of diabetes has increased faster in low- and middle-income countries than in high-income countries.¹ A person is normally diagnosed with diabetes via symptoms like hyperglycaemia, which is characterized by a fasting blood glucose level of $\geq 7.0 \text{ mmol L}^{-1}$.^{1, 3} Hence, tight glycemic control is important for diabetes management which requires strict dietary control as well as frequent blood glucose measurement. Quantification of glucose using various platforms, thus, remains important and relevant. This chapter details the concept of coupling boronic acid to a metal carbonyl and a SERS planar substrate to develop a selective SERS-based glucose assay, and an attempt to extend it to an *in vivo* application.

3.1 Methods for glucose detection

Portable hand held glucose meter for home use was first available in the 1980s based on the ferrocene-mediated electrochemistry of glucose oxidase (GOx).⁴⁻⁶ The working principle involves the oxidation of glucose by glucose oxidase (GOx), followed by electron transfer facilitated by the mediator (ferrocene) to the electrode (figure 3.1).^{4, 7, 8} The protein layer surrounding the flavin adenine dinucleotide (FAD) redox center of GOx hinders direct electron transfer to the electrode, hence the need for a mediator.⁸ The method was subsequently modified to a ferricyanide-mediated version and since then, the method of detection has remained unchanged.

Figure 3.1. Sequence of reactions that occur in glucose oxidase-mediated biosensor. Adapted from ref. 8 with permission from the American Chemical Society.

In general, a drop of biofluid (in most cases, blood) is applied onto an enzymatic test strip and inserted into the device, which comprises a transducer-detector unit for electrochemical detection and quantification.⁶ Though the enzymatic reaction is specific, the electrochemical reaction can be interfered with by redox-active species present in the fluid. Another drawback is the pain associated with the frequent drawing of blood samples.

Progress has also been made on the use of optical tools for direct glucose detection. Polarimetric detection of glucose in the aqueous humor of the eye is a potentially useful non-invasive method which is built upon the chirality of glucose rotating the plane of polarization of incident light.⁹⁻¹¹ However, the presence of other chiral molecules in the biofluid interferes with the detection, resulting in a lack of selectivity. Another optical technique is Raman spectroscopy, which has been used for the direct *in-vitro* detection of glucose in blood and simulated aqueous humour, with quantification by partial least squares analysis.¹²⁻¹⁵ This spectroscopic method provides valuable structural and functional information, and samples in aqueous media can be used due to the low Raman scattering cross section of water. Unfortunately, high laser power and long data acquisition times are required due to the intrinsic low Raman scattering cross section of the glucose molecule.

Efforts have therefore been made to develop SERS substrate-based glucose sensors; the advantage of using SERS is the improved Raman signal which can lead to sensitive detection in clinical samples. This was pioneered by Van Duyne and co-workers in the development of silver or gold Metallic Film Over Nanospheres (MFON) substrates functionalized with self-assembled monolayer of alkanethiol or alkanethiolate tri(ethylene glycol) for partitioning glucose from the aqueous media for SERS sensing (figure 3.2).¹⁶⁻²¹ This was also subsequently demonstrated with SERS-active nanogap substrates functionalized with mixed monolayers.²²

Figure 3.2. Schematic showing the partitioning of glucose by the (1-mercaptoundeca-11-yl)tri(ethylene glycol) partition layer on the metallic SERS substrate. Adapted from ref. 17 with permission from the American Chemical Society.

3.2 Concept of selective detection of glucose on SERS planar substrate using boronic acid chemistry

Besides the use of nanoparticles, SERS enhancement can also be achieved on planar substrates. Gold nanoparticles are used extensively for the cellular detection of biomarkers but they are susceptible to aggregation and hence require the need for methods to stabilize them. Planar substrates, on the other hand, comprising patterned metallic surfaces immobilized onto a platform, are chemically more stable and resistant to aggregation. This makes them suitable for both *in-vivo* and *in-vitro* SERS biosensing.^{17, 21} Some of the patterned metallic substrates developed include sphere template films,²³ nanoparticle-anchored surfaces,²⁴ biomimetic substrates,²⁵ substrates with 3D Raman active volume,²⁶ and metallic film over nanosphere (MFON).¹⁷ All of these substrates, though differing in their physical appearance and performance, serve to enhance the Raman signals of molecules adsorbed onto their surface, with enhancement factors ranging from 10^6 to 10^8 .²⁷ As a result, ultrasensitive detection can be achieved and they have been employed in the detection of a wide array of biological and chemical molecules.²⁷⁻³⁶

There are various glucose-responsive systems which employ glucose oxidase, lectin and boronic acid chemistry for the detection of glucose.³⁷ In this study, we utilized boronic acid (BA) chemistry to selectively target glucose in a SERS-based assay. The small but robust boronic acid functional group ($R-B(OH)_2$) is simple and can be easily functionalized via the R group. The interaction of boronic acid with diols

has been well-studied; they are known to bind covalently and reversibly to diols to form boronate esters in aqueous media (scheme 3.1).³⁸⁻⁴⁰

Scheme 3.1. Interaction of the phenylboronate anion with 1,2- and 1,3-diols to form boronate esters complexes with five- and six-membered rings, respectively.⁴⁰

Saccharides contain many hydroxyl groups which can interact with the Lewis acidic boronic acid. This has formed the basis for fluorescence-based probes for saccharides sensing, whereby the fluorescence emission can be modulated with the binding of saccharides to boronic acid.^{41, 42} In most cases, selective detection of glucose over other saccharides can be achieved with specially designed systems that employed diboronic acid moieties.^{39, 43-54} The principle behind this is the formation of a stable boronic acid-diol complex which requires the presence of syn-periplanar hydroxyl groups for preferential binding; these diboronic acids are designed to straddle the two sets of syn-periplanar diol groups present in the α -D-glucofuranose ring (figure 3.3a).^{40, 44-46, 55} This results in a higher tendency for the glucose molecule to form a bidentate boronic acid complex; the other physiologically relevant saccharides form monodentate complexes (figure 3.3b).^{46, 55}

Figure 3.3. a) Interaction of a fluorescent diboronic acids anthracene conjugate with a glucose molecule.^{44, 46, 55} b) Illustration of glucose-bidendate boronic acid complex while other saccharides form a monodendate complex.

Building on this concept, the strategy was to develop a selective glucose assay analogous to that of the sandwich-based enzyme-linked immunosorbent assay (ELISA), with a metal carbonyl-boronic acid conjugate as the label.^{56, 57} Two saccharide receptors were used; the first (primary) receptor comprised 4-mercaptophenylboronic acid (BA) attached onto a SERS substrate, and the second (secondary) receptor was a metal carbonyl-boronic acid conjugate. Glucose was first captured by the primary saccharide receptor, followed by labeling by the conjugate (figure 3.4). This allowed selective quantification of the glucose via the CO stretching vibrations of the metal carbonyl, enhanced by the SERS substrate.

Figure 3.4. Schematic for the selective detection of glucose on the SERS-active substrate. The presence of a glucose molecule brings the metal carbonyl-boronic acid conjugate to the substrate via formation of a bidentate complex.

3.3 Fabrication of boronic acid functionalized SERS substrate

The SERS-active substrate used in this study was Bimetallic Film Over Nanospheres (BMFON).⁵⁸⁻⁶¹ This is a substrate, fabricated in-house, that does not require clean room environment or sophisticated instruments for fabrication, and it can give excellent Raman signal enhancement with an enhancement factor of 2.0×10^7 ; it has been employed in the detection of biomolecules within an extremely low sample volume ($\sim 20 \mu\text{L}$).^{58, 59, 61} The BA was immobilised onto BMFON via its thiol (-SH) group, with its boronic acid group $-\text{B}(\text{OH})_2$ serving as the saccharide receptor. This eliminated the need to introduce the saccharide receptor ($-\text{B}(\text{OH})_2$) and the substrate-binding moiety (-SH) separately. The distribution of BA prior to incubation with glucose was first examined by SERS mapping, using an absorption peak of the phenyl group at 1580 cm^{-1} which showed an even distribution of BA on BMFON (figure 3.5). The BA layer was very stable as the substrate soaked in PBS showed only slight variations in the signals and distribution of BA over a period of three days.

Figure 3.5. (a) Bright field image of BMFOM. SERS mapping images at 1580 cm⁻¹ before (b) and after (c) immobilization of BA. Stability test of BA-functionalized BMFON in PBS solution - SERS mapping images at 1580 cm⁻¹ on (c) day 1, (d) day 2 and (e) day 3. (f) SERS spectra of BA-functionalized BMFON from days 1 - 3.

3.4 Synthesis and characterization of cluster **2b**-B(OH)₂

The secondary saccharide **2b**-B(OH)₂ is a triosmium carbonyl cluster-boronic acid conjugate. Osmium carbonyl clusters are often air- and moisture-stable, and they have been shown to be sufficiently robust for bio-imaging applications.⁶² Cluster **2b**-B(OH)₂ was prepared by the reaction of Os₃(CO)₁₀(NCCH₃)₂, **1**, with 4-mercaptophenylboronic acid to form the $\mu,\kappa S$ -thiolate bridged cluster (scheme 3.2). It has been completely characterized spectroscopically by IR, HR-MS, ¹H and ¹³C NMR (appendix A3.1-5). A sample of **2b**-B(OH)₂ incubated with an excess of glucose at pH 9.0 showed the presence of an adduct of **2b**-B(OH)₂ with glucose in the HR-MS, demonstrating its ability to bind glucose (appendix A3.6) .

Scheme 3.2. Synthesis of **2b-B(OH)₂** and its reaction with glucose.

The structure of **2b-B(OH)₂** was also confirmed by a single-crystal X-ray crystallographic study which showed it to be a condensation trimer, **2b**-boroxine, comprising a six-membered cluster-decorated boroxine ring (figure 3.6); this is commonly observed in solid-state characterization of boronic acid moieties.⁶³ Boroxine is an important precursor for the synthesis of covalent organic frameworks and polymeric materials.⁶³⁻⁶⁵ Organometallic-decorated boroxines containing a triply ferrocene-bridged boroxine cyclophane has been reported,⁶⁶ but to the best of our knowledge, this is the first example of a metal carbonyl-boroxine.

Scheme 3.3. Formation of **2b-boroxine** from **2b-B(OH)₂**.

Figure 3.6 ORTEP plot of the molecular structure of **2b-boroxine**. Thermal ellipsoids are drawn at the 50% probability level.

3.5 Selective detection and quantification of glucose

Fructose and galactose are the most abundant monosaccharides after glucose. It is thus useful to examine if the assay could discriminate glucose in the presence of these other monosaccharides.⁶⁷⁻⁶⁹ Indeed, BA-functionalized BMFON substrate incubated with a small amount ($\sim 20\ \mu\text{L}$, 20 mM) of a solution of glucose, fructose or galactose prior to treatment with **2b**-B(OH)₂ showed significant CO stretching vibrations at $\sim 2000\ \text{cm}^{-1}$ only with glucose; it was very modest with fructose or galactose (figure 3.7).

Figure 3.7. The Raman responses for BA-functionalized BMFON incubated with (a) glucose, (b) fructose, (c) galactose and (d) control, prior to incubation with **6**. Boxed region shows CO stretching vibrations of **2b**-B(OH)₂ which is free of interference from other functional groups.

The intensity of the sharp peak at $2111\ \text{cm}^{-1}$ as a function of incubation with varying concentrations of the three saccharides showed an increase in the SERS signal intensity as a function of the concentration of glucose (figure 3.7a-b). The limit of detection was estimated at 0.1 mM, with a detection range (0.1 – 10 mM) that covered the physiological concentration (5 mM). The responses for fructose and galactose were, in contrast, much lower. The greater affinity of **2b**-B(OH)₂ for glucose may be attributed to the higher tendency of glucose to form a bidentate complex with boronic acid. This is reminiscent of a recent report which utilized the greater affinity of glucose for binding to boronic acids to form 1:2 (glucose:boronic acid) adducts, while fructose only forms 1:1 adducts.⁵⁴ Presumably, all three saccharides interacted with the BA-functionalized BMFON, but there was preferential binding of **2b**-B(OH)₂ with the surface-bound glucose.

Glucose monitoring for diabetes is usually carried out on urine or blood samples; the former is not a substitute but rather, an alternative or complement, which can provide very valuable information where blood glucose monitoring is not accessible, affordable, or desirable. To demonstrate the practicality of our platform, we determined glucose in urine samples doped with a standard glucose solution (figure 3.8a, c). The concentration of glucose in the urine sample was determined to be 5.1 mM, in good agreement with the amount of glucose added (5.0 mM).

Figure 3.8. (a) Plot represents the intensity of CO stretching frequency versus different concentrations of (●) glucose, (▲) fructose, and (■) galactose, with the interpolated value for the spiked urine sample shown in red. The first data points are at 0.1 mM. (b) SERS spectrum of **2b**-B(OH)₂ obtained from different concentrations of glucose. (c) SERS spectrum of **2b**-B(OH)₂ after BMFON has been incubated with a urine sample spiked with glucose.

3.6 Cytotoxicity of 2b-B(OH)₂ and analogues towards cancer and non-cancer cells

Sugars play an essential role, not just as the energy source, but also as an integral part of the cell in the form of glycans – oligo- or polysaccharides linked to proteins and lipids via glycosylation.^{70, 71} Alterations in glycan expression is a hallmark of malignant transformation, with the expression of sialic acid on the cell surface being the most widely reported.^{72, 73} Sialic acid is a class of *N*- or *O*-substituted neuraminic acid (nine-carbon monosaccharide) and some common examples found on the terminal ends of glycans are Neu5Ac and Kdn (figure 3.9).⁷⁴ Overexpression of the enzymes, *N*-acetylglucosaminyltransferase V and glycosyltransferase, in cancer cells can lead to increased branching of the glycans and glycosylation at the terminal ends, respectively, resulting in an overall increase in sialylation.^{75, 76} Examples of glycan epitopes (parts of a proteins for antibodies recognition) commonly found in cancer cells include sialyl Lewis x (sLe^x) and polysialic acid (PSA) (figure 3.9).⁷⁷

Figure 3.9. Molecular structures of sialic acid and glycan epitopes commonly found in cancer cells.

Other than having a strong affinity for saccharides, boronic acid is reported to interact with Neu5Ac at physiological pH (7.4), with a significantly higher binding constant than glucose.^{78, 79} From this has spawned applications that utilized boronic acid to target cell surface glycans. An interesting example is the use of polymer functionalized with phenylboronic acid to selectively capture and release cancer cells that overexpressed sialic acid.⁸⁰ Other work involved the use of imaging agents,

including quantum dots,⁸¹ fluorescence probe,⁸² and gold nanoparticles,^{83, 84} functionalized with phenylboronic acid to selectively target and image cancer cells.

It was therefore of interest to examine if metal carbonyl-boronic acid conjugates such as cluster **2b**-B(OH)₂ may also be applied to cell-based and, potentially, *in vivo* applications like the identification of cancer cells via their surface glycans. As a preliminary, however, the cytotoxicity of cluster **2b**-B(OH)₂ and other analogues had to be evaluated. This was carried out on the MDA-MB-231 breast cancer cell line via an MTS assay. This metastatic cell line was chosen as it has one of the highest expression of sialic acid among breast cancer.⁸⁵

To our surprise, cluster **2b**-B(OH)₂ was found to demonstrate cytotoxicity comparable to that of the positive control, cluster **1**, with IC₅₀ values of 3.8 ± 0.9 and 4.0 ± 0.6 μM, respectively.⁸⁶ This result is surprising as it has previously been found that alkylthiolato-triosmium carbonyl clusters, which have been employed for bioimaging, exhibited little effect on cell viability.^{62, 87} For example, the viability of HL-60 leukemia cell (with respect to control) treated with 30 μM of [Os₃(CO)₁₀(μ-H)(μ-S(CH₂)₁₀COO)]Na and a phosphatidylcholine analogue were 89% and 77%, respectively.⁶²

To gain an understanding of the reasons behind the cytotoxicity of **2b**-B(OH)₂, a number of other phenylthiolato-triosmium carbonyl cluster analogues were synthesized (scheme 3.4).

Scheme 3.4. Synthesis of cluster **2b**-H, -OH and -COOH.

Figure 3.10. ORTEP plot of the molecular structure of **2b-OH** (top) and **2b-COOH** (bottom). Thermal ellipsoids are drawn at the 50% probability level.

Cluster **2b**-H was synthesized according to the reported method,⁸⁸⁻⁹⁰ and the syntheses of **2b**-OH and -COOH were adapted from that. The structures of **2b**-OH and -COOH were also confirmed crystallographically (figures 3.10). Cluster **2b**-B(OH)₂ showed similar toxicity to the -OH and -COOH analogues, while the ligand BA was found to have little effect on cell viability, even at a high concentration of 50 μ M (table 3.1 and figure 3.11). The cytotoxicity of cluster **2b**-H could not be determined as it was found to be highly insoluble in aqueous medium. The differences in cytotoxicity can be attributed to the solubility and not the nature of the functional groups on the phenyl ring. The study also eliminated the ligand BA as the source of toxicity; the triosmium carbonyl cluster, or in combination with the aromatic group, may have been responsible.

