Electronic Supporting Information

Amphiphilic Phenylalanine derivatives that temporally generate Reactive Oxygen Species from Water in presence of Au(III) ions

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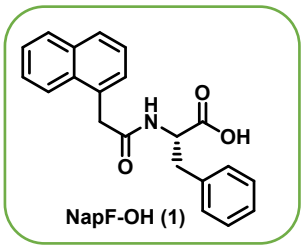
Material and Methods:

1-Naphthylacetic acid (1-NAA) was purchased from Spectrochem Chemicals, 1,1′-carbonyldiimidazole (CDI), acetic acid, sodium acetate and acetic anhydride were purchased from Alfa Aesar, sodium hydroxide, hydrochloric acid, nitric acid, phosphoric acid, sodium nitrate, sodium dihydrogen phosphate and triethylamine were obtained from Merck. 5-tert-Butoxycarbonyl-5-methyl-1-pyrroline-N-oxide (BMPO, Enzo Life Sciences, ALX-430-141-M050) were purchased from Enzo Life Sciences. Sulphuric acid and sodium sulphate were procured from Rankem. All solvents used in synthesis were purified, dried, or distilled, as required. FTIR spectra were recorded on dry samples pelletized with KBr by using PerkinElmer Spectrum BX FT-IR spectrometer. UV-vis absorption spectra were recorded on Specord 210 Plus spectrophotometer from Analytik Jena. NMR spectra were recorded using Bruker Ultra shield (400 and 500 MHz) spectrometers. Scanning Electron microscopy (SEM) was done on an Ultra 55 High resolution scanning electron microscope by Carl Zeiss. Circular dichroism (CD) studies were performed on a JASCO J815 CD instrument. Powder X-ray diffraction (PXRD) patterns were recorded on a PANalytical EMPYREAN diffractometer using Cu K_α (λ= 1.5418 Å) incident X-rays.

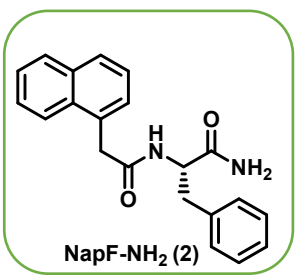
Synthesis and characterizations

2-(2-(naphthalene-1-yl)acetamido-3-phenylpropanoic acid (1):



NapF-OH (1)

L-Phenylalanine hydrochloride methyl ester (2 g, 1 eq, 5.75 mmol) was dissolved in methanol (5 mL) followed by drop wise addition of 1 N NaOH (6.9 mL, 1.2 eq, 6.9 mmol) at 0 °C. This mixture was stirred for 3h at RT. Hydrolysis was monitored by TLC. Methanol was removed by rotary evaporator and reaction mixture was acidified by 1 N HCl to pH 2. A white colour solid was formed which was separated by simple filtration and dried overnight under vacuum desiccator to obtain 1.79 g (93%) of **1**, m.p. 160 °C. FTIR (KBr pellet, cm^{-1}): 2500-3300 (br., O-H str.), 1708 (acid, C=O str.), 1662 (amide, C=O str.), 1539 (N-H bend) 3282 (N-H str.). ^1H NMR (0.1 N NaOD): d= 2.77-2.99 (2H, Ar-CH_2), 3.95(2H, Np-CH_2), 4.36-4.40 (1H, CHCOO), 6.84-7.92 (12H, Ar-H).^{13}C NMR (0.1 N NaOD) δ= 177.94 (CO-Acid), 173.40 (CO-Nap), 55.97 (CH), 39.92 (C-Nap), 37.42 (C-ph) 137.18-123.36 (C-Aromatic). HRMS (ESI): $C_{21}H_{19}NO_3$ + Na calc.: 356.1257, found: 356.1256.

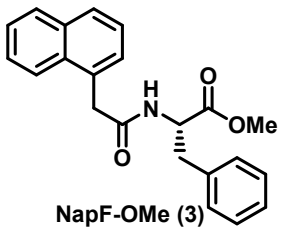


NapF-NH_2 (2)

(S)-2-(2-(naphthalen-1-yl)acetamido)-3-phenylpropanamide (2):

Compound **1** (2 g, 1 eq, 5.75 mmol) was dissolved in dry THF in presence of N_2 balloon (5 mL) and followed by portion wise addition of CDI (6.9 mL, 1.2 eq, 6.9 mmol) at 0 °C. This mixture was stirred for 30 min. at RT. 3 mL of ammonia in methanol (30% v/v) was added dropwise to this solution. The reaction was monitored by TLC. A white precipitate was observed in to reaction flask. THF and excess ammonia was removed by rotary evaporator and reaction mixture was washed by diethyl ether (thrice). A white colour solid was obtained which was dried under vacuum to obtain 1.79 g (96%) of **2**, m.p. 160 °C. FTIR (KBr pellet, cm^{-1}): 2500-3300 (br., O-H str.), 1708 (acid, C=O str.), 1662 (amide, C=O str.), 1539 (N-H bend) 3282 (N-H str.). ^1H NMR (0.1 N NaOD): δ = 2.77-2.99 (2H, Ar-CH_2), 3.95 (2H, Np-CH_2), 4.36-4.40 (1H, CHCOO), 6.84-7.92 (12H, Ar-H). ^{13}C NMR (0.1 N NaOD) δ= 177.94 (CO-Acid), 173.40 (CO-Nap), 55.97(CH), 39.92(C-Nap), 37.42 (C-ph), 137.18-123.36 (C-Aromatic). HRMS (ESI): $C_{21}H_{20}N_2O_2$+Na^+ calc.: 332.4030, found: 355.3922.

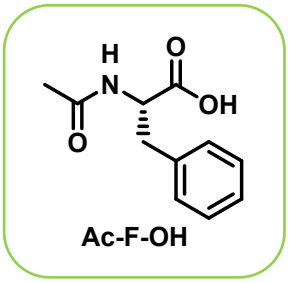
Methyl-2-(2-(naphthalene-1-yl) acetamido-3-phenylpropanoate (3):



NapF-OMe (3)

L-Phenylalanine hydrochloride methyl ester (1.49 g, 1 eq, 6.95 mmol) was taken in dry THF (15 mL) and triethylamine (1.5 mL, 1.5 eq, 10.42 mmol) was added drop wise to it at 0 °C followed by stirring for 10 mins. In a separate flask, 1-NAA (1.42 g, 1.1 eq, 7.64 mmol) and CDI (1.69 g, 1.5 eq, 10.42 mmol) were dissolved in dry THF (15 mL) and stirred for 5 mins. This activated acid solution was added drop wise at 0 °C to the basic mixture of L-Phenylalanine hydrochloride methyl ester followed by stirring under N_2 atmosphere for 1.5 h at RT. Reaction was monitored by TLC (eluent phase was 5:95 MeOH:$CHCl_3$). THF was removed from the reaction mixture by vacuum and 30 mL of CHCl3 was added to it. The $CHCl_3$ layer was washed with dilute HCl and sat. $NaHCO_3$, dried with sodium sulphate. Removal of $CHCl_3$ by rotary evaporator yielded **2**, which was purified by column chromatography (stationary phase was silica 100-200 mesh and eluent phase was $CHCl_3$). Obtained 2.2 g (91%) of **3** as white solid, m.p. 125 °C. FTIR (KBr pellet, cm^{-1}): 1751 (ester, C=O str.), 1647 (amide, C=O str.), 1535 (N-H bend), 3305 (N-H str). ^1H NMR (400 MHz, $CDCl_3$, ppm): 2.90-2.92 (2H, Ar-CH_2), 3.95 (3H, COOMe), 3.96-4.11 (2H, Nap-CH_2), 4.83-4.88 (1H, CHCOO), 5.75 (1H, NH), 6.56-7.98 (12H, Ar-H). HRMS (ESI): $C_{22}H_{21}NO_3$ + Na^+ calc.: 370.1414, found: 370.1415.

