Multiwalled Carbon Nanotubes for Combination Therapy: a Biodistribution and Efficacy Pilot Study

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Supporting material

1. *General information*

1.1 *Materials and Methods*

MWCNTs were purchased from Sigma-Aldrich, O.D. x L.= 6-9 nm x 5 μm, carbon > 95%, CoMoCat©, NOTA-NCS was purchased from Macrocyclics Inc. All the other reagents, whose synthesis is not described, were commercially available and have been used without any further purification, if not specified otherwise. R_f values are referred to TLC on silica gel plate (0.25 mm, Merck silica gel 60 F_{254}). NMR spectra were recorded on Varian Gemini 200 MHz or Varian Mercury 400 MHz at room temperature. Chemical shifts were reported in parts per million (ppm) relative to the residual solvent peak rounded to the nearest 0.01 for proton and 0.1 for carbon (reference: $CHCl_3$ [^{1}H:7.26, ^{13}C:77.0], DMSO [^{1}H:2.50, ^{13}C:39.7], MeOH [^{1}H:3.35, ^{13}C:49.3]. Coupling constants *J* were reported in Hz to the nearest 0.01 Hz. Peak multiplicity was indicated as follows s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and br (broad signal). IR spectra were recorded on a Perkin-Elmer FT-IR 881 or Shimadzu FT-IR 8400s or Shimadzu IRAffinity-1s spectrometer. IR data are reported as frequencies in wavenumbers (cm^{-1}). Mass spectra were recorded on a Thermoscientific LCQ-Fleet. UV-Vis spectra were recorded on Varian Cary 4000 Uv-vis spectrophotometer using 1cm cell or a BioTeK Synergy H4 microplate reader. Fluorescence spectra were registered on a Jasco FP750 spectrofluorimeter using 1 cm cell. Thermogravimetric analysis (TGA) are run under N_2 atmosphere (50 or 100 mL min^{-1}) on an EXSTAR Seiko 6200 analyzer coupled with a ThermoStarTM GSD 301T (TGA-MS) for MS gas analysis of volatiles. Elemental analyses were performed with a Thermofinnigan CHN-S Flash E1112 analyzer. ICP analysis were made using an Optima 2000 Perkin Elmer Inductively Coupled Plasma (ICP) Dual Vision instrument after acidic mineralization. TEM images were acquired at the electronic microscopic center CNR Florence (CE.M.E.) with a Philip CM12 with CRYO-GATAN UHRST 3500 technology, digital camera and EDAX microanalysis. Radio-TLCs were run on Instant Thin Layer Chromatography Medium (*i*TLC) chromatography paper (Agilent) and read on an AR-2000 radio-TLC Imaging Scanner (Bioscan Inc.). PET/CT images were recorded on a Bruker Albira PET/CT/SPECT Preclinical Imaging System and reconstructed using an iterative MLEM algorithm. Ga-68 and Cu-64 were acquired as HCl solutions from the MD Anderson Cyclotron Radiochemistry Facility (CRF).

2. *Synthetic procedures*

2.1 *Oxidation of Multiwalled Carbon Nanotubes*

Pristine nanotubes (500 mg) were dispersed in a 3:1 mixture of 95% sulfuric acid and 65% nitric acid (30 mL) and heated at 100°C under stirring for 30 minutes. The mixture was cooled at room temperature and quenched with 130 mL of milliQ water, the obtained solution was centrifuged at 1500 rcf (relative centrifugal force) for 30 minutes, the supernatant was removed, and the precipitate dispersed with milliQ water (130 mL) and centrifuged again. The CNT slurry recovered from centrifugation (still acidic) was filtered on a 0.2 µm polycarbonate membrane and washed with water until neutral pH of the filtered solution. The process provided 200 mg of oxidized material, elemental analysis: C 79.1%, N 0.15%, H 0.62% and O 20.3%. FT-IR showed the C=O absorption peak at 1700 cm^{-1}.

2.2 *Separation of Oxidized Carbon Nanotubes by length.*

Bulk oxidized CNT (140 mg) were dispersed using ultrasound bath in milliQ water (280 mL) to obtain a 0.5 mg/mL solution. The solution was centrifuged at 10k rcf for 1h and supernatant and precipitate were collected. The supernatant was centrifuged again at 15k rcf for 1h providing a new precipitate and a new supernatant. The procedure was repeated using the supernatant other 3 times using different G force (20, 25 and 30 K rcf) as reported in the figure S1. The supernatant of the 30 k rcf centrifuge was dried and 65 mg of material were obtained, the length was measured with TEM (Figure S2) giving an average value of 130 nm accordingly with the literature.[3]

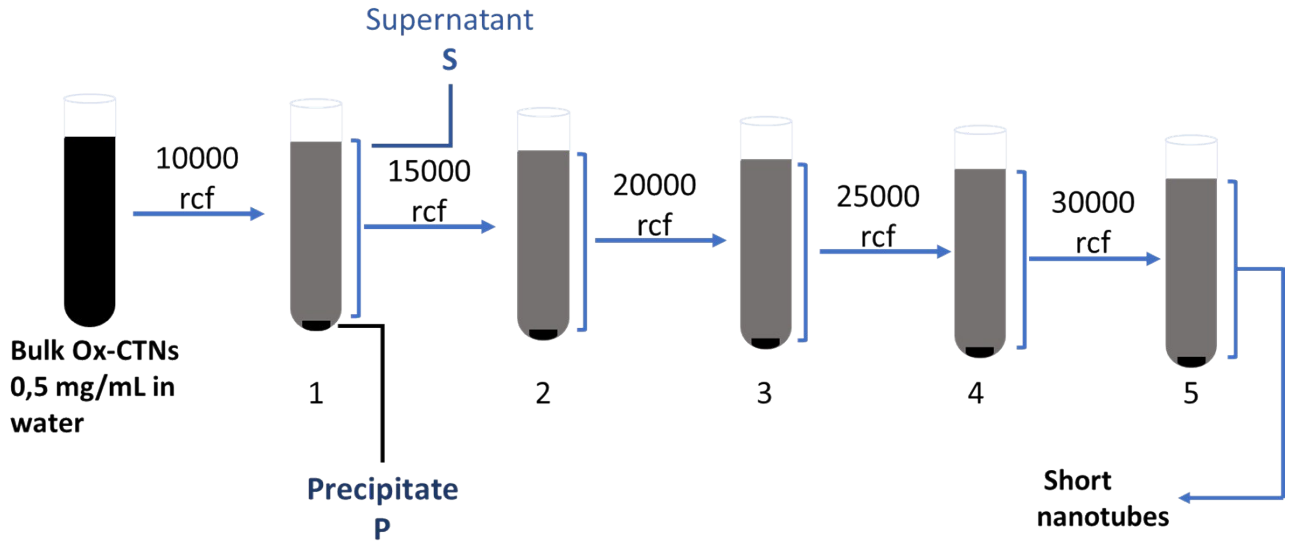


Figure S1. Schematic representation of the CNT separation procedure using centrifuge cycles.