Together with earlier work on the alkylthiolato clusters, this indicated that the cytotoxicity of Os₃(CO)₁₀(μ -H)(μ -SR) clusters are dependent on the R groups which may play an important role in modulating the lipophilicity and electronic property of the clusters. It may be speculated that the phenyl group, being more electron-withdrawing than the alkyl group, can result in the sulphur lone pairs less easily donated, thereby forming the η^1 -SR more easily; this creates a vacant site on the osmium centre which allows the reaction with biologically relevant targets.

Table 3.1. Inhibition of cell growth on MDA-MB-231 cells under serum-free conditions, after 24 h, as determined by MTS assay.

Compounds	IC ₅₀ / μ M
1	4.0 $\pm$ 0.6
BA	> 50
2b -B(OH) ₂	3.8 $\pm$ 0.9
2b -OH	2.6 $\pm$ 0.4
2b -COOH	4.0 $\pm$ 0.4

Figure 3.11. Comparison of the antiproliferative activity among the clusters (5 μM) and ligand BA (50 μM) on MDA-MB-231 cells, using **1** as the positive control. Control: DMSO (0.5 %, v/v).

An effective anti-cancer drug is one that selectively targets cancer cells over normal cells; this is important for the reduction of any side effects due to the indiscriminate nature of the drug. To understand the selectivity, **2b-B(OH)₂** and **–OH** were further evaluated with the estrogen receptor positive (ER+) breast cancer cell line MCF-7, as well as on the non-cancerous breast epithelial cell line MCF-10A.

The IC₅₀ values obtained showed that **2b-OH** exhibited higher toxicity than the **–B(OH)₂** analogue and, together with **1**, they were more cytotoxic towards MDA-MB-231 (ER-) than MCF-7 (ER+) cells (table 3.2). This suggests that the compounds were not acting through the estrogen receptors but via a different target. Unfortunately, all the tested osmium compounds showed higher toxicity towards the normal cells than the cancer cells, with no selectivity observed. The cell death was also verified visually under a bright-field microscope with significant changes to the cell morphologies observed in comparison to the negative control (figure 3.13).

The compounds were also evaluated on the respective cell lines in the presence of serum. Substantial increase in the IC₅₀ for all compounds were observed; this was especially the case for cluster **1** (table 3.2). Though the potencies of cluster **2b-B(OH)₂** and **–OH** were rather similar to **1** under serum-free conditions, they were less susceptible to interference by serum and demonstrated better efficacy than **1** in the

presence of serum; the IC_{50} of **1** increased 5-8 folds in the presence of serum, reflecting its low efficacy in serum conditions (figure 3.12).⁹¹ The increase can be attributed to the high reactivity of **1** towards thiols and carboxylic acid in serum proteins to form the stable μ -S and μ, κ^2O, O' clusters respectively.⁸⁹⁻⁹² It is precisely this high chemical reactivity which allowed the bioconjugation of trisodium clusters to biomolecules via such functionalities.⁶² Clusters **2b**-B(OH)₂ and –OH, which are intrinsically more stable, can be better drug candidates as compared to **1**.

Table 3.2. Inhibition of cell growth on breast cell lines, after 24 h in serum and serum-free condition, as determined by MTS assay.

Compounds	IC_{50} / μ M					
	MDA-MB-231		MCF-7		MCF-10A	
	Serum-free	With serum	Serum-free	With serum	Serum-free	With Serum
1	4.0 $\pm$ 0.6	29.2 $\pm$ 0.6	7.9 $\pm$ 1.2	53 $\pm$ 2	2.0 $\pm$ 0.3	19 $\pm$ 4
BA	> 50	> 100	> 50	> 100	> 50	> 100
2b -B(OH) ₂	3.8 $\pm$ 0.9	15 $\pm$ 3	6 $\pm$ 1	24 $\pm$ 1	2.35 $\pm$ 0.09	7 $\pm$ 1
2b -OH	2.6 $\pm$ 0.4	6 $\pm$ 1	3.1 $\pm$ 0.8	16.1 $\pm$ 0.6	2.2 $\pm$ 0.5	9.2 $\pm$ 0.1

Figure 3.12. Comparison of the IC_{50} of **1**, **2b**-B(OH)₂ and –OH on MDA-MB-231 and MCF-7 cells under serum and serum-free conditions.

Figure 3.13. Bright-field images of MDA-MB-231, MCF-7 and MCF-10A cells, in serum-free condition, after 24 h incubation with 10 μ M solutions of **1**, **2b-B(OH)₂** and **2b-OH**.

3.7 Induction of apoptosis by **2b**-B(OH)₂ and -OH

Cell death can be broadly categorized into either apoptosis or necrosis.^{93, 94} Apoptosis, also known as programmed cell death, is an active and controlled process of cellular self-destruction that can be triggered internally or externally, while necrosis is regarded as a form of passive, accidental cell death triggered by external stimuli. Apoptosis is tightly regulated within the cell and plays critical role in cell growth and maintenance of healthy cell population. Alternatively, this form of cell death has been utilized by many for the development of apoptosis-inducing drugs for targeting cancer cells.^{95, 96} Some examples include doxorubicin,⁹⁷ palitaxel,⁹⁸ cisplatin,⁹⁹ and more recently osmium carbonyl clusters.^{86, 100} Hence, it is of interest to investigate the mode of action of **2b**-B(OH)₂ and -OH whether they triggered cell death via the apoptotic pathway.

3.7.1 Cell nuclear staining using Hoechst 33342

Morphological changes in the nuclei are regarded as hallmarks of apoptosis and some of these include shrinkage of the nuclei, chromatin condensation and nuclei fragmentation.¹⁰¹⁻¹⁰³ These characteristics can be visualized in pre-stained cells via fluorescence microscopy. Cell nuclear staining can be carried out using Hoechst stains (developed by the German company Hoechst AG) – a class of bisbenzimidazole fluorescent dye widely used as nuclear counterstain.¹⁰⁴ In particular, the water-soluble and cell membrane permeable Hoechst 33342, is one of the most frequently used DNA counterstain suitable for cell cycle and apoptosis studies (figure 3.14).¹⁰⁵

Figure 3.14. Molecular structure of Hoechst 33342.

Live MCF-7 cells treated with **1**, **2b**-B(OH)₂ and -OH were stained with Hoechst 33342 and imaged with fluorescence microscopy (figure 3.15). The nuclear morphology of cells treated with **2b**-B(OH)₂ and -OH showed nuclei of reduced size and distorted shape, a sign of early apoptosis. Cells with nuclei showing chromatin

condensation were observed, similar to that of positive control (cluster **1**); in comparison, in the negative control (0.5% DMSO, v/v), the nuclei appeared round and intact.

Figure 3.15. Fluorescence images of MCF-7 cells stained with Hoechst 33342 after 20 h treatment with: a) negative control, 0.5% DMSO (v/v), b) **1** (4 μM), c) **2b-B(OH)₂** (6 μM) and d) **2b-OH** (4 μM). The arrows indicate apoptotic cells.

Changes in the nuclear morphology were quantified through image-based single cell phenotypic analysis of the nuclear area and fluorescence intensity of the cell population.¹⁰⁶ Typically, the fluorescence images of the cells were processed using an in-house image-processing software (cellXpress) which segments the cells singly and extracts the relevant cellular features for quantification.¹⁰⁷ This well-established technique has also been used in a high-throughput image-based nephrotoxicity prediction platform for toxic compounds.¹⁰⁸ Apoptotic cells generally display reduced nuclear area and fluorescence intensity in comparison to normal cells. MCF-7 cells treated with increasing concentrations of both **2b-B(OH)₂** and **2b-OH** resulted in increased population of cells with smaller nuclear size and lower DNA intensity (lower left quadrant of the contour-scatter plots) in comparison to the negative control (figure 3.16), suggestive of apoptosis. The contour plot of the negative control showed two

distinct cell populations characterized by the G1 and G2 phases of the cell cycle, respectively (figure 3.16a). Treatment with the clusters resulted in a shift in the cell population to the G2 phase (top right quadrant) which may suggest G2/M arrest; the cell cycle was halted and the cells were not able to undergo mitotic cell division.

Figure 3.16. Contour-scatter plots showing the nuclear area vs total DNA fluorescence intensity of single MCF-7 cells after 20 h treatment: a) negative control, 0.5% DMSO (v/v), b-c) **2b-B(OH)₂** (3 and 6 μ M respectively) and d-e) **2b-OH** (2 and 4 μ M respectively). The dots represent single-cell measurements quantified from the images.

3.7.2 Phosphatidylserine detection by Annexin V-FITC conjugate

Another feature of apoptosis is the externalization of phosphatidylserine (PS) at the cell surface, which is found in the inner side of the membrane of live cells.^{109, 110} The exposure of PS on the surface provides recognition sites for macrophages to remove the apoptotic body and also allows the binding of the lipid binding protein, Annexin V, in the presence of Ca^{2+} ions (figure 3.17).^{111, 112} Thus, Annexin V conjugated to a fluorescence probe, fluorescein isothiocyanate (FITC), is commonly used as a label for detecting PS in apoptotic cells.¹¹³

Figure 3.17. Externalization of phosphatidylserine at the plasma membrane of apoptotic cells and detection by Annexin V-FITC conjugate. Adapted from:

http://www.bdbiosciences.com/br/instruments/accuri/articles/archive/2015_02/index.jsp.

Accessed on 25 Jul 2016.

MCF-7 cells treated with **1**, **2b**-B(OH)₂ and –OH were stained with Hoechst 33342 and Annexin V-FITC and visualized under a fluorescence microscope (figure 3.18). The images showed significant increase in Annexin V-FITC staining (green) in cells treated with **1**, **2b**-B(OH)₂ and –OH in comparison to the negative control. The intensity also increased with treatment at higher concentration of **2b**-B(OH)₂ and –OH; image-based quantification of the the membrane (non-DNA region) also showed substantial Annexin V-FITC staining with the increase in concentration (figure 3.19). Together with the information obtained from the nuclear staining, these suggest that **2b**-B(OH)₂ and –OH were apoptosis inducing.

Figure 3.18. Fluorescence images of MCF-7 cells stained with Hoechst 33342 (blue) and Annexin V-FITC (green) after 20 h treatment with: a) negative control, 0.5% DMSO (v/v), b-c) $2b\text{-B(OH)}_2$ (3 and 6 μM respectively), d-e) $2b\text{-OH}$ (2 and 4 μM respectively) and f) **1** (4 μM).

Figure 3.19. Image-based quantification of total fluorescence intensity of Annexin V-FITC from MCF-7 cells treated with various clusters. Negative control, 0.5% DMSO (v/v).

3.8 Chemical oxidation of **2b**-B(OH)₂ to **2b**-OH and electrochemical studies

The study above has shown that **2b**-B(OH)₂, -OH and -COOH exhibit similar toxicities (IC₅₀ values). The source of cytotoxicity is likely to be from the phenylthiolato triosmium core, with the substituents on the phenyl ring helping to improve water solubility. Further chemical and electrochemical studies were carried out in attempts to understand the mode of action of these clusters.

The boronic acid functionality of **2b**-B(OH)₂, which was intended to interact with sialic acid, can undergo oxidation by H₂O₂ to form the hydroxyl group;¹¹⁴ this forms the basis for the development of boronic acid or boronate esters-based sensors for H₂O₂ sensing (scheme 3.5).¹¹⁵⁻¹¹⁷ The possible chemical oxidation of **2b**-B(OH)₂ to **2b**-OH was thus investigated.

Scheme 3.5. Oxidation of boronic acid or boronate ester by H₂O₂.¹¹⁵

The conversion of **2b**-B(OH)₂ to **2b**-OH in the presence of H₂O₂ and imidazole as base was confirmed by IR spectroscopy (figure 3.20). The shift in the CO vibrational bands to lower wavenumber, in the presence of imidazole, was indicative of the conversion to **2b**-OH, as verified with an authentic sample of **2b**-OH. Monitoring the reaction by ESI-HRMS revealed a decline in the ratio of **2b**-B(OH)₂ (*m/z* 1004) to **2b**-OH (*m/z* 976) over time (figure 3.21). A control setup without the addition of H₂O₂ showed a consistent high ratio of peaks at 1004 to 976 throughout the reaction, indicating little or no loss of **2b**-B(OH)₂.

Figure 3.20. a) Overlays of IR spectra of **2b**-B(OH)₂ incubated with 5 eq. of H₂O₂ and 1 eq. of imidazole in H₂O:MeOH (1:3, v/v) over time. Authentic spectra of **2b**-B(OH)₂ and -OH were overlaid for comparison. All spectra were taken in MeOH. b-d) Expanded regions of various bands in the spectra.

Figure 3.21. ESI-HRMS of **2b**-B(OH)₂ incubated with 5 eq. of H₂O₂ and 1 eq. of imidazole in H₂O:MeOH (1:3, v/v) taken at a) 1 h and b) 3 h. c) ESI-HRMS of **2b**-B(OH)₂ incubated with 1 eq. imidazole but without the addition of H₂O₂. The *m/z* isotopic pattern corresponding to **2b**-B(OH)₂ was highlight in blue, while the **2b**-OH was in red.

Organometallic-based anti-cancer drugs like ferrocifen (ferrocene analogue of tamoxifen) can undergo phenol oxidation as part of its activation process; ferrocene-modulated phenol oxidation to the reactive quinone methide is believed to be

responsible for their cytotoxicity (scheme 3.6).¹¹⁸⁻¹²⁰ Cluster **2b**-OH draws similarity with ferrocifen as it contains a phenol moiety which can potentially undergo phenol oxidation. The electrochemical behavior of **2b**-OH was thus investigated by cyclic voltammetry (CV), using $n\text{Bu}_4\text{NPF}_6$ as the supporting electrolyte.

Scheme 3.6. Proposed mechanism for the oxidation and formation of quinone methides from ferrocenyl phenol.¹¹⁸

The cyclic voltammogram of **2b**-OH showed a chemically irreversible oxidation process at 0.80 V vs. Fc/Fc^+ . The CV of the analogues, **2b** and **2b**-COOH, showed similar chemically irreversible oxidation processes at 0.82 V and 0.85 V, respectively, under the same conditions (figure 3.22). This implies that oxidation occurred on the triosmium cluster core, since the oxidation potential appears to be independent of the functional groups on the phenyl ring. The oxidized species are unstable, resulting in the corresponding reduction process not being observed when the scan direction is reversed. This is in agreement with other reports that the oxidation of the triosmium pyridyl cluster $[\text{Os}_3(\mu\text{-H})(\text{CO})_{10}(\mu\text{-NC}_5\text{H}_4)]$, and analogues, were irreversible; the oxidation was attributed to the cluster and was not affected by substituents on the pyridyl ligand.¹²¹

Figure 3.22. Cyclic voltammograms of 2 mM of **2b-OH**, **2b** and **2b-COOH** in CH_3CN at 22 ± 0.2 °C with 0.1 M $n\text{Bu}_4\text{NPF}_6$, recorded at a 1 mm diameter planar glassy carbon electrode at a scan rate of 0.1 V s^{-1}

3.9 Conclusion

A triosmium carbonyl cluster-boronic acid conjugate, **2b**-B(OH)₂ was utilized as a SERS probe for selective glucose detection using BMFON as the SERS-active substrate in a sandwich assay. The assay showed good selectivity towards glucose over fructose and galactose. Quantification was carried out using the CO stretching vibration of the conjugate **2b**-B(OH)₂, and had a detection range of 0.1-10 mM. This glucose assay exhibited several advantages: (i) no prior purification of the sample was needed, (ii) an extremely low sample volume was required, (iii) there is very high specificity for glucose, and (iv) the spectroscopic handle for glucose quantification was relatively free from interference by other biomolecules. There remain improvements to be made, however, including the use of a cheaper transition metal and making the detection label-free.

Extension of the application of **2b**-B(OH)₂ towards cell-based detection was attempted. However, the unexpected discovery that **2b**-B(OH)₂ is cytotoxic made it inappropriate for its intended use as an intracellular imaging agent, but potentially suitable as a therapeutic agent. Further investigation on other analogues, **2b**-OH and –COOH, revealed that the cytotoxicity is independent on the substituents on the phenyl ring, but is probably related to the presence of the aromatic ring. Clusters **2b**-B(OH)₂ and –OH were deduced to induce cell death via the apoptotic pathway. Hence, future improvements can be made in enhancing the selectivity of the clusters and to understand the mode of action in greater details.

