Electronic Supplementary Material (ESI) for RSC Advances.

SUPPORTING INFORMATION

Red-Emitting, EtTP-5-Based Organic Nanoprobes for Two-Photon Imaging in 3D Multicellular Biological Models

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Contents:

Acronyms:

σ_2	Two-photon action cross section
2P	Two-Photon
3D	Three-Dimensional
BCP	Block CoPolymer
EtTP-5	2,2,10,10-Tetraethyl-6,14- bis(triisopropylsilylethynyl)-1,3,9,11-tetraoxadicyclopenta [b,m]- pentacene
FNP	Flash NanoPrecipitation
MCS	MultiCellular Spheroid
NIR	Near Infra-Red
NP	NanoProbe
PEG	Poly(Ethylene Glycol)
PS	Poly(Styrene)
TPEM	Two-Photon Excited Microscopy
ZP	Zeta Potential

Materials and Experimental Procedures

Materials

Tetrahydrofuran (THF, HPLC grade), toluene (reagent grade) and sodium chloride (99.5%) were purchased from Fisher Scientific. RPMI media, DMEM F12 media, DMEM media, B27, EGF, penicillin, streptomycin, 10 mM phosphate buffered saline (PBS) and fetal bovine serum were purchased from Life Technologies. Rubrene (99.9%), Rhodamine B, insulin, oestradiol, iodine (99.8%), potassium iodide (99.5%) and neutral buffered formalin solution (10%) were purchased from Sigma Aldrich. MCF7, HCT116 and MDA-MB231 cells were purchased from ATCC. A sample of synthetic EtTP-5 was provided by the group of J Anthony.[1] Polystyrene (PS) 1.6k, and polystyrene-polyethylene glycol (PS-*b*-PEG) 1.6k-*b*-5.0k, were purchased from Polymer Source, Inc.. Deionized water (DI water) 18.2 MΩ·cm was generated using a NANOpure Diamond UV water system (Barnstead International). Phosphotungstic acid stain was provided by the Centre de Microscopie Electronique Appliquée à la Biologie (CMEAB) at the Paul Sabatier University (Toulouse).

Formation and Characterization of Nanoprobes

Nanoprobes were formed *via* Flash NanoPreciptation (FNP) using a multi-inlet vortex mixer (MIVM). A THF stream containing 7 mg/mL PS 1.6k, 28 mg/mL PS-*b*-PEG 1.6k-*b*-5.0k and 0.3 mg/mL EtTP-5 was pumped into a MIVM at a flow rate of 42 mL/min along with three DI water streams with a combined flow rate of 168 mL/min using a syringe pump (Harvard Apparatus, PHD 2000 programmable). To remove the THF after NP formation, samples were dialyzed against DI water using a Spectra/Por® regenerated cellulose membrane with a molecular weight cut off of 6-8 kD. To sterilize the NPs, the solution was filtered with a 0.22 µm sterile syringe filter (PALL) in a sterile hood. NP size was determined *via* dynamic light scattering (DLS) using a Zetasizer Nano-ZS equipped with a green laser (Malvern Instruments). The reported particle size is the volume weighted diameter as determined by the Malvern deconvolution software. Zeta potential measurements were done on NPs in a 3 mM NaCl solution using the aforementioned Zetasizer Nano-ZS. NP concentration was determined *via* thermogravimetric analysis (TGA) using a Perkin Elmer Diamond TG/DTA. TEM samples were stained with phosphotungstic acid and imaged on a Hitachi HT-7700. TEM images were analyzed with ImageJ. To determine the NP

PEG surface density, the Baleux assay was performed as previously described by D'Addio *et al*.[2]

Photophysical Characterization

The steady state absorption and emission spectra of the EtTP-5 dye in solution and nanoprobe form was measured on a Xenius XM spectrofluorometer (SAFAS). The relative fluorescence quantum yields of the EtTP-5 solution and nanoprobes were measured using rubrene in toluene as a reference (ϕ = 98%).[3] The two-photon excitation spectra were obtained by upconverted fluorescence measurements using a Ti:sapphire femtosecond laser Insight DS with pulse width <120 fs and a repetition rate of 80 MHz (Spectra-Physics). The excitation beam was collimated over the cell length (10 mm) and the fluorescence, collected at 90° of the excitation beam, was focused into an optical fiber connected to a spectrometer. The incident beam intensity was adjusted to ensure an intensity-squared dependence of the fluorescence over the whole spectral range investigated. Calibration of the spectra was performed by comparison with the published rhodamine B TPA spectrum.[4]

