Organometallic Carbonyl Clusters: A New Class of Contrast Agents for Photoacoustic Cerebral Vascular Imaging

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1. General procedure.
All manipulations for chemical syntheses were carried out using standard Schlenk techniques under an argon or nitrogen atmosphere.\textsuperscript{[1]} The triosmium carbonyl cluster Os$_3$(CO)$_{10}$(NCMe)$_2$ (I) was prepared according to the reported procedure from the precursor Os$_3$(CO)$_{12}$,\textsuperscript{[2]} which was purchased from Oxkem; all other chemicals were purchased from other commercial sources and used as supplied. IR spectra were obtained using a Bruker Alpha Fourier transform infrared spectrometer. $^1$H NMR spectra were recorded in CDCl$_3$ on a Bruker Avance 500 spectrometer and referenced to residual solvent resonances. The Raman spectral measurements were carried out using a Renishaw InVia Raman (UK) microscope with a Peltier cooled CCD detector and an excitation wavelength of 785 nm, where the laser excitation is directed onto the sample via a 50x objective lens. All Raman spectra were processed with the WiRE3.0 software. The maximum laser power at the sample was measured to be 300 mW and the exposure time was set at 10 s for all measurements. Prior to each measurement, the instrument was calibrated using the standard Raman spectrum of silicon with a peak centered at 520 cm$^{-1}$.

2. Synthesis of Os$_3$(CO)$_{10}(\mu$-$H)\mu$-$S$(CH$_2$)$_2$COOH.
Os$_3$(CO)$_{10}$(NCCH$_3$)$_2$ (200 mg, 0.22 mmol) was stirred with 3-mercaptopropionic acid (48 mg, 0.22 mmol) in dichloromethane (30 ml) at room temperature for 12 h. After the reaction, the solvent was removed in vacuo and the residue purified by column chromatography using hexane/DCM as the eluant to yield a yellow solid. Yield: 114 mg (57%).

IR (CH$_2$Cl$_2$): $\nu$CO 2106 (w), 2066 (s), 2057(s), 2020 (s), 1997 (w).

$^1$H NMR (CDCl$_3$): $\delta$ 2.58-2.69 (m, 4H, CH$_2$CH$_2$), -17.40 (s, 1H, OsHOs)

3. Synthesis of [Os$_3$(CO)$_{10}(\mu$-$H)(\mu$-$S$(CH$_2$)$_2$COO)]$^+Na^+$ (Os salt)
To Os$_3$(CO)$_{10}(\mu$-$H)(\mu$-$S$(CH$_2$)$_2$COOH) (100 mg, 0.09 mmol) in methanol (20 ml) was added sodium carbonate (18.7 mg, 0.19 mmol). The mixture was refluxed for 1 h, cooled to room temperature, and the excess sodium carbonate filtered off. The solvent was then removed under reduced pressure to afford 2 as a water-soluble, yellow solid. Yield: 95 mg (95%).

IR (KBr): $\nu$CO 2110 (w), 2061 (s), 2099 (s), 1928 (w).

$^1$H NMR (300 MHz, D$_2$O): $\delta$ 2.47-2.69 (m, 4H, CH$_2$CH$_2$), -17.11 (s, 1H, OsHOs)

ESI (m/z): 957.1 [M$^+$]
4. Dark-field confocal photoacoustic microscopy system and experimental set-up

The 50-MHz dark-field confocal fPAM system used and the experimental set-up for in vivo imaging, is depicted below. The microscopy system consists of laser pulse generation and delivery, PA signal reception, and image reconstruction and display. Laser pulses, 4 ns wide, were generated at a frequency of 10 Hz by using an optical parametric oscillator (Surlite OPO Plus, Continuum, USA). The laser was pumped by a frequency-tripled Nd:YAG Q-switched laser (Surlite II-10, Continuum, USA). The incident wavelength was set at 410 nm. The custom-made 50-MHz ultrasonic transducer (Acoustic Sensor, Taiwan) has a -6 dB fractional bandwidth of 57.5%, focal length of 9 mm, and a 6 mm active element, offering an axial resolution of 32 µm and a lateral resolution of 61 µm.