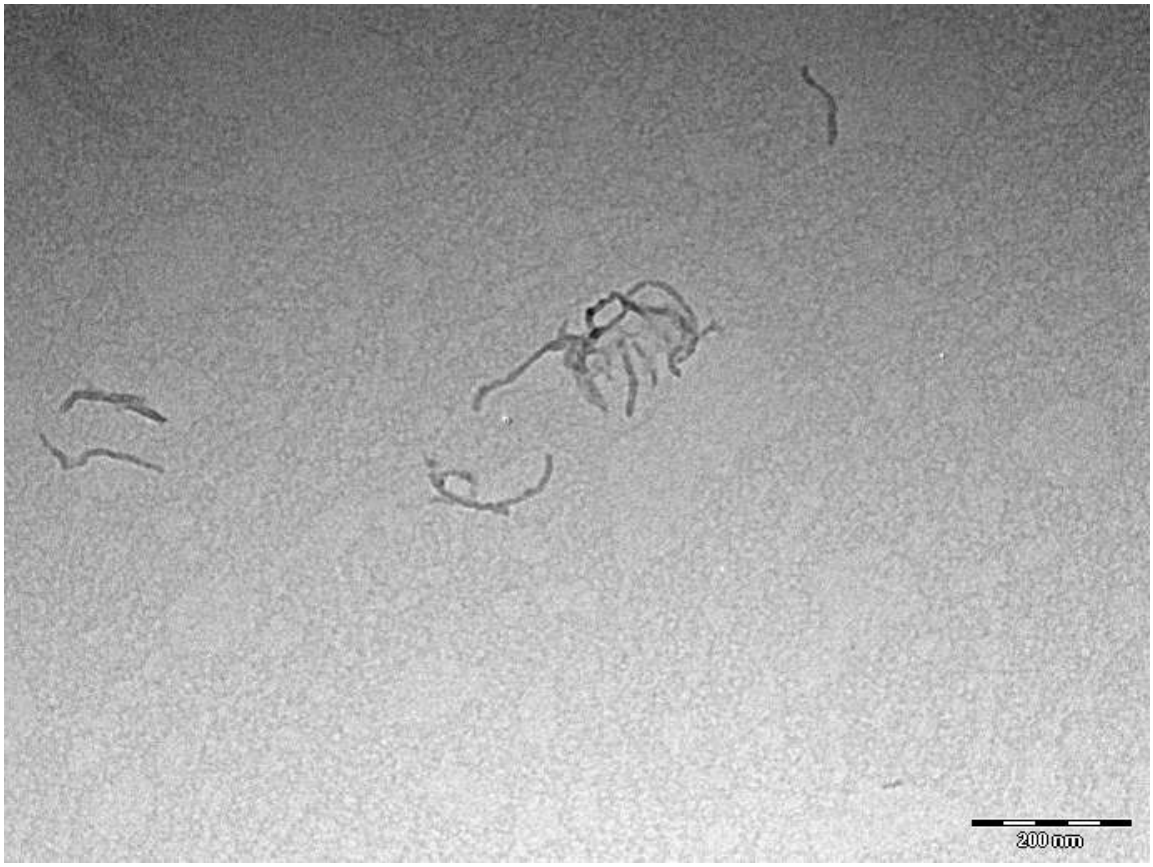
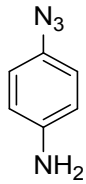


Figure S2. TEM image of short ox-MW-CNT **2**

2.3 *Synthesis of MET-CNT (3)*

Short ox-CNT (**2**) (35 mg) were dispersed in freshly distilled oxalyl chloride (10 mL) under nitrogen. Then the dispersion was stirred at reflux for 24 h, after that the oxalyl chloride was accurately removed under vacuum. The acyl chloride CNT directly dispersed in dry DMF (9.5 mL), using an ultrasound bath, metformin (170 mg) was added to the dispersion and the mixture stirred at 120 °C under nitrogen for 40 h. CNT-MET were recovered by filtration over a 0.2 μm nylon membrane washing several times with DMF and methanol to remove the unreacted metformin. Metformin loading ranged from 1.9 to 2.0 mmol/g as evaluated from elemental analysis on three different samples.

2.4 *Synthesis of 4-azidoaniline (4)*

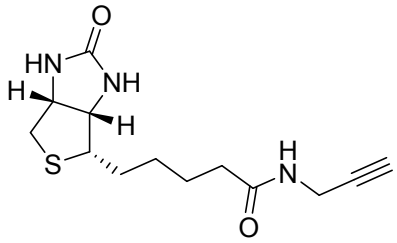


Compound **4** was synthesized from 4-bromoaniline in quantitative yield following a procedure reported in the literature.[4]

2.5 *Synthesis of MET-N_3-CNT (5) (Tour reaction)*

Material **3** (20 mg) was dispersed in dry DMF (2 mL) and sonicated for 15 minutes, then 4-azidoaniline (61.5 mg, 0.46 mmol) and isoamylnitrite (38.6 mg, 0.33 mmol) were added and the mixture stirred at 60°C under nitrogen for 24 h. CNT **5** was recovered by filtration on a 0.2 μm nylon membrane, washing with DMF until a colorless solution obtained and then with methanol to remove the unreacted materials. FT-IR peak at 2100 cm^{-1} confirmed the azide decoration (figure S22).

2.6 *Synthesis of biotin propargylamide (6)*

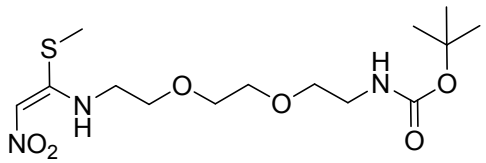


Compound **6** was synthesized accordingly to a procedure reported in literature with a yield of 90%.[5]

2.7 *Synthesis of MET-B-CNT (7)*

Compound **7** was synthesized from material **5** accordingly to a procedure previously reported. [5] The biotin content was evaluated measuring the sulfur content via ICP-AES, biotin was found to be 0.210 to 0.277 mmol/g on different samples.

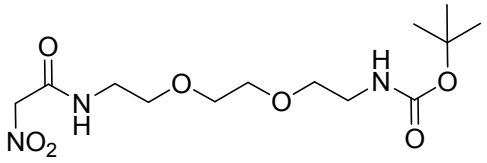
2.8 *Synthesis (E)-tert-butyl (3-(nitromethylene)-7,10-dioxa-2-thia-4-azadodecan-12-yl)carbamate*



1,1-*bis*(methylthio)-2-nitroethylene (798.4 mg, 4.83 mmol, 1.2 eq) and *p*-toluensulfonic acid monohydrate (34.7 mg, 0.202 mmol, 0.05 eq) were dissolved in 40 mL of ethanol and heated at 35 °C. The solution was added with tert-butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate (1,00 g, 4.03 mmol, 1 eq) dissolved in 13 mL of ethanol and the mixture stirred at 35 °C for 72 h. The resulting methyl thiol, developed during the reaction, was removed with a nitrogen flow bubbling through bleach. The solvent was, finally, removed under vacuum and the product recovered after flash chromatography on silica gel. R_f = 0.35 (dichloromethane/ethyl acetate 1:1). ^{1}H-NMR (200 MHz, $CDCl_3$): δ 10.6 (bs, 1 H, N*H*), 6.57 (s, 1 H, C*H*NO_2), 5.5 (bs, 1 H, N*H*Boc), 3.69 - 3.51 (m, 10 H, 5 x

CH_2), 3.30 (q, J = 6.3 Hz, 2 H, CH_2NHBoc), 2.44 (s, 3 H, CH_3S), 1.42 (s, 9 H, (CH_3)$_3$C) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ 156.0 (s, *C*O_2*t*Bu), 106.6 (d, *C*HNO_2), 79.1 (s, $(CH_3)_3$*C*), 70.7 (t, *C*H_2O), 70.3 (t, *C*H_2O), 70.2 (t, *C*H_2O), 68.6 (t, *C*H_2O), 44.2 (t, *C*H_2NH), 40.4 (t, *C*H_2NHBoc), 28.4 (q, 3 C, (*C*H_3)$_3$C), 14.4 ppm (q, *C*H_3S), =C-S carbon atom was not detected,. MS-ESI: 388 $[M + Na]^+$. HRMS-ESI: calculated for $C_{14}H_{27}N_3O_6S$ 366.16933 $[M + H]^+$, positive ionization measured 366.16973 ± 0.0001 $[M+H]^+$.

