A novel fully water-soluble Cu(I) probe for detection in live cell imaging

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Electronic Supporting Information

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General experimental methods. The NMR experiments were carried out at 27° C on a Varian UNITY Inova 500 MHz spectrometer (^{1}H at 499.88 MHz, ^{13}C-NMR at 125.7 MHz) equipped with pulse field gradient module (Z axis) and a tuneable 5 mm Varian inverse detection probe (ID-PFG). ESI mass spectra were acquired on a ES-MS Thermo-Finnigan LCQ-DECA using MeOH (positive ion mode). A JASCO V-560 UV-Vis spectrophotometer equipped with a 1 cm path-length cell was used for the UV-Vis measurements. Luminescence measurements were carried out using a Cary Eclipse Fluorescence spectrophotometer with a λ_{ex} of 515 and 542 nm and a 0.5 nm resolution, at room temperature. The emission was recorded at 90° with respect to the exciting line beam using 5:5 slit-widths for all measurements. All chemicals, including $[Cu(CH_3CN)_4]PF_6$, were reagent grade and were used without further purification. Millipore water was used to prepare all aqueous solutions. All spectroscopic measurements in solution were performed in 10 mM buffer (HEPES, MOPS or PBS), pH 7.2. Tetra-tia-azaundecene was synthesized according to the literature protocol.[1]

Procedure for fluorescence titrations. Two mother solutions of **OBEP-CS1** (receptor) and Cu^+ (guest) (7.0 x 10^{-3} M) in 10 mM of buffer, pH 7.2, were prepared. From these, different solutions with different ratio receptor/guest were prepared as reported below, and emission spectra, normalized to eliminate dilution effect, were recorded. Fluorescence titration was carried out using λ_{exc} 515 nm and recording at λ_{em} 575 nm 25°C. The stability constants for the aqueous Cu^+–CH_3CN system have been accurately determined[2] and can be used in the nonlinear least-squares data analysis to account for competitive binding by CH_3CN.[3] With this data treatment, the apparent binding affinity of **OBEP-CS1** was estimated using HypSpec (version 1.1.33),[4] a software designed to extract equilibrium constants from potentiometric and/or spectrophotometric titration data. HypSpec starts with an assumed complex formation scheme and uses a least-squares approach to derive the spectra of the complexes and the stability constants. χ^2 test (chi-square) was applied, where the residuals follow a normal distribution (for a distribution approximately normal, the χ^2 test value is around 12 or less). In all of the cases, $\chi^2 \leq 10$ were found, as obtained by 3 independent measurements sets. The $\Phi_F$value **of OBEP−CS1** change from 0.15 to 0.11 by addition of 1 equivalent of Cu^+. The fluorescence quantum yields of **OBEP-CS1** was determined according to the literature:

$$\Phi_x=\Phi_s(F_x/F_s)(A_s/A_x)(\lambda_{exs}/\lambda_{exx})(n_x/n_s)^5$$

where Φ is quantum yield; F is the integrated area under the corrected emission spectrum; A is the absorbance at the excitation wavelength; λ_{ex} is the excitation wavelength; n is the refractive index of the solution; the subscripts x and s refer to the unknown and the standard, respectively. Fluorescein (Φ_F= 0.90) in 0.1 mol/L NaOH was used as the standard.

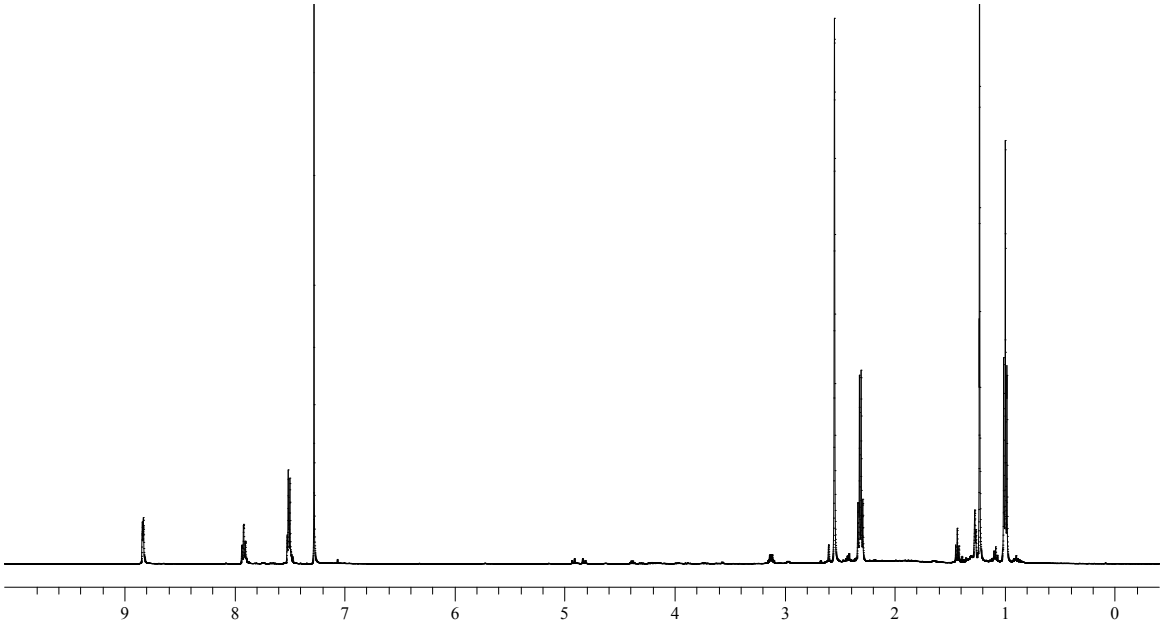
Determination of Stoichiometry. Stoichiometry of the complex was investigated by the Job's plot method using spectrophotometric measurements. The samples were prepared by mixing equimolecular stock solutions (2 x 10^{-4} M) of **OBEP-CS1** and Cu^+ to cover the whole range of molar fractions keeping constant the total concentration (1×10^{-5} M). The changes in emission intensity at 575 nm (λ_{exc} 515 nm), compared to uncomplexed receptor species ($\Delta I \times \chi^{-1}$) were calculated and reported versus the receptor mole fraction (χ). These plot show invariably a maximum at 0.5 mol fraction of receptor indicating its 1:1 complex formation with the Cu^+ ion.

