Electronic Supplementary Information

Membrane-disrupting Iridium(III) Oligocationic Metallopeptides

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General

All reagents were acquired from commercial sources. Dimethylformamide (DMF), Trifluoroacetic acid (TFA) and 2,2,2-Trifluoroethanol (TFE) were purchased from Scharlau, CH_2Cl_2 from Panreac and CH_3CN from Merck. $IrCl_3$ and 2-phenylpyridine (ppy) were from Sigma-Aldrich. All other chemicals were purchased from Sigma-Aldrich or Fluka. All solvents were dry and of synthesis grade, unless specifically noted. Reactions were followed by analytical RP-HPLC with an Agilent 1100 series LC/MS using a Luna C_{18} (250 x 4.60 mm) analytical column from Phenomenex. Peptides were purified on a Phenomenex Luna C_{18} (250 × 10 mm) semi-preparative reverse phase column. The standard gradient used for analytical and semi-preparative HPLC was 1% to 75% over 35 min (water/acetonitrile, 0.1% TFA). The fractions containing the products were freeze-dried, and their identity was confirmed by ESI−MS with an Agilent 1100 Series LC/MSD VL model in positive scan mode using direct injection of the purified peptide solutions into the MS. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI/MS) was performed with a Bruker Autoflex MALDI/TOF model in positive scan mode by direct irradiation of the matrix-absorbed peptide.

Synthesis of the unnatural coordinating residue Fmoc-βAla-bpy-OH (1)

The coordinating residue Fmoc-βAla-bpy-OH (**1**) was synthetized following a procedure previously reported.[1]

Peptide Synthesis

The coupling agents HBTU (O-Benzotriazole-*N,N,N′,N′*-tetramethyl-uronium-hexafluorophosphate) and HATU (2-(1*H*-7-azabenzotriazol-1-*yl*)-1,1,3,3-tetramethyluronium hexafluorophosphate methanaminium) were from *GL Biochem* (Shanghai). PyAOP ((7-Azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate) was purchased from *IRIS Biotech.* Fmoc amino acids derivatives were purchased with the standard side chain protecting scheme from *GL Biochem* (Shanghai) Ltd. or Novabiochem, except for Fmoc-β-Ala-OH (Cat. #: FAA1300), which was from *IRIS Biotech*.

C-terminal amide peptides were synthesized by following standard SPPS protocols on a 0.1 mmol scale using rink amide resin. Amino acids were coupled in 4-fold excess (vs. mmol of resin load) using HBTU (4 eq) and DIEA/DMF 0.2 M (8 eq). Fmoc–βAla-Bpy–OH (**1**) was coupled in four-fold excess using HATU (4 eq) and DIEA/DMF 0.2 M (8 eq). Each amino acid was activated for 30 seconds in DMF before being added onto the resin. Peptide bond-forming couplings were conducted for 45 min to 1h. The deprotection of the temporal Fmoc protecting group was performed by treating the resin with 20% piperidine in DMF solution for 30 min.

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[1] I. Gamba, I. Salvadó, G. Rama, M. Bertazzon, M. I. Sánchez, V. M. Sánchez-Pedregal, J. Martínez-Costas, R. F. Brissos, P. Gamez, J. L. Mascareñas, M. Vázquez López, M. E. Vázquez, *Chem. Eur. J.*, **2013**, *19*, 13369–13375

Linear peptides were cleaved from the resin, and side-chain protecting groups were simultaneously removed using a standard TFA cleavage cocktail (50 μL of CH_2Cl_2, 25 μL of H_2O, 25 μL of TIS and 900 TFA μL for 40 mg of resin). The resulting suspension was shaken for 2 h. The resin was filtered, and the TFA filtrate was concentrated with a nitrogen gas stream to an approximate volume of 1 mL, which was added to ice-cold diethyl ether (10 mL). After 10 min, the precipitate was centrifuged and washed again with 15 mL of ice-cold ether. The solid residue was dried under argon and redissolved in acetonitrile/water 1:1 (1 mL) and purified by semi-preparative reverse-phase HPLC.

Cyclic peptides (C-terminal carboxylates) were cleaved from the 2-Chlorotrityl Chloride resin (from Iris Biotech) using AcOH/TFE/DCM (1:1:8) during 1 h. The resin was filtered, and the filtrates added to ice-cold diethyl ether (10 mL). After 10 min, the precipitate was centrifuged, washed again with 15 mL of ice-cold ether, and dried under vacuum. For the cyclization step, the isolated peptide was treated overnight with PyAOP (2 eq) DIEA (6 eq) in DMF. The solvent was concentrated *in vacuo*, and the resulting solid was washed with 15 mL of EtOH and centrifuged. The side-chain protecting groups were cleaved, and the final peptide isolated and purified following standard procedures.

(βAla-bpy–R_3)$_2$, 52.1 mg obtained, 71% yield for a 0.05 mmol scale; **(βAla-bpy–R_3)$_3$**, 51.2 mg obtained, 68% yield for a 0.05 mmol scale; ***cyclo*-(βAla-bpy–R_3)$_2$**, 52.1 mg obtained, 71% yield for a 0.05 mmol scale; ***cyclo*-(βAla-bpy–R_3)$_3$**, 50.2 mg obtained, 68% yield for a 0.05 mmol scale; ***cyclo*-(W–R_3)$_2$**, 45.3 mg obtained, 69% yield for a 0.05 mmol scale.

Synthesis of the Ir(III) organometallopeptides

Once the linear and cyclic peptide ligands were synthesized, the peptide ligand was suspended in 10 mL H_2O/MeCN (1:1) in the dark for 15 min. $[Ir(ppy)_2Cl]_2$ was added in the selected ratio (2.5 eq/number of Bpy moieties in the peptide sequence) and the resulting mixture was stirred under argon for 4 hours at 80 ºC. The reaction mixture was directly purified by semi-preparative HPLC to give the desired products. $[Ir(ppy)_2Cl]_2$ was synthetized from $IrCl_3$ following a reported procedure.[2] We previously reported the synthesis of **Ir_2-R_8**.[3]

(Ir-R_3)$_2$, 62.5 mg obtained, 50.2% yield for a 0.05 mmol scale; **(Ir-R_3)$_3$,** 70.1 mg obtained, 55.3% yield for a 0.034 mmol scale; ***cyclo*-(Ir-R_3)$_2$**, 62.4 mg obtained, 50.4% yield for a 0.05 mmol scale; ***cyclo*-(Ir-R_3)$_3$**, 77.2 mg obtained, 41.6% yield for a 0.05 mmol scale.