2.9 ***Synthesis of tert-butyl (2-(2-(2-(2-nitroacetamido)ethoxy)ethoxy)ethyl)carbamate (9)***



Compound **9** was synthesized accordingly to the literature with minor changes.[6] (E)-tert-butyl (3-(nitromethylene)-7,10-dioxa-2-thia-4-azadodecan-12-yl)carbamate (256 mg, 0.7 mmol, 1 eq) was dissolved in 700 µL of a 3 : 1 acetonitrile water solution and heated at 30°C. Then a solution of mercury chloride (190.19 mg, 0.70 mmol, 1 eq, 1 mL of CH_3CN) was added dropwise and the mixture stirred at 30 °C for 48 h. The reaction mixture was than filtered over celite to remove the solid mercury salts and the product recovered after flash chromatography on silica gel. R_f = 0.32 (ethyl acetate/dichloromethane 2:1). ^{1}H-NMR (400 MHz, CD_3OD): δ = 5.20 (s, 2 H, CH_2NO_2), 3.66 (s, 4 H, OCH_2CH_2O), 3.63 (t, *J* = 5.2 Hz, CH_2O), 3.56 (t, *J* = 5.6 Hz, CH_2O), 3.48 (t, *J* = 5.2 Hz, CH_2NH), 3.26 (t, *J* = 5.6 Hz, CH_2NH), 1.36 (s, 9H, *(CH$_3$)$_3$C*) ppm. ^{13}C NMR (101 MHz, CD_3OD): δ = 164.0 (s, C=O), 158.8 (s, C=O), 80.4 [s, $(CH_3)_3$*C*], 71.6 (t, *C*H_2O), 71.5 (t, *C*H_2O), 71.4 (t, *C*H_2O), 70.5 (t, *C*H_2O), 41.5 (t, *C*H_2NH), 41.1 (t, *C*H_2NH), 29.0 (q, 3 C, (*C*H_3)$_3$C) ppm. IR: 3695 (N-H), 2974 (C-H), 2931 (C-H), 2875 (CH), 1723 (s, C=O, carbamate), 1693 (s, C=O, amide), 1563 (s, N-O), 1603 (w, N-O), 1392, 1255, 1172 cm^{-1}. MS-ESI: positive ionization 358 $[M + Na]^+$, negative ionization 334 $[M - H]^-$. HRMS-ESI: calculated for $C_{13}H_{25}N_3O_7$ m/z = 358.15887 $[M + Na]^+$, positive ionization measured m/z = 358.15847 (± 0.0001) $[M + Na]^+$.

2.10 ***Synthesis of DDS 1***

The loading of DOXO was carried out following the procedure already reported.[5] For characterization see section **4.2**.

2.11 ***Synthesis of material 10***

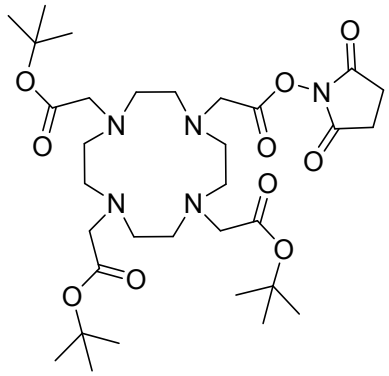
Material **7** (10 mg) and compound **9** were dispersed in a mixture of methanol and water 1:1 (1.2 mL) using an ultrasound bath (15 min). Then 12 µL of 4.24 M NaOH were added and the mixture stirred

five days at 60°C. Material **10** was recovered after filtration on a 0.2 μm PC membrane washing with water and methanol to remove all the impurities. Loading of protected amine 1.40 mmol/g based on TGA-MS section **4.5** and **6**. FT-IR peaks: 3452 and 3331 O-H and N-H stretching, 1695 doublet CO stretching of amides and carboxylic groups, 1571, 1527, 1419, 1203, 1056.

2.12 *Synthesis of material 11*

Material **10** (10 mg) was dispersed in 4 M HCl in dioxane sonicating for 5 min then the dispersion was stirred for 4 h at room temperature. The acid media was removed by precipitating the CNT in centrifuge (15 min at 1500 rcf) and pipetting the supernatant, then the material was re-dispersed in isopropanol and precipitated in centrifuge to remove any residue of HCl. Then to obtain the free amine group the material was dispersed in aqueous 0.01 M NaOH and precipitated in centrifuge using the program described above. Finally the material was washed with isopropanol to remove the NaOH solution and recovered. The amount of free amine group was evaluated with a semiquantitative Kaiser Test, section **4.4**.

2.13 *Synthesis of DOTA-OSU 15*



Compound **15** was synthesized accordingly to the literature with minor changes to the procedure.[7] Tri-*tert*-butyl ester DOTA (40 mg, 0.0698 mmol, 1eq) was dissolved in 1 mL of dry acetonitrile, then 5 mg of *N*-hydroxi-succinimide (0.039 mmol, 1.1 eq) and HBTU 14.8 mg (0.039 mmol, 1.1 mmol) were added and the mixture stirred under nitrogen for 24 h. The product purified by flash chromatography on alumina gel (ethyl acetate: acetonitrile 4:1, *Rf* 0.8). Yield 30%. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 3.6 to 2 ppm (serie of broad peaks 28H) and 1.44 ppm (s, 27H *t*-Bu).^{13}C NMR (101 MHz, $CDCl_3$) δ = 173.3 (3C, COO*t*Bu), 173.1 (COON), 169.8 (CON), 82.3 (3C, C_q), 55.9 (CH_2COON), 55.7 (3C, CH_2COO*t*Bu), 53.2 (2C, CH_2 succinimide), 38.7 (4C, CH_2N macrocycle) and 28.0 ppm (3C, CH_3 *t*Bu). ESI-MS+ m/z= 692.50 $[M+Na]^+$.

2.14 *Synthesis of DDS 12*

Material **11** and DOTA-OSU **15** in ratio 1.5:1 in weight were dispersed in dry, amine free, DMF (900 µL) under nitrogen and sonicated for 5 min. Then TEA was added, and the mixture stirred for 48 at room temperature under nitrogen. Then crude mixture was diluted with 5 mL of methanol and centrifuged for 15 min at 1500 rcf, the supernatant removed and replaced with 15 mL of fresh methanol. The procedure was repeated twice then the solid was washed with 40 mL of a 1:3 mixture of methanol/isopropanol and, finally, isopropyl ether. The products were characterized via TGA-MS. Then *t*-butyl group were removed by suspending the product in a solution of trifluoroacetic acid/acetonitrile 2:1 (10 mg/mL conc.) and stirring at room temperature for 5 h. Then the solution was diluted with acetonitrile, filtered over PTFE membrane (0.2 µm pores), and washed firstly with methanol and then with phosphate buffer solution at pH 7.4 to neutralize the residual acidity. Finally Doxorubicin was loaded as reported in point **2.1** and quantified as reported in point **4.2** and **4.6**. FT-IR: 3319 broad band, 1717 C=O stretching, 1610, 1573, 1406, 1281, 1207, 1113, 986 cm^{-1} doxorubicin fingerprints.

2.15 *Synthesis of DDS 13*

DDS **13** was synthesized with the same protocol of DDS **12** with the exceptions that NOTA-Bn-NCS (Macrocyclics inc.) was used instead of compound **15**, and on the coupling product was directly loaded the doxorubicin. For characterization see point **4.2** and **4.6**.

2.16 Synthesis of DDS 14

DDS **14** was prepared according to a previously reported protocol[3] starting from short ox-CNT. For characterization see point **4.6**.

3. *Radiochemistry*

3.1 *Formation of the ^{68}Ga-NOTA-CNTs-MET complex*

^{68}Ga eluate (567 µCi, 200 µL) was buffered at pH 5.6 with sodium acetate buffer 1 M (35 µL), then CNTs-NOTA-Met (50 µL of 1.5 mg/mL solution) was added. The mixture was kept at 37°C for 10 minutes, quenched with 380 µL of PBS. Chelated metal 100%. Radioactive yield 65% not decay corrected.

3.2 *Formation of the ^{68}Ga-NOTA-CNTs-MET-DOXO complex*

Ga-68 eluate (0.05M in HCl, 370 µCi, 100 µL) was buffered at pH 5.6 with sodium acetate buffer 1M (35 µL), then material **13 (**83 µL of 1.5 mg/mL solution) was added. The mixture was kept at 37°C for 10 minutes, quenched with PBS (380 µL) and the efficiency of the labeling monitored by

*i*TLC (PBS). [^{68}Ga]**13** was obtained with a 93.5% radiochemical purity and radioactive yield 60% (not decay corrected).

3.3 *Formation of the [^{68}Ga]DOTA-CNTs-MET-DOXO complex*

The standard complexation conditions required heating at 85°C due to the different size of Ga(III) ions respect to Cu(II),[8–10,11] a temperature not compatible with the presence of the DOXO-CNT complex. For this reason, the procedure was modified lowering the temperature. Ga-68 eluate (0.05 M in HCl, 370 μCi, 100 μL) was buffered at pH 5.6 with sodium acetate buffer 1M (35 μL), then material **12**(50 μL of 1.5 mg/mL solution) was added. The mixture was kept at 37 °C for 10 minutes and quenched with PBS (380 μL) with an efficiency of 70%. The free metal was removed in centrifuge, the CNT were precipitated, and the supernatant replaced with fresh PBS 3 times, after purification the sample reached 95% of radiochemical purity with a radioactive yield 60% not decay corrected.