Determination of Selectivity. The solutions of the metal ions were prepared from $NiCl_2 \cdot 2H_2O$, $CaCl_2$, $FeCl_2 \cdot 4H_2O$, $MgCl_2$, $CuSO_4$, $Co(OAc)_2 \cdot 4H_2O$, KPF_6, $ZnCl_2 \cdot 2H_2O$, NaCl and $[Cu(CH_3CN)_4]PF_6$, respectively.

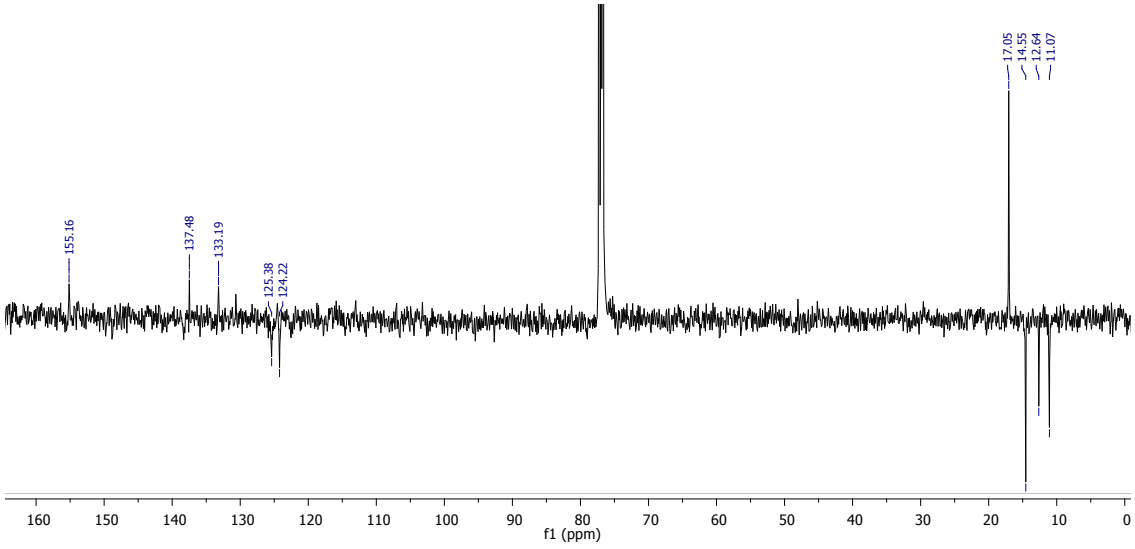
DOSY experiments. Diffusion-Ordered SpectroscopY (DOSY) NMR has been particularly used in host–guest chemistry to rule out the possibility of 2:2 complexes in the form of a molecular capsule, or other higher order species. In particular, in D_2O at 298 K we obtained a diffusion coefficient of 3.51×10^{-10} m^2/s for **OBEP-CS1** (1 mM). The DOSY technique provides information about the size of the molecular aggregate in solution. In fact, by means of the Stokes–Einstein equation, the diffusion coefficient of the compound can be converted into its hydrodynamic radius R_h and this value can be compared with the calculated radius obtained by Hyperchem-minimized structure of the complex. Thus, combining the diffusion coefficient of the complex of **OBEP-CS1** with the viscosity of D_2O at 298 K in the Stokes–Einstein equation ($R = k_BT/2\pi\eta D$; where k_B is the Boltzmann constant, T is the absolute temperature, and η is the viscosity of D_2O at 298 K, a hydrodynamic radius $R_h(exp)$ = 6.3 Å was obtained. This value is in good agreement with the hydrolytic radius $R_h(calcd)$ = 8.5 Å calculated from the Hyperchem minimized structure in the maximal extension. Furthermore, diffusion coefficient value can be associated to the molecular weight, by the mathematic treatment recently described.[6] The diffusion coefficient value founded (3.51×10^{-10} m^2/s) is correlated in water solution with a theoretical molecular weight of 548, which preclude the aggregates formation.

Synthesis of 1

To a solution of 2,4-dimethyl-3-ethylpyrrole (1.19 mL, 8.8 mmol, 2 eq) in 250 mL of dry dichloromethane, 0.42 mL of 2-pyridinecarboxaldehyde (4.4 mmol, 1 eq) was added, followed by a catalytic amount of trifluoroacetic acid. After overnight stirringunder a nitrogen atmosphere, the mixture was concentrated to 50.0 mL under vacuum, and 1.50 g of 2,3-dichloro-5,6-dicyanobenzoquinone (1.5 eq) was added and stirred for 2 h. Triethylamine (6.0 mL) and $BF_3.OEt_2$ (7.0mL) were then added. After 2h the mixture was concentrated under vacuum and washed with CH_2Cl_2 and water. The organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The mixture was purified by silica flash column chromatography ($CHCl_3$) to yield **1** (0.40 g, 24% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.82 (d, *J* = 4.5 Hz, 1H, ArH *orto* to N), 7.90 (d of t, *J* = 1.5, 7.5 Hz, 1H, ArH *para* to N), 7.49 (m, 2H, ArH *meta* to N), 2.53 (s, 6H, -CH_3 near pyridine), 2.30 (q, *J* = 7.5 Hz, 4H, -$\underline{CH_2}$-CH_3), 1.21 (s, 6H, -CH_3 near BF_2), 0.98 (t, *J* = 7.0 Hz, 6H, -CH_2-$\underline{CH_3}$). ^{13}C NMR (125.7 MHz, $CDCl_3$): δ 155.16, 137.47, 133.18, 125.38, 124.22, 17.05, 14.55, 12.64, 11.07.ESI-MS: *m/z* 382.2 $[M+H]^+$. Anal. Calcd. For $C_{22}H_{26}BF_2N_3$: C, 69.30; H, 6.87; B, 2.84; F, 9.97; N, 11.02. Found: C, 69.15; H, 6.77; B, 2.81; F, 9.91; N, 10.92.