Photostability studies

The photostability of EtTP-5 in solution and EtTP-5 in NP form was determined by continuously illuminating a stirred EtTP-5 THF solution or a NP solution for a desired time at either 633 nm (132 μW) in mono-photon mode or 850 nm in two-photon mode (27 mW) using a Zeiss LSM 510 NLO microscope equipped with a helium-neon laser and a femto-second pulsed Chameleon laser from Coherent. The laser power was the same as that used for cell imaging. Sample fluorescence was then measured on a Xenius XM spectrofluorometer (SAFAS).

2D and 3D Cell Culture and Imaging

Monolayer MCF7 cells were cultured in RPMI media containing 10% fetal bovine serum, 1 μM insulin and 1% of penicillin/streptomycin at 37 °C with 5% CO_2 in a humid environment. To form spheroids, MCF7 cells were deposited into MicroTissue® devices as described by Napolitano *et al*.[5] and incubated in DMEM F12 media with 5% B27, 5% EGF, 1 μM insulin, 10 nM oestradiol and 1% of penicillin/streptomycin at 37 °C with 5% CO_2 in a humid environment for 5 days.

Monolayer cells and MCS were incubated with a solution of 0.5 mg/mL EtTP-5 NPs in DMEM media with 10% fetal bovine serum and 1% of penicillin/streptomycin for 3 or 24 hours. The cells and MCS were subsequently washed three times with PBS and then stained prior to imaging. All imaging was performed on a Zeiss LSM 510 NLO microscope equipped with a helium-neon laser and a femto-second pulsed Chameleon laser from Coherent.

Cytotoxicity evaluation

The EtTP-5 NP cytotoxicity was evaluated using the Annexin V apoptosis detection assay (Invitrogen). MCF7 cells were incubated with solutions of 0, 0.05, 0.1, 0.5, 0.75 or 1 mg/mL EtTP-5 NPs in DMEM media with 10% fetal bovine serum and 1% of penicillin/streptomycin for 24 hours in 12 well plates. Cells were washed three times with PBS and then with the annexin-binding buffer prior to incubation with the annexin V conjugate. Subsequently, cells were analyzed *via* flow cytometry (Accuri C6, BD Biosciences).

Further Nanoprobe Size Characterization

The nanoprobe (NP) size, distribution and surface charge was further characterized *via* scanning ion occlusion sensing (SIOS), a novel technique for characterization of nanoparticle size and charge,[6] using a qNano (Izon Science Ltd.). The mean particle diameter was determined to be 55 nm (d10 = 44; d50 = 52 ;d90 = 71). The surface charge was determined to be -2.5 mV. These values are in good agreement with those determined via DLS. We would like to thank Dr. Camille Roesch (Izon Science Ltd.) for her help with these measurements.

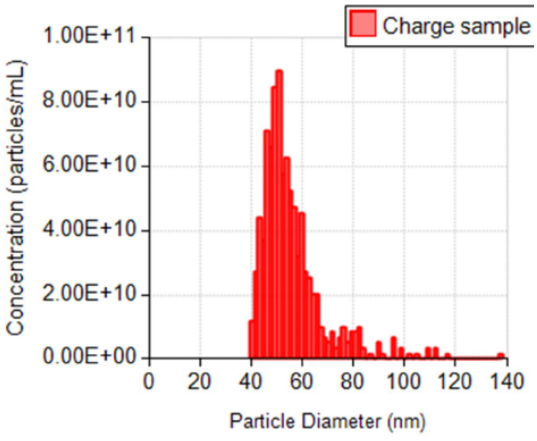


Figure S1. The particle size distribution determined by scanning ion occlusion sensing (SIOS) is shown.