Laser energy was delivered using a 1-mm multimodal fiber. The fiber tip was coaxially aligned with a convex lens, an axicon, a plexiglass mirror, and an ultrasonic transducer on an optical bench, forming dark-field illumination that was confocal with the focal point of the ultrasonic transducer. The incident energy density on the sample surface was well within American National Standards Institute (ANSI) safety limits. The transducer was immersed in an acrylic water tank during the imaging process, and the hole at the bottom of the tank was sealed with a piece of 15-µm thick polyethylene film. A thin layer of ultrasonic gel was applied as a PA conductive medium, which was then attached to the thin polyethylene film to ensure reliable coupling of the PA waves with the water tank. The PA signals received by the ultrasonic transducer were pre-amplified by a low-noise amplifier (noise figure 1.2 dB, gain 55 dB, AU-3A-0110, USA), cascaded to an ultrasonic receiver (5073 PR, Olympus, USA) and then digitized and sampled by a computer-based 14-bit analog to digital (A/D) card (CompuScope 14220, GaGe, USA) at a 200-MHz sampling rate for data storage.

Fluctuations in the laser energy were monitored with a photodiode (DET36A/M, Thorlabs, USA). The recorded photodiode signals were measured prior to the experiment to compensate for PA signal variations caused by laser-energy instability. The achievable penetration depth of the current PA microscopy system was 3 mm with approximately 18-dB SNR, where SNR is defined as the ratio of the signal peak value to the root-mean-square value of the noise. Three scan types can be provided by this system: A-line (i.e., one-dimensional images where the axis represents the imaging depth), B-scan (i.e., two-dimensional images where one axis is the lateral scanning distance and the other is the imaging depth), and C-scan (i.e., projection images from the three-dimensional images). The amplitude of the envelope-detected PA signals was used in the subsequent functional imaging analysis.

For both in vivo and in vitro imaging, the laser was pulsed with a pulse repetition rate of 10 Hz and coupled by a lens to an optical fiber to illuminate the ROI. A window at the bottom of the water container was sealed with an optically and ultrasonically transparent, disposable polyethylene film. After a commercially available ultrasound gel was applied to the brain for acoustic coupling, the brain was
placed between the water container and the custom-made stereotaxic apparatus for imaging.

5. Cell viability study.
Five thousand oral squamous cell carcinoma (OSCC) cells were seeded in a 96-well plate for 24 h before a solution of Os salt in DMEM (1 mL) at the indicated concentration (0.01 – 30 \(\mu\)M) was introduced into each well. After incubation for 24 h, 10 \(\mu\)L of CCK8 (cell counting kit-8, Sigma-Aldrich) was added to each well. Cell absorbance was measured with a SpectraMax 384 Plus spectral analyser after 4 h, with 450 nm excitation.

A polyethylene tubing (~20 cm in length) was filled with the sample solution and then positioned at the focus of the transducer, i.e., at a depth of 9 mm with respect to the transducer in the water tank. The system was maintained in a 25 °C water bath throughout the experiment. Imaging was carried out with the photoacoustic microscopy system at 32 \(\times\) 61-\(\mu\)m resolution, with a scanning step size of 20 \(\mu\)m for each B-scan.

Six male Wistar rats (NUS-CARE, Singapore), weighing 250 - 300 grams each, were used. Animal experiments were conducted in accordance with guidelines from the Institutional Animal Care and Use Committee (IACUC) at the National University of Singapore. The rats were fasted for 24 h prior to the imaging experiments, but were given water ad libitum. They were anesthetized with isoflurane (2-3% in 100% O\(_2\)), mounted in a dorsal position over a custom-made acrylic stereotaxic holder, and the skin and muscle on the skull were removed to expose the bregma landmark. The anteroposterior (AP) distance between the bregma and the interaural line was directly surveyed. The bregma was 9.3 ± 0.12 mm (mean ± standard deviation [SD]) anterior to the interaural line.

A craniotomy was also performed for each animal, and a bilateral cranial window of approximately 6 (horizontal) \(\times\) 4 (vertical) mm size was made with a high-speed drill. After the rat was secured to the stereotaxic frame and placed on the bed pallet, the pallet was moved into position at the bregma, which was 9 mm anterior to an imaginary line drawn between the centers of each ear bar (the interaural line). The interaural and bregma references were then used to position the heads in the PAM system. After bregma positioning, a PA C-scan (projection image from the three-dimensional images) was performed to acquire reference images of the cortical vasculature, at \(\lambda_{410}\). An aqueous solution of Os salt or SWNT (200 \(\mu\)L, 30 \(\mu\)M) was administered via retro-orbital injection prior to imaging.
8. Photoacoustic plot, IR and NMR spectrum.

**Figure S1-1.** (a) Plot of photoacoustic B-scan signal strength versus concentrations of Os₃(CO)₁₂. (b) False-colour images representing the relative photoacoustic B-scan signal strengths.

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References in Supporting Information