3.10 Experimental section

3.10.1 General methods

All reactions and manipulations were carried out under an argon atmosphere using standard Schlenk techniques. Solvents that were used for reaction were distilled over the appropriate drying agents under argon before use. Purification of compounds was generally carried out by column chromatography on silica gel, or by preparative thin-layer chromatography (TLC) using 20 cm x 20 cm plates pre-coated with silica gel 60 F₂₅₄. Infrared (IR) spectra were recorded on a Bruker Alpha FT-IR spectrometer. Solution spectra were recorded in DCM solution, unless otherwise stated, in a solution IR cell with NaCl windows and a path length of 0.1 mm, at a resolution of 2 cm⁻¹. For compounds in MeOH solution, a solution IR cell with CaF₂ windows and a path length of 0.1 mm was used. ¹H and ¹³C{¹H} NMR spectra were recorded on a Bruker Advance BBFO (400 MHz), JEOL ECA 400 or ECA 400SL (400 MHz) spectrometer, in (CD₃)₂CO. ¹H and ¹³C{¹H} chemical shifts are referenced to the residual proton and carbon resonance of the solvent. HRMS were recorded in ESI mode on a Waters UPLC-Q-ToF MS mass spectrometer. The Raman spectral measurements were carried out using a Renishaw InVia Raman (UK) microscope with a Peltier cooled CCD detector and an excitation wavelength at 785 nm, where the laser beam was directed to the sample through a 50× objective lens, which was used to excite the sample and also to collect the return Raman signal. All Raman spectra were processed with the WiRE3.4 software. The laser power at the sample was measured to be 20.8 mW and the exposure time was set at 10 s, throughout the measurements. Prior to each measurement, the instrument was calibrated against a silicon standard with a Raman peak centered at 520 cm⁻¹.

Cluster **1** and **2b** were prepared according to reported procedures with slight modifications.^{89, 122} Os₃(CO)₁₂ was purchased from Oxxem Ltd, and all other reagents were purchased from other commercial sources and used as supplied without further purification.

3.10.2 Synthesis of cluster **2b**-B(OH)₂

Cluster **1** (26 mg, 0.028 mmol) and 4-mercaptophenylboronic acid (5 mg, 0.034 mmol) were dissolved in THF (6 mL) and left to stir at room temperature overnight. Solvent was removed in vacuo and the residue was purified by TLC using ethyl acetate:hexane (1:1, v/v) as eluant to yield one major yellow band as cluster **2b**-B(OH)₂.

Yield: 18 mg (63 %)

$R_f = 0.36$

IR (DCM, cm^{-1}): ν_{CO} 2110w, 2069s, 2060m, 2023s, 2001m, 1987w.

^1H NMR (400 MHz, $(\text{CD}_3)_2\text{CO}$) δ 7.84 (2H, d, $J=8.4$, Ar), 7.43 (2H, d, $J=8.4$, Ar), 7.32 (2H, s, $\text{B}(\text{OH})_2$), -16.93 (1H, s, OsHOs).

$^{13}\text{C}\{^1\text{H}\}$ NMR (100 MHz, $(\text{CD}_3)_2\text{CO}$): δ 182.20, 181.57, 177.39, 175.29, 172.74, 170.64, 170.61, 148.12, 135.17, 131.43.

HRMS-ESI m/z calculated for $\text{C}_{16}\text{H}_6\text{BO}_{12}\text{S}^{190}\text{Os}_3 [\text{M-H}]^-$ 1002.8427, found 1002.8414.

3.10.3 Incubation of **2b-B(OH)₂** with glucose

Cluster **2b-B(OH)₂** (2 mg, 2.0×10^{-3} mmol) dissolved in MeOH (200 μL) was added to D-glucose (2 mg, 0.011 mmol) dissolved in pH 9.0 sodium hydroxide solution (200 μL). The reaction mixture was incubated at room temperature overnight. The mixture was further diluted with MeOH prior to HRMS-ESI analysis.

HRMS-ESI m/z calculated for $\text{C}_{22}\text{H}_{16}\text{BO}_{17}\text{S}^{187}\text{Os}^{192}\text{Os}_2 [\text{M}]^-$ 1165.8989, found 1165.8999.

3.10.4 Fabrication of BMFON substrate

Monodisperse PS colloidal suspension ($\varnothing = 384$ nm, 2.5 wt%) stored at 4 °C was purchased from Kisker. During preparation, the PS sphere solution was added with 15wt% surfactant sodium dodecyl sulphate (SDS), to composite solution containing 2.36 wt% PS spheres and 0.85 wt% SDS. Clean microscope glass slides used as supporting substrate were cut into square pieces (10 mm x 10 mm x 1 mm). The glass slides were first sonicated in a bath of ethanol for 20 min before being dried with argon gas. Using the spin-coating method, PS sphere monolayers were fabricated onto the clean glass slides. This was done by dispersing 10 μL of the prepared colloidal solution onto the center of a glass slide. Each glass slide was spin coated in the spin coater preset at 2000 rpm for 20 s. Due to the Bragg diffraction given by the closely-packed PS spheres, a green reflective surface was formed straight after. The coated glass slides were then dried in vacuum desiccators overnight at 0.6 pa pressure. The substrates were first coated with Ag (99.999% purity, JEOL) at thickness that amount to 120 s of sputtering (JEOL JFC-1600 Auto fine coater) before being sputtered with a

layer of Au (99.999% purity, JEOL) for 80 s. Each metal layer was deposited at a rate of 1.33 nm/s.

3.10.5 Immobilization of 4-mercaptophenylboronic acid on BMFON

Fabricated BMFON was incubated in 4-mercaptophenylboronic acid ethanolic solution (10 mM) for duration of 2 hours. The substrates were then washed thoroughly with pure ethanol and dry in air.

3.10.6 Detection of saccharides (glucose, fructose and galactose)

BA-functionalized BMFON was incubated with varied concentration of saccharides (~20 μ L) for 1 h. The substrate was then washed thrice with water (3 x 2 mL) prior to incubation with a solution of **2b**-B(OH)₂ (2 mM) for 1 h. The solution was removed and the substrate was washed thrice with water (3 x 2 mL). Subsequently, the substrate is placed on a glass slide and under the Raman microscope objective lens for measurements. An excitation wavelength of 785 nm was used and the exposure time of 10 s was used throughout the measurement.

3.10.7 Detection of glucose in clinical urine sample

Samples used for the study were from clinical specimens, stored at -20°C. Specimens were used in accordance to procedures with approval of the local ethics committee (CIRB Re 2011/558/C) and informed consent was given by all patients. In this experiment, urine sample was added a known amount of glucose and was mixed well. BA-functionalized BMFON was incubated with the doped urine for 1 h. The substrate was then washed thrice with water (3 x 2 mL) prior to incubation with a solution of **2b**-B(OH)₂ (2 mM) for 1 h. The substrate was washed thrice with water (3 x 2 mL), prior to SERS measurement.

3.10.8 Synthesis of cluster **2b-OH and **2b**-COOH**

In a typical reaction, **1** (51 mg, 0.055 mmol) and 1.2 eq. of the respective ligands (4-mercaptophenol or 4-mercaptobenzoic acid) were dissolved in THF (6 mL) and left to stir at room temperature overnight. Solvent was removed in vacuo and the residue was purified by TLC to yield the product as the major yellow band. The characterization data is summarized as follow:

	2b-OH	2b-COOH
Yield	44 mg (82 %)	35 mg (63 %)
R_f	0.48 (DCM as eluant)	0.54 (ethyl acetate as eluant)
IR (DCM, cm⁻¹), ν_{CO}	2109w, 2067s, 2058m, 2022s, 1999m, 1985w.	2110w, 2070s, 2061m, 2024s, 2003m, 1987w.
¹H NMR, (CD₃)₂CO)	δ 8.86 (1H, s), 7.28 (2H, d, <i>J</i> =9.2, Ar), 6.82 (2H, d, <i>J</i> =9.2, Ar), -16.86 (1H, s, OsHOs).	δ 8.01 (2H, d, <i>J</i> =7.4, Ar), 7.61 (2H, d, <i>J</i> =9.2, Ar), -16.95 (1H, s, OsHOs).
¹³C{¹H} NMR (100.46 MHz, (CD₃)₂CO):	δ 182.29, 181.73, 177.52, 175.48, 173.01, 170.89, 170.79, 159.27, 135.62, 133.78, 116.30.	δ 182.19, 181.48, 177.36, 175.20, 172.70, 170.56, 170.45, 166.73, 151.94, 132.65, 131.93, 130.61.
HRMS-ESI	<i>m/z</i> calculated for C ₁₇ H ₅ O ₁₁ S ¹⁸⁴ Os ¹⁸⁶ Os ¹⁹⁰ Os [M-H] ⁻ 976.8201, found 976.8148.	<i>m/z</i> calculated for C ₁₇ H ₅ O ₁₂ S ¹⁸⁹ Os ¹⁹² Os ₂ [M-H] ⁻ 1005.8313, found 1005.8364.

3.10.9 Cell culture

Experimental cultures of breast (MDA-MB-231, MCF-7 and MCF-10A) were obtained from the American Type Culture Collection (ATCC) and cultured in tissue culture dishes (Corning, NY) at 37 °C in 5 % CO₂ atmosphere. Phosphate-buffered saline (PBS) was obtained from Gibco. Experiments were performed on cells within 20 passages. The breast cancer cells MDA-MB-231 and MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Biowest L0106, France) supplemented with 10 % fetal bovine serum (FBS; Gibco, NY), 1 % L-glutamine (PAA Laboratories, Austria), and 1 % penicillin/streptomycin (HyClone, UT). The MCF-10A cells were maintained in DMEM-F12 medium (Biowest L0093, France) supplemented with 7.5 % FBS (Gibco, NY), epidermal growth factor (Invitrogen), insulin (Sigma Aldrich), hydrocortisone (Sigma Aldrich) and 0.4 % gentamicin (Gibco, NY), and 1 % penicillin/streptomycin (HyClone, UT). Bright-field images of the adhered cells were taken using Nikon Eclipse TS100 inverted microscope, with a 10x objective and Nikon digital Sight DS-L3 camera.

3.10.10 Cell proliferation assay

The anti-proliferation activity of the compounds on the various cell lines was screened using a MTS assay modified from one reported previously.^{86, 100} Stock solutions of the compounds in sterile-filtered dimethyl sulfoxide (DMSO) were prepared and serially diluted to lower concentrations. For treatment with the compounds, cells were seeded into 96-well plate in serum-supplemented medium at the same initial density of 10 000 cells (80 μ L) per well, and allowed to adhere and grow for 24 h. Sterile water (110 μ L) was added to the wells at the perimeter to prevent evaporation of the media from the enclosed wells. For serum-free experiments, the cells were allowed to adhere and grow for 18 h, followed by serum-starving in serum-free medium for 6 h before treatment with the indicated concentrations of compounds in serum-free medium (0.5 % DMSO) for 24 h. Control cells were treated with vehicle (0.5 % DMSO). To each well, 10 μ L of MTS reagent (MTS Cell Proliferation Assay Kit, BioVision, CA) was added and then left to incubate in a 37 °C incubator with 5% CO₂ for 2 h. The absorbance intensities at 490 nm were then measured and the cell proliferation relative to the control sample was calculated. Each sample was analyzed in triplicates and was corrected with background intensities from same incubation conditions without the cells. IC₅₀ was determined from a sigmoidal dose response (variable slope) equation using the GraphPad Prism 5 software. The values were obtained from at least two separate experiments with the mean value calculated.

3.10.11 Apoptosis assay

MCF-7 cells were seeded into 96-well optical-grade glass black plates with transparent bottom (Nunc Inc, IL), pre-coated with fibronectin (0.5%, Santa Cruz Biotechnology) in serum-supplemented medium at the same initial density of 10 000 cells (80 μ L) per well and allowed to adhere and grow for 18 h. This was followed by serum-starving in serum-free medium for 6 h before treatment with the indicated concentrations of compounds in serum-free medium (0.5 % DMSO) for 20 h. Control cells were treated with vehicle (0.5 % DMSO). Three replicates were performed for each treatment condition. Apoptotic/necrotic/healthy cells detection kit (PromoKine, Heidelberg, Germany) was used to identify apoptotic cells. The adhered cells were washed twice with 1x binding buffer (2 x 50 μ L), followed by staining with Hoechst 33342 and

Annexin V-FITC at room temperature for 15 min. The stained cells were washed twice with 1x binding buffer (2 x 50 μ L) before proceeding to imaging in the buffer (70 μ L).

3.10.12 Image acquisition, segmentation and features extraction

Fluorescence imaging was performed with a 20x objective using the Axio Observer Z1 (Zeiss), with coolSNAP HQ2 (Photometrics, SONY) camera and Definite laser focus (Zeiss) focusing system. Nine sites per well were imaged. The images were saved in 16-bit TIFF format and corrected using the “rolling ball” algorithm implemented in ImageJ (NIH, v1.48) to reduce non-uniform background illuminations.¹²³ Cell segmentations and feature measurements were performed using the cellXpress software platform (Bioinformatics Institute, A*STAR, v1.4).¹⁰⁷

3.10.13 Chemical oxidation of **2b**-B(OH)₂

A solution of **2b**-B(OH)₂ (4.4 mg, 0.0044 mmol) dissolved in MeOH (3 mL) was added to 1 eq. of imidazole dissolved in deionized water. Hydrogen peroxide solution (2.2 μ L, 30 %, w/w) was added to the mixture and left to incubate at room temperature over 3 h. For analysis by IR spectroscopy, aliquot of the mixture was removed and dried under a stream of nitrogen. The yellow residue was dissolved in MeOH and analyzed by IR spectroscopy in a CaF₂ IR solution cell. For ESI-HRMS analysis, the mixture was diluted with MeOH prior to analysis.

3.10.14 Cyclic voltammetry

Cyclic Voltammetry (CV) experiments were performed with a computer controlled Eco Chemie Autolab PGSTAT302N potentiostat in a three-electrode cell where a 1 mm diameter planar glassy carbon (GC) disk working electrode (Cypress Systems) was used together with a Pt auxiliary electrode (Metrohm) and an Ag wire miniature reference electrode (Cypress Systems) which was connected to the test solution via a salt bridge containing 0.5 M *n*Bu₄NPF₆ in CH₃CN. Accurate potentials were obtained via the addition of ferrocene (Fc) as an internal standard at the end of the measurements. All solutions used for the voltammetric experiments were deoxygenated by purging with argon gas and measurements were performed in a Faraday Cage.

3.10.15 X-ray crystallographic analysis of **2b**-boroxine, -OH and -COOH

X-ray crystallographic analysis was carried out by Dr Rakesh (CBC, NTU). Diffraction-quality single crystal was obtained by slow evaporation of a toluene solution of **2b**-B(OH)₂ and DCM solution of **2b**-OH and -COOH. The crystal was mounted onto quartz fibers and the diffraction intensity data was collected on a Bruker Kappa diffractometer with the SMART suite of programs,¹²⁴ equipped with a CCD detector and using Mo K α radiation ($\lambda=0.71073$ Å). Data were processed and corrected for Lorentz and polarization effects with SAINT, and for absorption effects with SADABS.¹²⁵ Structural solution and refinement were performed with the SHELXTL suite of programs.¹²⁶ The structure was solved by direct methods to locate the heavy atoms, followed by successive difference maps for the light, non-hydrogen atoms. All non-hydrogen atoms were refined with anisotropic thermal parameters in the final model. The metal hydrides were placed by potential energy calculations using the XHYDEX program,¹²⁷ with fixed isotropic thermal parameters, while organic hydrogen atoms were placed in calculated positions, assigned isotropic thermal parameters and refined with a riding model. The crystallographic data for the structure is summarized in table 3.3.

Table 3.3. Crystallographic data for **2b**-boroxine, -OH and -COOH.