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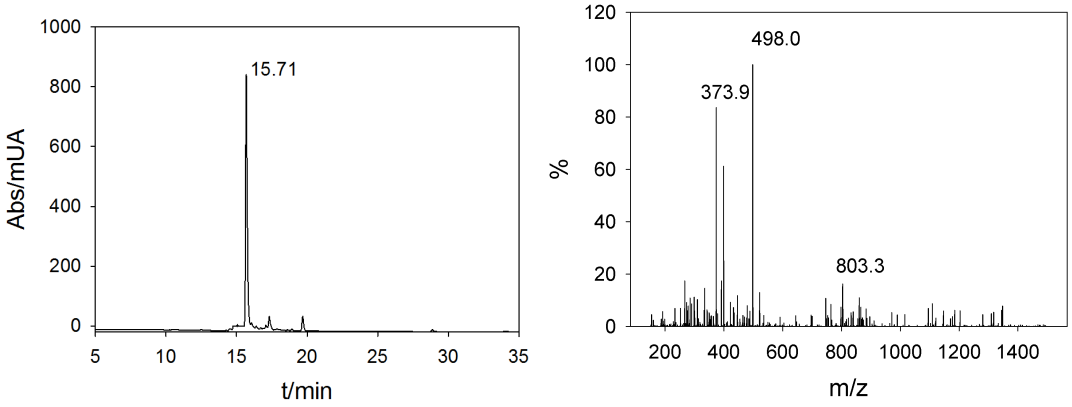
[2] S. Sprouse, K. A. King, P. J. Spellane, R. J. Watts, *J. Am. Chem. Soc.*, **1984**, *106*, 6647–6653

[3] I. Gamba, I. Salvadó, R. F. Brissos, P. Gamez, J. Brea, M. I. Loza, M. E. Vázquez, M. Vázquez López, *Chem. Commun.*, **2016**, *52*, 1234-1237

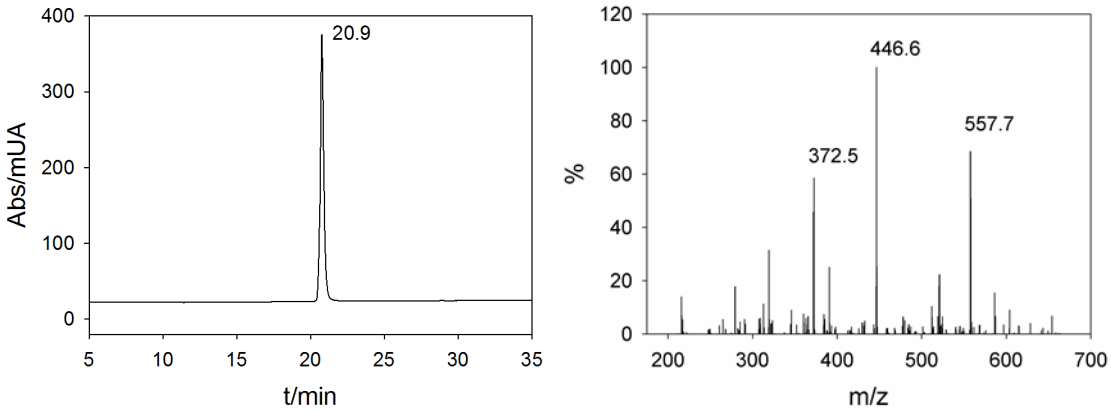
HPLC chromatograms and mass spectra of the peptides

(βAla-bpy–R_3)$_2$,



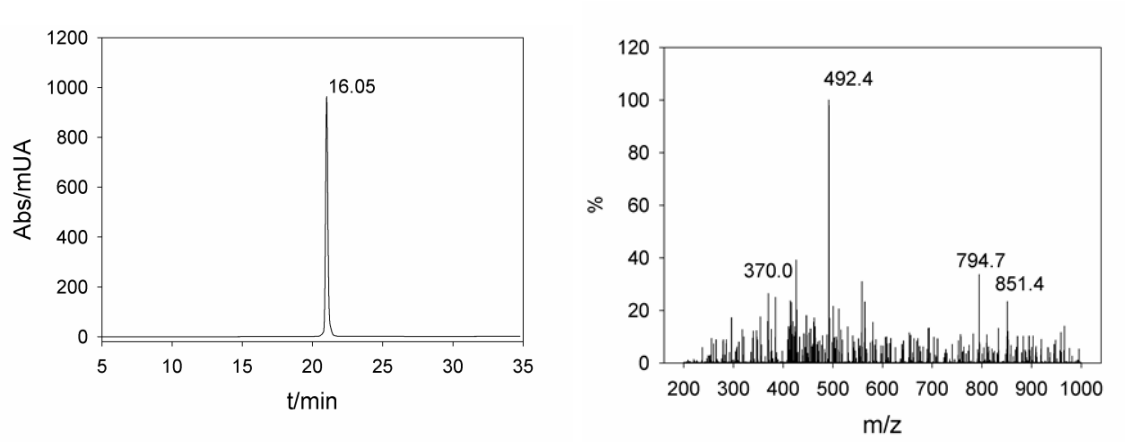
MS(ESI) (m/z): Calculated ($[M+4H]^{4+}$, $[C_{64}H_{102}N_{32}O_{11}]^{4+}$) = 373.7, found= 373.9; calculated ($[M+3H]^{3+}$, $[C_{64}H_{101}N_{32}O_{11}]^{3+}$)= 497.9, found= 498.0; calculated ($[M+TFAH+2H]^{2+}$, $[C_{66}F_3H_{101}N_{32}O_{13}]^{2+}$) = 803.4, found= 803.3.

(βAla-bpy–R_3)$_3$,



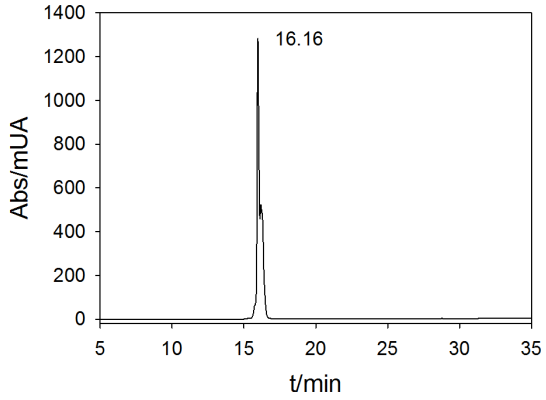
MS(ESI) (m/z): Calculated ($[M+6H]^{6+}$, $[C_{96}H_{152}N_{48}O_{16}]^{6+}$) = 372.2, found= 372.5; calculated ($[M+5H]^{5+}$, $[C_{96}H_{151}N_{48}O_{16}]^{5+}$)= 446.4, found= 446.6; calculated ($[M+4H]^{4+}$, $[C_{96}H_{150}N_{48}O_{16}]^{4+}$)= 557.8, found = 557.7.

