Electronic Supplementary Information for:

A new strategy of improving cytotoxicity of copper complex toward metastatic melanoma cells unveiled by EPR spectroscopy

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Experimental section

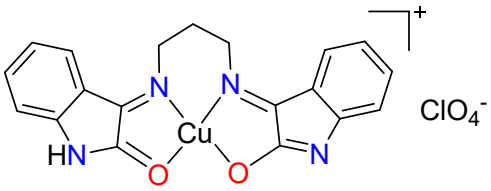
The chemicals 3-amino-1,2,4-triazole-5-thiol (att), 3-amino-1,2,4-triazole-5-carboxylic acid (atzac), isatin (2,3-dioxoindole) (isa), 1,3-diaminopropane (pn), and copper(II) perchlorate hexahydrate, were purchased and used as received from Sigma Aldrich/Merck Co.

Instrumentation

^{13}C and ^{29}Si solid-state NMR analysis were accomplished with a Bruker Avance 500 spectrometer, at Institute of Chemistry of UNESP/Araraquara-SP. FT infrared spectra, elemental and metal analyses were carried out at Central Analítica/IQ-USP, by using a Perkin Elmer – Frontier, CHN- Perkin Elmer-2400 and ICP-OES instrument, from Spectro Arcos, respectively, at University of São Paulo, Brazil. S- and X-band continuous-wave EPR measurements were made on a Bruker EMX plus with a rectangular 4122SHQ and ER106QT cavities. Pulsed EPR measurements at the X-band were carried out on a Bruker ElexSys E580 spectrometer equipped with a rectangular EN 4118X-MD4 Bruker resonator. The temperature was stabilised with an Oxford MercuryiTC within ~0.1 K.

Syntheses of [N,N'[bis-(3,3'-indolin-2-one)]-1,3-diiminopropane]-copper(II) perchlorate ([Cu(isapn)]ClO_4) complex

The [Cu(isapn)]ClO_4 complex (Figure S1) were obtained and characterized in our previous studies.[1,2] In general, the imine ligand is obtained by condensation reaction of isatin with the suitable ligand at pH round 6.4, followed by metalation with perchlorate or chloride metal salt. This complex was isolated as a keto-enol tautomer. [Cu(isapn)]$ClO_4 \cdot 2H_2O$ ($C_{19}H_{19}N_4O_8ClCu$, 530.38 g/mol): Yield 76%, Calculated C 43.03, H 3.80, N 10.56%; Found C 43.08, H 3.67, N 10.34%. FTIR (KBr pellet, cm^{-1}): ν(C=N) 1595, ν(C=O) 1728 and ν(C-H) 728; MS (ESI+): m/z found: 395.02 (calcd.: 395.07, for C19H16N4O2Cu).[2]



[Cu(isapn)]ClO_4

Figure S1. Structure of [N,N'[bis-(3,3'-indolin-2-one)]-1,3-diiminopropane]-copper(II) perchlorate ([Cu(isapn)]ClO_4).

Synthesis of tetrakis[(3-amino-1,2,4-triazole-5-carboxylic acid) chloropropyl] octasilsesquioxane (POSS-atzac) and tris[3-amino-1,2,4-triazole-5-thiol) chloropropyl] octasilsesquioxane (POSS-att)

The precursor Octakis(3-Chloropropyl) octasilsesquioxane (POSS-Cl) was synthetized and characterised in previous work.[3,4] Moreover, the preparation of octa[3-amino-1,2,4-triazole-5-carboxylic acid) propyl] silsesquioxane (POSS-atzac) was firstly presented in a previous study.[5] However, a new synthesis of the POSS-atzac was performed with some modifications, in order to obtain a compound with three or four units of the 3-amino-1,2,4-triazole-5-carboxylic acid ligand bonded to the POSS-Cl surface. Additionally, the synthesis of a new tris[3-amino-1,2,4-triazole-5-thiol) chloropropyl] octasilsesquioxane (POSS-att) is reported here for the first time.