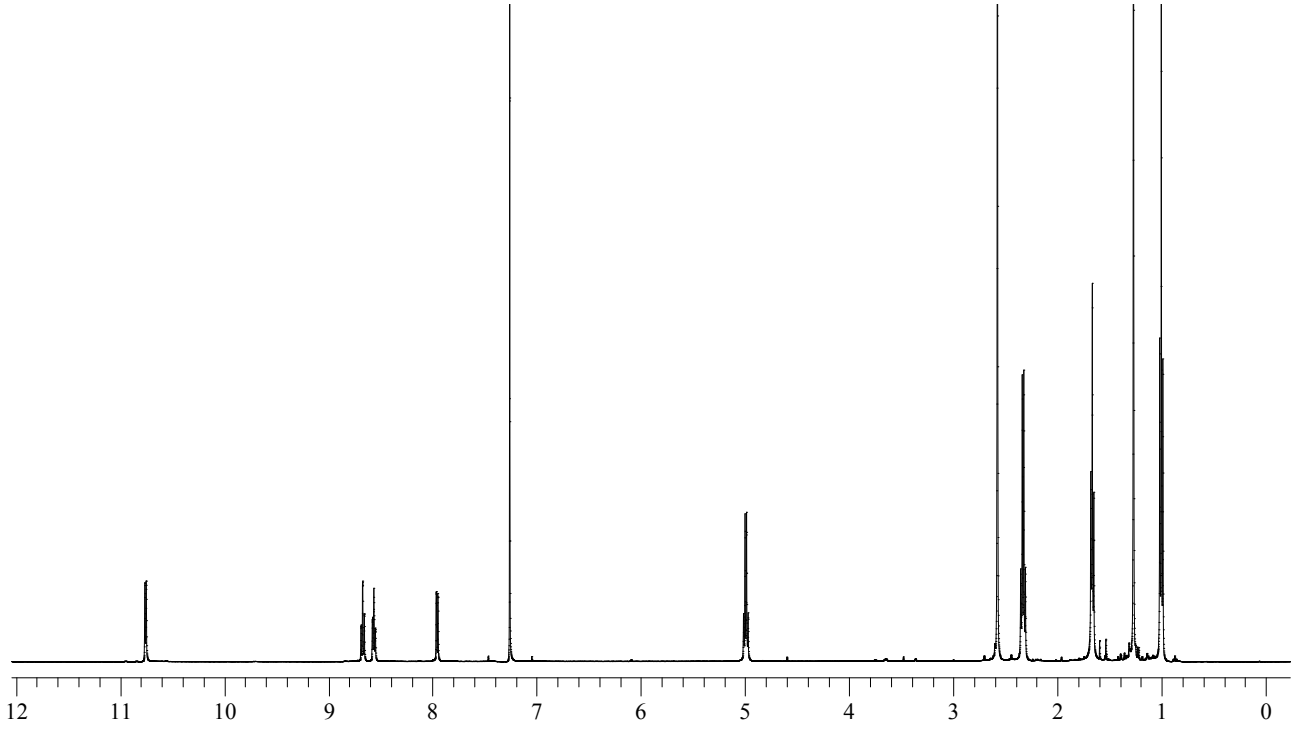
^{1}H NMR spectrum of **1** in $CDCl_3$



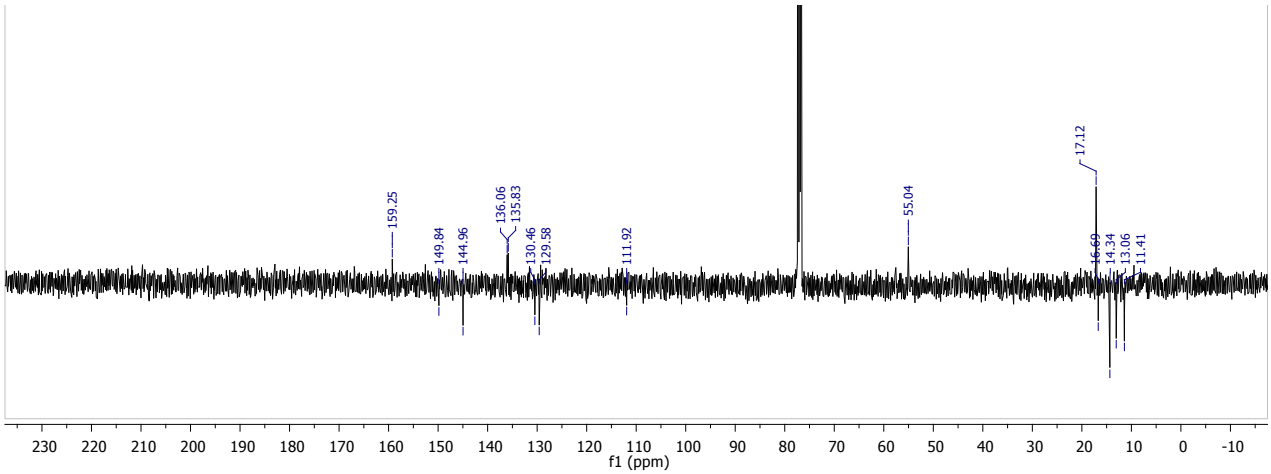
APT spectrum of **1** in $CDCl_3$

Synthesis of 2

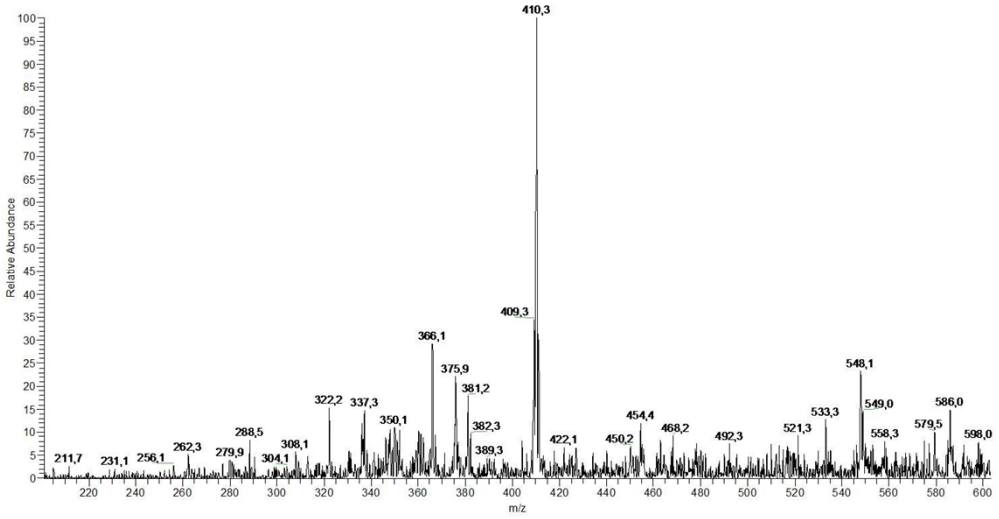
To a solution of **1** (0.40 g, 1.5 mmol) in dry acetonitrile, iodoethane (3.9 g, 20 mmol) was added under nitrogen atmosphere. The mixture was then stirred at 50 ºC for 2 days. The mixture was cooled to room temperature and concentrated under vacuum. The residue was purified by neutral aluminum oxide flash column chromatography (CH_2Cl_2 / CH_3OH from100/0 to 95/5) recovering starting compound **1** (conv. 20%) and obtaining 98.1 mg of **2** (61% yield).^{1}H NMR (500 MHz, $CDCl_3$): δ 10.76 (d, *J* = 6.5 Hz, 1H, ArH *orto* to N^+-Et), 8.67 (d of t, *J* = 1.5, 7.5 Hz, 1H, ArH *para* to N^+-Et), 8.56 (t of d, *J* = 1.5, 7.5 Hz, 1H, ArH *meta* to N^+-Et), 7.96 (dd, *J* = 1.5, 7.5 Hz, 1H, ArH *meta* to N^+-Et), 4.99 (q, *J* = 7.5 Hz, 2H, N^+-$\underline{CH_2}$-CH_3), 2.58 (s, 6H, -CH_3 near pyridine), 2.33 (q, *J* = 7.5 Hz, 4H, -$\underline{CH_2}$-CH_3), 1.67 (t, *J* = 7.5 Hz, 3H, N^+-CH_2-$\underline{CH_3}$), 1.27 (s, 6H, -CH_3 near BF_2), 1.05 (t, *J* = 7.5 Hz, 6H, -CH_2-$\underline{CH_3}$). ^{13}C-NMR (125.7 MHz, $CDCl_3$): δ 144.96, 136.06, 135.83, 130.46, 129.58, 57.60, 55.04, 17.12, 16.69, 14.35, 13.05, 11.41. ESI-MS: *m/z* 410.3 $[M]^+$. Anal. Calcd. For $C_{24}H_{31}BF_2IN_3$: C, 53.66; H, 5.82; B, 2.01; F, 7.07; I, 23.62; N, 7.82. Found: C, 53.59; H, 5.78; B, 2.00; F, 7.01; I, 23.59; N, 7.78.