	2b -boroxine	2b -OH	2b -COOH
Empirical formula	C ₉₆ H ₃₀ B ₆ O ₆₆ Os ₁₈ S ₆ • 2.8C ₇ H ₈	C ₆₄ H ₂₄ O ₄₄ Os ₁₂ S ₄ • 2.5CH ₂ Cl ₂	C ₁₇ H ₆ O ₁₂ Os ₃ S • CH ₂ Cl ₂
Formula weight	6177.99	4119.79	1089.80
Temperature (K)	103(2)	103(2)	153(2)
Wavelength (Å)	0.71073	0.71073	0.71073
Crystal system	Triclinic	Triclinic	Triclinic
Space group	P $\bar{1}$	P $\bar{1}$	P $\bar{1}$
<i>a</i> (Å)	9.0414(4)	8.4267(6)	8.4854(5)
<i>b</i> (Å)	20.8788(8)	14.1473(9)	12.0178(6)
<i>c</i> (Å)	22.4877(10)	21.5478(13)	12.8840(7)
α (deg)	65.347(2)	89.327(3)	81.639(3)
β (deg)	86.861(3)	87.798(4)	77.714(3)
γ (deg)	81.045(3)	75.631(4)	76.102(3)
<i>V</i> (Å ³)	3810.9(3)	2486.6(3)	1240.01(12)
Z value	1	1	2
ρ_{calc} (g/cm ³)	2.692	2.751	2.919
μ (Mo K α) (mm ⁻¹)	15.103	15.561	15.692
F(000)	2768	1841	980
Crystal size (mm ³)	0.160 x 0.080 x 0.040	0.120 x 0.100 x 0.060	0.420 x 0.380 x 0.280
Theta range for data collection	2.281 to 25.796°	2.403 to 26.372°	2.270 to 26.372°
Index ranges	-10 ≤ <i>h</i> ≤ 10, -22 ≤ <i>k</i> ≤ 25, 0 ≤ <i>l</i> ≤ 27	-12 ≤ <i>h</i> ≤ 12, -20 ≤ <i>k</i> ≤ 20, -30 ≤ <i>l</i> ≤ 30	-10 ≤ <i>h</i> ≤ 10, -14 ≤ <i>k</i> ≤ 15, 0 ≤ <i>l</i> ≤ 16
Reflections collected	41406	55963	49433
Independent reflections	14383 [R(int) = 0.1204]	10175 [R(int) = 0.1125]	5058 [R(int) = 0.1026]
Completeness to $\theta = 25.242^\circ$ (%)	99.9	99.9	99.9
Absorption correction	Semi-empirical from equivalents		
Max. and min. transmission	0.58 and 0.20	0.46 and 0.16	0.0960 and 0.0580
Refinement method	Full-matrix least-squares on F ²		
Data / restraints / parameters	14383 / 350 / 946	10175 / 9 / 611	5058 / 0 / 325
Goodness-of-fit on F ²	1.011	1.075	1.081
Final R indices [I > 2σ(I)]	R1 = 0.0648, wR2 = 0.1591	R1 = 0.0557, wR2 = 0.1430	R1 = 0.0392, wR2 = 0.1021
R indices (all data)	R1 = 0.0912, wR2 = 0.1798	R1 = 0.0753, wR2 = 0.1590	R1 = 0.0442, wR2 = 0.1054
Largest diff. peak and hole (e ⁻ Å ⁻³)	3.235 and -2.692	3.779 and -3.544	3.037 and -1.795

3.11 References

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Chapter 4: High Nuclearity Metal Carbonyl Clusters as Near-IR Contrast Agents for Photoacoustic *In Vivo* Imaging

Photoacoustic (PA) imaging, a hybrid imaging modality which combines optical and acoustic imaging, is emerging as an important tool for deep tissue imaging. The development of various PA contrast agents which serve to improve the contrast for site-specific imaging and disease diagnosis is therefore of great relevance. The first report on a metal carbonyl as a PA contrast agent was by Leong and Olivo *et. al.*; a water-soluble triosmium carbonyl cluster was used for *in vivo* imaging of the rat cerebral cortex vasculature.¹ The highly stable contrast agent allowed the brain vasculature, especially the superior sagittal sinus (SSS), to be imaged at higher photoacoustic contrast compared to their intrinsic contrast or the use of carbon nanotubes. However, a major drawback lies in the incident wavelength of 410 nm; tissue penetration is low at this wavelength and thus an invasive procedure is required to carry out the imaging. A metal carbonyl that absorbs in the near-IR (NIR) range, from 680 to 1000 nm, is therefore more desirable as it will offer deeper tissue penetration and less scattering. This chapter details investigations on the optical and PA properties of NIR-absorbing metal carbonyls, and their application in non-invasive *in vivo* PA imaging.

4.1 Optical properties of group 8 metal carbonyl clusters - low to high nuclearity

Three ruthenium carbonyl complexes (**12'a**, **20** and **21**, the last as the [PPN] salt; PPN = [(Ph₃P)₂N]⁺) of low nuclearity (Ru_n, n = 1, 3, 6) were synthesized and identified in accordance to reported methods (figure 4.1a).²⁻⁴ Their absorption spectra showed strong absorptions in the UV-visible region (figure 4.1b), and they are shifted towards longer wavelengths with an increase in nuclearity.

Figure 4.1. a) Molecular structures of low nuclearity ruthenium complexes **12'a**, **20** and **21**. Counter cation has been omitted for clarity. b) Optical absorption spectra of the complexes in DMSO at their respective concentrations.

The study was extended to a number of previously reported high nuclearity carbonyl clusters (HNCCs) of ruthenium, **22-25**, as well as one of osmium, **26** (**24-26** were synthesized as the [PPN] salt).⁵⁻⁸ They included homoleptic, as well as carbido, hydrido, and phosphine-substituted derivatives, with different metal core structures (figure 4.2a). All the HNCCs exhibited absorption in the NIR. In particular, there were distinct absorption maxima (λ_{max}) at 719 and 768 nm for clusters **22** and **26**, respectively; the others showed broad shoulders tapering towards longer wavelengths (figures 5.2). This latter broad and continuous absorption from the UV-visible to the NIR region has been attributed to the large number of “interband” transitions, leading to a high degree of overlap of absorption bands.⁹ These absorptions have been assigned to M-CO $\sigma^* \leftarrow \pi$, as well as M-M $\sigma^* \leftarrow \sigma$ transitions,^{9, 10} and they are correlated to the HOMO-LUMO gap. For clusters **22-26**, absorption is negligible at ≥ 900 nm, which suggests a HOMO-LUMO gap of ~ 1.0 eV.¹⁰⁻¹² Cluster **23** shows the highest molar extinction coefficient for its λ_{max} in the NIR region (appendix A4.1, table 4.1).

Figure 4.2. a) Molecular structures of HNCC **22-26**. Counter cations and CO ligands in the HNCCs have been omitted for clarity. b) Optical absorption spectra of HNCCs in DMSO at 200 μ M. Boxed area represents the NIR region of interest.

4.2 Photoacoustic properties of high nuclearity carbonyl clusters 22-26

The PA activity for the five HNCCs were measured in a phantom, which mimics tissue optical properties. Strong PA signals were observed for all the clusters, with the PA intensity at their corresponding λ_{max} varying linearly with concentration (figure 4.3c). The quantum yields (slope of the graph), a useful indicator of the PA strength, were comparable, with the highest being that for **23** (figure 4.3c, table 4.1). With the exception of **25**, the PA spectral intensity followed a similar trend to their optical spectra, indicating little or no decomposition (figure 4.3, appendix A4.2).

Figure 4.3. i) PA (red) and optical absorption (black) spectra of a) cluster **24** and b) **26**. c) Combined plot of PA amplitude as a function of concentrations of **22-26** in DMSO.

4.3 Biocompatible sodium salt of cluster **26**

All the ruthenium HNCCs showed moderate stability and decomposed easily in solution over a few days, in contrast to the more stable osmium cluster **26**. The PPN⁺ counterion, a common cation for stabilizing the HNCCs and other organometallic complexes, was evaluated against an oral squamous cell carcinoma (OSCC) cell line in an MTS assay. It exhibited very high cytotoxicity with an IC₅₀ value of 1.1±0.4 μM (figure 4.4, appendix A4.3). To increase biocompatibility, the synthesis of [Os₁₀(μ₆-C)(CO)₂₄]²⁻ was modified to afford the sodium salt Na₂.**26** (Scheme 4.1).⁸ This had a significantly lower toxicity, with an IC₅₀ of 83±2 μM (figure 4.5, appendix A4.4).

Scheme 4.1. Microwave-assisted synthesis of cluster Na₂.**26**. CO ligands are omitted for clarity.

Figure 4.4. Cell viability (MTS) assay of a) [PPN]Cl and b) Na₂.**26** on OSCC cells, using 1 % DMSO as vehicle.

The molar extinction coefficient (at the λ_{max}) of Na₂.**26** in three different solvent compositions were found to be similar: (1) DMSO, (2) 10% DMSO in phosphate buffer saline (PBS) (v/v), and (3) 10% DMSO in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 1% L-glutamine and 1%

penicillin/ streptomycin (v/v) (appendix A4.5). This was, however, not reflected in the PA response as the signal amplitude was higher in DMSO than in the aqueous biological media (figure 4.5, appendix A4.6, table 4.1). That a stronger PA signal is found in organic solvents than in aqueous media has been reported for a number of contrast agents, and attributed to differences in the Grüneisen parameter.^{13, 14} This multi-component parameter, which describes the solvent environment, is temperature-dependent and governs the thermoelasticity of materials, a key contributor towards the PA effect.¹⁵⁻¹⁷ Different solvent compositions result in different Grüneisen parameters which will affect the amplitude of the PA signal. Nevertheless, the PA signal of Na₂.**26** in modified DMEM was sufficiently strong and remained detectable down to 12.5 μ M.

Figure 4.5 a) Optical absorption (black) and PA spectra (red) of Na₂.**26** (200 μ M, 10% DMSO in modified DMEM). b) PA response of Na₂.**26** as a function of concentration in three different solvent compositions. c) Single wavelength PA image of the phantom at 760 nm. The right channel contains a solution of Na₂.**26** (200 μ M, 10% DMSO in modified DMEM), while the left channel contains only the DMSO in medium.

The salt Na₂.**26** exhibited good chemical and PA stability in 10% DMSO in modified DMEM; the absorbance at $\lambda_{\text{max}} = 760$ nm remained consistent, and the

CO vibrations at 2034 and 1988 cm^{-1} remained unchanged with consistent peak integrals, over a 7-day period (figure 4.6a-c). The salt incubated in modified DMEM for various time points (10 min, 1, 3, 5 and 7 days) were subjected to gel electrophoresis. The gel showed a similar dark red band for all time-points, indicating little or no decomposition (figure 4.6d). Furthermore, the solution was stable to irradiation at 760 nm over a period of 120 min; the CO peaks remain unchanged with consistent peak integrals (appendix A4.7).

Figure 4.6. Time course study of a solution of $\text{Na}_2.26$ (200 μM) in 10% DMSO in modified DMEM, over a period of 7 days: a) FTIR spectra (vCO) obtained from the dried samples, b) integrals of the vCO absorbance, c) optical absorption spectra, d) electrophoresis gel.

In a comparative study with indocyanine green (ICG), we found that the latter has low PA stability although it has better optical and PA properties (appendix A4.9, table 4.1); the PA signal decreased sharply upon irradiation at 790 nm, and a similar decline was observed in its optical spectrum (figure 4.7, appendix A4.8). ICG is an organic tricarbocyanine NIR dye which has been approved by the U.S. Food and Drug Administration for clinical use in angiography, oncology and lymphatic vasculature imaging.^{18, 19} Furthermore, there have also been many reports on its use in PA imaging, including through development into nano-sized constructs.²⁰⁻²³

Figure 4.7. Time course studies of Na₂.26 and ICG over 120 min with irradiation at the respective λ_{max} : a) PA amplitude as a function of time, b) optical absorbance as a function of time.

Table 4.1. Optical absorption and PA properties of clusters **22-26** and indocyanine green (ICG).

Entry	Compounds	$\lambda_{\text{max}} / \text{nm}^{[a]}$	$\epsilon / \text{M}^{-1} \text{cm}^{-1}$	PA quantum yield
1 ^[b]	22	719	4.52×10^3	329 (± 20)
2 ^[b]	23	680 ^[f]	4.81×10^3	644 (± 19)
3 ^[b]	24 ^[e]	680 ^[f]	1.46×10^3	342 (± 12)
4 ^[b]	25 ^[e]	680 ^[f]	1.87×10^3	431 (± 24)
5 ^[b]	26 ^[e]	768	7.75×10^2	308 (± 15)
6 ^[b]	Na ₂ . 26	770	9.08×10^2	315 (± 35)
7 ^[c]	Na ₂ . 26	727	9.69×10^2	50 (± 3)
8 ^[d]	Na ₂ . 26	760	8.41×10^2	87(± 5)
9 ^[d]	ICG	789	1.14×10^5	5097 (± 157)

[a] Within the NIR region of 680-900 nm.

[b] In DMSO.

[c] In 10% DMSO in PBS (v/v).

[d] In 10% DMSO in DMEM containing 10% FBS, 1% L-glutamine and 1% penicillin/ streptomycin (v/v).

[e] As [PPN] salt

[f] Absorption spectra showed only a broad shoulder tapering towards longer wavelengths in the 680-900 nm region, so 680 nm was chosen as it gave the highest absorption in the region.

4.4 Photoacoustic *in vivo* imaging and biodistribution of **26**

The potential of Na₂**26** as an exogenous contrast agent was assessed on three mice with OSCC xenografts; they were injected intravenously through the tail vein with a solution of Na₂**26** in 10% DMSO in heparinized saline (200 µL, 500 µM), giving an effective concentration of approximately 50 µM upon dilution with blood in the animal. Abdominal scans were made before, and up to 24 h after, the injection using real-time multispectral photoacoustic tomography (MSOT).²⁴ In MSOT, the specimen is irradiated with light pulses of different wavelengths. The PA signals generated are collected to afford reconstructed ultrasound images, and spectral unmixing to identify the spectral signatures of molecules of interest present.²⁵

The use of Na₂**26** as the contrast agent for whole body imaging was achieved as evident from the increase in PA contrast in various major abdominal organs, therefore, allowing them to be visualized and outlined easily. MSOT reconstructed images of the mice showed an increase in PA contrast in the liver, spleen and kidneys up to the first hour; by up to 4-, 3- and more than 2-folds, respectively (figure 4.8-4.9). This was reversed by the third hour, however, and almost reached baseline at 6 h, suggesting fast clearance or breakdown of Na₂**26** in the body. As expected, there was no significant accumulation in the tumor in comparison to other tissues as Na₂**26** does not bear any tumor targeting ligand, although the tumor-to-muscle ratio of the PA signal intensity showed an apparent 5-fold contrast at 3 h followed by a gradual decrease at 6 h (appendix A4.10).

Figure 4.8. *In vivo* PA imaging of mouse OSCC xenograft model using $\text{Na}_2.26$ (200 μL , 500 μM) as a contrast agent. a) PA signal from $\text{Na}_2.26$ across a single slice of the mouse abdomen showing part of the liver, spleen (S), kidneys (K) and tumor (T) before and after injection up to 24 h. All PA images are overlaid on their corresponding anatomy images at 800 nm. b) PA signal from $\text{Na}_2.26$ across a single slice of the mouse abdomen showing a greater portion of the liver before and after injection up to 24 h. All PA images are overlaid on their corresponding anatomy images at 800 nm. Bottom left corner shows the reference anatomy of a single abdominal slice depicting in particular, the liver of a frozen sectioned mouse, as obtained elsewhere.²⁶

Figure 4.9. *In vivo* PA imaging of mouse OSCC xenograft model using Na₂.26 (200 μ L, 500 μ M) as a contrast agent. a) Transverse sections of the mouse abdomen, at 2 mm interval, before and at 1 h after the injection of Na₂.26. b) Fold change in the PA intensity in the liver, spleen and kidneys over that at t=0.

The biodistribution of Na₂.**26** inferred from the MSOT experiment has been corroborated by an analysis of the osmium contents through inductively coupled plasma mass spectrometry (ICP-MS), after the mice were sacrificed at 24 h and 96 h. Comparison of the values obtained at 24 h and 96 h reveal that accumulation of osmium exists even after 96 h, mostly in the liver, spleen and kidneys, while other organs showed a significant drop in osmium content. The amount of osmium per tissue mass is significantly higher in the liver, spleen, lungs and kidneys than that in the control mice; it is highest in the liver, followed by the spleen (figure 4.10, appendix A4.11). This is similar to what has been found with the more extensively studied gold nanoparticles;²⁷⁻²⁹ while there have been variations across the studies, in most of the cases, accumulation has been observed to be highest in the liver, followed by tumor, spleen or kidneys.

Figure 4.10. ICP-MS analysis of the amount of Os per tissue mass in various major organs of mice, 24 h and 96 h after injection of Na₂.**26**, with that of control samples. Error bars represent standard error of the mean, n = 3 for samples with Na₂.**26** (24 h) and n = 2 for samples with Na₂.**26** (96 h) and control samples. *Represented as mass of Os per fluid volume / ng μL⁻¹.

To further study the effect of the accumulation of the osmium metal in the various organs (liver, spleen, liver, lungs and heart), the mice were sacrificed at the 24 h and 96 h and the tissue morphology of the organs analyzed by histopathology.

Hematoxylin and eosin (H & E) staining of liver and spleen tissues displayed no obvious damage between the samples treated with Na₂.26 and that of control (figure 4.11), despite the higher accumulation of osmium content. Furthermore, no visible abnormalities were observed in the tissue of kidneys, heart and lungs (appendix A4.12). The condition and weight of the mice were also monitored over 24 h and 96 h. The mice administered with cluster Na₂.26 were well-conditioned and showed no sign of weight loss after 96 h (appendix A4.13). However, the deposition of osmium metal in the liver and spleen is still of concern which requires a long-term study in the future.