POSS-Cl (5 g, 4.82 mmol) was added into two reaction flasks containing 100mL dimethylformamide and stirred under reflux until its complete solubilisation. Next, the ligands 3-amino-1,2,4-triazole-5-carboxylic acid (atzac) (2.78 g, 21.96 mmol) and 3-amino-1,2,4-triazole-5-thiol (att) (2.52 g, 21.69 mmol) were added slowly into each flask over a

period of 3h and stirred for 3 more days. At the end of the reaction time, the solids were obtained after the addition of ethanol to the medium to precipitate the materials tetrakis[3-amino-1,2,4-triazole-5-carboxylic acid) chloropropyl] octasilsesquioxane (POSS-atzac, white solid) and tris[3-amino-1,2,4-triazole-5-thiol) chloropropyl] octasilsesquioxane (POSS-att, yellow solid). The solids were separated by filtration and washed with ethanol/H_2O. ***POSS-atzac*** ($Si_8O_{20}C_{36}H_{60}N_{16}Cl_4$, 1400.10 g/mol): Yield 95%, Calculated C 30.80%, H 4.31, N 15.96%; Found H 5.01, N 14.96%. FTIR (KBr pellet, cm^{-1}): ν(C-H) 2942, ν(C=O) 1674, ν(C=N) 1574, ν(C-N) 1449, ν_{as}(Si-O-Si) 1114 and ν(C-Cl) 696. ^{13}C solid-state NMR (100.61 MHz, ppm): 9.47 (CH_2-Si), 23.39 (CH_2-CH_2Si), 48.77 (CH_2-N), 42.30 (CH_2-Cl), 141.13-148.99 (aromatic ring), 154.84 (carboxylic acid group). ^{29}Si solid-state NMR (79.48 MHz, (Si-O), ppm): -67.38. ***POSS-att*** ($Si_8O_{12}C_{30}H_{57}N_{12}S_3Cl_5$, 1271.99 g/mol): Yield 91%, Calculated C 28.24%, H 4.50%, N 13.17%; Found H 4.84, N 12.26%. FTIR (KBr pellet, cm^{-1}): ν(C-H) 2934, ν(S-H) 2500, ν(C=O) 1636, ν(C=N) 1541, ν(C-N) 1442, ν(C-S) 1250, ν_{as}(Si-O-Si) 1115, and ν(C-Cl) 692. ^{13}C solid-state NMR (100.61 MHz, ppm): 11.79 (CH_2-Si), 25.26 (CH_2-CH_2Si), 37.40 (CH_2-Cl) 50.99 (CH_2-N), 152.69-159.48 (aromatic ring). ^{29}Si solid-state NMR (79.48 MHz, (Si-O), ppm): -67.55.

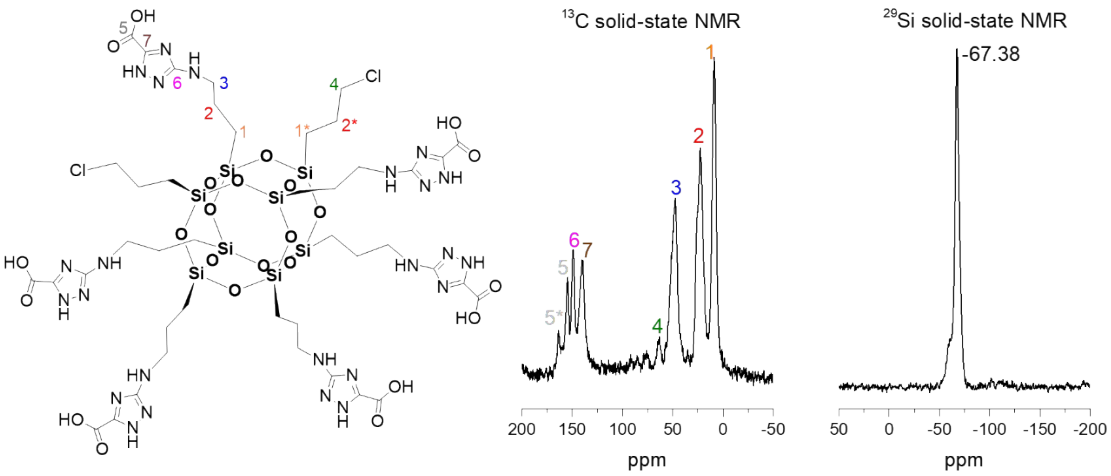


Figure S2. ^{13}C **(A)** and ^{29}Si **(B)** solid-state NMR spectra of the hexa[3-amino-1,2,4-triazole-5- carboxylic acid) chloropropyl] octasilsesquioxane (**POSS-atzac**).