3.4 *Formation of the [^{64}Cu]DOTA-CNTs-MET-DOXO complex*

Cu-64 solution (0.1M in HCl, 1 mCi, 1 μL) was buffered at pH 5.6 with sodium acetate buffer 1M (50 μL), then material **12** (20 μL of 1.5 mg/mL solution in water) was added. The mixture was kept at 37°C for 30 minutes, quenched with 430 μL of PBS. Chelated metal 93.5%. Radioactive yield 77.2% not decay corrected.

4. *Characterization of the synthesized compounds*

4.1 *General procedure for the quality control of the labeling reactions*

The quality control was accomplished via *i*TLC, 1 to 3 μL of the reaction mixture were diluted with 100 μL of EDTA 0.1 M in PBS to chelate all the free metal, a 3-5 μL drop was eluted with PBS on *i*TLC paper and the strip read with the ITLC scanner. The radiolabeled CNT stay at baseline and the free metal move with the front of the solvent. The instrument displays the results as a chromatogram (as reported in figure S4). The quantification was done integrating the area under the peaks.

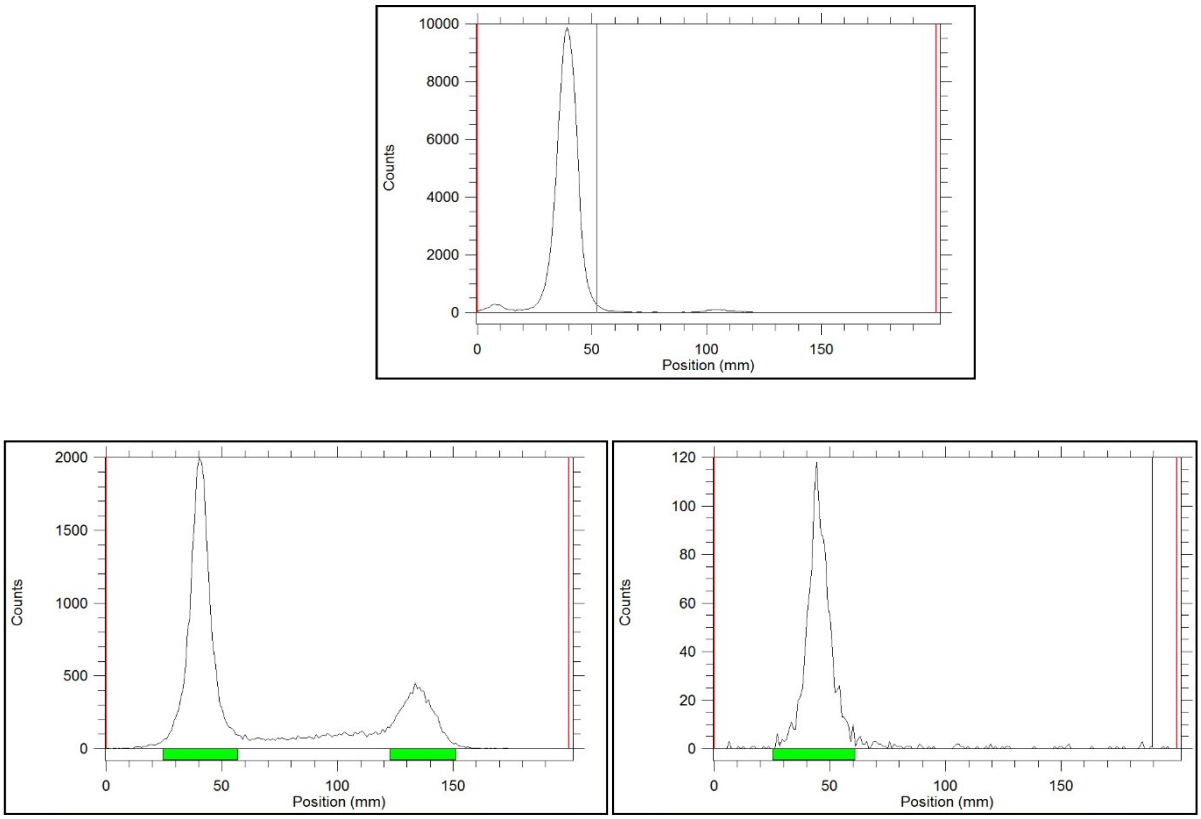


Figure S4. Quality control *i*TLC chromatograms: on the top DOTA-CNT **12** labeling with Cu-64, on the bottom DOTA-CNT **12** with Ga-68, crude mixture (left side) and after purification (right side).

4.2 *Quantification of Doxorubicin loading*

The quantification was made measuring the UV-Vis absorption (peak at 480-490 nm) of the non-loaded DOXO after work-up, and back-calculating the concentration with a calibration curve, as reported in the literature.[12–14]

The calibration curve was built reading the absorbance of five solution at different concentration as reported in the table of figure S5.

Conc. mg/mL	**Abs**
0,0015	0,024
0,006	0,093
0,012	0,183
0,024	0,353
0,048	0,714

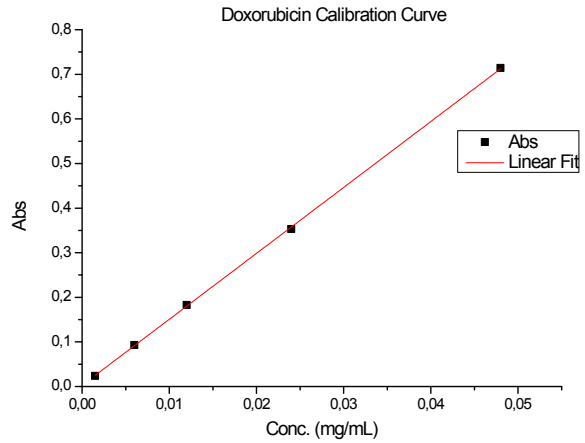


Figure S5. Calibration curve of doxorubicin used for the quantification of the loading of CNT, equation y=14.78488+0.00226, r^2=0.99985.

The solutions of work up were collected and dilute to known volume, then the absorbance was measured and the concentration back-calculated giving the amount of non-loaded Dox. The loading on the nanotube was calculated by difference between the non-loaded and the used Dox. The release of Dox from the DDS at acidic pH was evaluated as reported in ref 16 of the article with the same results.

4.3 *Doxorubicin loss during the labeling*

The loss of doxorubicin during the labeling reaction was quantified by UV-Vis absorption spectroscopy. A stock solution of CNTs-MET-B-DOXO **1** in PBS 1.5 mg/mL was prepared, then 200 µL of this solution were buffered at pH 5.6 with 400 µL of sodium acetate buffer 1M and heated at 37°C for 1 h. Aliquots of 100 µL of the reaction solution were collected after 10, 20, 30 and 60 minutes and quenched with 100 µL of PBS. The nanotubes were precipitated in centrifuge and the absorbance of the supernatant was measured.