***cyclo*-(Ir-R_3)$_2$**

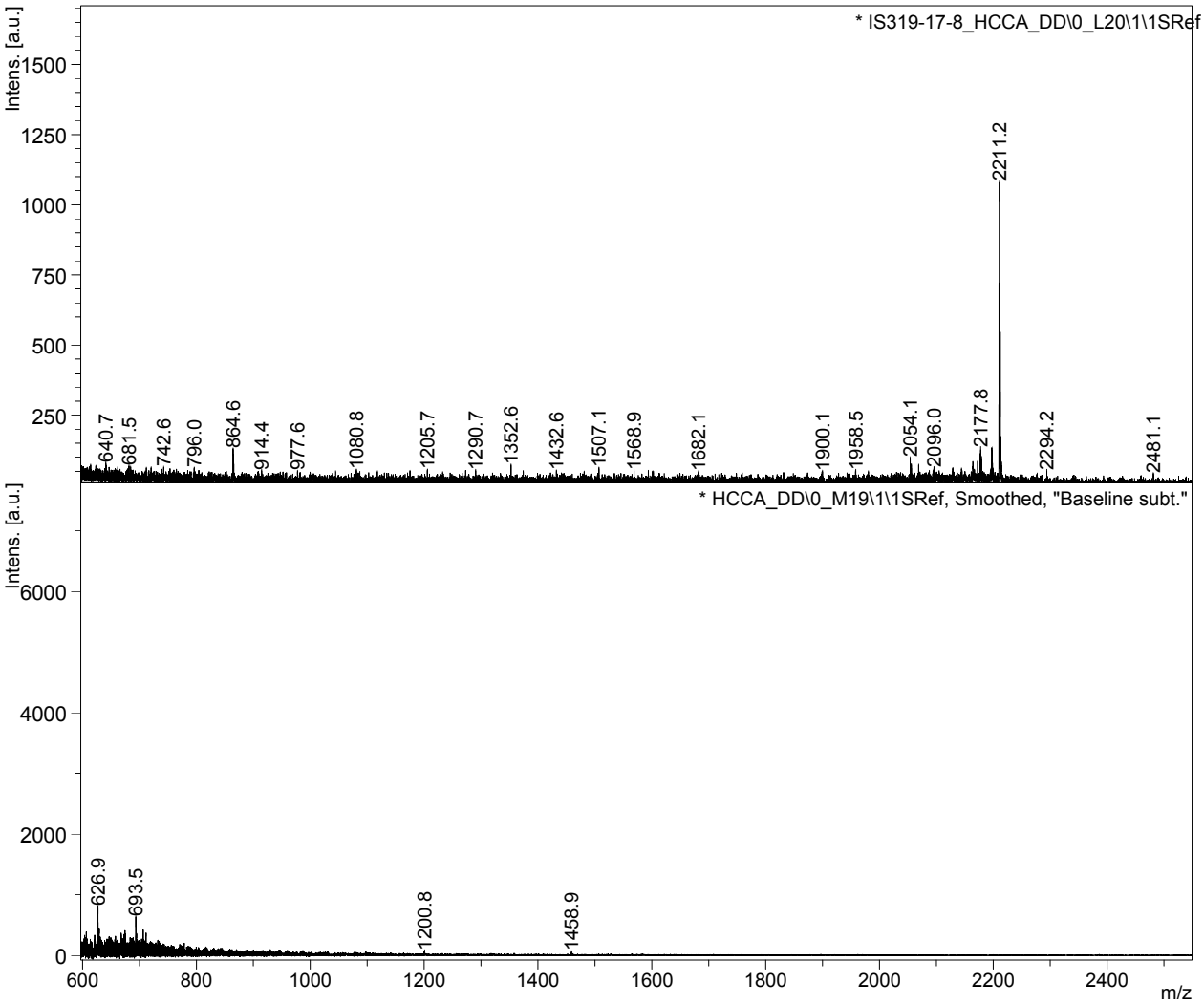


MS(ESI) (m/z): Calculated ($[M+4H]^{4+}$, $[C_{64}H_{100}N_{32}O_{10}]^{4+}$) = 369.2, found= 370.0; calculated ($[M+3H]^{3+}$, $[C_{64}H_{99}N_{32}O_{10}]^{3+}$)= 491.9, found= 492.4; calculated ($[M+TFAH+2H]^{2+}$, $[C_{66}F_3H_{99}N_{32}O_{12}]^{2+}$)= 794.4, found= 794.7; calculated ($[M+2TFAH+2H]^{2+}$, $[C_{68}F_6H_{100}N_{32}O_{14}]^{2+}$) = 851.4, found = 851.4.