Synthesis of metal-bioconjugated materials: Bis-[N,N'[bis-(3,3'-indolin-2-one)]-1,3-diiminopropane]-copper(II)-tetra[3-amino-1,2,4-triazole-5-carboxylic acid) chloropropyl] octasilsesquioxane (POSS-atzac-[Cu(isapn)]) and Bis-[N,N'[bis-(3,3'-indolin-2-one)]-1,3-diiminopropane]-copper(II)-tris[3-amino-1,2,4-triazole-5-thiol) chloropropyl] octasilsesquioxane (POSS-att-[Cu(isapn)])

The synthesis of new metal-bioconjugated materials were accomplish by solubilization of both [Cu(isapn)]ClO_4 complex (4.5 mmol) in DMSO/H_2O (20:80 v/v), at pH 6.4. Next, 3g of POSS-atzac and POSS-att (2.13 and 2.21 mmol, respectively) were added slowly over a period of 2h into the solutions containing the solubilized complexes and stirred for 24h at room temperature. Then, new metal-organic-inorganic hybrid nanomaterials POSS-atzac-[Cu(isapn)] and POSS-att-[Cu(isapn)] were filtrated and washed three times with a mixture of DMSO/H_2O. The samples were dried at 70°C overnight and grinded before storage in glass flasks.

POSS-atzac-[Cu(isapn)] ($Si_8O_{28}C_{112}H_{124}N_{32}Cl_4Cu_4$, 2980.33 g/mol): Yield 96%, Calculated C 45.03%, H 4.18%, N 15.01%, Cu 8.51%; Found H 4.01%, N 15.56%, Cu 7.98%. FTIR (KBr pellet, cm^{-1}): ν(C-H) 2935, ν(C=O) 1656, ν(C=N) 1568, ν(C-N) 1454, ν_{as}(Si-O-Si) 1111, and ν(C-Cl) 703. ***POSS-att-[Cu(isapn)]*** ($Si_8O_{18}C_{87}H_{105}N_{24}S_3Cl_5Cu_3$, 2457.16 g/mol): Yield 90%, Calculated C 42.41%, H 4.30%, N 13.64%, Cu 7.74%; Found H 3.36%, N 12.82%, Cu 6.88%. FTIR (KBr pellet, cm^{-1}): ν(C-H) 2934, ν(S-H) 2500, ν(C=O) 1636, ν(C=N) 1541, ν(C-N) 1442, ν(C-S) 1250, ν_{as}(Si-O-Si) 1115, and ν(C-Cl) 692.

Synthesis of binuclear complex [Cu(isapn)(μ-triazole)Cu(isapn)]

The synthesis of the binuclear complex was carried out by solubilizing the 3-amino-1,2,4-triazole-5-carboxylic acid ligand (2.25 mmol) in hot water. Then, the [Cu(isapn)]ClO_4 complex (6.2 mmol) solubilized in DMSO/H_2O (20:80) v.v at pH 7 was added to the reaction medium and left under stirring for 2h. Next, binuclear complex [Cu(isapn)(μ-triazole)Cu(isapn)] was filtrated and washed several times with a mixture of DMSO/H_2O. The sample was dried at 70°C overnight and stored in glass flasks.

[Cu(isapn)(μ-triazole)Cu(isapn)] $C_{41}H_{36}Cu_2N_{12}O_6$ 918.15 g/mol: Yield 94%, Calculated (%) C 53.53, H 3.94, N 18.27, Cu 13.82; Found (%): C 53.39, H 3.88, N 18.16, Cu 13.75.