Conc. mg/mL	Abs	STDV
0,093	0,174	0,01391
0,049	0,09	0,00589
0,025	0,044	9,43E-4
0,013	0,024	0,00309
0,006	0,013	0,00471

0.001	0	0,00455

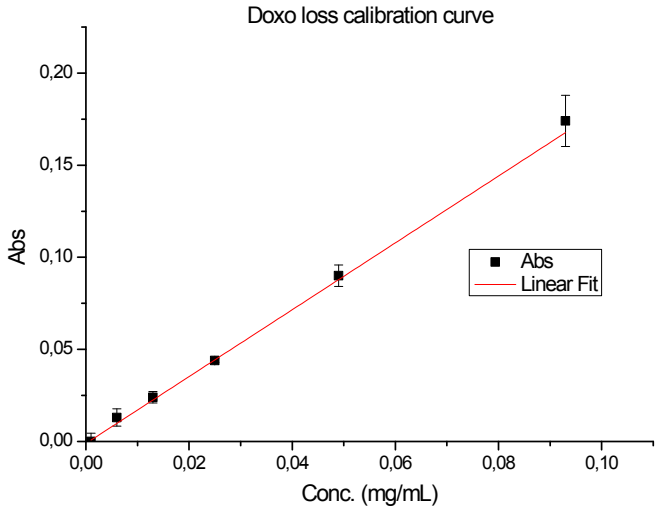
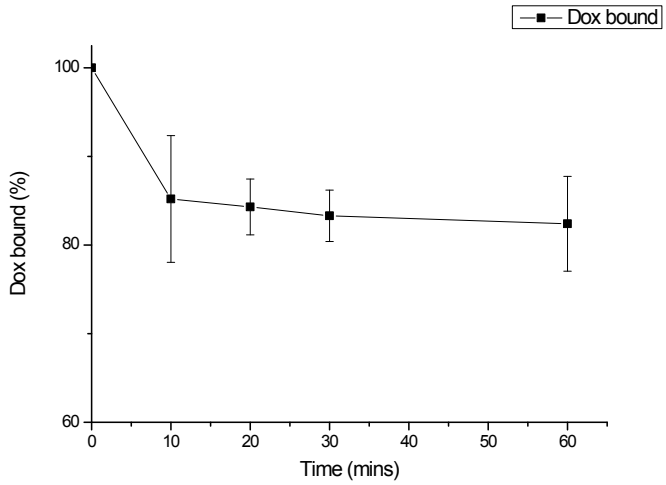


Figure S6. Doxorubicin UV-Vis calibration curve for DOXO loss experiment, equation: y=1.81497x0.00106, R^2=0.99548. Error bars, where not visible, are smaller than symbols.

DOXO loss was quantified to be around 15 % during the reaction time, this loss should not affect the biodistribution, providing a pharmacokinetic profile similar to the original DDS.



Time min	Abs	Conc.	Dox loss %
10	0,027	0,030	14,8
20	0,028	0,032	15,7
30	0,029	0,034	16.7
60	0,031	0,036	17.6

Figure S7. Doxorubicin loss at pH 5.6 and 37°C at different time point.

This experiment served also as release test for the Dox at acidic pH to demonstrate its release in the acidic environment of lysosome

4.4 *Kaiser test*[1,2]

The Kaiser test was carried out following a reported procedure. Three solutions were prepared:

A. 500 mg of ninhydrin in 10 mL of ethanol.
B. 80 mg of phenol in 20 mL of ethanol.
C. 2 mL of 0.001 M potassium cyanide dilute to 100 mL with pyridine.

The calibration curve was built using a stock solution of L-valine 1.5 mg/mL in ethanol and taking different aliquots and adding different volumes of the reactive solutions:

A. 75 µL
B. 75 µL
C. 100 µL

The obtained solution was heated at 120°C for 10 minutes, then diluted to 0.5 mL of volume with milliQ water and absorbance at 580 nm of each solution was measured.

Vol. µL	mg	mmol	mmol/mL	Abs	STDV
66	0,099	0,000845	0,00169	3,864	0,097278
33	0,0495	0,000423	0,000845	1,885	0,059152
16	0,024	0,000205	0,00041	0,938	0,011269
8	0,012	0,000102	0,000205	0,333333	0,008622
4	0,006	5,12E-05	0,000102	0,146333	0,007638
2	0,003	2,56E-05	5,12E-05	0,060333	0,004933

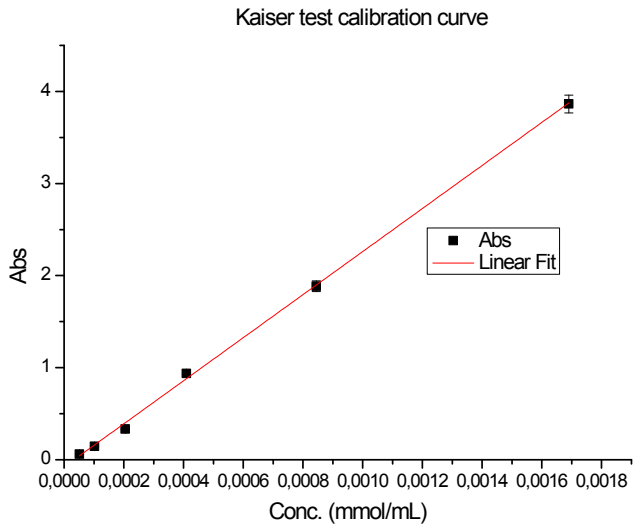


Figure S8. UV-vis calibration curve Kaiser Test equation: y= 2338.3744x+0.02549, R^2=0.98253. Error bars where not visible are smaller than symbols.

Measurements on Materials

Material	Abs	M	mmol	mmol/g of NH_2
11	0.099	7,75E-05	3,87677E-05	0.129

13	0.088	7.32E-05	3.66E-05	0.120

4.5 *General procedure to calculate the payload from TGA spectra*

The amount of payload was calculated from spectra using a graphical approach: the time-point for the weight loss due to the decoration was evidenced by dTG spectrum, then it was measured as percentage directly from the TGA spectrum.

For material **10** was chosen the range 160-260 °C were the loss of weight compared to compound **7**was found to be 41%. Instead two different intervals were evidenced for tri-*t*-BuDOTA-CNTs: 160-240 °C with a loss of weight of 2% and 240-370°C with a loss of 2.9%.

From the weight loss % the mmol of the functionalities were calculated dividing by the molecular weight of the fragment and finally expressed as mmol/g of material. TGA spectra are reported in section **6.**

4.6 *Characterization of final materials*

Characterization of materials was accomplished using different technique:

- metformin loading was evaluated measuring the increment of nitrogen content before and after functionalization using elemental analysis.
- Biotin functionalization was estimated measuring the increment of sulfur content using ICP-AES.
- Doxorubicin loading was estimated using UV-Vis as described above.
- DOTA was quantified by TGA-MS analysis.
- NOTA was quantified indirectly measuring the drop of free amine groups with the Kaiser Test.

Construct	Metformin mmol/g	Biotin mmol/g	Doxorubicin %wt	DOTA mmol/g	NOTA mmol/g
11	2.00	0.21	41.6	-	-
12	1.82	0.137	38.9	0.1	-
13	1.82	0.228	40	-	0.01
14	-	0.27	38.6	-	-

Table S9. Characterization of main products.

5. *Biological studies*

5.1 *In Vitro studies of toxicity*

Cytotoxic effects of CNTs loaded with metformin, doxorubicin, or both drugs, were evaluated by using the MTT method. MCF7 and 4T1 cancer cell lines were seeded in 96 multiwell plates and then treated with increasing concentration of CNTs for 48 hours at 37°C. After this time, cells were extensively washed with PBS and incubated in the presence of 0.5 mg/ml MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for 2 hours at 37°C. Insoluble formazane crystals obtained were dissolved by adding DMSO to plate dishes. The absorbance of samples was quantified by using a microplate reader (Biorad) measuring the absorbance of samples at 570 nm. All experiments were carried out in triplicate. Data reported in the figure represent the mean values S.E.M.

5.2 *In Vivo* stability of [^{64}Cu]CNT-DOTA and [^{68}Ga]NOTA-CNT complexes.

Mouse care, treatments, and imaging were conducted under The University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee (IACUC) protocols 00001179 and followed all appropriate regulations and laws.

Samples of blood and urine were collected 1.5 h after injection, samples of blood were diluted with 30 µL of PBS and 10 µL of the obtained solution dropped on the *i*TLC strip; urine samples were directly deposed (10 µL) on the strip. The *i*TLC strip were eluted with a EDTA 0.1M solution and then read to measure the amount of bound metal

Cu-64	**Blood %**	**Urine %**
Mouse 1	100	-
Mouse 2	100	83
Mouse 3	72	71
Mouse 4	83	75
Mean	89	76
stdv	12	5

Ga-68	**Blood %**
Mouse 1	70
Mouse 2	57
Mouse 3	43
Mouse 4	83
Mean	57
stdv	11

Figure S10. Percentage of chelated metal for the Cu[64]-DOTA-CNTs in blood (n=4) and urine (n=3) and for Ga[68]-NOTA-CNTs (n=3), mean value and standard deviation.