Acetyl-L-phenylalanine (4):



Phenylalanine (53.4 mmol) was suspended in 5% aq. $NaHCO_3$ (150 mL) and cooled to 0 ˚C. Acetic anhydride (6.1 mL, 64.5 mmol, 1.2 eq.) was added dropwise over a period of 1 h. The mixture was stirred at room temperature for 2 h and the reaction was monitored by TLC (9:1 ethanol/1 M acetic acid, ninhydrin stain) until the consumption of starting material was observed. The mixture was then acidified to pH 2-3 with 6 M HCl and cooled overnight. The resulting precipitate was filtered off, washed with cold water (2 x 10 mL) and dried to give the N-acetylated amino acid as a white solid.

Gelation

1 mL water was added to a glass vial containing 5 mg of ADF-**1**. This mixture was heated to boil until the solid dissolved. The resulting solution was allowed to cool to room temperature. Hydrogel was considered to have formed if no flow of solvent was observed upon inversion of the vial. If gelation was required at a particular pH, water was replaced with aqueous buffer. For example, to obtain a gel at pH 6, ADF-**1** was suspended in 0.1 M acetate buffer (pH = 6) and heated to boil to obtain clear solution and kept undisturbed at RT for gelation.

Gel-melting temperature (T_m)

The melting temperatures of gels prepared in normal and heavy water were determined by the 'inverse flow method' in which the samples were gelled in sealed tubes and attached to a thermometer near the bulb end in an inverted position. This assembly was immersed in a water bath at room temperature and the temperature was raised at ca.1 °C·min^{-1} on a hot plate. The temperature at which the gel mass fell was recorded as T_m.

Preparation of 1+Au(III)

0.25 mg of **1** (0.75 μmol) was dissolved in distilled in 900 μL of methanol. Then 100 μL of $HAuCl_4$ (stock solution = 0.75 mM) was added to it and mixed well. The solvent was then removed in vacuum. A crystalline yellow colored solid of **1+ Au(III)** was obtained which was analysed by APCI-MS and FTIR.

UV-vis spectroscopy

UV-vis absorption spectra of ADF-**1** and **1+Au(III)** were recorded in the region of 190-700 nm in water and methanol respectively at 100 μM concentration of each samples.

Field Emission Scanning Electron Microscopy (FE-SEM)

For FE-SEM, the samples were scooped from the surface of the vial using a spatula and spread on SEM stubs having double sided carbon tape on them. The stubs were dried inside a vacuum desiccator for 48 h and subsequently gold coated for 120 s for sample ADFs **1-4** and without gold coated for **1+Au(III)** sample. SEM images were recorded on Carl Zeiss (Ultra plus) FE-SEM at an accelerating voltage of 10 kV and 15 kV.

Energy dispersive X-ray spectroscopy (EDS)

EDS was examined using a spectrometer (Oxford Instruments X-MaxN) attached to FESEM. Measurements were performed at a working voltage of 20 kV and elemental Co was used as reference.

Circular Dichroism

CD spectra of ADF-**1** and **1+Au(III)** are recorded in water and methanol, respectively. Baseline was corrected by using water: CH_3OH (1:1, v/v) solvent. Transparent hydrogel of ADF-**1** (0.25 mg/ mL) in water was prepared and CD was recorded, which corresponded to an absorbance value at 295 nm (A_{295}) = 0.1. Data were averaged over at least three accumulations; blank subtracted and smoothened. The sample of **1+Au(III)** (100 μM) was prepared in methanol.

Rheology

Rheological measurements were carried out on a Rheoplus MCR302 (Anton Paar) rheometer with parallel plate geometry and obtained data was processed with start rheometer software. For the oscillatory shear measurements, parallel top plate with a 25 mm diameter and 1.0 mm gap distance were used. Gels (prepared in H_2O and D_2O at 2x MGC) were transferred on the bottom plate of the rheometer. The shear modulus (storage modulus, G', and loss modulus, G'') were plotted against %strain from 0.1 % to 100 %. Frequency sweep experiment was performed from 0.1 to 100 rad/s at constant strain of 1 % (Fig. S7).

Field Emission Scanning Electron Microscopy (FESEM)

For FESEM, images were recorded on Carl Zeiss (Ultra plus) FE-SEM at an accelerating voltage of 10 and 15 kV. For sample preparation, 0.25 mg of powdered samples of ADFs **1-4** were dissolved separately in 2 mL water and D_2O respectively in hot water bath and then cooled

at room temperature. Samples of ADFs **1-4** + Au(III) were similarly prepared. Same samples were prepared in H_2O:D_2O solvents also. After cooling at room temperature, samples were scooped from the surface of the vial using a spatula and spread on SEM stubs having double sided carbon tape on them. The stubs were dried inside a vacuum desiccator for 48 h and subsequently ADFs **1-4** samples gold coated for 120 s before imaging and ADF 1+Au(III) complex without gold coated. (Fig. S8).

Transmission electron microscopy (TEM)

The morphology of samples was examined using FEI TALOS 200S instrument at a working voltage of 200 kV. The samples for TEM analysis were prepared by drop casting a homogeneous dilute dispersion of ADF-**1** and **1+Au(III)** complex over a carbon coated 400 mesh Cu grid. For sample preparation, 0.25 mg of powdered samples of ADFs **1-4** were dispersed separately in 2 mL water. 10 µL of this dispersion was drop-cast on carbon-coated copper grid and stained with 0.1% phosphotungstic acid for 20 s. Samples of ADF + Au(III) were similarly prepared and 10 µl of each sample was drop-cast on Cu-grid and analysis was performed without staining. High-angle annular dark-field scanning transmission electron microscopy (HAADF-STEM) energy dispersive X-ray spectroscopy (EDS) mapping was carried out at an accelerating voltage of 200 kV (Fig. S8).

Powder X -ray diffraction (PXRD)

The dried powdered samples were put on a sample holder and diffraction data was collected in a 2θ range of 5-60º. Diffraction patterns for dried ADF **1** and the wine red colored flocculates of **1+Au(III)** in water were recorded in a 2θ range of 5-60° (degree).

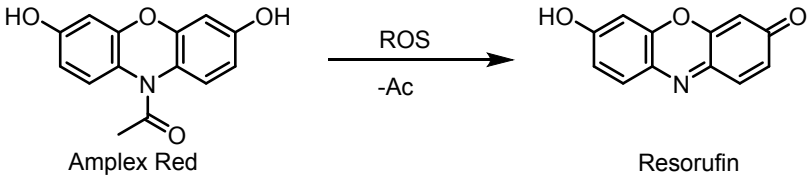
Reactive Oxygen Species: Investigation from 1+Au(III) Complex

H_2DCFDA Assay[1,2]

To assess the generation of ROS from the **1+Au(III)** complex H_2DCFDA molecule was used. It converts to its fluorescent form (DCF) in the presence of ROS such as H_2O_2. Therefore, 20 µM (stock solution = 1 mM) solution of H_2DCFDA in methanol was mixed with 1 mL of 0.0075 mM **1+Au(III)** complex in a glass vial (5 mL, vial size) protected from light and stirred for 10 min at room temperature. The resulting solution contains 20 µM H_2DCFDA. In a quartz cuvette, 500 µL of the 20 µM H_2DCFDA were mixed and UV was recorded (Fig. S9).