Continuous-wave and pulsed EPR spectroscopy

Continuous-wave (CW) electron paramagnetic resonance (EPR) measurements were performed on a Bruker EMXplus EPR spectrometer operating at X- (*ca.* 9.5 GHz) or S-band (*ca*. 4 GHz) microwave frequencies in room and low temperatures at EPSRC UK National EPR Facility in Manchester. The magnetic field values were corrected against Bruker's strong

pitch standard sample (g = 2.0023). The spectra were recorded in solution and also in polycrystalline sample.

Pulse experiments were done on a Bruker ELEXSYS E580 spectrometer operating at X-band (*ca.* 9.7 GHz) band. Echo-detected field swept (EDFS) spectra were collected using a two-pulse Hahn-echo sequence ($\pi/2 - \tau - \pi - \tau$ – echo) at fixed interpulse delay time τ and varying the static magnetic field. Cryogenic temperatures were achieved using a cryogen-free closed-cycle helium circuit and the EasySpin software was used to simulate the experiments.[5]

Hyperfine sublevel correlation (HYSCORE) spectroscopy was performed using a four-pulse sequence ($\pi/2 - \tau - \pi/2 - t_1 - \pi - t_2 - \pi/2 - \tau$ – echo) with $\pi/2$ pulses of 16 ns and fixed τ and magnetic field. The intervals t_1 and t_2 started at 100 ns and were incremented independently to give a two-dimensional correlation pattern and the experiments were done at different τ-values to avoid blind spot effects inherent to ESEEM spectroscopy. The acquired time-domain signal was background corrected with polynomial functions, zero-filled to 1024 points, apodised with Hamming window and Fourier transformed to give the frequency-domain spectra. EasySpin package was also used for modelling.[5]

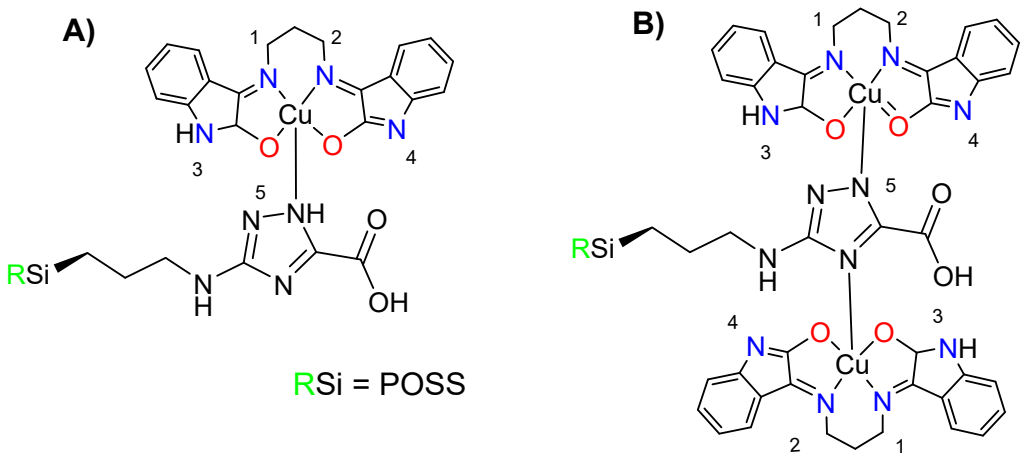


Figure S3. Proposed structures for A) the major mononuclear complex [POSS-atzac-[Cu(isapn)], andB) the secondary binuclear species [Cu(isapn)RSi(atzac)Cu(isapn)] detected inside the POSS matrix.

Cells and cell culture

SKMEL-147 (wild type, Cellosaurus, CVCL 3876 - Human melanoma cells) was friendly given by Professor Roger Chammas from ICESP - Cancer Institute of São Paulo State. The human

foreskin fibroblasts P4 cells (non-tumourogenic) were isolated from tissues at Faculty of Pharmaceutical Sciences-USP at Professor Silvya Stuchi Maria-Engler's group. Dulbecco's Modified Eagle's medium - DMEM (Vitrocell) supplemented with 10% fetal bovine serum – FBS (Vitrocell) was used to cultivate both cell lines in an oven with 5% CO_2 atmosphere and 37°C.