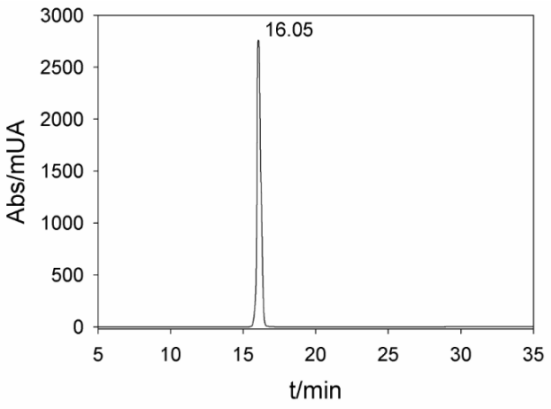
***cyclo*-(Ir-R_3)$_3$**



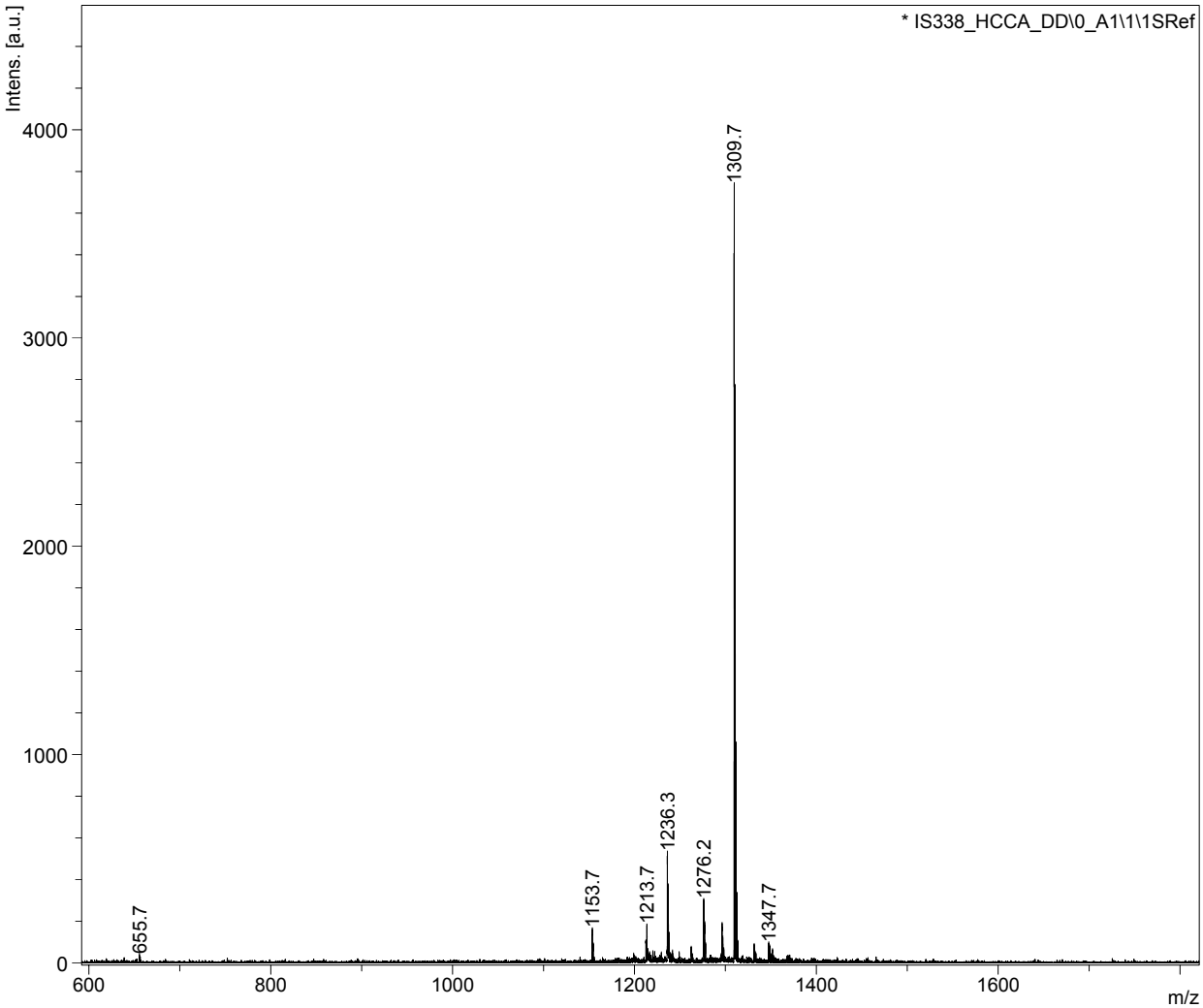
MS-(MALDI-TOF) (m/z): Calculated ([M], [$C_{96}H_{144}N_{48}O_{15}$]) = 2209.2, found = 2211.2.



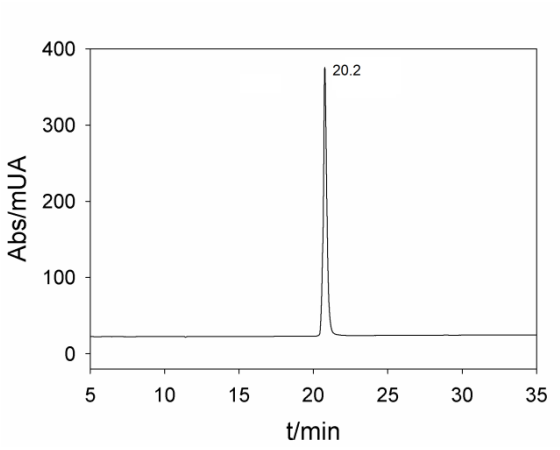
***cyclo*-(W-R_3)$_2$**



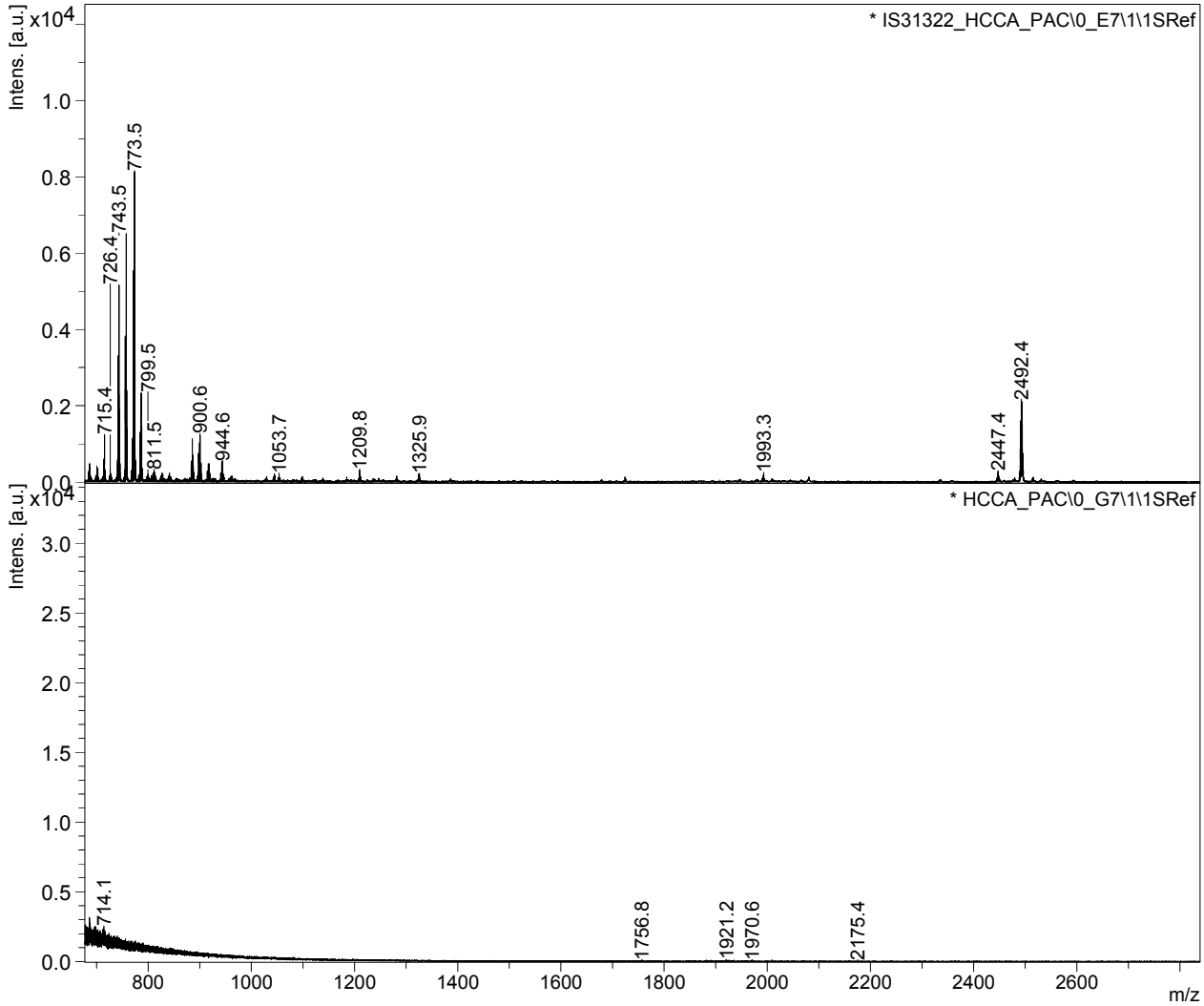
MS-(MALDI-TOF) (m/z): Calculated ([M], [$C_{58}H_{97}N_{28}O_8$]) = 1308.7, found = 1309.7.