Quantification of ROS (H_2O_2) by Amplex Red Assay [3,4]

H_2O_2 production from **1+Au(III)** complex was measured by the Amplex Red assay. The absorbance was recorded from 200 to 700 nm wavelength on a Specord 210 spectrometer. The slope of the increase in absorbance at 572 nm is converted to the rate of H_2O_2 production with a standard curve. Complete titrations were performed at 22 °C. The similar titration was performed with heavy water (D_2O). The activation of amplex red in presence of ROS can be



Scheme S1: Schematic showing the activation of Amplex red in presence of ROS.

seen in synthetic scheme 1.

Hydroxyl Radical (•OH) Analysis: DMSO Oxidation

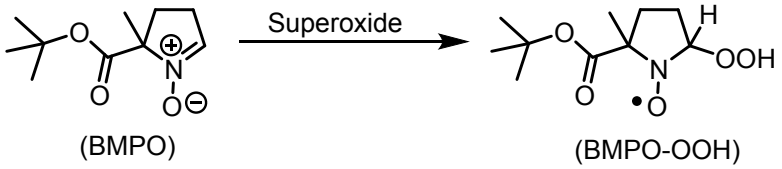
The *in situ* generated •OH by the action of **1+Au(III)** on DMSO, were analysed by LCMS. The oxidation of DMSO produces many intermediates (Fig. S12a top panel). The *in situ* produced methanesulfinic acid (DMS), the reaction product of dimethyl sulfoxide (DMSO) oxidation by the •OH, has been studied by LC-MS which is useful tool for the detection of hydroxyl radical. The **1+Au(III)** complex (500 μL, 0.75 μM) was treated with DMSO for 30 min and direct injected into HPLC for the analysis of oxidised products[6,7].

Electron Paramagnetic Resonance (EPR) of Hydroxyl Radicals

EPR spectra were recorded at 180 K on a Bruker EMXMicroX continuous wave X-band spectrometer operating at a frequency of 9.4 GHz. The modulation frequency and modulation amplitude were 100 KHz and 2.0 G, respectively. The unfilled clean tubes were checked for the absence of any EPR activity prior to loading the samples. EPR experiment was performed with Au(III), **1** and **1+Au(III)** in water. The EPR tube was evacuated and backfilled with argon and then water was added to the mixture. The reaction was performed with mixture of ADF-**1** (0.75 μM) and $HAuCl_4$ (0.75 μM) in water (0.5 mL) at 20 °C for 0.5 h. The solution was then added to a tube and examined by using EPR analysis at RT. EPR spectra for pure **1** and $HAuCl_4$ (0.75 μM, 0.5 mL, water) were also recorded separately for comparison.

Superoxide (O_2^-) Generation Detection by EPR with BMPO

The formed superoxide (O_2^-) from **1+Au(III)** complex in H_2O was detected by using 5-tert-butoxycarbonyl 5-methyl-1-pyrroline N-oxide (BMPO) is a nitrone spin trap, which forms distinct adducts with O_2- (BNPO-O_2^-) with a long half-life ($t_{1/2}$) of 23-25 minutes[8,9] (Scheme



Scheme S2: BMPO spin-trapping for superoxide (O_2) in the presence of H_2O. (BMPO = 5-tertbutoxycarbonyl-5-methyl-1-pyrroline N-oxide).

S2, Fig. S12).

Cell Culture: Cell imaging with H_2DCFDA

Cells were seeded into the 6-well tissue culture plate in the presence of 500 µL presence of complete DMEM media, incubated for 24 h and then treated with **1**, **Au(III)** and **1+Au(III)** at 0.75 µM concentrations and H_2DCFDA (100 nM) different time (0.5, 2, 6 h). Then cells were washed with PBS buffer and 500 µL supplemented DMEM medium was added. Over a period, cells were washed and then analysed under fluorescent microscope.

MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide] Assay: *In vitro* cytotoxicity was measured using the colorimetric methyl thiazolyl tetrazolium (MTT) assay on HeLa cells and HEK-293T cells. The cells (HEK-293T, HeLa) were seeded at 1 x 10^4 cells per well in a 96-well plates in Dulbecco's modified eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C and 5% CO_2 atmosphere for overnight and then incubated overnight for the attachment. Further for 24 h, cells in triplicates were treated with different time (0.5, 2, 6 h) and constant concentrations (0.75 µM) of the **1**+**Au(III), 1** and **Au(III)** ions. Then cells were treated with 20 µL of 5 mg/mL solution of MTT (MP Biomedicals) in 20 mM PBS, after 4 h, all of the medium including MTT solution (5 mg/mL) was aspirated from the wells. The remaining formazan crystals were dissolved in 150 µL of DMSO and the absorbance was measured at 570 nm using microplate reader. The cytotoxicity index was determined using the untreated cells as negative control (Fig. S14).

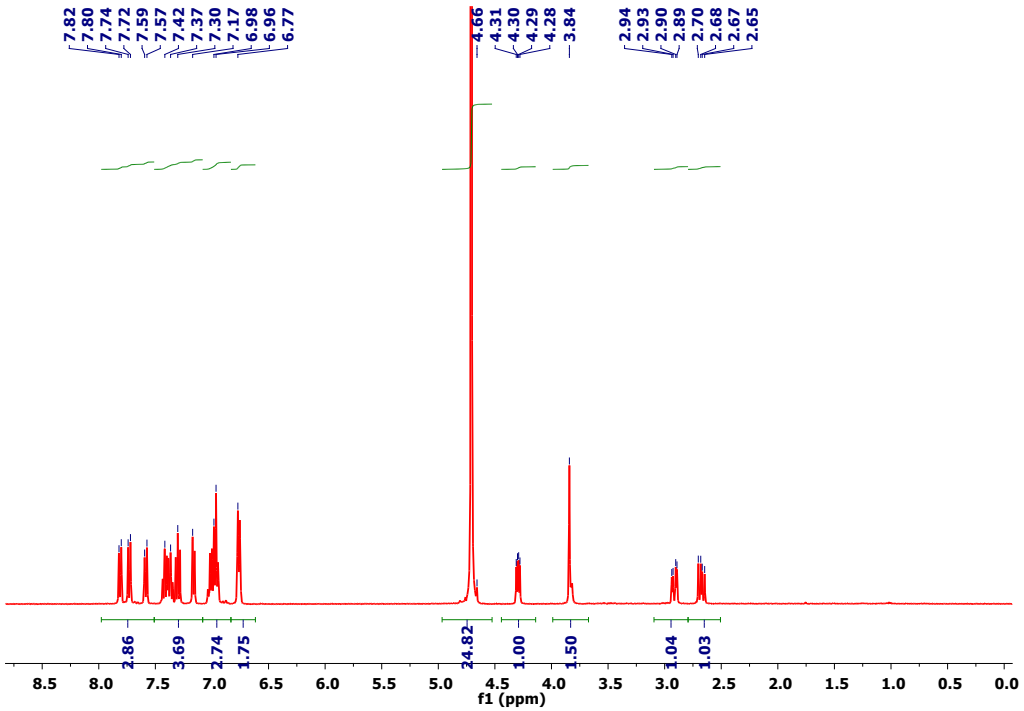


Figure S1a. ^{1}H NMR (400 MHz) of ADF-**1** in NaOD/ D_2O.