Cell viability analysis

Cell viability was evaluated by the tetrazolium dye reduction assay (MTT) to verify the cytotoxicity of the free and inserted complexes on two modified-POSS. 1.10^4 of melanoma (SKMEL-147) and non-tumour cells (fibroblast P4), were placed in a 96-well plate and growth for 24h. Next, the medium was removed, and each well was washed three times with phosphate buffered saline buffer (PBS), pH 7.4. The solutions and suspensions of each compound were prepared in culture medium, and directly added to the cells, which were treated for 24 or 48h. Subsequently, the medium was replaced by 200 µL of a solution containing 0.2 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent (MTT, Sigma Aldrich), and the plates incubated for 3h. After this period, it was possible to observe the formation of formazan crystals under a microscope. These crystals were solubilized by addition of 100 µL DMSO, and the absorbance of resulting solution was measured at 570 nm on a microplate reader (Tecan Infinite M200, Switzerland). Cytotoxicity was expressed as IC_{50} values, which was determined from the concentration-response curve, and corresponding to 50% amount of viable cells.

Cytotoxicity tests

Table 1: Comparative results of MTT assays for the cytotoxicity of pure and bonded complexes on POSS or MCM matrices towards tumour and non-tumour cell lines.

Compounds	**IC_{50} , µM or µg/L**			
	SKMEL-147		**Fibroblast**	
	24h	**48h**	**24h**	**48h**
[Cu(isapn)]ClO4	> 100 µM	> 100 µM	> 100 µM	> 100 µM
POSS-atzac-[Cu(isapn)]	78 ± 4 µg/mL (35.5 µM)*	60 ± 6 µg/mL (27.3 µM)*	255 ± 8 µg/mL (> 100 µM)*	241 ± 11 µg/L (> 100 µM)*
MCM-[Cu(isapn)] #	155 ± 5 µg/mL	150 ± 6 µg/mL	418 ± 11 µg/mL	298 ± 9 µg/mL

	(73.2 ± 2.4μM)*	(70.8 ± 2.4μM)*	(197.2± 5.2μM)*	(140.7±4.3μM)*
MCM-atzac-[Cu(isapn)] #	34 ± 1 μg/mL (9.1 ± 0.3μM)*	35 ± 1 μg/mL (9.3 ± 0.3μM)*	186 ± 9 μg/mL (49.6 ± 2.4μM)*	80 ± 7 μg/mL (21.3 ± 1.9μM)*
POSS-atzac	161 ± 10μg/mL (> 100 μM)	141 ± 5 μg/mL (> 100 μM)	> 500 μg/mL (> 100 μM)	> 500 μg/mL (> 100 μM)
MCM-atzac #	53 ± 3 μg/mL	49 ± 2 μg/mL	347 ± 11 μg/mL	93 ± 3 μg/mL

***** considering the percentage of complex immobilized in the matrix and 100% release.

previous results in **ref. 6** - New J. Chem. 2019, **43**, 386–398.

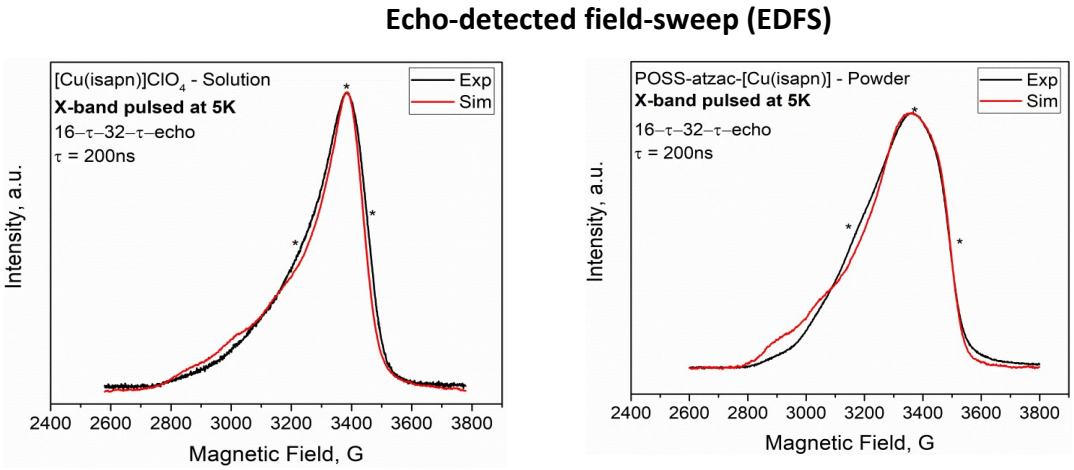


Figure S4. Echo-detected absorption spectra for pure complex in solution, and immobilised copper(II) species in solid complex **2**, measured at 5 K.