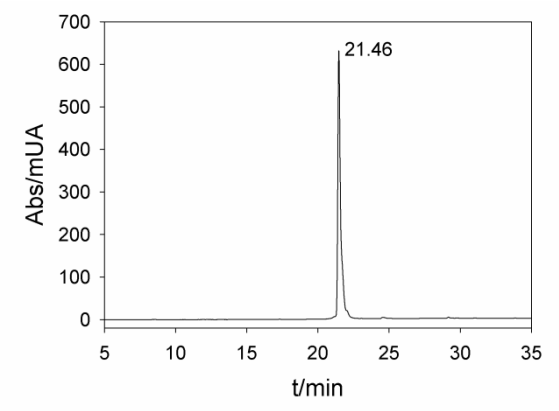
(Ir-R_3)$_2$:



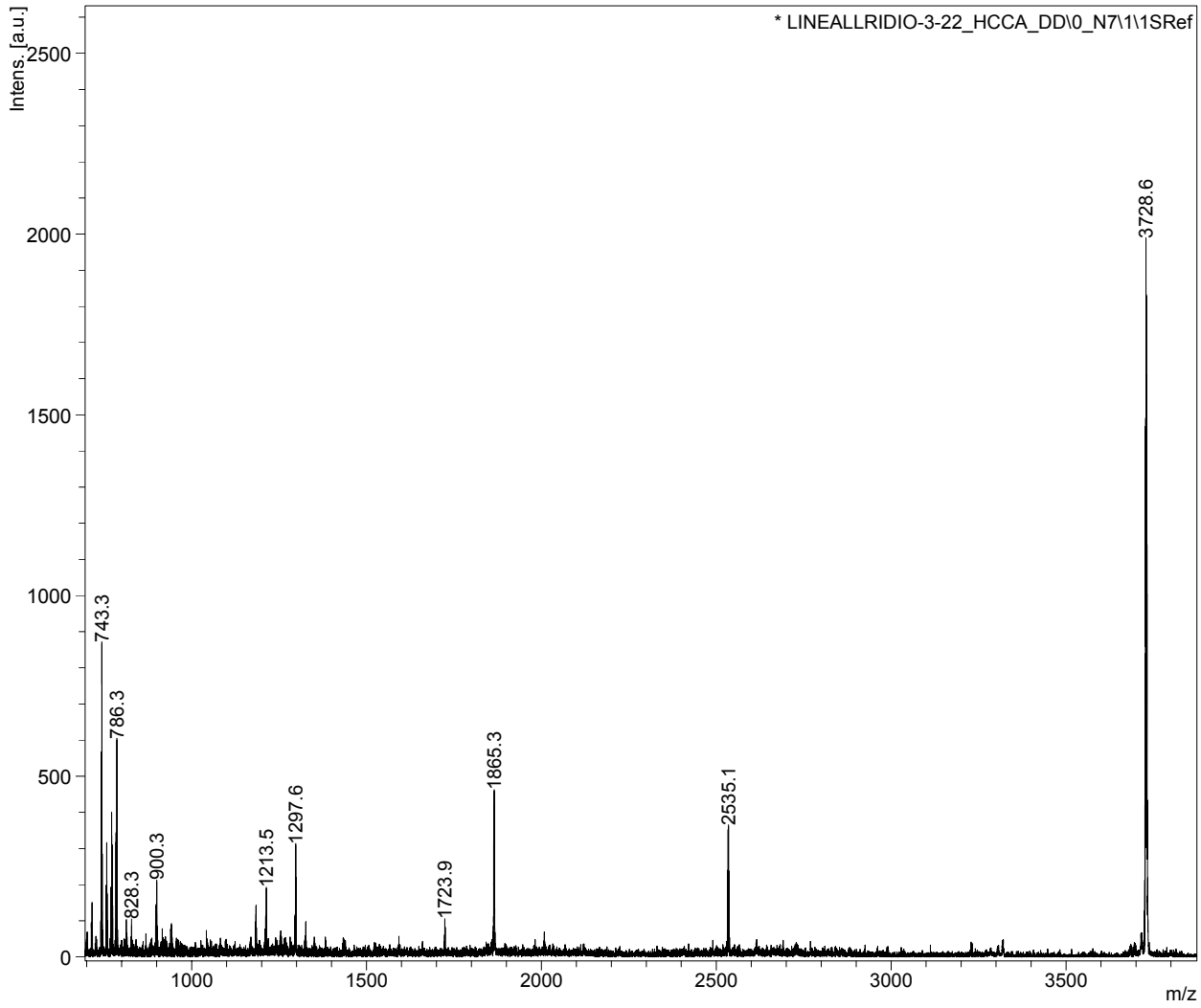
MS-(MALDI-TOF) (m/z): Calculated ([M]$^{2+}$, [$C_{108}H_{130}Ir_2N_{36}O_{11}$]$^{2+}$) = 2493.0, found = 2492.4.