^{1}H NMR spectrum of **2** in $CDCl_3$



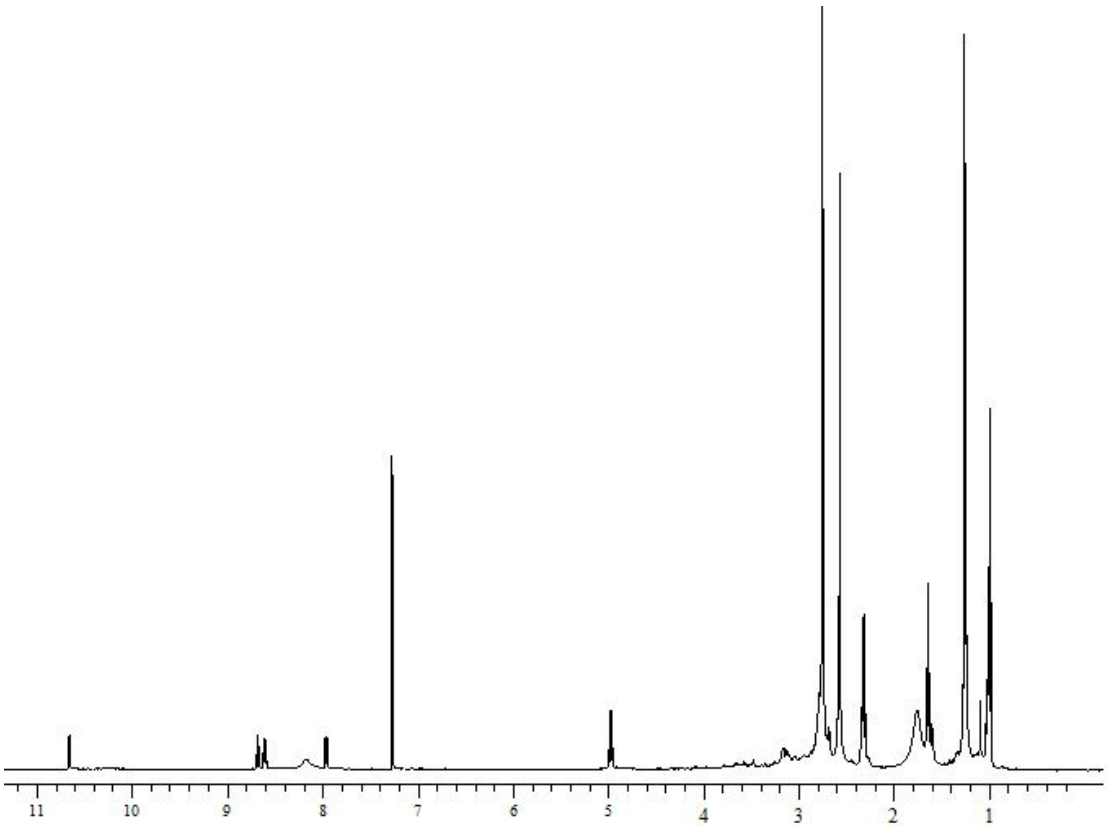
APT spectrum of **2** in $CDCl_3$



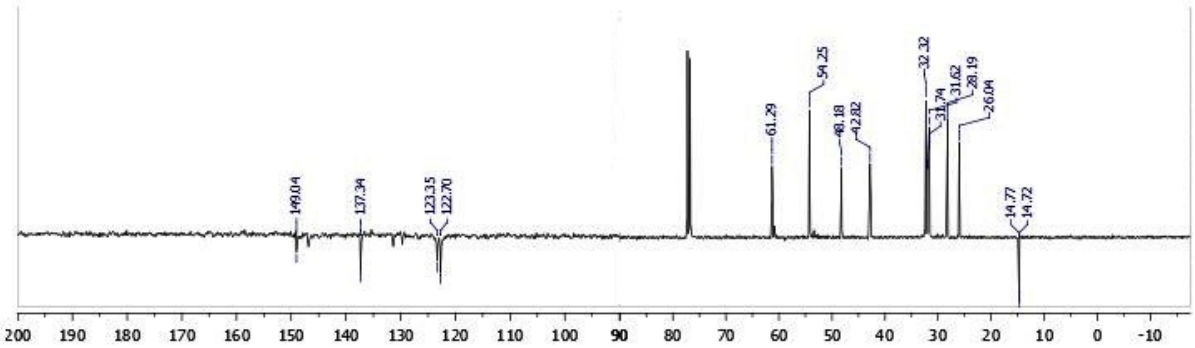
ESI-MS spectrum of **2**

Synthesis of OBEP-CS1

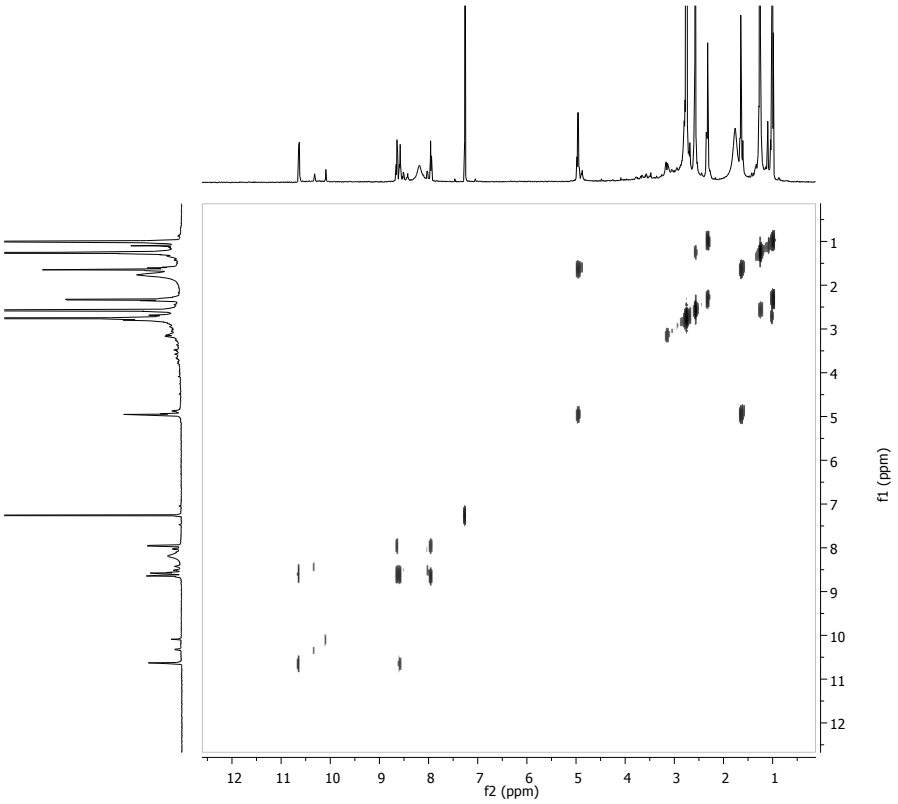
To a solution of **2** (30 mg, 0.06 mmol) in dry dichloromethane (5 mL), 10 mg (0.057 mmol) of *N*-bromosuccinimide were added under nitrogen atmosphere. The mixture was stirred at room temperature for 1h, then150 mg (0.48 mmol) of tetra-tia-azaundecene were added. Reaction was stirred overnight at room temperature and solvent was removed under reduced pressure. **OBEP-CS1** was isolated by neutral aluminum oxide column chromatography (CH_2Cl_2 / CH_3OH from 100/0 to 95/5) obtaining 30.5 mg of probe (yield 60%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 10.63 (d, *J* = 6.5 Hz, 1H, ArH *orto* to N^+-Et), 8.64 (t, *J* = 8.0 Hz, 1H, ArH *para* to N^+-Et), 8.59 (t, *J* = 6.5 Hz, 1H, ArH *meta* to N^+-Et), 7.95 (d, *J* = 8 Hz, 1H, ArH *meta* to N^+-Et), 4.97 (q, *J* = 7.5 Hz, 2H, N^+-$\underline{CH_2}$-CH_3), 2.72-2.81 (m, 18H, Bodipy-$\underline{CH_2}$-N + N-$\underline{CH_2CH_2}$-S), 2.58 (m, 10H, -CH_3 near pyridine + S-$\underline{CH_2}$-CH_3), 2.33 (q, *J* = 8 Hz, 4H, Bodipy-$\underline{CH_2}CH_3$), 1.65 (t, *J* = 7.5 Hz, 3H, N^+-CH_2-$\underline{CH_3}$), 1.26 (m, 9H, -CH_3 near BF_2 + S-CH_2-$\underline{CH_3}$), 1.00 (t, *J* = 8 Hz, 6H, Bodipy-$CH_2\underline{CH_3}$). ^{13}C-NMR (125.7 MHz, $CDCl_3$): δ 149.04, 137.34, 123.35, 122.70, 61.29, 54.25, 48.18, 42.82, 32.32, 32.18, 32.06, 31.74, 31.62, 28.19, 26.04, 14.77, 14.72.ESI-MS: *m/z* 721.3 $[M]^+$. Anal. Calcd. For $C_{36}H_{56}BF_2IN_4S_4$: C, 50.94; H, 6.65; B, 1.27; F, 4.48; I, 14.95; N, 6.60; S, 15.11. Found: C, 50.90; H, 6.61; B, 1.25; F, 4.45; I, 14.92; N, 6.54; S, 15.08.



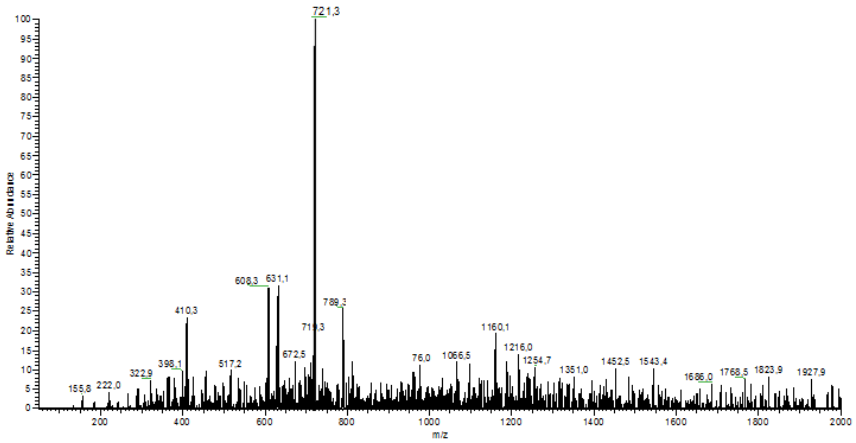
^{1}H NMR spectrum of **OBEP-CS1** in $CDCl_3$