Figure 4.11. Histopathology (H & E staining) of liver and spleen tissues of mice, 24 h and 96 h after intravenous injection of Na₂.26, with that of control samples. Scale bar = 100 μ m.

4.5 Development of biocompatible HNCC via polymer encapsulation

The moderate water solubility of the HNCCs, however, necessitated the use of a high concentration of DMSO for delivery into the biological system. This is a concern as DMSO is cytotoxic. One possible strategy to address this is to encapsulate the HNCCs in a micelle. Amphiphilic diblock copolymers like polystyrene-*block*-poly(acrylic acid) (PS-*b*-PAA) can form water soluble and biocompatible micelles which have been used to encapsulate a wide range of materials like gold nanoparticles,^{30, 31} carbon nanotubes,³² iron oxide nanoparticles,^{33, 34} and cadmium sulfide quantum dots.³⁵

Encapsulation by PS-*b*-PAA can be carried out via either a kinetic or a thermodynamic approach (figure 4.12). The kinetic approach is suitable for polymers with shorter PAA chains, like PS₂₅₀-*b*-PAA₁₃, and involves the slow addition of a non-solvent to a solution of the polymer.³⁰ This induces self-assembly of the polymer, with controlled exclusion of polymer from the solution. Polymers with longer PAA chains, like PS₁₅₄-*b*-PAA₄₉, can self-assemble into micelles on heating followed by slow cooling in the presence of hydrophobic nanostructures. This thermodynamic approach involves allowing the micelles to form homogeneously as the critical micelle concentration (CMC) is gradually reached on cooling.³¹

Figure 4.12. Graphical representation of the encapsulation of HNCC by PS-*b*-PAA via the thermodynamic and kinetic approach.

An attempt at the thermodynamic approach was made with the polymer PS₁₅₄-*b*-PAA₄₉ which contains long PAA chains. To enhance interaction with the polystyrene block of the polymer, cluster **23** was used; this tetrahedral-shaped cluster has PPh₃ ligands substituted at the four apices of the metal core. The cluster and the polymer were dissolved in DMF: water (4.5:1, v/v), heated at 110 °C for 15 min, and then allowed to slowly cool to room temperature in the oil bath. The sample was then diluted with a large amount of water to de-swell the polymer. A black precipitate, a sign of cluster decomposition, was observed. With a shorter heating time (5 min), TEM images of the sample showed electron-rich spherical particles surrounded by a dense network of the polymer (figure 4.13). However, no well-defined encapsulated particles were observed. Characterization of the polymer-bound clusters by ATR-FTIR showed that the CO vibrational frequencies were shifted, indicating that the cluster has changed (figure 4.14). The frequency shifts were greater with the longer heating time (15 min), pointing to thermal instability of the cluster.

Figure 4.13. TEM images of PS₁₅₄-*b*-PAA₄₉ encapsulation of **23** heated at 110 °C for 5 min.

Figure 4.14. ATR-FTIR spectra of polymer bound cluster **23** heated at 110 °C for 5 min (red) and 15 min (blue) respectively, in comparison with cluster **23** (black).

Encapsulation of cluster **23** via the kinetic approach was investigated with PS₁₄₄-*b*-PAA₂₂, a polymer with the shorter PAA chain. A fixed amount of the cluster (1 mg) with different amounts of the polymer (from 0.48 to 0.08 mg) were dissolved in DMF, and then water was slowly added. A higher amount of polymer (0.48 mg) was required to achieve homogeneity as lower concentrations resulted in precipitation of the cluster on standing overnight (figure 4.15). The TEM images showed dark spherical structures in a dense network of polymer (figure 4.16). However, there were no observable changes in the spherical structures with the polymer concentration.

Figure 4.15. Photograph of cluster **23** incubated with various amount of polymer (0.48, 0.40, 0.24, 0.16 and 0.08 mg from samples 1 to 5 respectively). Control sample contains no polymer.

Figure 4.16. TEM images of (a-c) samples 1, 2 and 5, and d) control sample.

The homogeneous mixture of sample 1, with 0.48 mg of polymer, was characterized by ATR-IR and the CO vibrational signals were in good agreement with that of cluster **23** (figure 4.17). This showed that the addition of polymer to cluster **23** did not affect the integrity of cluster, which would otherwise have shown up as changes in the CO vibrational frequencies. Nevertheless, precipitation occurred upon standing overnight. An attempt was made to stabilize the micelles with amines; this is known to fix and stabilize the micelles by crosslinking the carboxylic ends.^{30, 34} The homogeneous mixture was first dialyzed overnight against deionized water. The sequential addition of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), followed by 2,2-(ethylenedioxy)bis (ethylamine) and $\text{NH}_2\text{-PEG-COOH}$, led to precipitation after incubating for 2 h; the micelles could not be stabilized.

Figure 4.17. IR spectra of cluster **23** (red) and the cluster with polymer (black).

4.6 Conclusion

In this chapter, the cluster nuclearity was shown to be a useful parameter for fine-tuning the absorption wavelength. The HNCC $[\text{Os}_{10}(\mu_6\text{-C})(\text{CO})_{24}]^{2-}$, **26**, was shown to exhibit good PA properties in the NIR region and was applied successfully in PA imaging of live mice. Although no significant pathological changes were observed in the major organs, the accumulation of osmium metal in the liver and spleen is of concern and would require a more comprehensive, long-term study. Attempts to make the HNCCs more biocompatible through delivery as polymer encapsulated micelles were unsuccessful. Nevertheless, these results have demonstrated that HNCCs are reliable NIR contrast agents with potential use in deep tissue imaging, although further improvements to their biocompatibility and performance will be desirable.

4.7 Experimental section

4.7.1 General methods

All reactions and manipulations were carried out under an argon atmosphere using standard Schlenk techniques. Solvents that were used for reaction were distilled over the appropriate drying agents under argon before use. Purification of compounds was generally carried out by column chromatography on silica gel, or by preparative thin-layer chromatography (TLC) using 20 cm x 20 cm plates pre-coated with silica gel 60 F₂₅₄. Infrared (IR) spectra were recorded on a Bruker Alpha FT-IR spectrometer. Solution spectra were recorded in DCM solution, unless otherwise stated, in a solution IR cell with NaCl windows and a path length of 0.1 mm, at a resolution of 2 cm⁻¹. Alternatively, IR spectra of aqueous samples were recorded by drop casting the mixture (10 µL) onto a CaF₂ disc or onto the ZnSe ATR attachment, followed by drying under a stream of nitrogen before recording on the spectrometer. Optical absorption spectra were recorded using a Hitachi U-2900 double-beam spectrophotometer using quartz cuvette with path length of 10 mm. For samples of smaller volume (100 µL), the optical absorption spectra were recorded using a Beckman Coulter DU 730 spectrometer. HRMS were recorded in ESI mode on a Waters UPLC-Q-ToF MS mass spectrometer.

Copper specimen grids (300 mesh) with formvar/carbon support film were purchased from Beijing XXBR Technology Co. TEM images were collected from a JEM-1400 (JEOL) Transmission Electron Microscopy operated at 100 kV. TEM grids were treated with oxygen plasma in a Harrick® plasma cleaner/sterilizer for 1 min to improve the surface hydrophilicity. A sample solution (10 µL) was dropped onto the hydrophilic side of the TEM grid. Filter paper was used to wick off the excess solution on the TEM grid, which was then left to dry in air for 5 min.

Compounds **11'a**, **20-26** were synthesized and identified according to reported methods with slight modifications.²⁻⁸ Their respective IR spectra can be found in the appendix A4.14-5.21. Os₃(CO)₁₂ and Ru₃(CO)₁₂ were purchased from Oxkem Ltd. [PPN]Cl and indocyanine green (ICG) were purchased from Sigma Aldrich. Amphiphilic diblock copolymer polystyrene-*block*-poly(acrylic acid) (PS₁₅₄-*b*-PAA₄₉, M_n = 16000 for the polystyrene block and M_n = 3500 for the poly(acrylic acid) block, M_w/M_n = 1.15; PS₁₄₄-*b*-PAA₂₂, M_n = 15000 for the polystyrene block and M_n = 1600 for the poly(acrylic acid) block, M_w/M_n = 1.11) was purchased from Polymer Source,

Inc. All other chemical reagents were purchased from other commercial sources and used without further purification.

4.7.2 Synthesis of cluster **Na₂.26**

The synthesis was adapted from the microwave-assisted synthesis of cluster PPN salt of **26**.⁸ A Discover-SP microwave reactor (2450 MHz, CEM Corp., Matthews, NC) and thick-walled 35 mL glass vessel with Teflon-lined caps (CEM Corp., Matthews, NC) were used. In a typical reaction, a 35 mL microwave vessel was charged with Os₃(CO)₁₂ (150 mg, 0.1654 mmol) and diglyme (7 mL). The mixture was irradiated with 300 W with high stirring rate, till the temperature reached 230 °C. The power was subsequently reduced to 100 W for additional heating for 10 min. The reaction mixture was cooled to 50 °C and vented, before irradiated at 300 W for 4 min. The cooling, venting and irradiation at 300 W for 4 min was repeated once. The solvent was removed by evaporation over a hot water bath and the residue was extracted with acetone (20 mL) and filtered over a thin layer of celite. The solvent was removed and the residue dissolved in MeOH (10 mL). NaCl (20 mg, 0.34 mmol) was added to the solution and stirred at 60 °C for 1 h. Solvent was removed and the residue was purified by TLC using acetone-hexane (3:2, v/v) as eluant to give cluster **Na₂.26** as a dark red band.

Yield: 33 mg (24 %)

R_f = 0.23

IR (acetonitrile, cm⁻¹): ν_{CO} 2038s, 1990s. (see appendix 5.21, lit: 2038s, 1990s)³⁶

HRMS-ESI: *m/z* 1294.0946 [M]²⁻ (see appendix A4.22 for isotopic pattern)

4.7.3 Cell culture and cell viability assay

Experimental cultures of oral squamous cell carcinoma (OSCC) cell line were obtained from the American Type Culture Collection (ATCC) and cultured in tissue culture dishes (Nunc Inc., IL). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Biowest, France) supplemented with 10 % fetal bovine serum (FBS; Gibco[®], NY), 1 % L-glutamine (PAA Laboratories, Austria), and 1 % penicillin/streptomycin (HyClone, UT) at 37 °C in 5 % CO₂ atmosphere. Phosphate-buffered saline (PBS) was obtained from PAA Laboratories. Stock solutions of cluster **Na₂.26** and [PPN]Cl in sterile-filtered dimethyl sulfoxide (DMSO) were prepared and

serial diluted to lower concentrations. For treatment with the compounds, cells were seeded in wells within 96-well plate in growth medium at the same initial density of 10 000 cells per well, and allowed to adhere and grow for 24 h. They were serum-starved in serum-free DMEM for 24 h, followed by treatment with the indicated concentrations of compounds in serum-free DMEM (1 % DMSO) for 24 h. Control cells were treated with vehicle (1 % DMSO). To each well, 10 μ L of MTS reagent (MTS Cell Proliferation Assay Kit, BioVision, CA) was added and then left to incubate in a 37 °C incubator with 5% CO₂ for 2 h. The absorbance intensities at 490 nm were then measured and the cell proliferation relative to the control sample was calculated. Each sample was analyzed in triplicates and was corrected with background intensities from same incubation conditions without the cells. IC₅₀ was determined from three separate experiments and the mean value calculated.

4.7.4 Gel electrophoresis

Agarose gel (1%, UltraPure™ Agarose, Invitrogen) was cast with and immersed in 0.5 x TAE (Tris-acetate-EDTA buffer, prepared from 50 x stock solution, pH 8.0, 1st BASE). Cluster Na₂.**26**, incubated in 10% DMSO in modified DMEM (30 μ L) for different duration (10 min, 1, 3, 5 and 7 days), was loaded into the respective wells of the gel and electrophoresed in a horizontal electrophoresis system (Wide Mini-sub® cell GT Basic System, Biorad, electrode spacing of 15 cm) at 150 V for 30 min.

4.7.5 Multispectral optoacoustic tomography (MSOT) experimental parameters and protocol

All phantom and *in vivo* mouse imaging experiments were performed using a real-time MSOT imaging system; inVision 128 (ithera Medical GmbH, Germany).²⁴ Optical excitation was provided by an optical parametric oscillator with a tunable NIR wavelength range from 680 to 900 nm, which was in turn pumped by a Q-switched Nd:YAG laser with a pulse duration of 10 ns and repetition rate of 10 Hz. Light was delivered by a fiber bundle divided into 10 output arms to illuminate the sample from multiple angles around the imaging plane. PA signals were acquired using a 128-element concave transducer array spanning a circular arc of 270°. This transducer array had a central frequency of 5 MHz, which provided a transverse spatial resolution in the range of 150–200 μ m. One transverse image slice was acquired from each laser

pulse, resulting in a frame rate of 10 Hz. During image acquisition, the sample was translated through the transducer array along its axis across the volume region of interest (ROI), in order to capture the corresponding transverse image slices.

4.7.6 Evaluation of PA activity of clusters 22-26 in phantom model

The polyurethane phantom is cylindrical in shape with a diameter of 2 cm; it is specially designed to mimic the shape, size, and optical properties of the mouse. In addition, it has two inner cylindrical channels, each with an inner diameter of 3 mm, one for holding the control medium and the other for holding the dissolved contrast agent in the same medium. The control medium and contrast agent suspension (170 μ L each) were pipetted into the respective channels of the phantom, and placed in the MSOT imaging chamber using a phantom holder. For data acquisition, an ROI of multiple transverse slices with a step size of 1 mm was set up across the channel portion which contained the probe and control, applied excitation wavelengths from 680 to 900 nm with an interval of 10 nm for each transverse slice, and recorded the averaged PA signals from 10 frames for each wavelength and position. The acquired images were reconstructed using model based approach and PA signals were quantified using ViewMSOT at the respective λ_{max} of each probe from the reconstructed images.³⁷ The PA amplitude of the probe was determined by subtracting the values of the channel containing the control from that of the probe, and the mean was calculated from 10 different transverse slices.

4.7.7 *In vivo* longitudinal monitoring of probe biodistribution in mouse xenograft model

Dr Ghayathri (SBIC, A*STAR) prepared the mouse xenograft model and performed the animal imaging experiments with my assistance. All animal experiments were performed in compliance with the protocol #120774 and #151085 approved by the Institutional Animal Care and Use Committee, Agency for Science, Technology and Research (A*STAR). Xenograft models were established on three Balb/C nude mice by injecting subcutaneously into the right flank of each mouse, a cell suspension (0.1 mL) containing 5×10^6 oral squamous carcinoma cells (OSCC) and matrigel (BD Biosciences, San Jose, CA) in a 1:1 volume ratio. When the tumor volume reached a palpable size, a solution of Na₂.**26** (200 μ L, 500 μ M) in 10% DMSO in heparinized

saline (v/v) was injected intravenously through the tail vein of each mouse anaesthetized under isoflurane. Ultrasound gel was applied on the mouse skin surface for *in vivo* imaging, and measurements were recorded in temperature-controlled water medium for good acoustic coupling. An animal holder with a thin polyethylene membrane was used to prevent direct contact between the mouse and the water. Seven laser excitation wavelengths (680, 700, 730, 750, 810, 850, and 900 nm) were chosen for multispectral imaging on the basis of the absorption maxima and minima of the contrast agent, and oxy- and deoxyhemoglobin. PA signals were acquired across multiple transverse slices from the liver to the lower abdomen region inclusive of the tumor at a step size of 0.5 mm and averaged over 10 frames for each wavelength.

4.7.8 Image processing and analysis for *in vivo* experiments

Images acquired from the *in vivo* experiments were reconstructed using direct back projection approach for offline analysis. After image reconstruction, spectral unmixing was performed to resolve individual components from different chromophores in the system. For each pixel, the method fits the total measured optoacoustic spectrum to the known absorption spectra of the individual chromophores, based on least-squares linear regression. Images were exported out from ViewMSOT and further analyzed using Fiji (version 2.0.0), an open source image-processing package.³⁸ Regions of Interest (ROIs) were chosen from liver, spleen, kidney and tumor and quantified for multispectrally unmixed PA signals before and after probe injection. PA signals at all time-points were normalized against that of the pre-injection scan and expressed as fold change over the same.