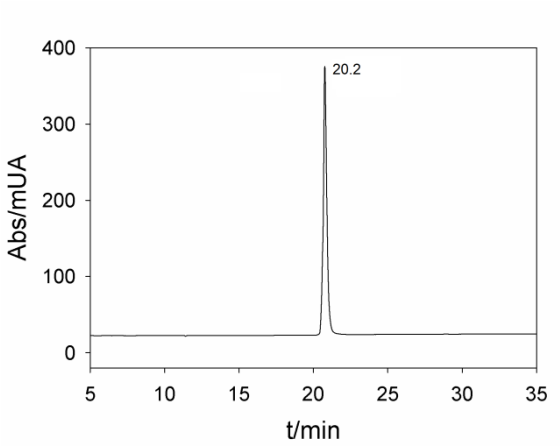
$(Ir\text{-}R_3)_3$



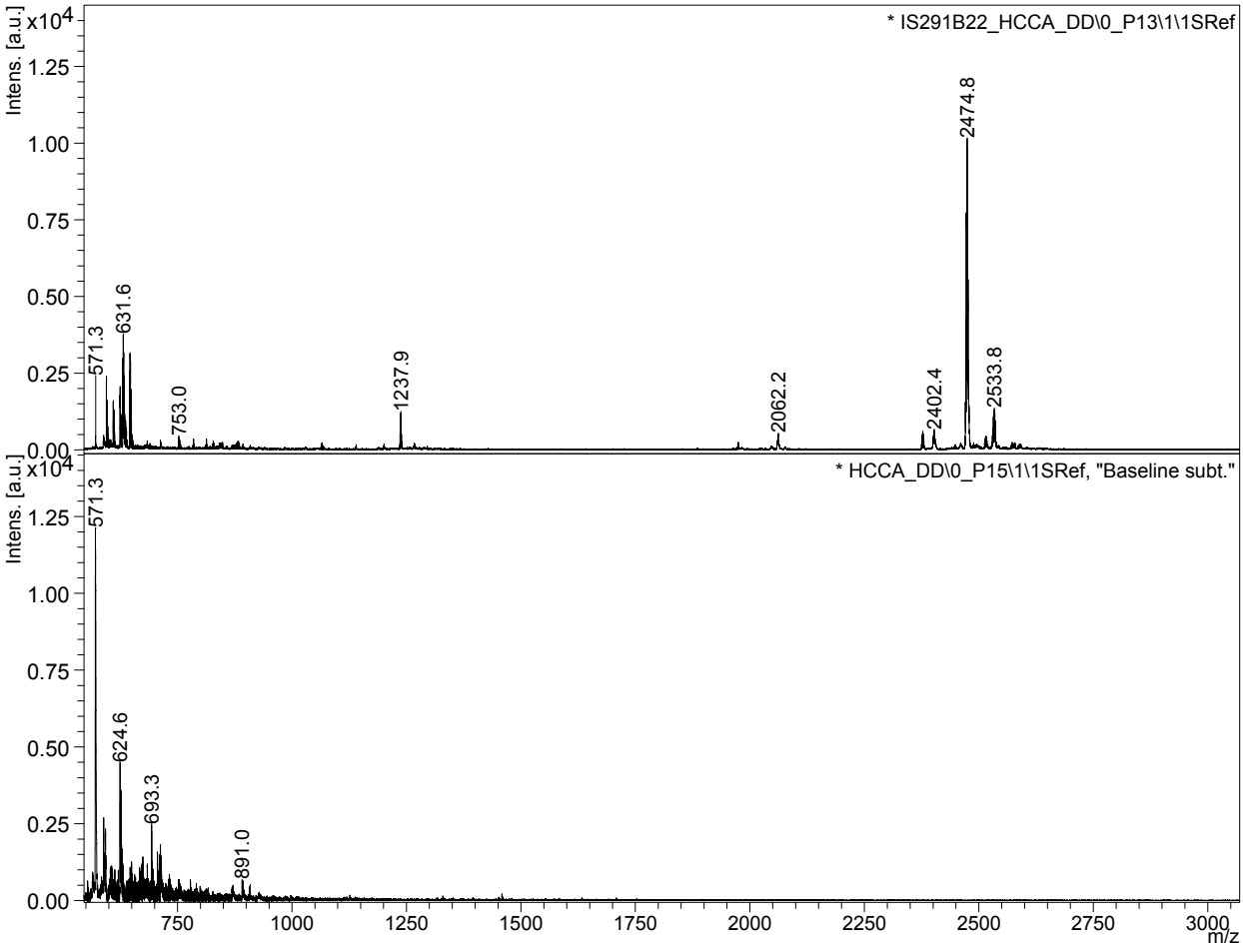
MS-(MALDI-TOF) (m/z): Calculated ($[M]^{3+}$, $[C_{162}H_{194}Ir_3N_{54}O_{16}]^{3+}$) = 3730.5, found = 3728.6.



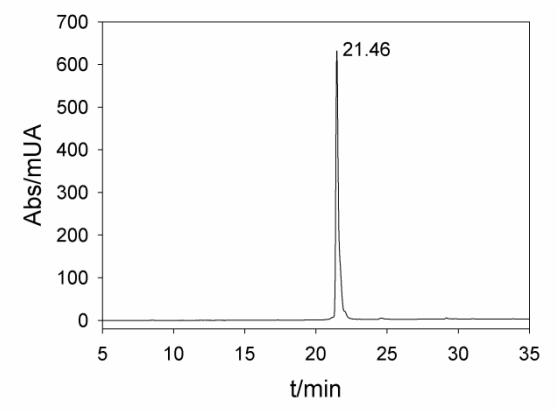
***cyclo*-(Ir-R_3)$_2$**



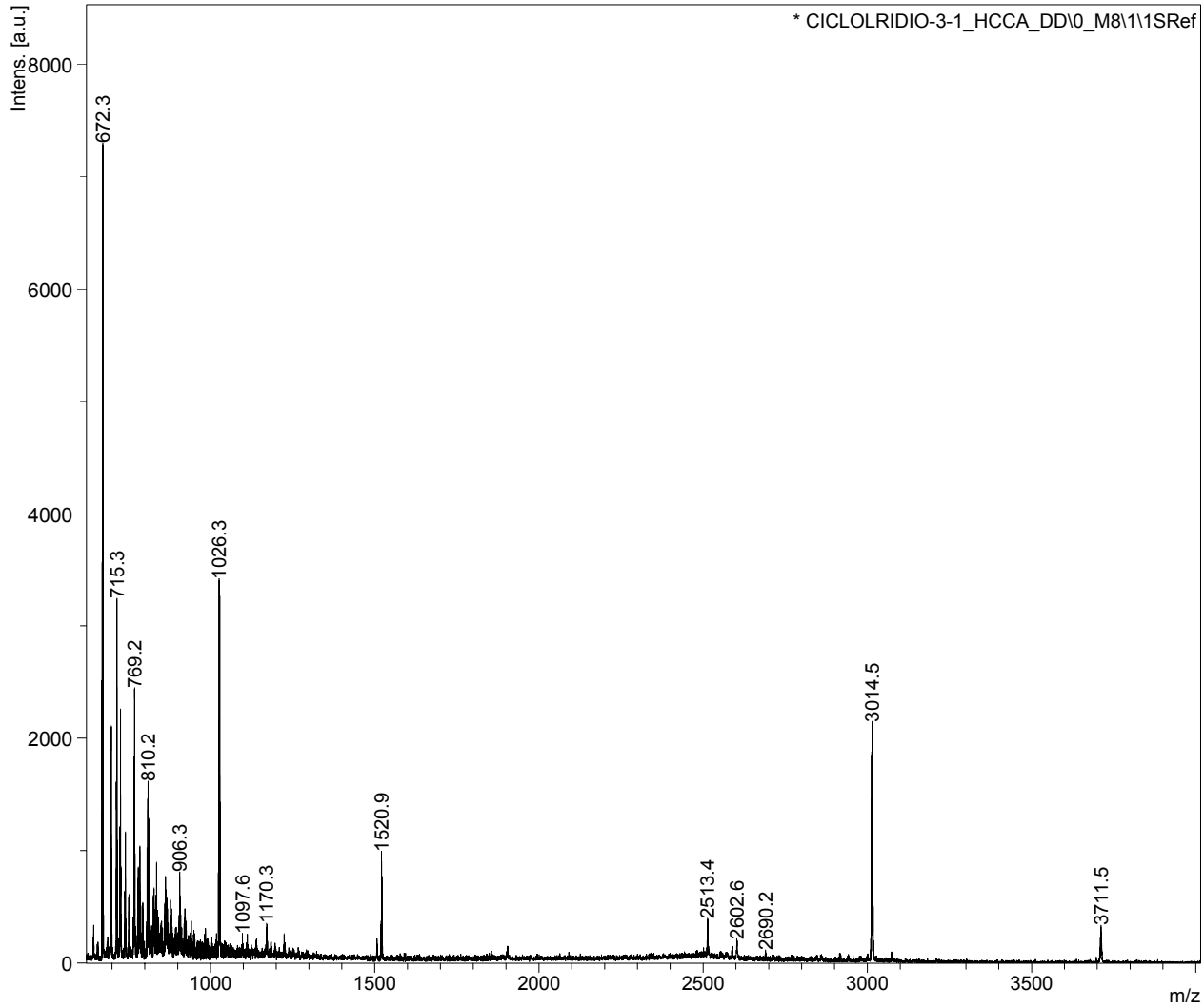
MS-(MALDI-TOF) (m/z): Calculated ($[M]^{2+}$, $[C_{108}H_{128}Ir_2N_{36}O_{10}]^{2+}$) = 2474.9, found = 2474.8.