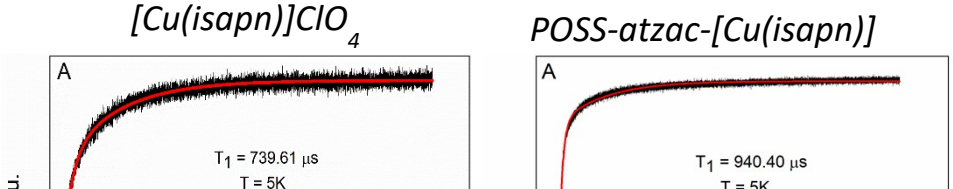


Figure S5. Spin–lattice relaxation (T_1) for **[Cu(isapn)]ClO_4** and **POSS-atzac-[Cu(isapn)]** at three different fields at 5K. T_1 were measured by inversion recovery with the pulse sequence π–τ–π/2–τ–π–τ-echo. The lengths of the mw π/2 and π pulses were 16 and 32 ns, respectively, and the interpulse delay τ = 200 ns.

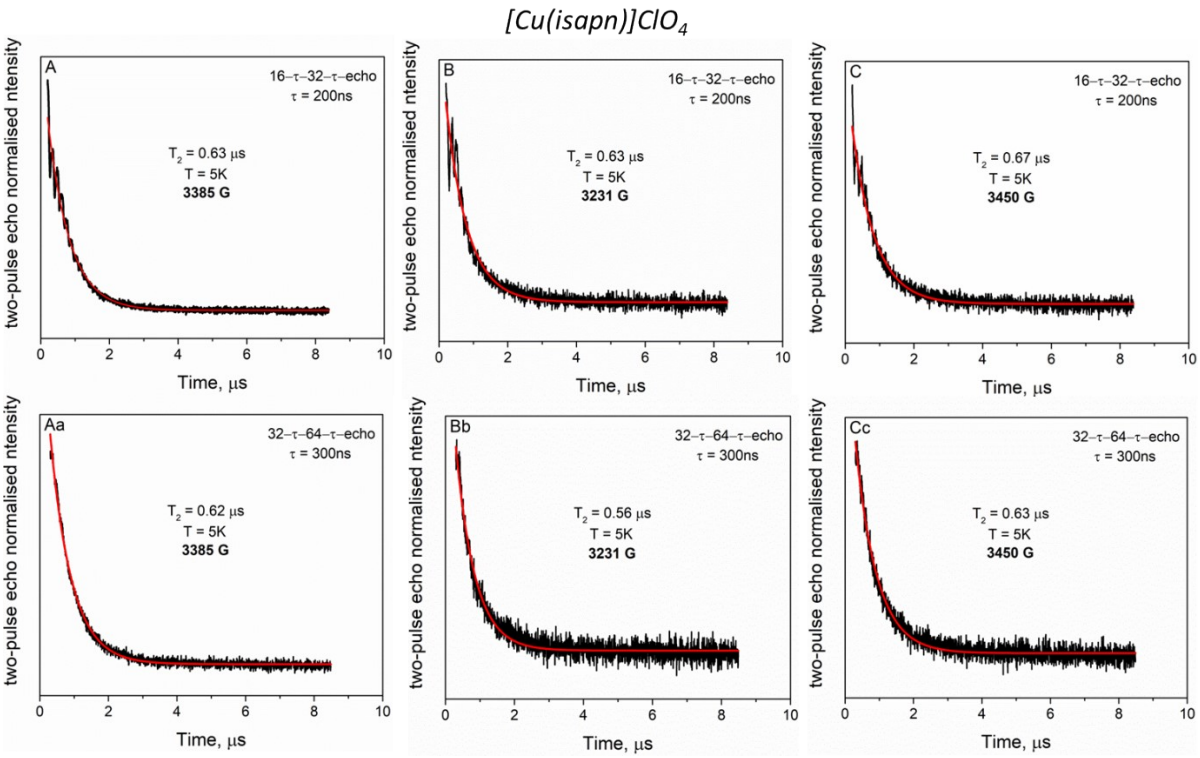