5.3 *PET/CT imaging of tumour bearing mice and analysis*

Mice were briefly anaesthetized (<5 min) using 1% to 2% isoflurane with O_2 as a carrier. Mice were injected *i.v., i.c.* or *i.p.* with of [^{64}Cu]**12** or [^{68}Ga]**13** in sterile phosphate-buffered saline (PBS) with a target of 3.7 MBq per mouse. Actual injected dose was calculated based on measuring the pre- and post-injection activity in the syringe with a dose calibrator (Capintec). Mice were then returned to their cages, quickly became ambulatory and could move freely, eat and drink *ad libitum* for ~45 min. Mice were then anaesthetized using 1% and 3% isoflurane, transferred to a pre-clinical PET/SPECT/CT system (Albira PET/SPECT/CT, Bruker) and maintained at 0.5% to 2% isoflurane with continuous monitoring of respiration during the acquisition. PET images were acquired for 10 min using a 15 cm FOV centred on the tumour; CT images were acquired for fusion using a 7 cm FOV also centred on the tumour. The same procedure was repeated for the 3h, 24h and 48 h PET/CT scans. The 10 min PET/CT dynamic scan was recorded immediately after injection of the tracer, and then mice were allowed to awake and freely move around their cages until the 3 h time point. Images were reconstructed using MLEM and FBP for PET and CT, respectively, and automatically fused by the software. Image data were decay corrected to injection time (Albira, Bruker) and expressed as %ID/cc (PMOD, PMOD Technologies). Tumour-to-muscle ratios (T/M) were calculated by dividing the activity present in the tumour by the activity present in the muscle.

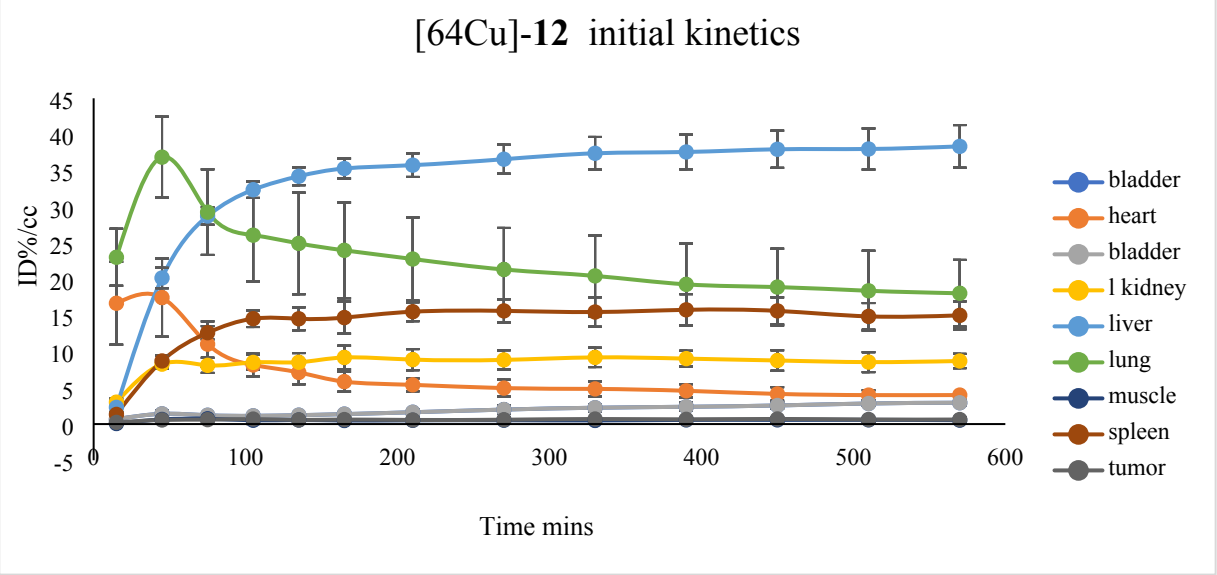


Figure S11. ^{64}Cu-DOTA-CNT initial kinetics.

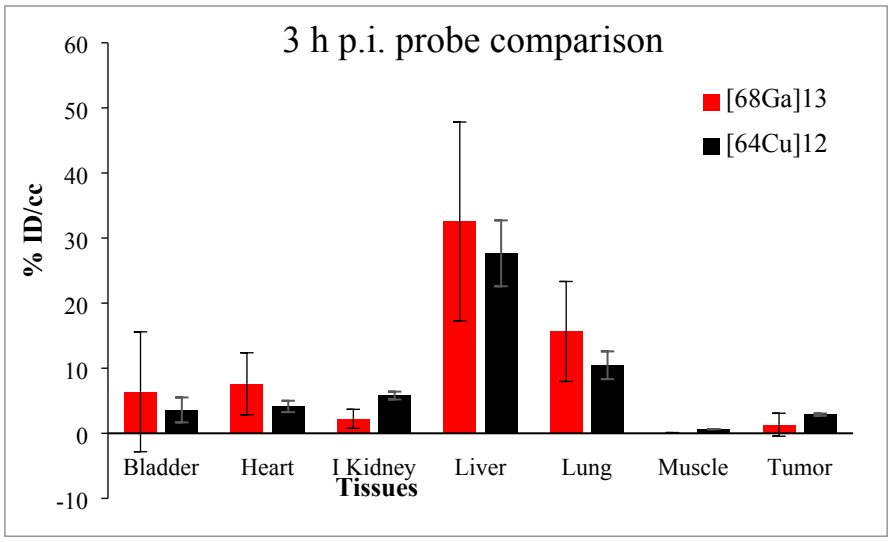


Figure S12 Biodistribution at 3 h post injection using the two different labelled materials and intravenous injection.

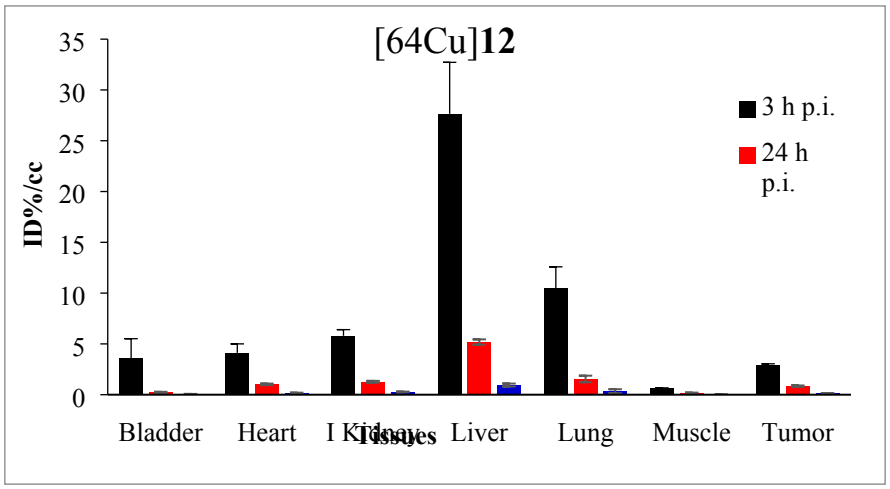


Figure S13. Biodistribution at different time point of [^{64}Cu]**12** after intravenous injection.