***cyclo*-(Ir-R_3)$_3$**



MS-(MALDI-TOF) (m/z): Calculated ($[M]^{3+}$, $[C_{162}H_{192}Ir_3N_{54}O_{15}]^{3+}$) = 3712.5, found = 3712.2.



Cytotoxicity studies (MTT assay)

General method: Cell lines and grown conditions

MRC-5, NCI/ADR-RES and NCl-H460 cell lines, as well as Eagle's Minimal Essential Medium (EMEM) and Roswell Park Memorial Institute medium (RPMI) were purchased from the American Tissue Culture Collection (ATCC). Frozen cells were thawed in a 75 cm^2 cell culture flask in RPMI supplemented with 2-mM L-glutamine (NCI/ADR-RES) and 10% FBS and maintained at 37 ºC in a 5% CO_2 atmosphere.

Cytotoxicity assay

The inhibition of cell proliferation induced by the samples was carried out by using MTT method. Cells were seeded in a 96-well microplate (10000 and 15000 cells/well for MCR-5 and NCI-H460, NCI/ADR-RES respectively) in 100 µl of growth medium and maintained at 37 ºC during 24 hours. Then growth medium was replaced by fresh medium containing different concentrations of the samples to be assayed and maintained at 37 ºC in a 5% CO_2 atmosphere for different times (168 hours for MCR-5 and and 48 hours for NCI/ADR-RES and NCI-H460 cells). After this time, 10 µL MTT (5 mg/ml in PBS) were added to each well and maintained for 4 hours at 37 ºC in a 5% CO_2 atmosphere. The 100 µL of 10% SDS in 0.01 M HCl were added to each well an incubated for 12-14 hours under the same experimental conditions. Absorbance due to formation was detected in a Tecan Ultra Evolution reader using a wavelength of 595 nm. All assays were performed with triplicate points.

Data analysis

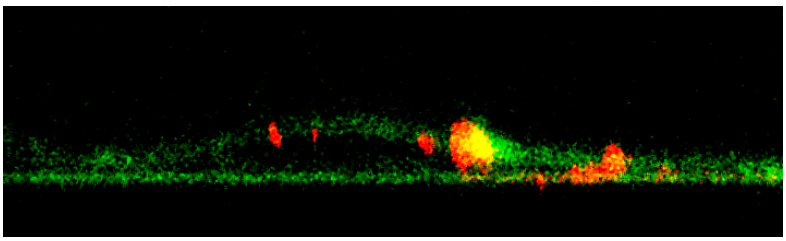
Data were expressed as the growth inhibition percentage calculated in basis on the equation: % inhibition= 100–[(AO × 100)/AT]. Where AT is the measured absorbance in wells containing compounds and AO is the absorbance measuredin blank wells (cells with medium and vehicle). The inhibitory potency of compounds was calculated by constructing concentration-% growth inhibition curves, and extrapolating IC50 values (concentration of compound that inhibits cells growth in a 50 %) from these curves.Curves were constructed by using *GraphPad Prism* software V2.01 (*GraphPad Inc*).

Fluorescence microscopy

Vero cells were maintained in monolayers in Dulbecco's Modified Eagle's medium (D-MEM, Invitrogen) containing 10% of Fetal Bovine Serum (FBS, Invitrogen). For the uptake experiments, cells grown glass coverslips (15 mm) were washed 3 times with PBS and overlaid with 1 mL of fresh DMEM with no added serum, and containing the probes at 20 µM. After 30 min incubation at 37 ºC, the medium was removed and the cells washed with DMEM (3 × 1 mL) and directly observed in DMEM without fixation. Images were obtained with an Olympus DP-71 digital camera mounted on an Olympus BX51 fluorescence microscope. Images were further processed (cropping, resizing and contrast global contrast and brightness adjustment) with Adobe Photoshop (Adobe Systems).

Confocal microscopy

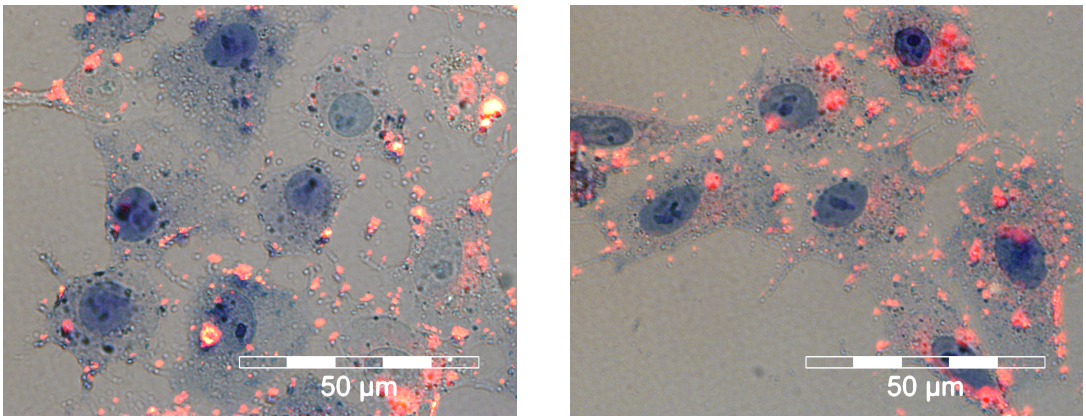
Monolayers of Vero cells were washed three times with DMEM in the absence of serum. Then, they were overlaid with DMEM containing 20 µM ***cyclo*-(Ir-R_3)$_2$** and 100nM of Concanavalin A from *Canavalia ensiformis* (Jack bean) FITC conjugate, Type IV (C7642, Sigma-Aldrich), and incubated for 30 minutes at 37 ºC. After incubation, the cells were washed again three times with DMEM and analyzed alive with a Leica AOBS-SP5X confocal microscopy equipped with an incubation chamber. Laser excitation and detection parameters were tuned to fit the maximum values for FITC and ***cyclo*-(Ir-R_3)$_2$** and are shown in green and red respectively.