APT spectrum of **OBEP-CS1** in $CDCl_3$

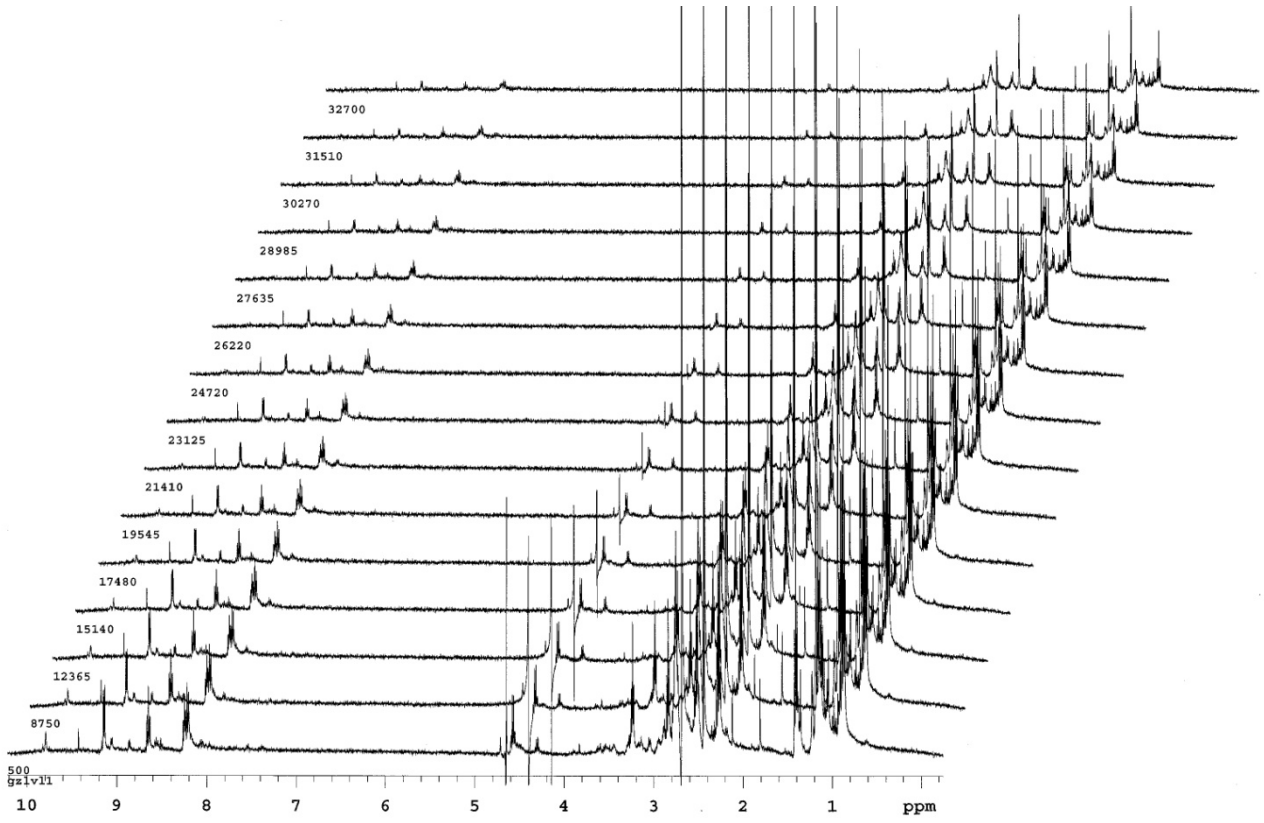


gCOSY spectrum of **OBEP-CS1** in $CDCl_3$



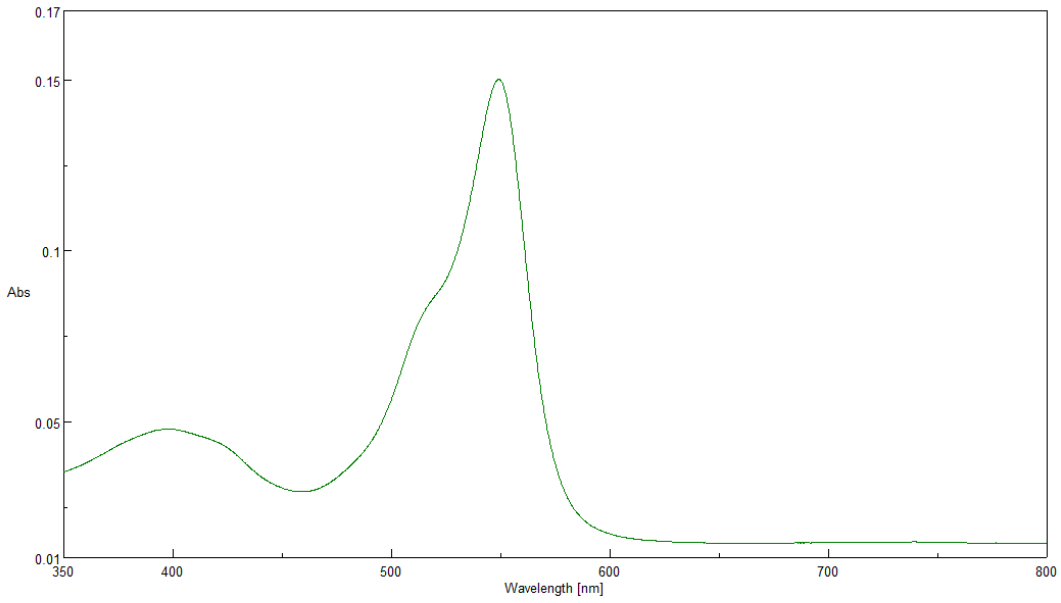
ESI-MS spectrum of **OBEP-CS1**

DOSY experiment

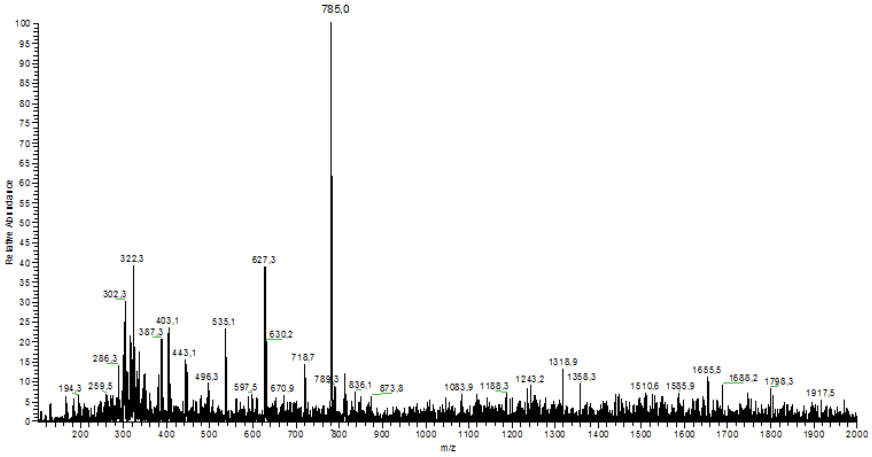


^{1}H NMR stack plot of the signal decay of **OBEP-CS1**(D_2O, 1mM, 298 K) as a function of the gradient strength (G).