4.7.9 Microwave acid digestion and ICP-MS analysis of osmium content of tissue samples

ICP-MS analysis was carried out by Dr Bahareh (CBC, NTU). The mice were euthanized after 24 and 96 h respectively, the organs extracted, washed with deionized water and snap-freezed in liquid nitrogen for future use. The selected organs were freeze-dried over a period of five days and the dried masses measured, prior to microwave acid digestion. A programmable Milestone microwave system (ETHOS EZ, Italy) was used to digest and prepare the samples for ICP analysis. Samples were placed inside the Teflon microwave vessels and 12 mL HCl (37%, ultrapure grade, J. T. Baker, Canada) were added. Ultra-pure water with 18.2 M Ω •cm resistivity was used

(ELGA, PURLAB Option – Q, UK) for sample and standard preparations. Using the microwave radiation (Power 1100W) the temperature was ramped to 185 °C in 20 min and then held at that temperature for 15 min. The vessels were opened after cooling down to room temperature for several hours, to prevent any sample loss. The extracts were diluted with ultrapure water, filtered through 0.2 µm syringe filters, and carefully transferred to ICP sample vials for elemental analysis. Samples were analyzed using an Agilent 7700 series ICP-MS (Japan) equipped with a third generation He reaction / collision cell (ORS3) to minimize interference. An external calibration curve for osmium was constructed, before each analytical run, from serial dilutions of an osmium standard for ICP-MS (1000 mgL⁻¹, Inorganic Venture, Christiansburg, VA, USA) with high purity deionized water.

4.7.10 Histopathology of organ tissues of mice

Six mice were intravenously injected with cluster Na₂.**26** (200 µL, 500 µM, 10% DMSO in saline) and euthanized after 24 h and 96 h (three mice at each time point) respectively. For the control sample, four mice were injected with 10% DMSO in saline instead. The mice were anaesthetized using ketamine (150 mg/mL) and xylazine (10 mg/kg) and euthanized by cervical dislocation to collect various organs for histopathology analysis. All organs were immediately fixed in 10% neutral buffered formalin (Sigma) and submitted to Advanced Molecular Pathology Laboratory (AMPL), IMCB for histopathology evaluation. The pathology report was prepared by an experienced pathologist. All bright field images were captured using the Nikon Ni-E upright microscope, with Plan Apo 20x DIC M N2 objective and Nikon DS-Ri2 camera. The images were viewed using NIS-Elements software.

4.7.11 Attempted polymer encapsulation of cluster 23 via the thermodynamic approach

In a typical reaction, PS₁₅₄-*b*-PAA₆₀ solution (10 µL, 8 mg mL⁻¹ in DMF) was added to a solution of cluster **23** (1 mg) dissolved in DMF (790 µL). Deionized water (180 µL) was then added and the mixture was vortexed for 10 s. The mixture was heated in a pre-heated oil bath at 110 °C for 5 or 15 min, before it was slowly cooled to room temperature in the oil bath. A portion of the sample (100 µL) was topped up with deionized water (1400 µL), mixed well and centrifuged at 6000g for 10 min. The

supernatant was removed and the pellet was dispersed in deionized water (100 μL) before proceeding to TEM analysis. For ATR-FTIR analysis, the pellet was dispersed in MeOH and was dropped onto the ATR sample stage and air-dried, before proceeding with IR analysis.

4.7.12 Attempted polymer encapsulation of cluster **23 via the kinetic approach**

In a typical reaction, PS₁₄₄-*b*-PAA₂₂ solution (60 μL , 8 mg mL⁻¹ in DMF) was added to a solution of cluster **23** (400 μL , 1 mg mL⁻¹ in DMF). The amount of polymer was varied with the use of 10-60 μL of the PS₁₄₄-*b*-PAA₂₂ solution. The volume of the mixture was topped up to 500 μL with DMF and vortexed briefly. The dark green solution was stirred vigorously while deionized water (3 mL) was added using a syringe pump at a rate of 0.05 mL/min. The mixture was left to stir for another 1 h. The carboxylic acid groups were first activated by the addition of EDC solution (13 μL , 40 mM) for 30 min, before the sequential addition of 2,2'-(ethylenedioxy)bis(ethylamine) solution (20 μL , 10 mM) and NH₂-PEG-COOH solution (13 μL , 40 mM). The mixture was stirred at room temperature for 2 h and precipitation was observed.

In another attempt, after the addition of water using the syringe pump, the mixture was dialyzed against deionized water using a cellulose membrane (Spectra Por 7 dialysis membrane, 15000 Da MWCO) overnight to remove DMF and other small organic compounds. Unfortunately, precipitation was observed in the tubing, indicating the instability of the micelles.

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Chapter 5: Conclusion

In this work, the application of transition metal carbonyls in bio-sensing and – imaging via two optical techniques, namely surface-enhanced Raman spectroscopy (SERS) and photoacoustic (PA) imaging, were examined (figure 5.1).

Figure 5.1. Summary of the work investigated in this thesis.

Two designs of metal carbonyl-based Raman reporters were examined for potential use in multiplexing – one based on transition metal carbonyl clusters and another on mononuclear half-sandwich metal carbonyls complexes. SERS multiplexing applications require non-overlapping signals, and changes to the ligand sphere and the charge (for cluster complexes) were found to be effective for tuning of the CO vibrational stretches over a wide frequency range. It was observed, however, that signal enhancement was dependent on the extent of aggregation and often led to large variations in the SERS enhancement. Vigorous study in developing controlled

aggregates involving metal carbonyls would therefore be required to determine the conditions needed to produce highly reproducible SERS tags. Nevertheless, incubation of the metal carbonyls with AuNS, followed by centrifugation and re-dispersion, was found to be convenient for qualitative screening of suitable metal carbonyls. Alternatively, the use of colloidal substrates with higher SERS enhancements can be employed, for example, gold nanostars and nanoflowers.^{1, 2} Further functionalization of the SERS tags with ligands for cell-labelling or for the detection of biomolecules of interest would be the next logical extension. Another would be in the study of the CO-releasing process of CO-releasing molecules (CORMs) and to monitor their fate in cellular applications.

The extension of metal carbonyl-based SERS reporters to a planar substrate eliminated the problem of aggregation associated with colloidal substrates, thereby ensuring reproducibility. Coupling boronic acid chemistry to metal carbonyl led to a selective glucose assay based on the conjugate, **2b**-B(OH)₂. This sandwich-based approach proved to be highly effective and may be extended to the detection of other biomolecules via the binding affinity between a binding group and the molecule, for example, through antigen-antibody and enzyme-substrate affinity (figure 5.2). A future worthwhile improvement would be replacement of the transition metal with a cheaper metal.

Figure 5.2. Future work for translating the glucose assay to the detection of other biomolecules and for the use of cheaper transition metals as SERS probes.

Transition metal carbonyl clusters were also demonstrated to be useful as contrast agents in PA imaging through their electronic spectral properties. The electronic spectra of these clusters showed optical absorption due to transitions relating to the cluster's metal core, and increasing the cluster nuclearity shifted the absorption into the NIR region. The HNCC $[\text{Os}_{10}(\mu_6\text{-C})(\text{CO})_{24}]^{2-}$, **26**, was shown to exhibit good PA properties in the NIR region, and its sodium salt was applied successfully for the whole body PA imaging of live mice. The need for improvement on the PA performance, biocompatibility and selectivity remain, however. Improvements to the NIR absorption, and hence the PA activity, of the HNCCs may be sought through a further increase in the cluster nuclearity, or through the use of conjugated ligands to enhance the NIR absorption. Improvements to the biocompatibility and selectivity may be carried out via functionalization of the HNCC with suitable bioligands that target the site of interest. Using nano-sized polymers, carbon nano-structures or mesoporous silica nanoparticles, as carriers for the HNCCs may also enhance biocompatibility and selectivity. Nevertheless, a comprehensive, long-term *in vivo* toxicity study will be required to better understand the effect of such clusters in biological systems.

Given the success of SERS sensing and PA imaging, it may also be worthwhile to investigate the use of metal carbonyls for multimodal imaging, which is, the use of more than one imaging technique simultaneously. Multimodal imaging has tremendous potential in clinical applications and can provide complementary information that is valuable at both cellular and molecular levels.^{3, 4} SERS-PA dual modal sensing is possible with the development of transition metal carbonyl-based SERS-PA active probes. For instance, the conjugation of HNCCs onto gold colloids can allow both the intrinsic NIR PA activity of the HNCCs and the enhanced CO vibrational frequency to serve as a dual handle, for both imaging and sensing.

The discovery of the cytotoxic nature of the metal carbonyl-boronic acid conjugate, **2b**-B(OH)₂, was quite unexpected as the alkylthiolato analogues were much less cytotoxic; that **2b**-B(OH)₂ was apoptosis-inducing was confirmed. It is now important to verify the role of the thiolate substituent in affecting the cytotoxicity. Examples of clusters that can be tested on to validate this point are the $\text{Os}_3(\text{CO})_{10}(\mu\text{-H})(\mu\text{-SCH}_2\text{C}_6\text{H}_4\text{X})$ clusters, where $\text{X} = \text{-B(OH)}_2$, -COOH and -OH (figure 5.3). Establishing this can allow us to modulate such clusters for their intended purposes accurately (for imaging or therapy) by virtue of the R groups. This could, for instance,

lead to improvements in selectivity towards cancer cells - the functional groups (-B(OH)₂, -OH and -COOH) can provide convenient conjugation points for ligands that target cancer cells (figure 5.3). In contrast to some of the cytotoxic osmium carbonyl clusters reported thus far, **2b** represent yet another class of clusters with anti-cancer potential and are more amenable to modification.⁵⁻⁸

Figure 5.3. Future work for the validation of the role of the R group in modulating the cytotoxicity of the phenylthiolatio clusters **2b** and bioconjugation of **2b** to cancer-targeting ligands via the X group.

We have demonstrated through the work in this thesis that transition metal carbonyls, in particular, clusters have great potential for two relatively new optical imaging modalities, *viz.*, SERS and PA imaging. These modalities have attracted much attention over the years as functional and robust tools in biology. The applications of metal carbonyls in these two techniques are still in its infancy, and the outlook is positive, with many more examples sure to emerge.

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44. **Figure A3.17.** Dose-response curves of a) **1**, b) **2b**-B(OH)₂, c) **2b**-OH and d) **2b**-COOH on MDA-MB-231 cells, after 24 h, in serum-free condition.
45. **Figure A3.18.** Dose-response curves of a) **1**, b) **2b**-B(OH)₂ and c) **2b**-OH on MCF-7 cells, after 24 h, in serum-free condition.
46. **Figure A3.19.** Dose-response curves of a) **1**, b) **2b**-B(OH)₂ and c) **2b**-OH on MCF-10A cells, after 24 h, in serum-free condition.
47. **Figure A3.21.** Dose-response curves of a) **1**, b) **2b**-B(OH)₂ and c) **2b**-OH on MCF-7 cells, after 24 h, in serum condition.
48. **Figure A3.22.** Dose-response curves of a) **1**, b) **2b**-B(OH)₂ and c) **2b**-OH on MCF-10A cells, after 24 h, in serum condition.

Chapter 4: High Nuclearity Metal Carbonyl Clusters as Near-IR Contrast Agents for Photoacoustic *In Vivo* Imaging

49. **Figure A4.1.** Optical absorption spectra of clusters **22**-[PPN]₂**26** at various concentrations (in DMSO) and plots of optical absorbance (at respective λ_{max}) as a function of concentration.
50. **Figure A4.2.** i) PA (red) and optical absorption (black) spectra, ii) PA amplitude as a function of concentration of cluster **22**-[PPN]₂**26**.
51. **Figure A4.3.** Cell viability assay of [PPN]Cl on OSCC cells and calculation of IC₅₀ from three replicates.
52. **Figure A4.4.** Cell viability assay of Na₂**26** on OSCC cells and calculation of IC₅₀ from three replicates.
53. **Figure A4.5.** Optical absorption spectra of Na₂**26** at various concentrations and plot of λ_{max} as a function of concentration of Na₂**26** in a) DMSO, b) 10% DMSO in PBS (v/v), and c) 10% DMSO in modified DMEM (v/v).
54. **Figure A4.6.** PA, optical absorption spectra and plot of PA amplitude (at respective λ_{max}) as a function of concentration of Na₂**26** in a) DMSO, b) 10% DMSO in PBS (v/v), and c) 10% DMSO in modified DMEM (v/v).
55. **Figure A4.7.** Irradiation of a solution of Na₂**26** (200 μ M) in 10% DMSO in modified DMEM at 760 nm for various time intervals: a) FTIR spectra (ν_{CO}) obtained from the dried samples, and b) integrals of the ν_{CO} absorbance.
56. **Figure A4.8.** a) Optical absorption spectra of ICG of various concentrations, b) plot of absorbance of ICG at $\lambda_{\text{max}}=790$ nm as
57. **Figure A4.9.** a) Optical absorption spectra of ICG of various concentrations, b) plot of absorbance of ICG at $\lambda_{\text{max}}=790$ nm as a function of concentration, c) PA (red) and optical absorption (black) spectra of ICG, d) plot of PA amplitude at $\lambda_{\text{max}}=790$ nm as a function of concentration of ICG in 10% DMSO in modified DMEM (v/v).
58. **Figure A4.10.** a) Enlarged images of the tumors at various time point with the PA intensities normalized (top). Fold change in PA intensity in the tumor over that at t=0 in the tumor (bottom). b) Tumor-to-muscle ratio of PA amplitude at various time points up till 24 h.
59. **Figure A4.11.** ICP-MS analysis of the percentage retention of injected dose (% ID) of Na₂**26** per organs, 24 h after injection. Error bars represent standard error of the mean, n = 3.
60. **Figure A4.12.** Histopathology (H & E staining) of kidneys, heart and lungs tissues of mice, 24 h and 96 h after intravenous injection of Na₂**26**, with that of control samples.
61. **Figure A4.13.** Percentage weight change of control mice and mice injected with Na₂**26** up till 24 h and 96 h with respect to initial weight. Error bars represent standard error of the mean, n = 3 for samples with **9** and n = 2 for control samples.
62. **Figure A4.14.** IR spectrum (ν_{CO}) of **12'a** in DCM.
63. **Figure A4.15.** IR spectrum (ν_{CO}) of cluster **20** in hexane.
64. **Figure A4.16.** IR spectrum (ν_{CO}) of cluster [PPN]**21** in DCM.
65. **Figure A4.17.** IR spectrum (ν_{CO}) of cluster **22** in DCM.
66. **Figure A4.18.** IR spectrum (ν_{CO}) of cluster **23** in DCM.

67. **Figure A4.19.** IR spectrum (ν_{CO}) of cluster $[\text{PPN}]_2\mathbf{24}$ in DCM.
68. **Figure A4.20.** IR spectrum (ν_{CO}) of cluster $[\text{PPN}]_3\mathbf{25}$ in DCM.
69. **Figure A4.21.** IR spectrum (ν_{CO}) of cluster $[\text{PPN}]_2\mathbf{26}$ in DCM.
70. **Figure A4.22.** IR spectrum (ν_{CO}) of cluster $\text{Na}_2\mathbf{26}$ in acetonitrile.
71. **Figure A4.23.** HRMS-ESI spectrum of cluster $\text{Na}_2\mathbf{26}$ with the calculated isotopic pattern.
72. **Table A4.1.** ICP-MS analysis of Os content in various organs 24 h after injection of $\text{Na}_2\mathbf{26}$.
73. **Table A4.2.** ICP-MS analysis of Os content in various organs 96 h after injection of $\text{Na}_2\mathbf{26}$.
74. **Table A4.3.** ICP-MS analysis of background Os content in various organs in control animals (without the introduction of $\text{Na}_2\mathbf{26}$).
75. **Table A4.4.** Calculation of the percentage retention of injected dose (% ID) of $\text{Na}_2\mathbf{26}$ in various organ tissues.
76. References

Chapter 2: Surface-Enhanced Raman Spectroscopy (SERS) of Metal Carbonyls on Gold Nanospheres

Figure A2.1. IR spectrum (v_{CO}) of **2a** in DCM.

Figure A2.2. IR spectrum (v_{CO}) of **3b** in DCM.

Figure A2.3. IR spectrum (ν_{CO}) of **4** in DCM.

Figure A2.4. IR spectrum (ν_{CO}) of **6** in DCM.

Figure A2.5. IR spectrum (ν_{CO}) of **6.H⁺** in DCM.

Figure A2.6. IR spectrum (ν_{CO}) of **7a** in DCM.

Figure A2.7. IR spectrum (ν_{CO}) of **7b** in DCM.

Figure A2.8. IR spectrum (ν_{CO}) of **8a** in DCM.

Figure A2.9. IR spectrum (ν_{CO}) of **9a** in DCM.

Figure A2.10. IR spectrum (ν_{CO}) of **10a** in DCM.

Figure A2.11. IR spectrum (ν_{CO}) of **10b** in THF.

Figure A2.12. IR spectrum (ν_{CO}) of **10c** in THF.

Figure A2.13. IR spectrum (ν_{CO}) of **11a** in DCM.

Figure A2.14. IR spectrum (ν_{CO}) of **12a** in DCM.

Figure A2.15. IR spectrum (ν_{CO}) of **12'a** in DCM.