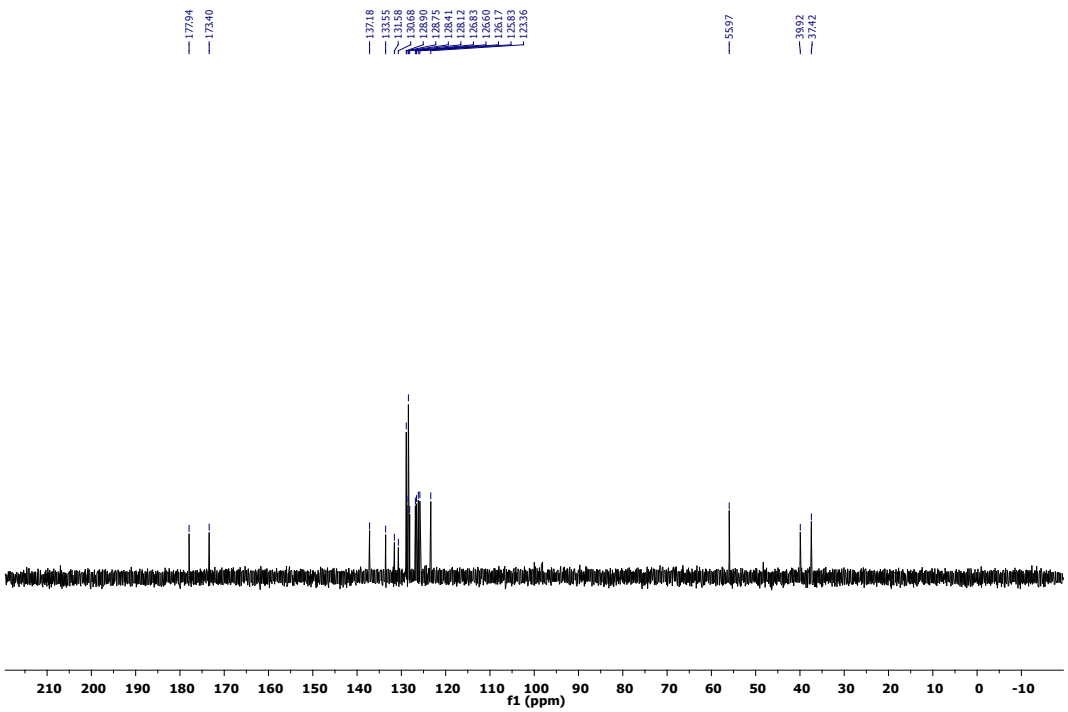


Figure S1b. ^{13}C NMR (100 MHz) of ADF-**1** in NaOD/ D_2O.

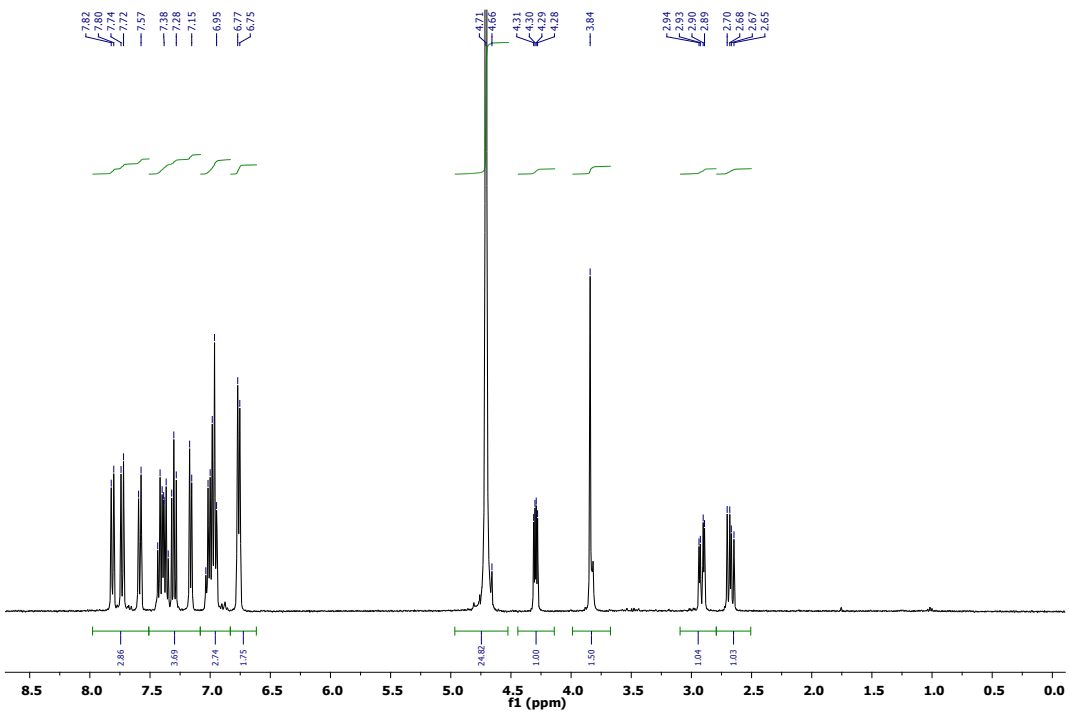


Figure S1c. ^{1}H NMR (400 MHz) of ADF-**2** in D_2O.