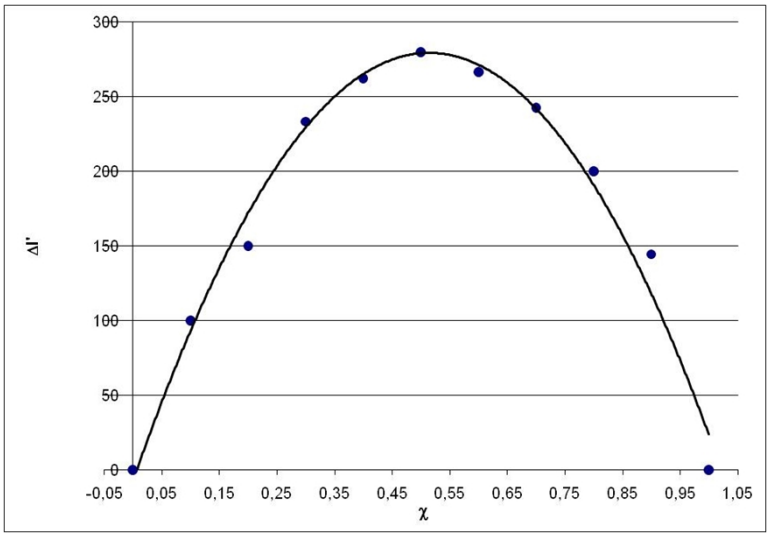
A diffusion coefficient of 3.56 x 10^{-10} m^2s^{-1} was obtained. Using this value in the Stoke-Einstein equation and in the equation reported by Morris and co-workers,[6] an hydrodynamic radius of 7.51 Å and a molecular weight of 571 is obtained. Considering that a miminized structure of **OBEP-CS1** (HyperChem 7.5, MM+) indicates a radius of 8.47 Å in the extended conformation and the ESI-MS spectrum shows a peak at 721 m/z, the formation of aggregates are excluded.



UV-Vis spectrum of **OBEP-CS1**, 5 x 10^{-6} M in 10 mM HEPES buffer, pH 7.2



ESI-MS spectrum of **OBEP-CS1** + 1 eq. Cu^{+}.



Job's Plot of **OBEP-CS1** and Cu^{+} in 10 mM HEPES buffer, pH 7.2

Fluorescence titrations

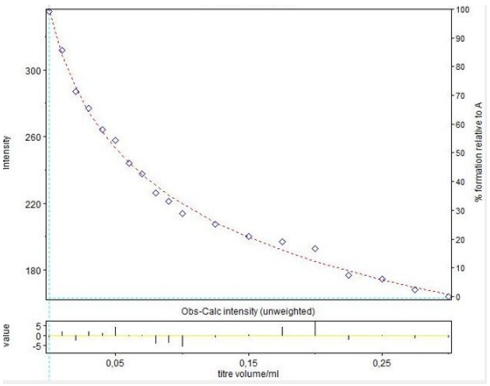
Binding constant values obtained in the buffer systems

Buffer[a]	logβ[b]
HEPES	13.39 ± 0.03
MOPS	12.1 ± 0.6
PBS	13.3 ± 0.2

[a] in all cases, buffer concentration is 10 mM (pH = 7.2);
[b] calculated with HypSpec v1.1.33

HypSpec plot for binding constant determination

Titration of **OBEP-CS1** with Cu^{+} in HEPES 10 mM, pH 7.2

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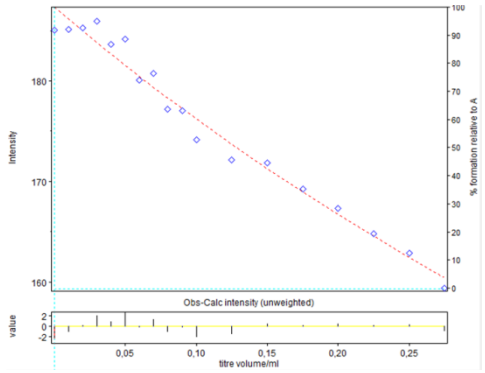
Iteration 1
relative
Parameter      shift      new value
Log beta AB   0,0000     13,39

standard
Log beta     value      deviation
BC            2,63
BC2           4,02
BC3           4,3
AB          13.39       0.03
```

Titration of **OBEP-CS1** with Cu^{+} in MOPS 10 mM, pH 7.2

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standard
Log beta     value      deviation
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BC3           4,3
AB          12.1        0.6
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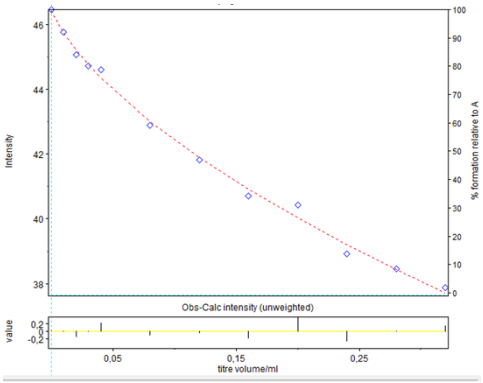
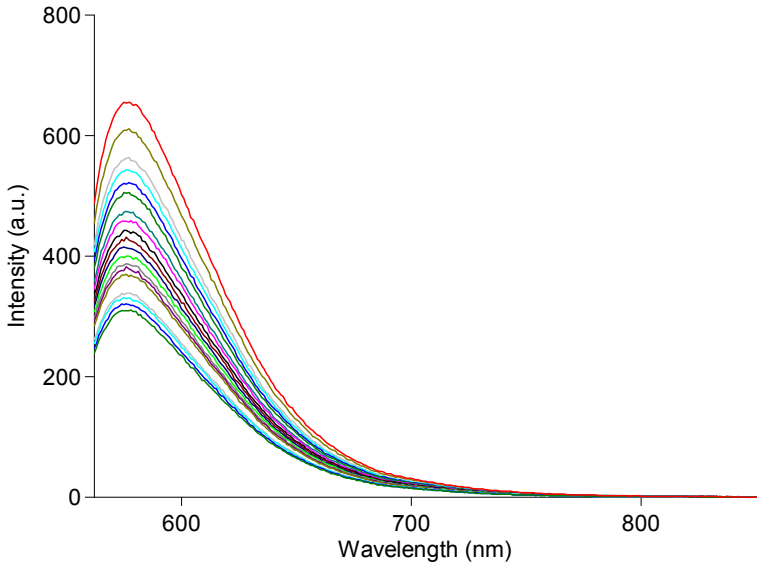
Titration of **OBEP-CS1** with Cu^{+} in PBS 10 mM, pH 7.2