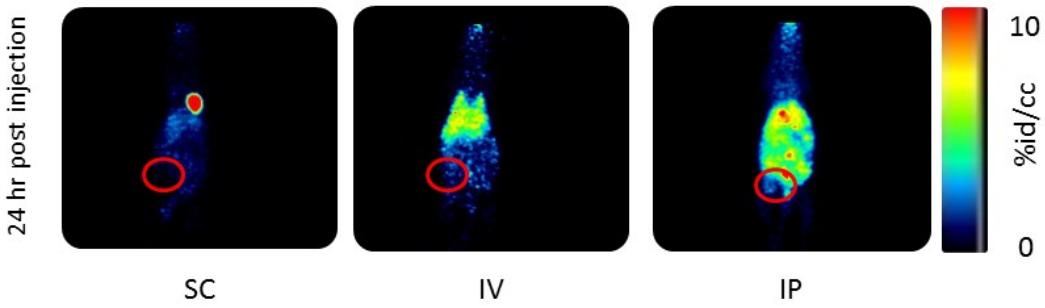


Figure S14: PET imaging of [^{64}Cu]**12** 24h post sub-cutaneous, intravenous and intraperitoneal injection

5.4 *Treatment*

15 Balb/c mice (Taconic) were implanted with 10K 4T1 Fluc-GFP tumors using an orthotopic implantation procedure to create a syngeneic immunocompetent model of triple negative breast cancer. Two weeks subsequent to implantation mice were treated, ip, twice weekly with either vehicle (PBS (Sigma Aldrich)), metformin+ dox (2 mg/kg) or DDS **1** (5 mg/kg). Tumor long and short axis were measured weekly and tumor volume calculated at 0.5*(long axis x short axis^2) . 12 days post treatment mice were imaged for bioluminescence signal (IVIS SPECTRUM, Perkin Elmer).[cite any one of our other papers]. Mice reached endpoint as defined by IACUC protocol 00001179 with tumor burden >= 1.5 or moribund status as determined by veterinary staff.

6. Supplementary Figures and Schemes

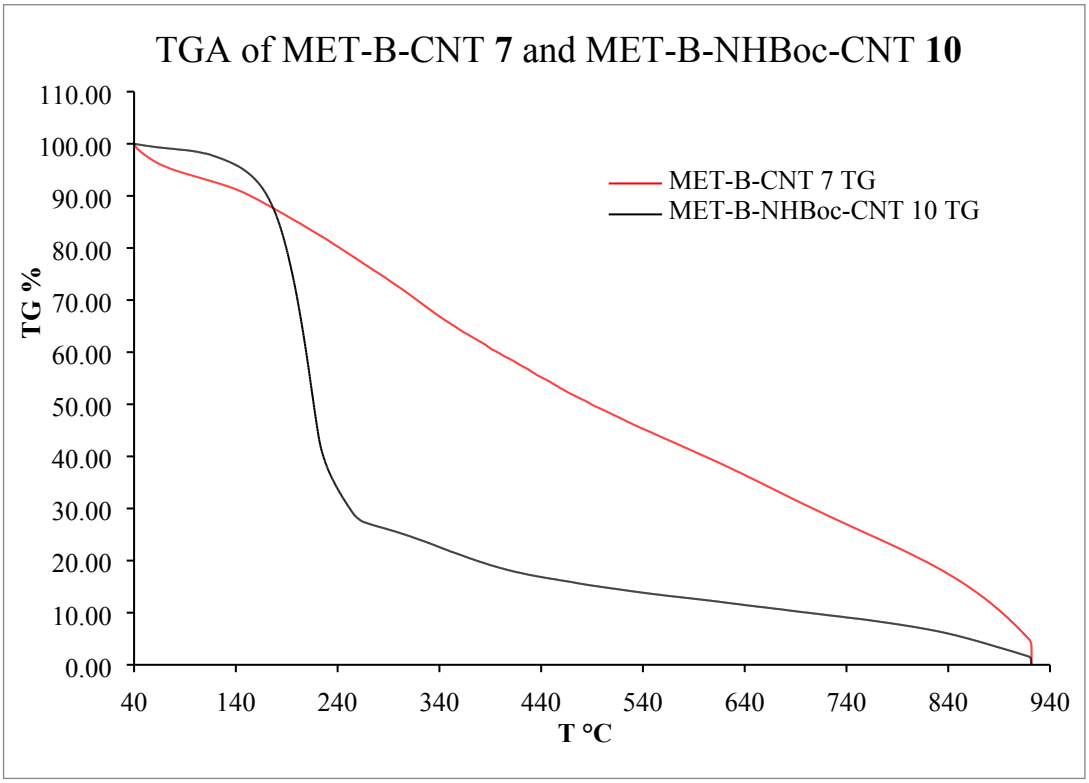


Figure S15. TGA of material 7 (Red) and material 10 (Black).