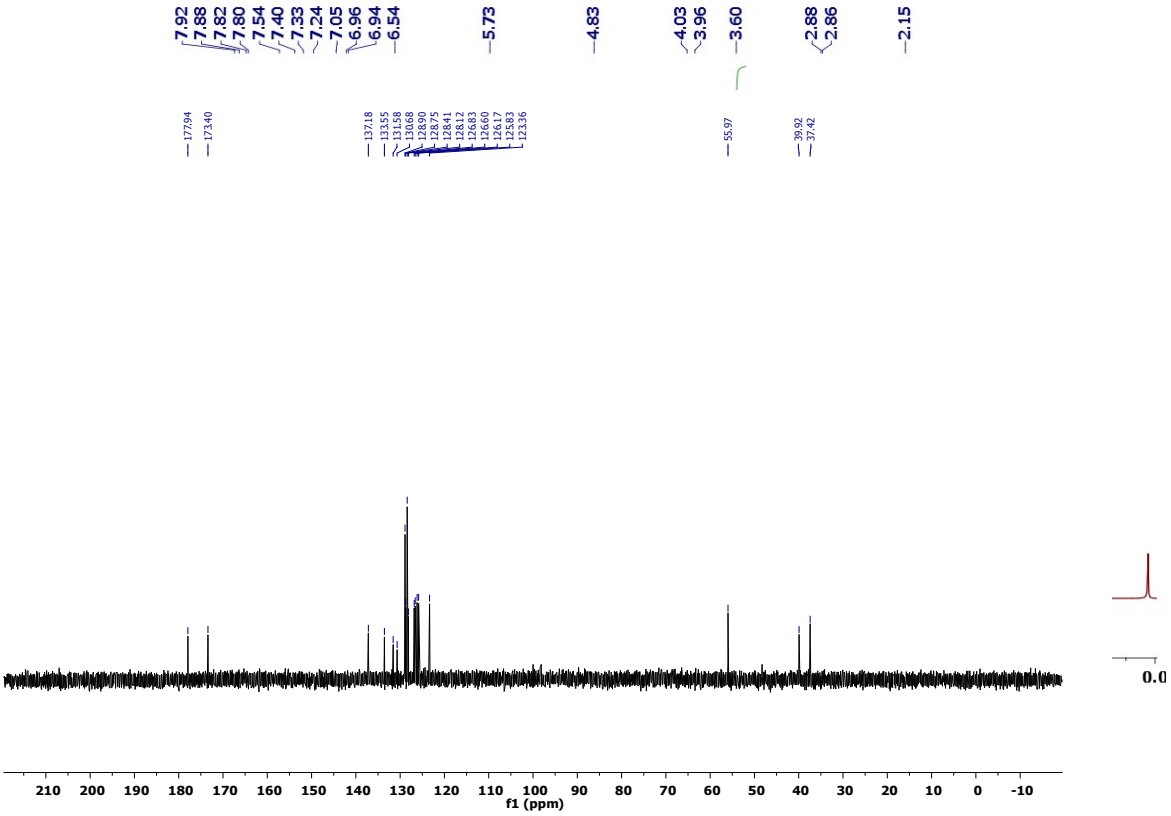


Figure S1d. ^{13}C NMR (100 MHz) of ADF-**2** in D_2O.

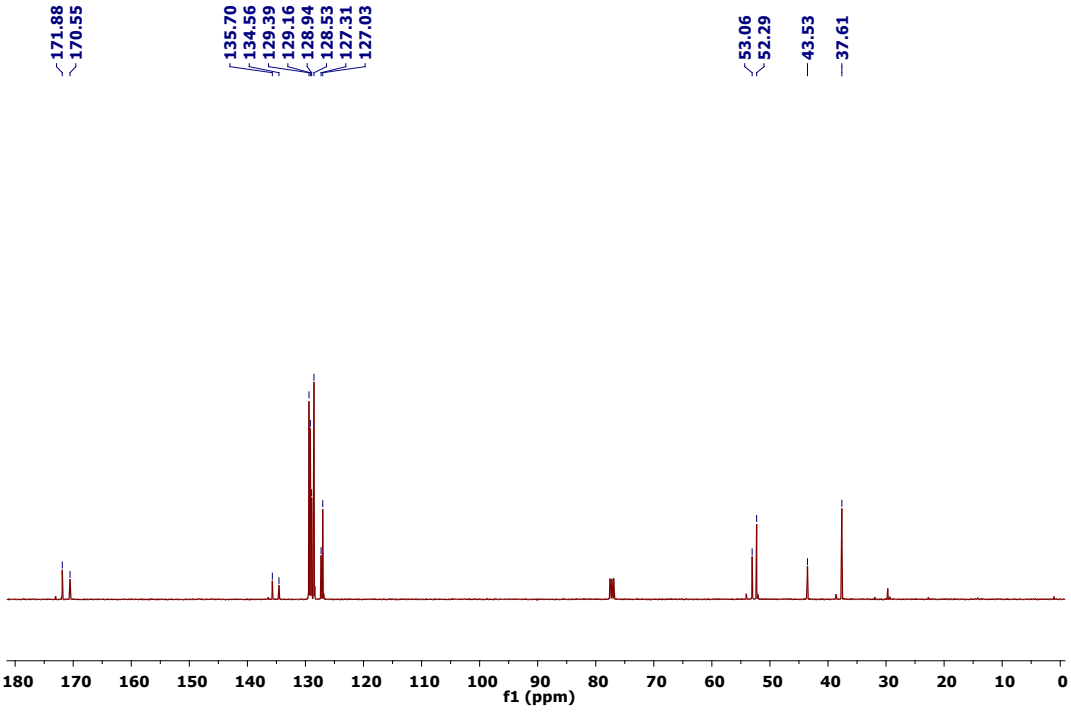


Figure S1f. ^{13}C NMR (100 MHz) spectra of ADF-**3** in DMSO-d_6.

Table S1: The specific optical rotation of **1** and **1+Au(III)**

Sample	$[\alpha]_D^{20}$
1	-27.4
1+Au(III)	+25.2
$CHCl_3$ extract of **1+Au(III)**	+24.9

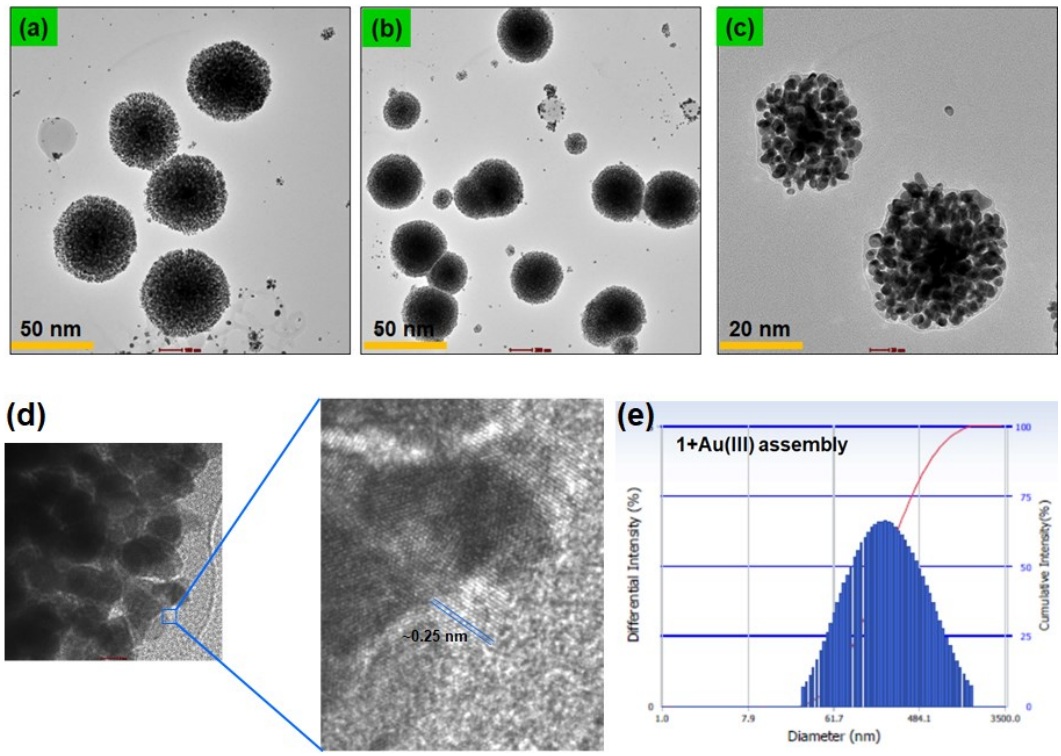


Figure S2. (a)-(d) TEM images and lattice fringes of AuNPs obtained from **1+Au(III)** in water; (e) Hydrodynamic diameter of the AuNP assemblies.