Figure S6: Two-pulse electron spin echo decays for **[Cu(isapn)]ClO_4 1** measured at three different fields (A) 3385G, (B) 3231G and (C) 3450G and pulse sequence at 5K as a function of the interpulse delay τ.

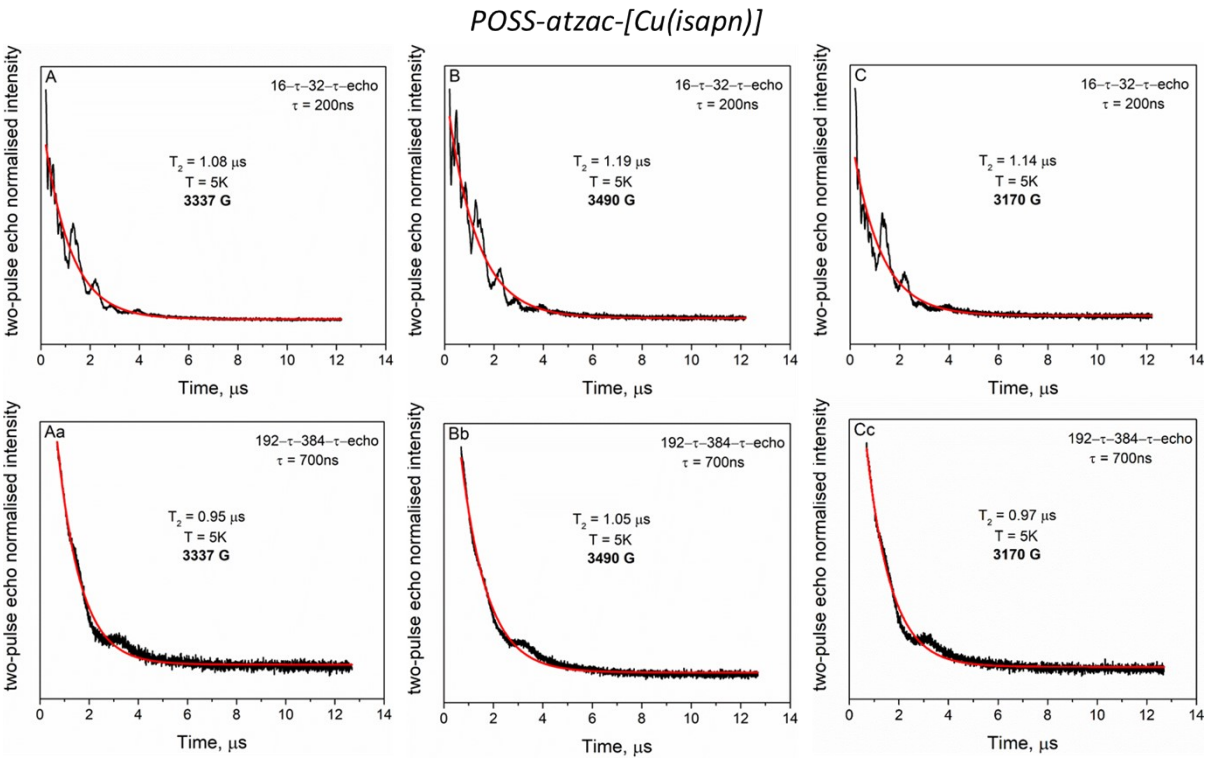


Figure S7: Two-pulse electron spin echo decays for **POSS-atzac-[Cu(isapn)] 2** measured at three different fields (A) 3337G, (B) 3490G and (C) 3170G and pulse sequence at 5K as a function of the interpulse delay τ.

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