Figure A2.16. IR spectrum (ν_{CO}) of **13a** in DCM.

Figure A2.17. ^1H NMR spectrum of **3b** in CDCl₃.

Figure A2.18. $^{13}\text{C}\{^1\text{H}\}$ NMR spectrum of **3b** in CDCl_3 .

Figure A2.19. ^1H NMR spectrum of **6** in CDCl_3 .

Figure A2.20. COSY NMR spectrum of **6** in CDCl_3 .

Figure A2.21. $^{31}\text{P}\{^1\text{H}\}$ NMR spectrum of **6** in CDCl_3 .

Figure A2.22. a) ESI-HRMS of **6** (top) with calculated isotopic pattern (bottom). b) ESI-HRMS single mass analysis of **6**.

Figure A2.24. COSY NMR spectrum of $6.H^+$ in $CDCl_3$.

Figure A2.25. $^{31}\text{P}\{^1\text{H}\}$ NMR spectrum of **6**.H⁺ in CDCl₃.

Figure A2.26. a) ESI-HRMS of **6.H⁺** (top) with calculated isotopic pattern (bottom). b) ESI-HRMS single mass analysis of **6.H⁺**

Figure A2.27. a) IR spectrum (ν_{CO}) of $\text{CpW(CO)}_3\text{X}$ in DCM and b) SERS spectra of respective complexes with AuNS, where X = Cl (black), Br (red) and I (blue), in DCM.

Chapter 3: Metal Carbonyl-Boronic Acid Conjugate - a SERS Probe for Glucose Detection and its Cytotoxicity

Figure A3.1. ^1H NMR spectrum of **2b**-B(OH) $_2$ in $(\text{CD}_3)_2\text{CO}$.

Figure A3.2. ^1H NMR spectrum of **2b-OH** in $(\text{CD}_3)_2\text{CO}$.

Figure A3.3. ^1H NMR spectrum of **2b**-COOH in $(\text{CD}_3)_2\text{CO}$.

Figure A3.4. $^{13}\text{C}\{^1\text{H}\}$ NMR spectrum of **2b**-B(OH)₂ in (CD₃)₂CO.

Figure A3.5. $^{13}\text{C}\{^1\text{H}\}$ NMR spectrum of **2b-OH** in $(\text{CD}_3)_2\text{CO}$.

Figure A3.6. $^{13}\text{C}\{^1\text{H}\}$ NMR spectrum of **2b**-COOH in $(\text{CD}_3)_2\text{CO}$.

Figure A3.7. IR spectrum (ν_{CO}) of **1** in DCM.

Figure A3.8. IR spectrum (ν_{CO}) of **2b**-B(OH)₂ in DCM.

Figure A3.9. IR spectrum (ν_{CO}) of **2b**-H in DCM.

Figure A3.10. IR spectrum (ν_{CO}) of **2b-OH** in DCM.

Figure A3.11. IR spectrum (ν_{CO}) of **2b-COOH** in DCM.

C16H7BO12Os3S
ZY-210-TLC 4 (0.101) Cm (4:20)

1: TOF MS ES-
637

Figure A3.12. ESI-HRMS of **2b-B(OH)₂**.

b Elemental Composition Report

Page 1

Single Mass Analysis

Tolerance = 1.5 PPM / DBE: min = -1.5, max = 50.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

12118 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)

Elements Used:

C: 16-16 H: 6-7 B: 0-1 O: 6-12 S: 0-2 184Os: 0-3 186Os: 0-3 187Os: 0-3 188Os: 0-3 189Os: 0-3 190Os: 0-3 192Os: 0-3

C16H7BO12Os3S

ZY-210-TLC 3 (0.083) Cm (1:8)

1: TOF MS ES-
1.12e+003

Minimum:							
Maximum:	5.0	1.5	-1.5				
			50.0				
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
1002.8414	1002.8427	-1.3	-1.3	17.5	21.8	0.0	C16 H6 B O12 S 190Os3

Figure A3.13. a) ESI-HRMS of **2b-B(OH)₂** (top) with calculated isotopic pattern (bottom). b) ESI-HRMS single mass analysis of **2b-B(OH)₂**.

b Elemental Composition Report

Page 1

Single Mass Analysis

Tolerance = 1.0 PPM / DBE: min = -1.5, max = 50.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

19839 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)

Elements Used:

C: 22-22 H: 16-16 B: 0-1 O: 10-17 S: 0-2 184Os: 0-3 186Os: 0-3 187Os: 0-3 188Os: 0-3 189Os: 0-3 190Os: 0-3 192Os: 0-3

C₁₆H₇BO₁₂Os₃S
ZY-GLUCOSE 3 (0.082) Cm (1:11)

1: TOF MS ES- 1.54e+002

Minimum:
Maximum:

		5.0	1.0	-1.5			
				50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
1165.8999	1165.8989	1.0	0.9	18.5	111.0	0.0	C ₂₂ H ₁₆ B O ₁₇ S 187Os 192Os ₂

Figure A3.14. a) ESI-HRMS of glucose adduct of **2b**-B(OH)₂ (top) with calculated isotopic pattern (bottom). b) ESI-HRMS single mass analysis of the adduct.

b Elemental Composition Report

Page 1

Single Mass Analysis

Tolerance = 10.0 PPM / DBE: min = -1.5, max = 50.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 2

Monoisotopic Mass, Even Electron Ions

255 formula(e) evaluated with 2 results within limits (up to 50 closest results for each mass)

Elements Used:

C: 16-17 H: 5-6 O: 11-12 S: 0-1 184Os: 0-1 186Os: 0-1 187Os: 0-1 188Os: 0-1 189Os: 0-1 190Os: 0-1

192Os: 0-1

C₁₆H₆O₁₁Os₃S

ZY-OsOH 12 (0.277)

1: TOF MS ES-
4.05e+000

Minimum:

Maximum:

5.0 10.0 -1.5

Mass Calc. Mass mDa PPM DBE i-FIT i-FIT (Norm) Formula

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
976.8148	976.8201	-5.3	-5.4	18.5	11.6	0.7	C17 H5 O11 S 184Os 186Os 190Os
	976.8217	-6.9	-7.1	18.5	11.6	0.7	C17 H5 O11 S 184Os 187Os 189Os

Figure A3.15. a) ESI-HRMS of **2b**-OH (top) with calculated isotopic pattern (bottom). b) ESI-HRMS single mass analysis of **2b**-OH.

b

Elemental Composition Report

Page 1

Single Mass Analysis

Tolerance = 10.0 PPM / DBE: min = -1.5, max = 50.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 2

Monoisotopic Mass, Even Electron Ions

492 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)

Elements Used:

C: 17-18 H: 5-6 O: 12-12 S: 0-1 184Os: 0-3 186Os: 0-3 187Os: 0-3 188Os: 0-3 189Os: 0-3 190Os: 0-3

192Os: 0-3

C₁₇H₆O₁₂O_s3S

ZY-OsCOOH 12 (0.277)

1: TOF MS ES-
2.03e+000

Minimum:									
Maximum:	5.0	10.0	-1.5	50.0					
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula		
1005.8364	1005.8313	5.1	5.1	18.5	12.0	0.0	C ₁₇ H ₅ O ₁₂ S 189Os 192Os ₂		

Figure A3.16. a) ESI-HRMS of **2b**-COOH (top) with calculated isotopic pattern (bottom). b) ESI-HRMS single mass analysis of **2b**-COOH.

a Dose-response curve of **1** on MDA-MB-231 cells in serum-free condition

log(inhibitor) vs. response -- Variable slope			
Best-fit values		95% Confidence Intervals	
Bottom	1.938	Bottom	-1.288 to 5.163
Top	88.98	Top	85.28 to 92.68
LogIC50	0.6851	LogIC50	0.6631 to 0.7071
HillSlope	-5.809	HillSlope	-10.31 to -1.311
IC50	4.843	IC50	4.604 to 5.094
Span	87.04	Span	81.75 to 92.34
Std. Error		Goodness of Fit	
Bottom	1.514	Degrees of Freedom	15
Top	1.737	R ²	0.9915
LogIC50	0.01032	Absolute Sum of Squares	249.9
HillSlope	2.111	Sy.x	4.082
Span	2.485	Number of points analyzed	19

b Dose-response curve of **2b**-B(OH)₂ on MDA-MB-231 cells in serum-free condition

log(inhibitor) vs. response -- Variable slope			
Best-fit values		95% Confidence Intervals	
Bottom	7.438	Bottom	2.647 to 12.23
Top	100.1	Top	96.12 to 104.1
LogIC50	0.6805	LogIC50	0.6172 to 0.7437
HillSlope	-8.377	HillSlope	-36.64 to 19.89
IC50	4.792	IC50	4.142 to 5.543
Span	92.65	Span	85.93 to 99.37
Std. Error		Goodness of Fit	
Bottom	2.233	Degrees of Freedom	14
Top	1.85	R ²	0.989
LogIC50	0.02949	Absolute Sum of Squares	349.4
HillSlope	13.18	Sy.x	4.996
Span	3.133	Number of points analyzed	18

c Dose-response curve of **2b**-OH on MDA-MB-231 cells in serum-free condition

log(inhibitor) vs. response -- Variable slope			
Best-fit values		95% Confidence Intervals	
Bottom	3.012	Bottom	-1.974 to 7.997
Top	99.44	Top	95.68 to 103.2
LogIC50	0.4793	LogIC50	0.3933 to 0.5654
HillSlope	-8.495	HillSlope	-17.02 to 0.03193
IC50	3.015	IC50	2.473 to 3.676
Span	96.43	Span	90.17 to 102.7
Std. Error		Goodness of Fit	
Bottom	2.288	Degrees of Freedom	12
Top	1.725	R ²	0.9927
LogIC50	0.03949	Absolute Sum of Squares	249.8
HillSlope	3.913	Sy.x	4.563
Span	2.872	Number of points analyzed	16

Figure A3.17. Dose-response curves of a) **1**, b) **2b**-B(OH)₂, c) **2b**-OH on MDA-MB-231 cells, after 24 h, in serum-free condition.

d Dose-response curve of **2b-COOH** on MDA-MB-231 cells in serum-free condition

log(inhibitor) vs. response -- Variable slope			
Best-fit values		95% Confidence Intervals	
Bottom	5.63	Bottom	3.738 to 7.522
Top	102.2	Top	99.89 to 104.5
LogIC50	0.5009	LogIC50	0.4804 to 0.5214
HillSlope	-3.764	HillSlope	-4.259 to -3.269
IC50	3.169	IC50	3.023 to 3.322
Span	96.59	Span	93.50 to 99.68
Std. Error		Goodness of Fit	
Bottom	0.8924	Degrees of Freedom	16
Top	1.097	R ²	0.9972
LogIC50	0.00966	Absolute Sum of Squares	101.2
HillSlope	0.2334	Sy.x	2.515
Span	1.458	Number of points analyzed	20

Figure A3.17 (cont'd). Dose-response curve of **2b-COOH** on MDA-MB-231 cells, after 24 h, in serum-free condition.

Figure A3.18. Dose-response curves of a) **1**, b) **2b-B(OH)₂** and c) **2b-OH** on MCF-7 cells, after 24 h, in serum-free condition.

Figure A3.19. Dose-response curves of a) **1**, b) **2b-B(OH)₂** and c) **2b-OH** on MCF-10A cells, after 24 h, in serum-free condition.

a Dose-response curve of **1** on MDA-MB-231 cells in serum condition

log(inhibitor) vs. response -- Variable slope			
Best-fit values		95% Confidence Intervals	
Bottom	0.571	Bottom	-1.963 to 3.105
Top	104.7	Top	102.1 to 107.3
LogIC50	1.475	LogIC50	1.451 to 1.499
HillSlope	-2.744	HillSlope	-3.112 to -2.377
IC50	29.84	IC50	28.25 to 31.53
Span	104.2	Span	100.2 to 108.1
Std. Error		Goodness of Fit	
Bottom	1.181	Degrees of Freedom	14
Top	1.215	R ²	0.9976
LogIC50	0.01113	Absolute Sum of Squares	85.06
HillSlope	0.1714	Sy.x	2.465
Span	1.841	Number of points analyzed	18

b Dose-response curve of **2b**-B(OH)₂ on MDA-MB-231 cells in serum condition

log(inhibitor) vs. response -- Variable slope			
Best-fit values		95% Confidence Intervals	
Bottom	-0.0985	Bottom	-3.830 to 3.633
Top	99.56	Top	95.98 to 103.1
LogIC50	1.26	LogIC50	1.219 to 1.302
HillSlope	-3.87	HillSlope	-4.841 to -2.900
IC50	18.21	IC50	16.57 to 20.02
Span	99.65	Span	94.23 to 105.1
Std. Error		Goodness of Fit	
Bottom	1.727	Degrees of Freedom	13
Top	1.656	R ²	0.9946
LogIC50	0.01903	Absolute Sum of Squares	186.3
HillSlope	0.4493	Sy.x	3.786
Span	2.513	Number of points analyzed	17

c Dose-response curve of **2b**-OH on MDA-MB-231 cells in serum condition

log(inhibitor) vs. response -- Variable slope			
Best-fit values		95% Confidence Intervals	
Bottom	0.3788	Bottom	-2.342 to 3.099
Top	99.49	Top	96.77 to 102.2
LogIC50	0.8857	LogIC50	0.8573 to 0.9141
HillSlope	-6.805	HillSlope	-8.268 to -5.341
IC50	7.686	IC50	7.199 to 8.206
Span	99.11	Span	95.26 to 103.0
Std. Error		Goodness of Fit	
Bottom	1.268	Degrees of Freedom	14
Top	1.266	R ²	0.9966
LogIC50	0.01325	Absolute Sum of Squares	134.7
HillSlope	0.6822	Sy.x	3.101
Span	1.795	Number of points analyzed	18

Figure A3.20. Dose-response curves of a) **1**, b) **2b**-B(OH)₂ and c) **2b**-OH on MDA-MB-231 cells, after 24 h, in serum condition.

Figure A3.21. Dose-response curves of a) **1**, b) **2b-B(OH)₂** and c) **2b-OH** on MCF-7 cells, after 24 h, in serum condition.

Figure A3.22. Dose-response curves of a) **1**, b) **2b**-B(OH)₂ and c) **2b**-OH on MCF-10A cells, after 24 h, in serum condition.

Chapter 4: High Nuclearity Metal Carbonyl Clusters as Near-IR Contrast Agents for Photoacoustic *In Vivo* Imaging

a) Cluster **22**

b) Cluster **23**

Figure A4.1. i) Optical absorption spectra of various concentrations (in DMSO), ii) plot of optical absorbance (at respective λ_{max}) as a function of concentrations of cluster a) **22** and b) **23** and.

Figure A4.1 (cont'd). i) Optical absorption spectra of various concentrations (in DMSO), ii) plot of optical absorbance (at respective $\lambda_{\max}$) as a function of concentrations of cluster c) [PPN]₂.24, d) [PPN]₃.25 and e) [PPN]₂.26.

Figure A4.2. i) PA (red) and optical absorption (black) spectra, ii) PA amplitude as a function of concentration of cluster a) **22**, b) **23** and c) **[PPN]₂.24**.