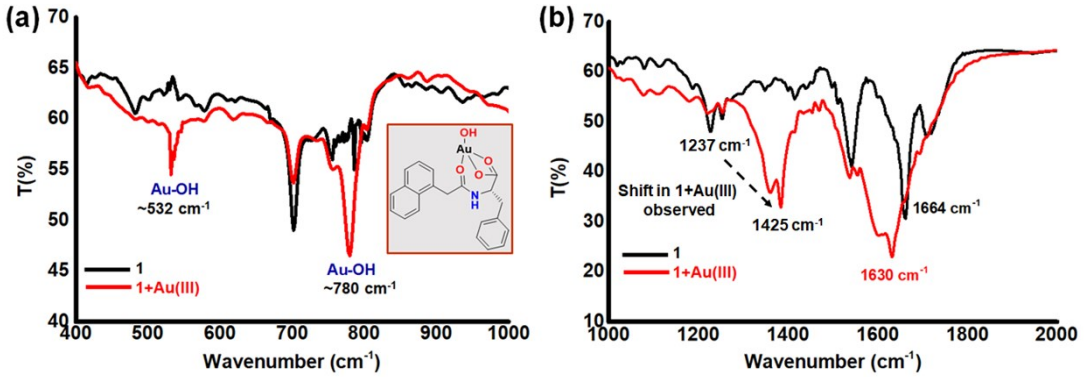


Figure S3. (a, b) FTIR spectra of **1** (black line) and **1+Au(III)** (red line). The obtained pink colored flocculates were dried and used for FT-IR analysis.

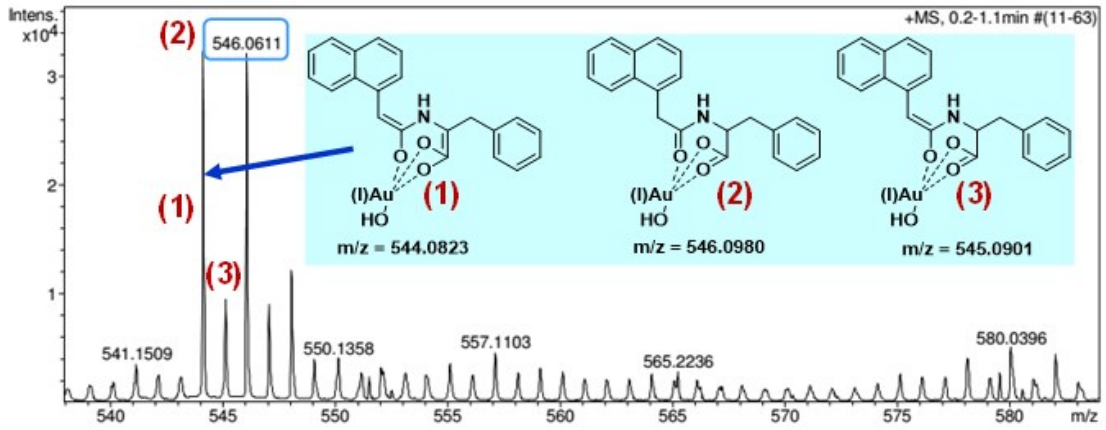


Figure S4. Characterization of **1+Au(III)** by APCI-MS.

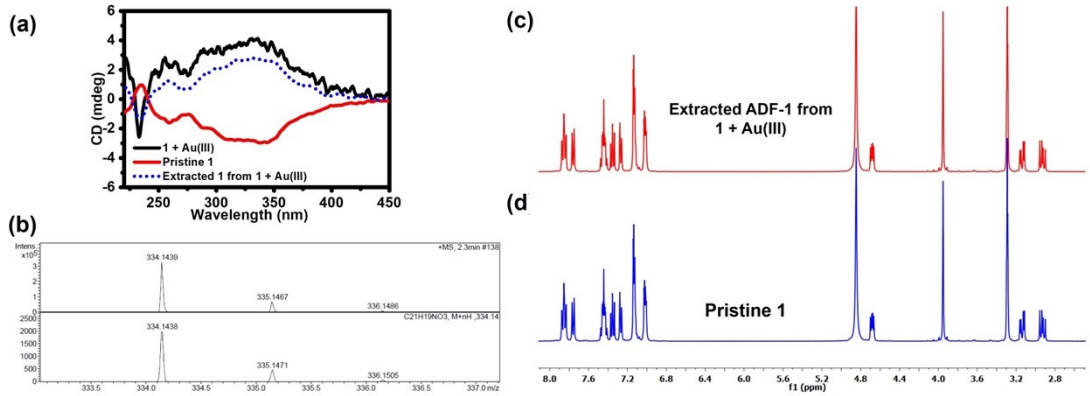


Figure S5: (a) Circular dichroism (CD) profiles in methanol for **1** (0.75 µM, red profile), for **1+Au(III)** (0.75 µM, blue solid line) and the material extracted into organic medium from **1+Au(III)** (blue solid line); (b) HRMS data for ADF-**1** extracted from **1+Au(III)** complex after AuNP synthesis; ^{1}H NMR spectra (500 MHz, $CDCl_3$) of ADF-**1** extracted after AuNP formation (b), and pristine ADF-**1** as a reference (c).

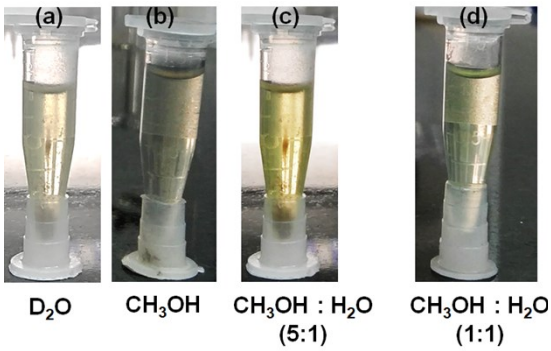


Figure S6. The effect of solvent on reduction of Au(III) in presence of ADF-**1**. (a-d) **1+Au(III)** in D_2O, CH_3OH, CH_3OH:H_2O (5:1), CH_3OH:H_2O (1:1) respectively. No pink coloration indicative of AuNP formation was observed in any of the tubes.

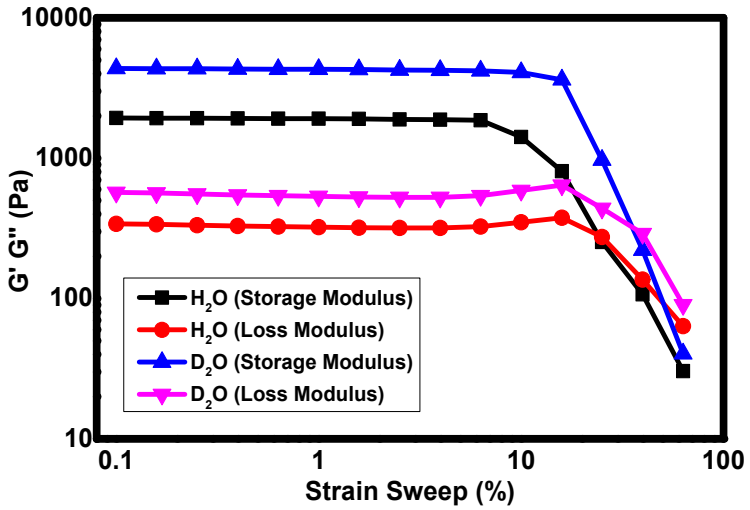


Figure S7. Comparative amplitude-sweep rheological study of the gels formed by ADF-**1** (1.5 mg/ml) in D_2O and H_2O.