```
 Iteration 1
relative
Parameter      shift      new value
Log beta AB   0,0001     13,3

standard
Log beta     value      deviation
BC            2,63
BC2           4,02
BC3           4,3
AB          13.3        0.2
```

Fluorescence titration (λ_{ex} 543 nm)



Fluorescent emission spectra of **OBEP-CS1** (5 μM) upon progressive addition of Cu^{+} (0 – 3 equivalents in HEPES buffer solution, 10 mM, pH 7.2), λ_{ex} 543 nm.

Cell cultures

The human neuroblastoma cell line SH-SY5Y, was grown in DMEM-F-12 (1 : 1) medium, supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 2 mM L-glutamine and maintained at 37°C, 5% CO_2 in a humidified incubator. For confocal experiments, cells were plated into glass-bottom dishes (Willco Wells B. V., The Netherlands) at a density of $1x10^5$ cells/dish. To induce differentiation of SH-SY5Y into neuronal- like cells, 5 µM of all-trans retinoic acid (RA) were added every two days in DMEM-F-12 (1 : 1) medium supplemented with 1% FBS, for 7-8 days.

MTT assays

To test the toxicity of **OBEPCS1**, differentiated SH- SY5Y cells were treated for 6 and 24 h with different concentration of copper sensor (0.1, 1, 5, 10, 20 µM). Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay: 20 µL MTT was added to each well of the multiwell plate and incubated at 37°C for 2 hours. The medium was then removed and 200 µL DMSO were added. The optical density was then read at 570 nm.

Confocal microscopy experiments

Live cell imaging experiments were carried out by using an Olympus FV1000 confocal laser scanning microscope (LSM) equipped UV/visible lasers: 405 nm (50 mW), 20 mW MultilineArgon laser (457 nm, 488 nm, 515 nm, total 30 mW), HeNe(G) laser (543 nm, 1 mW), HeNe(R) laser (633 nm, 1 mW); oil immersion objective (60xO PLAPO) and spectral filtering system. For all acquisitions, the detector gain was fixed at a constant value, emitted light was detected in sequential mode and images were taken for all of the samples at random locations throughout the area of the well.

Fluorescent probes addition was performed *in situ*, on the sample mounted on the microscope stage. Cells were rinsed with 10 mM PBS buffer, then Mitotracker Deep Red (50 nM in PBS, Invitrogen) and **OBEP-CS1** (1–20 μM) were added and cells imaged in xyt scan mode to track the probe cellular uptake and staining of mitochondria organelles and Cu(I), respectively.

Image analysis was carried out using the public domain, Java-based image processing program Image J (version 1.46e). The statistical analysis was performed with a one-way Anova test by using Microcal Origin (version 8.6).

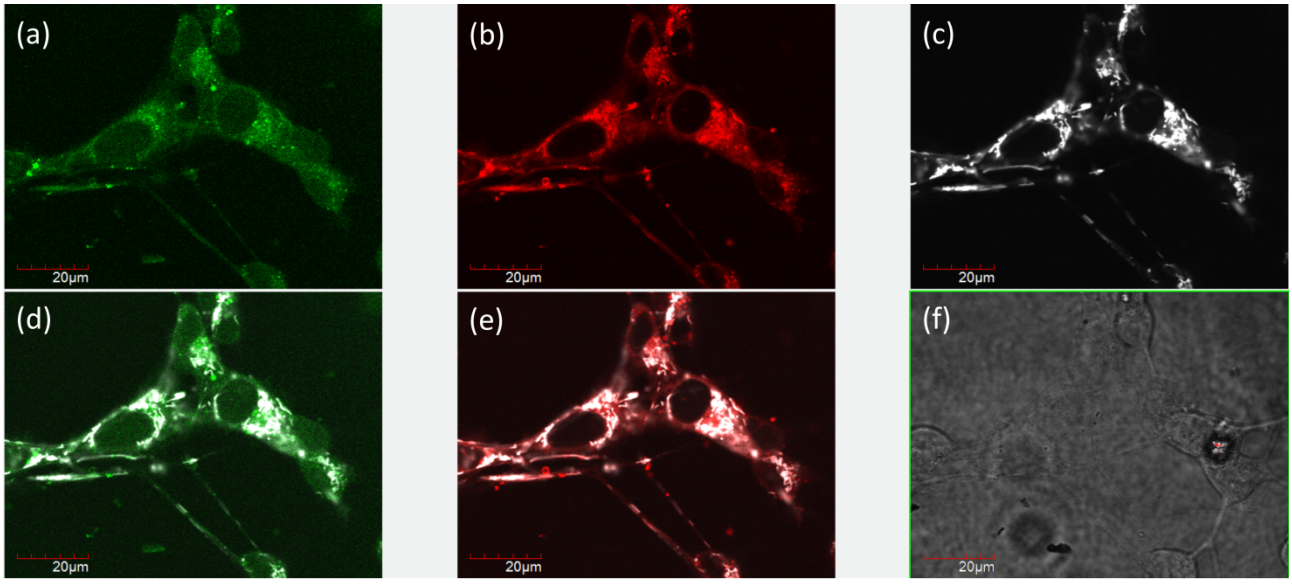


Figure S1. LSM of differentiated SH-SY5Y cells in DMEM after 4 hours of incubation with 5 μM **OBEP-CS1.** In (a) and (b) are shown respectively the green (λex/em= 488/500-550 nm) and red (λex/em= 543/550-605 nm) fluorescence of **OBEP-CS1**, while the mitochondria staining by Mitotracker Deep Red (λex/em= 633/650-700 nm) is displayed in (c). In (d) and (e) the merged fluorescence images respectively of (a+c) and (b+c), whereas in (f) the bright field optical image are shown.

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