Figure A4.2 (cont'd). i) PA (red) and optical absorption (black) spectra, ii) plot of PA amplitude as a function of concentration of cluster d) [PPN]₃.25 and e) [PPN]₂.26.

b)

Replicate 1 - MTS assay of [PPN]Cl on OSCC cells

Concentration (C) / μM	Cell viability (V) / %	100-Y	X=ln C	Y=ln [V/(100-V)]	Y ₀	X ₀
25.0	0.60	99.40	3.22	-5.103	0	0.3773
12.5	1.21	98.79	2.53	-4.406	IC ₅₀ =	1.46
6.3	6.55	93.45	1.84	-2.657

-0.5304402	0.3773
0.1336792	0.5593
0.9402810	0.2382
15.7450903	1
0.8931749	0.0567

Figure A4.3. a) First replicate of cell viability assay of [PPN]Cl on OSCC cells, using 1 % DMSO as vehicle, b) calculation of IC₅₀.

d)

Replicate 2 - MTS assay of [PPN]Cl on OSCC cells

Concentration (C) / μM	Cell viability (V) / %	100-V	X=ln C	Y=ln [V/(100-V)]	Y ₀	X ₀
20.0	0.72	99.28	3.00	-4.923	0	-1.1710
10.0	0.96	99.04	2.30	-4.635	IC ₅₀ =	0.31
5.0	3.24	96.76	1.61	-3.397

-0.8043229	-1.1710
0.2890845	1.2630
0.8855999	0.3316
7.7412536	1
0.8509783	0.1099

Figure A4.3 (cont'd). c) Second replicate of cell viability assay of [PPN]Cl on OSCC cells, using 1 % DMSO as vehicle, d) calculation of IC₅₀.

f)

Replicate 3 - MTS assay of [PPN]Cl on OSCC cells

Concentration (C) / μM	Cell viability (V) / %	100-V	X=ln C	Y=ln [V/(100-V)]	Y ₀	X ₀
20.0	0.40	99.60	3.00	-5.511	0	0.4231
10.0	0.62	99.38	2.30	-5.083	IC ₅₀ =	1.53
5.0	6.32	93.68	1.61	-2.697

-0.4242441	0.4231
0.1703612	0.7836
0.8611382	0.3653
6.2014057	1
0.8274729	0.1334

Mean IC ₅₀	1.10
Standard error	0.40

Figure A4.3 (cont'd). e) Third replicate of cell viability assay of [PPN]Cl on OSCC cells, using 1 % DMSO as vehicle, f) calculation of IC₅₀ and the mean value from three replicates.

b)

Replicate 1 - MTS assay of Na₂[Os₁₀(μ₆-C)(CO)₂₄] (Na₂.26) on OSCC cells

Concentration (C) / μM	Cell viability (V) / %	100-V	X=ln C	Y=ln [V/(100-V)]	Y ₀	X ₀
100.0	31.91	68.09	4.61	-0.758	0	4.3773
50.0	83.32	16.68	3.91	1.609	IC ₅₀ =	79.62
25.0	95.69	4.31	3.22	3.099

-0.3533478	4.3773
0.0463439	0.0956
0.9830888	0.1275
58.1325284	1
0.9446560	0.0163

Figure A4.4. a) First replicate of cell viability assay of Na₂[Os₁₀(μ₆-C)(CO)₂₄] (Na₂.26) on OSCC cells, using 1 % DMSO as vehicle, b) calculation of IC₅₀.

d)

Replicate 2 - MTS assay of Na₂[Os₁₀(μ_6 -C)(CO)₂₄] (Na₂.26) on OSCC cells

Concentration (C) / μM	Cell viability (V) / %	100-V	X=ln C	Y=ln [V/(100-V)]	Y ₀	X ₀
100.0	38.58	61.42	4.61	-0.465	0	4.4513
50.0	85.77	14.23	3.91	1.796	IC ₅₀ =	85.73
25.0	91.41	8.59	3.22	2.364

-0.4377199	4.4513
0.1513021	0.2625
0.8932715	0.3202
8.3695677	1
0.8583500	0.1026

Figure A4.4 (cont'd). c) Second replicate of cell viability assay of Na₂[Os₁₀(μ_6 -C)(CO)₂₄] (Na₂.26) on OSCC cells, using 1 % DMSO as vehicle, d) calculation of IC₅₀.

f)

Replicate 3 - MTS assay of Na₂[Os₁₀(μ₆-C)(CO)₂₄] (Na₂.26) on OSCC cells

Concentration (C) / μM	Cell viability (V) / %	100-V	X=ln C	Y=ln [V/(100-V)]	Y ₀	X ₀
100.0	38.47	61.53	4.61	-0.470	0	4.4205
50.0	78.99	21.01	3.91	1.324	IC ₅₀ =	83.14
25.0	89.95	10.05	3.22	2.192

-0.5006497	4.4205
0.1005357	0.1511
0.9612382	0.1930
24.7986260	1
0.9236596	0.0372

Mean IC ₅₀	82.83
Standard error	1.77

Figure A4.4 (cont'd). e) Third replicate of cell viability assay of Na₂[Os₁₀(μ₆-C)(CO)₂₄] (Na₂.26) on OSCC cells, using 1 % DMSO as vehicle, f) calculation of IC₅₀ and the mean value from three replicates.

Figure A4.5. i) Optical absorption spectra of $\text{Na}_2\text{.26}$ of various concentrations and ii) plot of λ_{max} as a function of concentration of $\text{Na}_2\text{.26}$ in a) DMSO, b) 10% DMSO in PBS (v/v), and c) 10% DMSO in modified DMEM (v/v).

Figure A4.6. i) PA (red) and optical absorption (black) spectra of $\text{Na}_2.26$, ii) plot of PA amplitude (at respective λ_{max}) as a function of concentration of $\text{Na}_2.26$ in a) DMSO, b) 10% DMSO in PBS (v/v), and c) 10% DMSO in modified DMEM (v/v).

Figure A4.7. Irradiation of a solution of Na₂.26 (200 μM) in 10% DMSO in modified DMEM at 760 nm for various time intervals: a) FTIR spectra (ν_{CO}) obtained from the dried samples, and b) integrals of the ν_{CO} absorbance.

Figure A4.8. Time course studies of Na₂.26 and ICG over 120 min with irradiation at the respective λ_{max}: a) optical absorption spectra of Na₂.26 and d) ICG.

Figure A4.9. a) Optical absorption spectra of ICG of various concentrations, b) plot of absorbance of ICG at $\lambda_{\text{max}}=790$ nm as a function of concentration, c) PA (red) and optical absorption (black) spectra of ICG, d) plot of PA amplitude at $\lambda_{\text{max}}=790$ nm as a function of concentration of ICG in 10% DMSO in modified DMEM (v/v).

Figure A4.10. a) Enlarged images of the tumors at various time point with the PA intensities normalized (top). Fold change in PA intensity in the tumor over that at t=0 in the tumor (bottom). Error bars represent standard error of the mean, n = 3. b) Tumor-to-muscle ratio of PA amplitude at various time points up till 24 h. Error bars represent standard error of the mean, n = 3.

Figure A4.11. ICP-MS analysis of the percentage retention of injected dose (% ID) of $\text{Na}_2.26$ per organs, 24 h after injection. Error bars represent standard error of the mean, $n = 3$.

Figure A4.12. Histopathology (H & E staining) of kidneys, heart and lungs tissues of mice, 24 h and 96 h after intravenous injection of Na₂.26, with that of control samples. Scale bar = 100 μ m.

Figure A4.13. Percentage weight change of control mice and mice injected with $\text{Na}_2.26$ up till 24 h and 96 h with respect to initial weight. Error bars represent standard error of the mean, $n = 3$ for samples with $\text{Na}_2.26$ and $n = 2$ for control samples.

Figure A4.14. IR spectrum (ν_{CO}) of **12'a** in DCM.

Figure A4.15. IR spectrum (ν_{CO}) of cluster **20** in hexane.

Figure A4.16. IR spectrum (ν_{CO}) of cluster **21** in DCM.

Figure A4.17. IR spectrum (ν_{CO}) of cluster **22** in DCM.

Figure A4.18. IR spectrum (ν_{CO}) of cluster **23** in DCM.

Figure A4.19. IR spectrum (ν_{CO}) of cluster **24** in DCM.

Figure A4.20. IR spectrum (ν_{CO}) of cluster **25** in DCM.

Figure A4.21. IR spectrum (ν_{CO}) of cluster **26** in DCM.

Figure A4.22. IR spectrum (ν_{CO}) of cluster **Na₂.26** in acetonitrile.

Figure A4.22. HRMS-ESI spectrum of cluster $\text{Na}_2\cdot\mathbf{26}$ (top). The calculated isotopic pattern is as shown at the bottom.

Table A4.1. ICP-MS analysis of Os in various organs of animals 24 h after injection of Na₂.26.

a) Animal 1 (mass = 23.1 g)

Tissue	Tissue mass (dried) / mg	Elemental concentration of Os / ppb	Mass of Os / µg	Mass of Os per tissue mass / ng mg⁻¹
Liver	363.28	859.50	85.95	236.6
Spleen	44.20	105.81	10.58	239.4
Lungs	36.07	10.47	1.05	29.0
Tumor	11.82	5.70	0.57	48.2
Kidneys	114.53	4.82	0.48	4.2
Heart	33.09	1.32	0.32	9.7
Brain	91.27	2.16	0.22	2.4
Blood (50 µL)	N. A.	1.79	0.18	3.6*

*Represented as mass of Os per fluid volume / ng µL⁻¹

b) Animal 2 (mass = 22.3 g)

Tissue	Tissue mass (dried) / mg	Elemental concentration of Os / ppb	Mass of Os / µg	Mass of Os per tissue mass / ng mg⁻¹
Liver	387.20	932.75	93.28	240.9
Spleen	38.01	48.55	4.86	127.7
Lungs	41.43	7.01	0.70	16.9
Tumor	27.03	1.25	0.13	4.6
Kidneys	117.58	5.19	0.52	4.4
Heart	44.31	1.76	0.18	4.0
Brain	94.73	1.01	0.10	1.1
Blood (50 µL)	N. A.	1.14	0.11	2.3*

*Represented as mass of Os per fluid volume / ng µL⁻¹

c) Animal 3 (mass = 21.0 g)

Tissue	Tissue mass (dried) / mg	Elemental concentration of Os / ppb	Mass of Os / μg	Mass of Os per tissue mass / ng mg^{-1}
Liver	361.55	927.50	92.75	256.5
Spleen	30.86	35.42	3.54	114.8
Lungs	37.22	25.79	2.58	69.3
Tumor	17.55	0.90	0.09	5.1
Kidneys	91.76	4.18	0.42	4.6
Heart	44.35	2.32	0.23	5.2
Brain	87.63	0.41	0.04	0.5
Blood (50 μL)	N. A.	1.14	0.11	2.3*

*Represented as mass of Os per fluid volume / $\text{ng } \mu\text{L}^{-1}$

d) Mean values

Tissue	Mass of Os per tissue mass / ng mg^{-1}
	Mean ($\pm$ SE of mean)
Liver	245 ± 6
Spleen	161 ± 40
Lungs	38 ± 16
Tumor	19 ± 14
Kidneys	4.4 ± 0.1
Heart	6.3 ± 3.0
Brain	1.3 ± 0.6
Blood*	$2.7 \pm 0.4^*$

*Represented as mass of Os per fluid volume / $\text{ng } \mu\text{L}^{-1}$

Table A4.2. ICP-MS analysis of Os in various organs of animals 96 h after injection of Na₂.26.

a) Animal 1 (mass = 20.1 g)

Tissue	Tissue mass (dried) / mg	Elemental concentration of Os / ppb	Mass of Os / μg	Mass of Os per tissue mass / ng mg^{-1}
Liver	311.45	777.31	77.73	249.6
Spleen	29.94	126.17	12.62	421.4
Lungs	26.56	3.56	0.36	13.4
Tumor	9.44	0.37	0.04	3.9
Kidneys	94.69	1.79	0.18	1.9
Heart	25.73	0.51	0.05	2.0
Brain	91.07	0.13	0.01	0.1
Blood (50 μL)	N. A.	0.28	0.03	0.6

*Represented as mass of Os per fluid volume / $\text{ng } \mu\text{L}^{-1}$

b) Animal 2 (mass = 20.0 g)

Tissue	Tissue mass (dried) / mg	Elemental concentration of Os / ppb	Mass of Os / μg	Mass of Os per tissue mass / ng mg^{-1}
Liver	314.38	732.42	73.24	233.0
Spleen	28.74	55.96	5.60	194.7
Lungs	31.75	1.82	0.18	5.7
Tumor	24.82	0.51	0.05	2.1
Kidneys	99.23	6.51	0.65	6.6
Heart	22.41	0.42	0.04	1.9
Brain	83.46	0.16	0.02	0.2
Blood (50 μL)	N. A.	0.23	0.02	0.5

*Represented as mass of Os per fluid volume / $\text{ng } \mu\text{L}^{-1}$

c) Mean values

Tissue	Mass of Os per tissue mass / ng mg^{-1}
	Mean ($\pm$ SE of mean)
Liver	241 $\pm$ 8
Spleen	308 $\pm$ 113
Lungs	10 $\pm$ 4
Tumor	3.0 $\pm$ 0.9
Kidneys	4 $\pm$ 2
Heart	1.93 $\pm$ 0.05
Brain	0.17 $\pm$ 0.02
Blood*	0.51 $\pm$ 0.05*

*Represented as mass of Os per fluid volume / $\text{ng } \mu\text{L}^{-1}$

Table A4.3. ICP-MS analysis of background Os content in various organs in control animals (without the introduction of Na₂.26).

a) Animal 1 (mass = 21.8 g)

Tissue	Tissue mass (dried) / mg	Elemental concentration of Os / ppb	Mass of Os / μg	Mass of Os per tissue mass / ng mg^{-1}
Liver	341.14	0.84	0.08	0.25
Spleen	33.86	0.39	0.04	1.15
Lungs	30.33	0.23	0.02	0.76
Tumor	13.15	0.22	0.02	1.67
Kidneys	103.46	0.46	0.05	0.44
Heart	36.89	0.31	0.03	0.84
Brain	90.78	0.28	0.03	0.31
Blood (50 μL)	N. A.	0.29	0.03	0.58

*Represented as mass of Os per fluid volume / $\text{ng } \mu\text{L}^{-1}$

b) Animal 2 (mass = 21.0 g)

Tissue	Tissue mass (dried) / mg	Elemental concentration of Os / ppb	Mass of Os / μg	Mass of Os per tissue mass / ng mg^{-1}
Liver	309.90	0.17	0.02	0.05
Spleen	24.06	0.13	0.01	0.54
Lungs	27.97	0.11	0.01	0.39
Tumor	5.89	0.16	0.02	2.72
Kidneys	102.63	0.15	0.02	0.15
Heart	37.17	0.12	0.01	0.32
Brain	88.43	0.13	0.01	0.15
Blood (50 μL)	N. A.	0.14	0.01	0.28

*Represented as mass of Os per fluid volume / $\text{ng } \mu\text{L}^{-1}$

c) Mean values

Tissue	Mass of Os per tissue mass / ng mg^{-1}
	Mean ($\pm$ SE of mean)
Liver	0.15 ± 0.1
Spleen	0.8 ± 0.3
Lungs	0.6 ± 0.2
Tumor	2.2 ± 0.5
Kidneys	0.3 ± 0.1
Heart	0.6 ± 0.3
Brain	0.23 ± 0.08
Blood*	$0.4 \pm 0.2^*$

*Represented as mass of Os per fluid volume / $\text{ng } \mu\text{L}^{-1}$

Table A4.4. Calculation of the retention of Os in various organ tissues.

a) Calculation of mass of Os administered into each animal

Percentage mass of Os in Na₂.**26** = 72.26%

No. of moles of Na₂.**26** administered into the animal = 200 μL x 500 μM = 0.100 μmol

Mass of Na₂.**26** administered into each animal = 0.100 μmol x 2632.53 g mol^{-1} = 263.25 μg

Mass of Os administered into each animal (M_T) = (72.26/100) x 263.25 = **190.23 μg**

b) Estimation of the amount of Os in blood based on projected total blood volume

The blood volume of mouse was estimated to be 79 (77-80) $\mu\text{L g}^{-1}$.^{1,2} Hence, this value was used for the calculation of blood volume:

Estimated blood volume of animal 1 (A1) = 79 $\mu\text{L g}^{-1}$ x 23.1 g = 1825 μL

$\therefore$ Mass of Os in blood of A1 = (0.179 $\mu\text{g} / 50 \mu\text{L}$) x 1825 μL = **6.53 μg**

Estimated blood volume of animal 2 (A2) = 79 $\mu\text{L g}^{-1}$ x 22.3 g = 1762 μL

$\therefore$ Mass of Os in blood of A2 = (0.114 $\mu\text{g} / 50 \mu\text{L}$) x 1762 μL = **4.02 μg**

Estimated blood volume of animal 3 (A3) = 79 $\mu\text{L g}^{-1}$ x 21.0 g = 1659 μL

$\therefore$ Mass of Os in blood of A3 = (0.114 $\mu\text{g} / 50 \mu\text{L}$) x 1659 μL = **3.78 μg**

Tissue	Mass of Os (M_O) / μg			Percentage retention of Os in the tissue ($M_O/M_T \times 100$) / %			
	A1	A2	A3	A1	A2	A3	Mean ($\pm$ SE of mean)
Liver	85.95	93.28	92.75	45.18	49.03	48.76	47.66 $\pm$ 1.24
Spleen	10.58	4.86	3.54	5.56	2.55	1.86	3.33 $\pm$ 1.14
Lungs	1.05	0.70	2.58	0.55	0.37	1.36	0.76 $\pm$ 0.30
Tumor	0.57	0.13	0.09	0.30	0.07	0.05	0.14 $\pm$ 0.08
Kidneys	0.48	0.52	0.42	0.25	0.27	0.22	0.25 $\pm$ 0.02
Heart	0.32	0.18	0.23	0.17	0.09	0.12	0.13 $\pm$ 0.02
Brain	0.22	0.10	0.04	0.11	0.05	0.02	0.06 $\pm$ 0.03
Blood[^]	6.53	4.02	3.78	3.43	2.11	1.99	2.51 $\pm$ 0.46

[^]Estimation based on total blood volume of animals

References

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2. J. E. Harkness and J. E. Wagner, *The Biology and Medicine of Rabbits and Rodents*, Lea and Febiger, Philadelphia, USA, 1989.