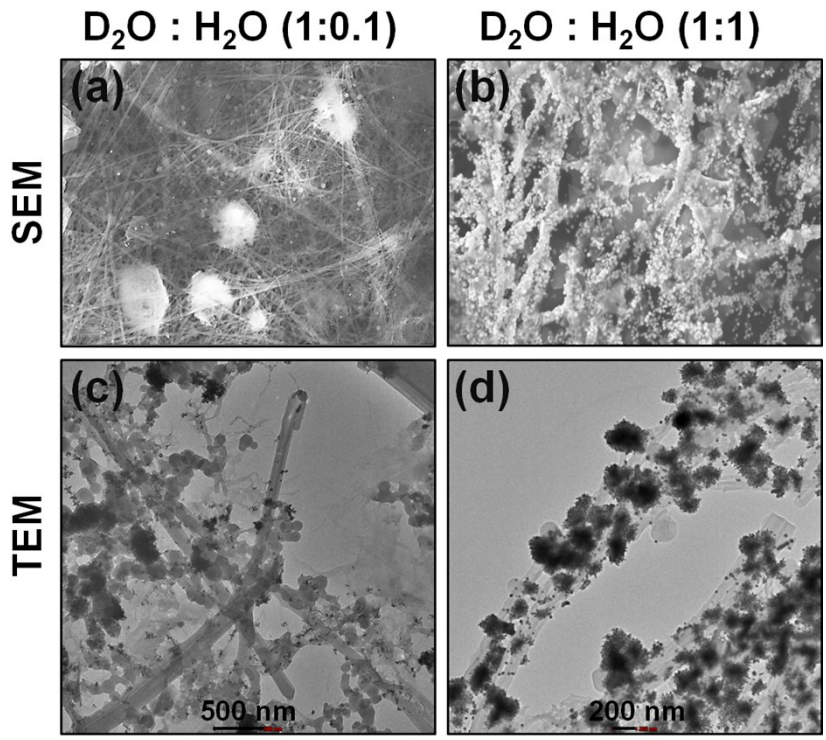


Figure S8: The morphological analysis by SEM (a, b) and TEM (c, d) of a mixture of ADF-**1** and Au(III) ions in different D_2O-water solvent systems. Scale bar = 50 μm for FE-SEM.

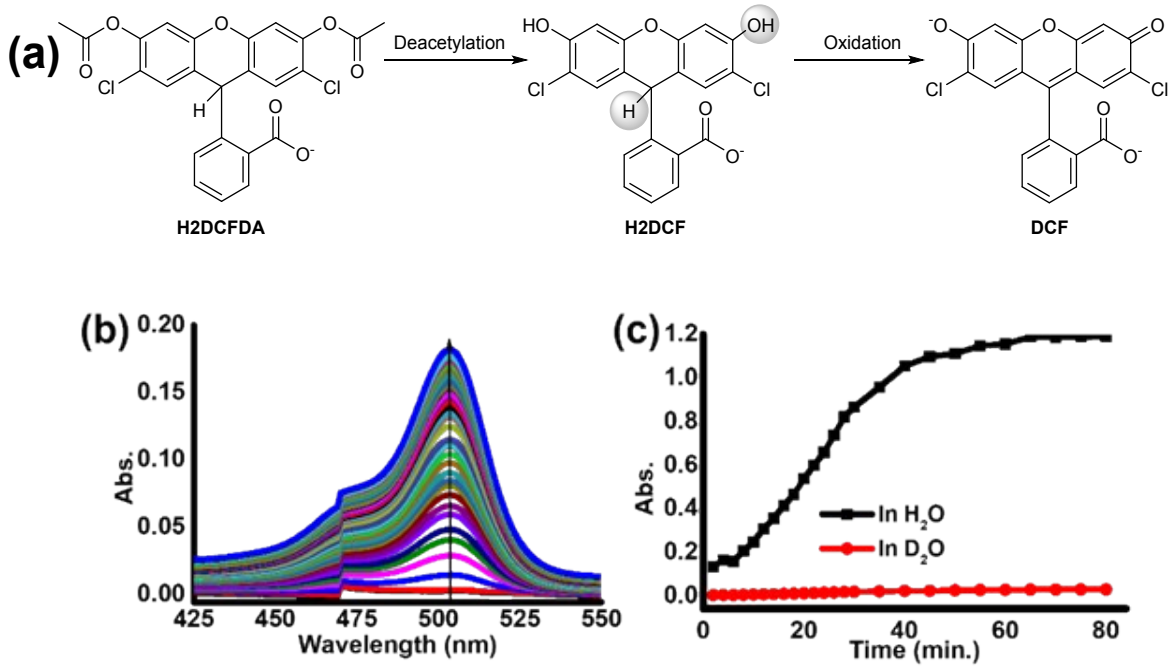


Figure S9 (a) The activation of H_2DCFDA to DCF in presence of reactive oxygen species (ROS); (b) UV-vis absorption spectra recorded for aqueous solution containing **1+Au(III)** (100 μM) and 100 μM H_2DCFDA; (c) Time-dependent increase in absorption at 500 nm corresponding to the conversion of H_2DCFDA to DCF by **1+Au(III)** in water (black line) and D_2O (red line).

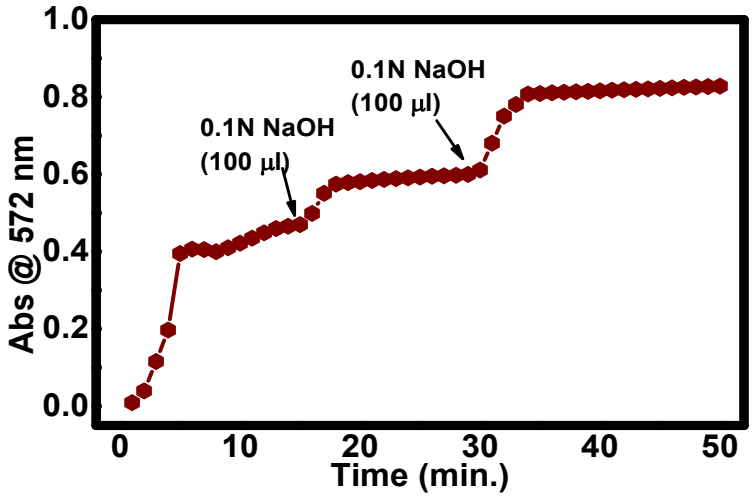


Figure S10: Generation of H_2O_2 (as followed by the conversion of Amplex Red to Resorufin) from **1+Au(III)** in water on addition of 0.1 N NaOH (100 µM) each time.