Nanoprobe Stability in Culture Media

A common problem with NPs is their instability in biological media due to the presence of salt and protein in solution. Proteins can adsorb onto the NP surface, causing aggregation. EtTP-5 NPs were incubated in DMEM cell culture media with 10% fetal bovine serum at 37 ^{o}C and their stability was monitored over 24 hours by eye and DLS. No precipitates were observed over time. The NP size increased minimally by 7 ± 4% after 24 hours of incubation.

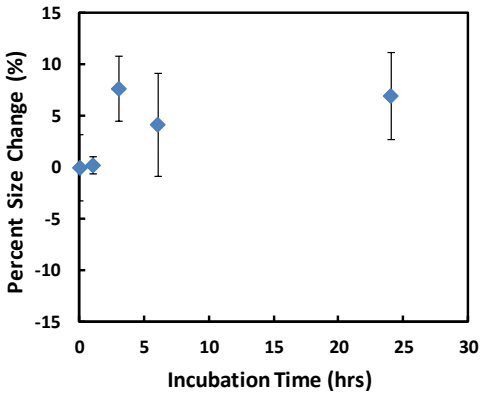
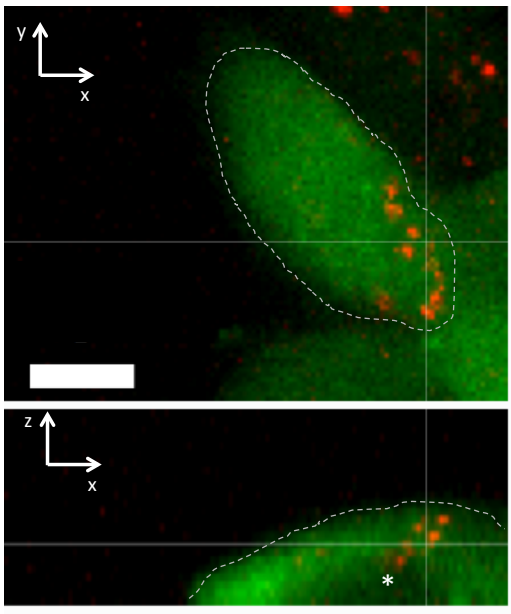


Figure S2. Percent change in nanoprobe (NP) size relative to the starting size as a function of NP incubation in DMEM cell culture media with 10% fetal bovine serum (n = 3).

3D Imaging of Multi-Cellular Tumor Spheroid Incubated with EtTP-5 Nanoprobe

Figure S3.



Live MCF7 cells were incubated with EtTP-5 nanoprobes (red) and then stained with the cytoplasmic CellTracker Green CMFDA (green). Images correspond to a x,y and x,z section through one cell. This sections were obtained from a region of the 3D z-stack shown in supplementary movie 1. The red dots correspond to the EtTP-5 NPs that have penetrated within the cytoplasm of the cell delineated by the dotted gray line. The non-fluorescent region at the bottom of the x,z image corresponds to the nucleus of the cell (star). Scale bar: 10μm.

Supplementary Movie 1. The movie corresponds to a two-photon z-stack through live MCF7 cells incubated with EtTP-5 nanoprobes and then stained with the cytoplasmic CellTracker Green CMFDA. The red dots visualized in the cytoplasm of the cells correspond to the EtTP-5 NPs that have penetrated within cells. Images from this z- stack are shown in figure 3 d.-f.

Supplementary Movie 2. The movie corresponds to a two-photon z-stack of a MCF7 multi-cellular spheroid stably expressing histone H2B-GFP (in green) incubated with EtTP-5 NPs (in red). Images from this z-stack are shown in figure 3 g.-i. Scale bar : 50 µm.

Supplementary Movie 3. The movie corresponds to a two-photon z-stack of a live HCT116 spheroid incubated with EtTP-5 NPs (in red) and then stained with the CellTracker Green CMFDA (in green).The CellTracker Green CMFDA allows to stain the outer cell layers of the spheroid. Scale bar : 100 µm.

Supplementary Movie 4. The movie corresponds to a two-photon z-stack of a MDA-MB-231 multi-cellular spheroid stably expressing histone H2B-GFP (in green) incubated with EtTP-5 NPs (in red). Scale bar : 50 µm.

References

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