Confocal XZ-section of Fluorescein-conjugated concanavalin A-stained Vero cell membrane (green), costained with ***cyclo*-(Ir-R3)$_2$** (red) showing the peptide colocalizing with the membrane marker.

Trypan Blue exclusion assay

Vero cells were prepared and incubated with 20 µM ***cyclo*-(Ir-R_3)$_2$** for 1h at 37 ºC as described before. Cells were then washed 3 times with PBS and incubated with trypan blue (1:1 mixture of trypan blue stock solution 0.25% in PBS and DMEM with no added serum) and then monitored under the microscope.



Trypan blue exclusion assay: Left: Vero cells incubated with 20 µM ***cyclo*-(Ir-R_3)$_2$** for 1h at 37 ºC observed right after incubation with trypan blue (note cells on the bottom right of the image that are not yet stained). Right, cells after 1 h incubation with trypan blue. In both cases the brightfield image is overlaid with the fluorescence emission of the metallopeptide forming the aggregates in the membrane (red).

Vesicle CF Leakage Experiments

Vesicles were prepared with a Mini-Extruder from Avanti Polar Lipids (pore size 100 nm). Fluorescence measurements were performed with a FluoroMax-2 spectrofluorometer (Jobin-Yvon) equipped with a stirrer and a temperature controller.

A thin lipid film was prepared by evaporating a solution of 25 mg EYPC in 1 ml MeOH/$CHCl_3$ (1:1) on a rotary evaporator (rt) and then *in vacuo* overnight. The resulting film was hydrated with 1.0 ml buffer (50 mM CF, 10 mM Tris, 10 mM NaCl, pH 7.4) for more than 30 min, subjected to freeze-thaw cycles (5×) and extrusions (15×) through a polycarbonate membrane (pore size, 100 nm). Extravesicular components were removed by gel filtration (Sephadex G-50) with 10 mM Tris, 107 mM NaCl, pH 7.4. Final conditions: ~5 mM EYPC; inside: 50 mM CF, 10 mM Tris, 10 mM NaCl, pH 7.4; outside: 10 mM Tris, 107 mM NaCl, pH 7.4.

EYPC-LUV stock solutions (25 µl) were diluted with a buffer (10 mM Tris, 107 mM NaCl, pH 7.4) and placed in a thermostated fluorescence cuvette (25 ˚C) and gently stirred (total volume in the cuvette, ~2000 µl; final lipid concentration, ~62.5 µM). CF efflux was monitored at λ_{em} 517 nm (λ_{ex} 492 nm) as a function of time after addition of the Iridium compound (**Irx**, 20 µl of the corresponding stock in buffer) at time t = 25 s and 1.2% aqueous triton X-100 (40 µl, 0.024% final concentration) at t = 225 s. Fluorescence intensities were normalized to fractional emission intensity I(t) using equation (S1).

$$I(t) = \frac{(I_t - I_0)}{(I_\infty - I_0)} \qquad \text{(S1)}$$

where $I_0 = I_t$ at pR addition, $I_\infty = I_t$ at saturation after lysis. Effective concentration for hydrazone EC_{50} and Hill coefficient n were determined by plotting the fractional activity Y (= I(t) at saturation just before lysis, t = ~190 s) as a function of the Iridium compound concentration $\boldsymbol{c_{Irx}}$ and fitting them to the Hill equation (S2).

$$Y = \frac{Y_0 + (Y_{max} - Y_0)}{1 + \left(\frac{EC_{50}}{C_{Irx}}\right)^n} \qquad \text{(S2)}$$

where Y_0 is Y without Iridium compound, Y_{MAX} is Y with an excess of Iridium compound at saturation, EC_{50} is the concentration of Iridium compound required to reach 50% of activity and n is the Hill coefficient.

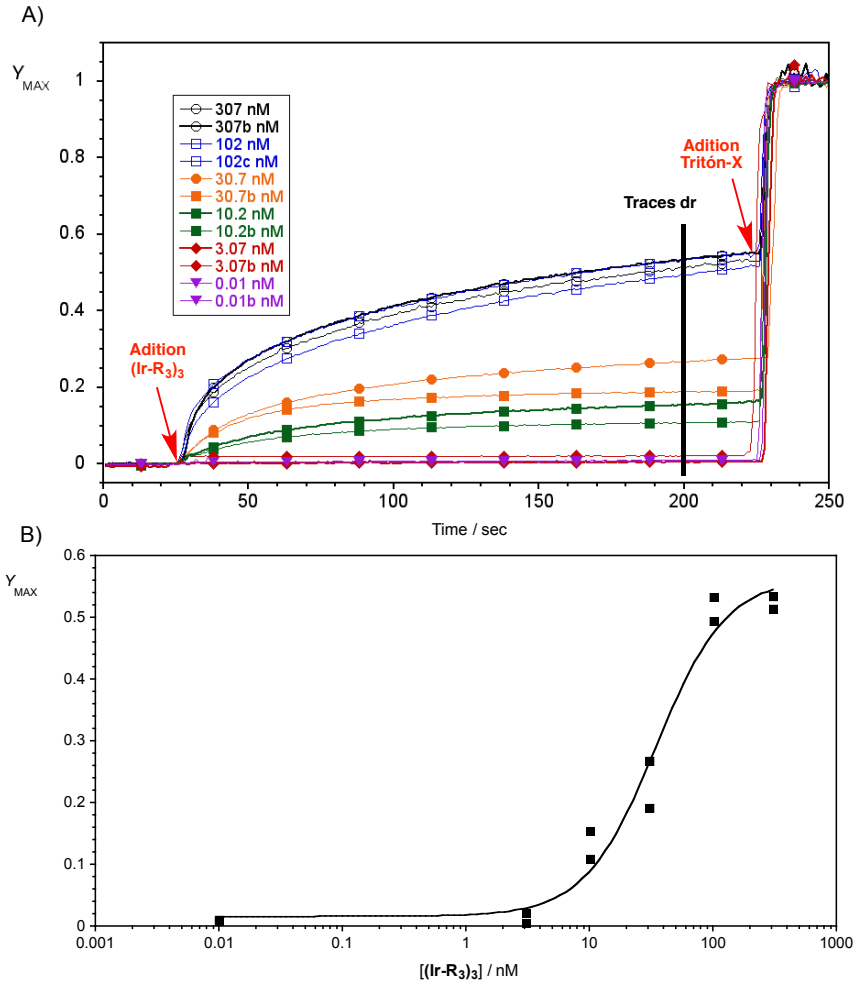


Figure for the Vesicle Transport Experiments. A) Kinetic traces of the CF fluorescence emission in LUVs with entrapped CF (50 mM) after the addition of the Iridium compound **(Ir-R$_3$)$_3$** at t = 25 s at different concentrations (307-0.01 nM) and 1.2% aqueous triton X-100 at t = 225 s. B) Representative dose-response curve, obtained by plotting the fractional activity Y (at time t = 200 s) as a function of **(Ir-R$_3$)$_3$** concentration and fitting the data to the Hill equation (S2).