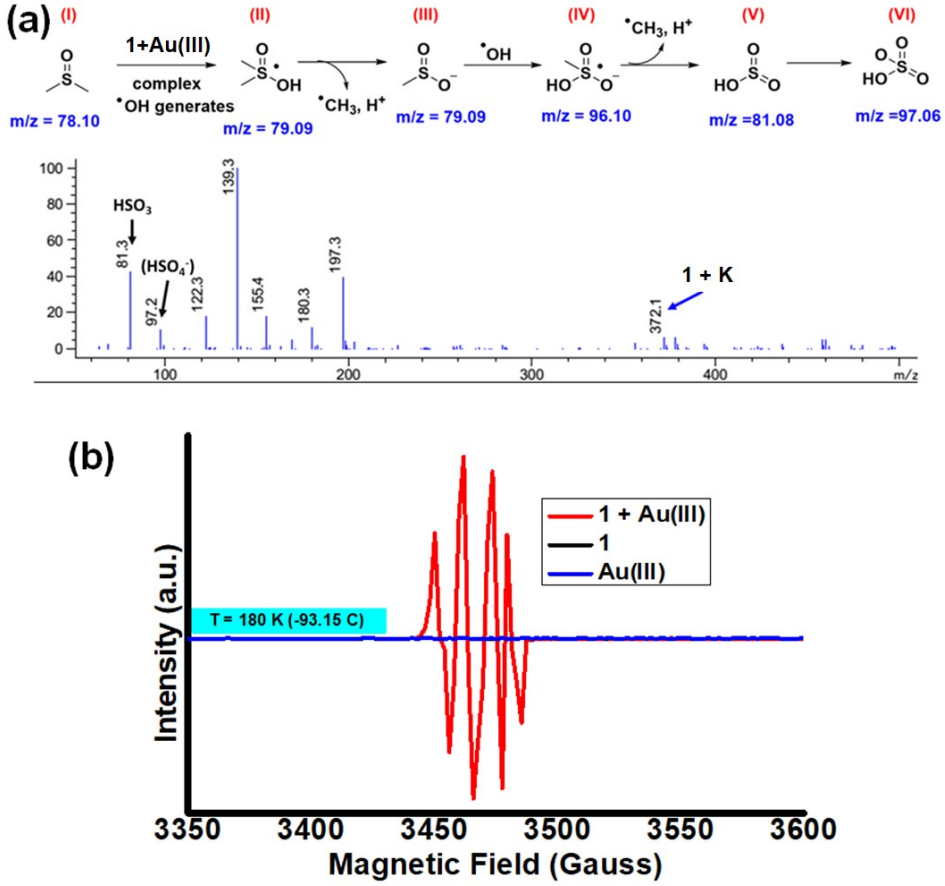


Figure S11. (a) Oxidation of DMSO by hydroxyl radical generates a variety of sulfur species that can be characterized with by LC-MS[6]; (b) EPR spectra of **1+Au(III)** (red line), ADF-**1** (black line) and only **Au(III)** (blue line) obtained at 180 K temperature.

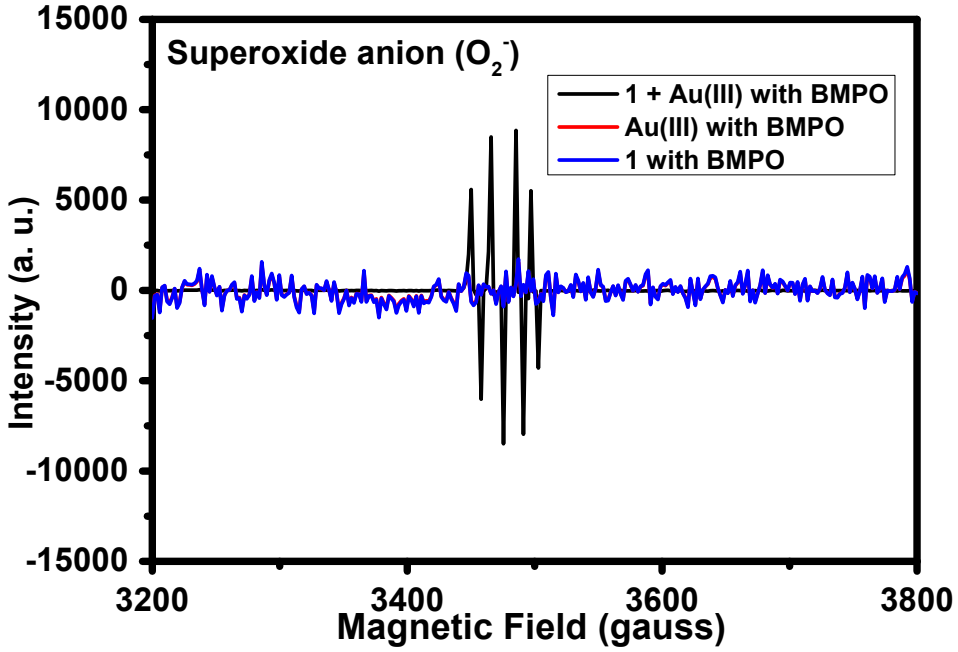


Figure S12. X-band EPR spectra of **1**+**Au(III)** (black line), ADF-**1** (blue line) and only Au(III) (red line) obtained with BMPO indicating the presence of superoxide anions.

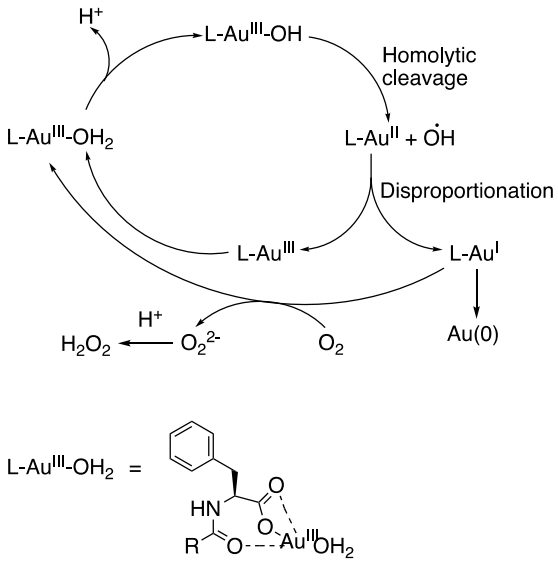


Figure S13. The proposed catalytic cycle for reduction of **Au(III)** ions by water in presence of ADF-**1** (L).

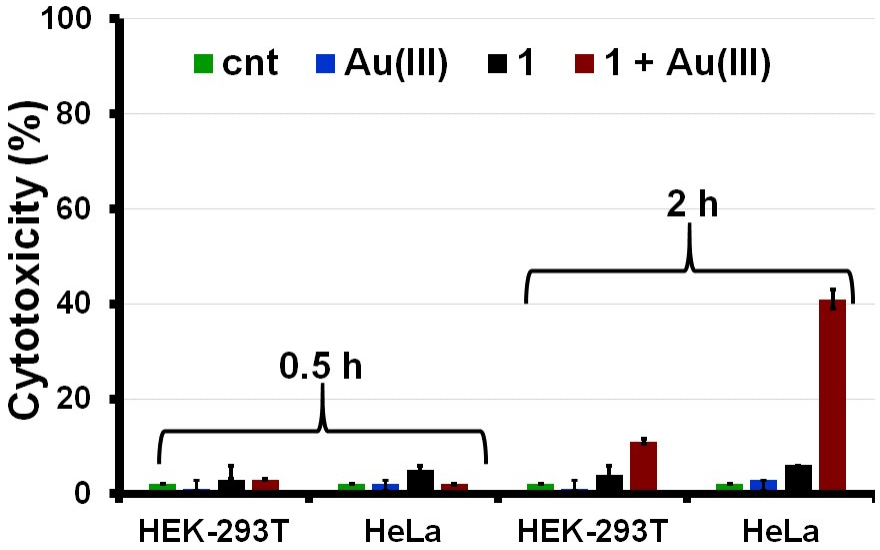


Figure S14. Cell viability of HEK-293T and HeLa cells upon exposure for 0.5 h and 2 h to **1** and Au(III) independently, and as a pre-formed mixture, **1+Au(III)**.

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