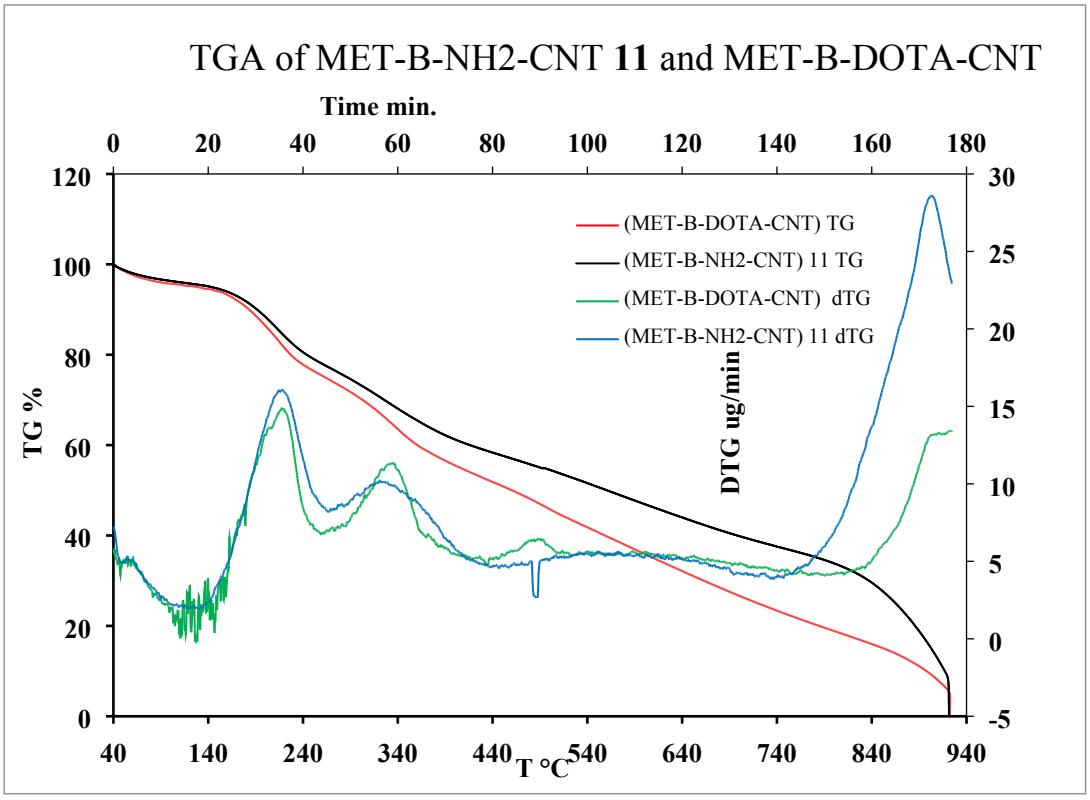


Figure S16. TGA of CNT-DOTA-*tert*butyl ester and material **11**

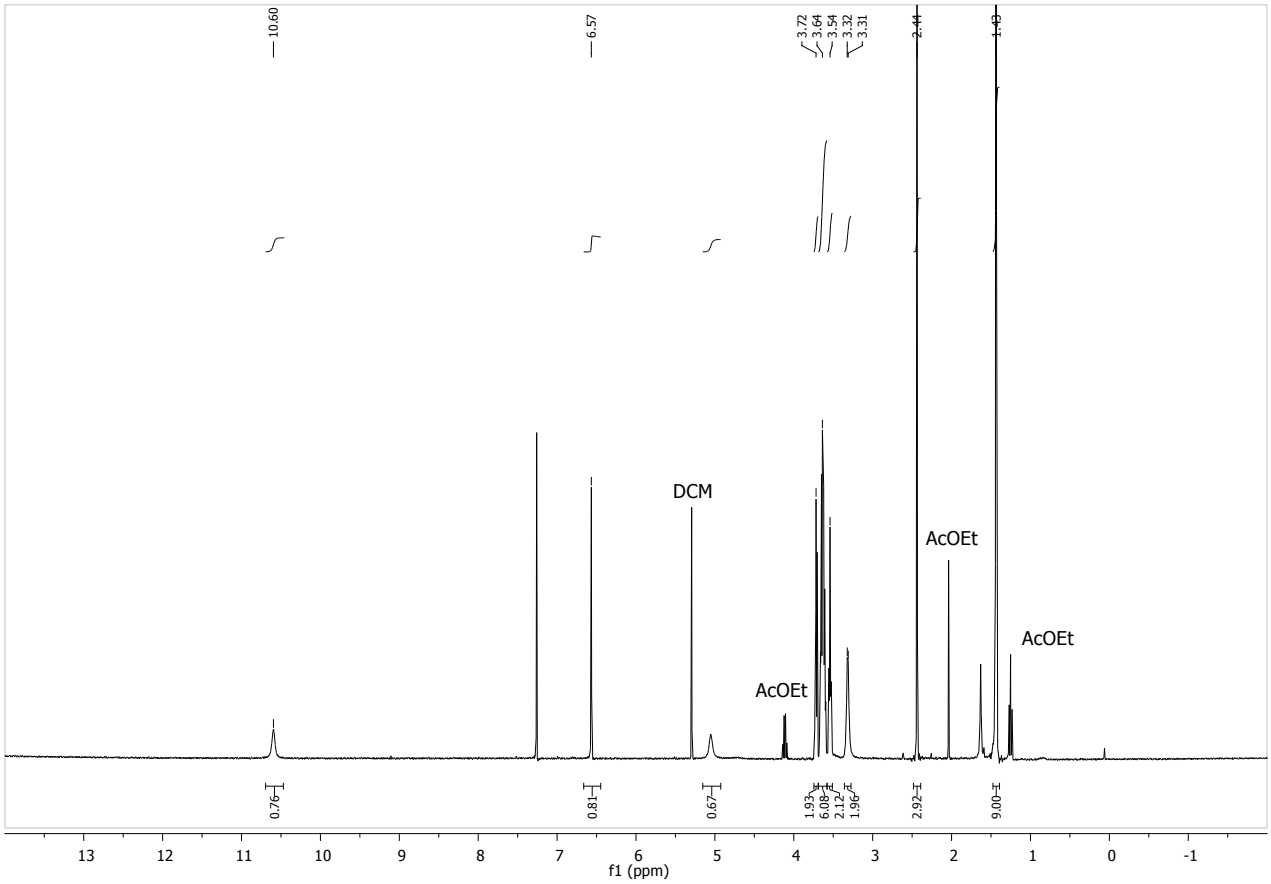


Figure S17. ^{1}H-NMR of (E)-tert-butyl (3-(nitromethylene)-7,10-dioxa-2-thia-4-azadodecan-12-yl)carbamate.

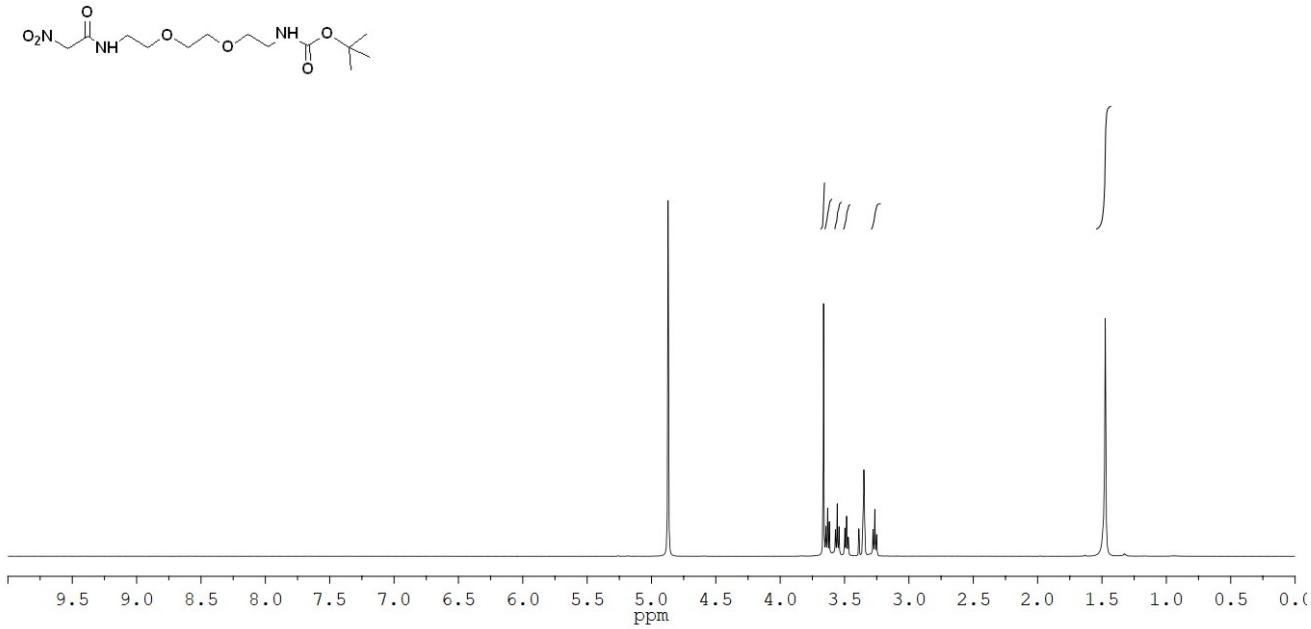


Figure S18. ^{1}H-NMR Compound **9**

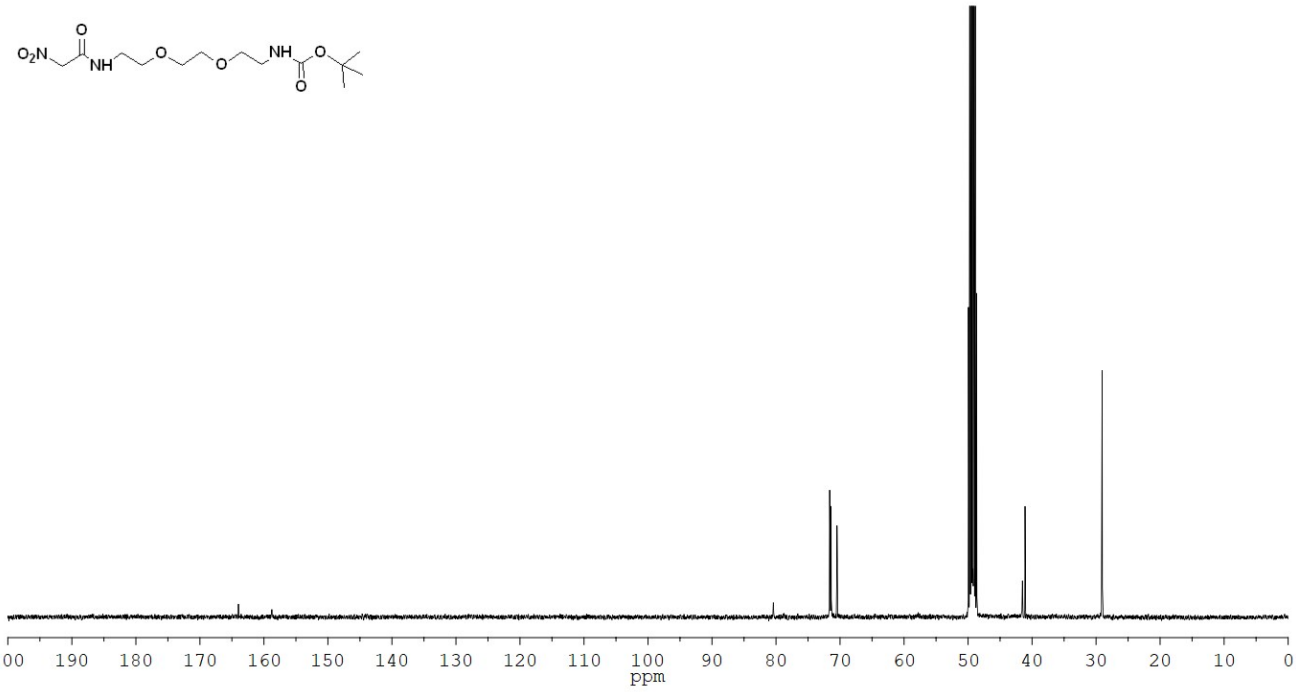


Figure S19. ^{13}C-NMR Compound **9**

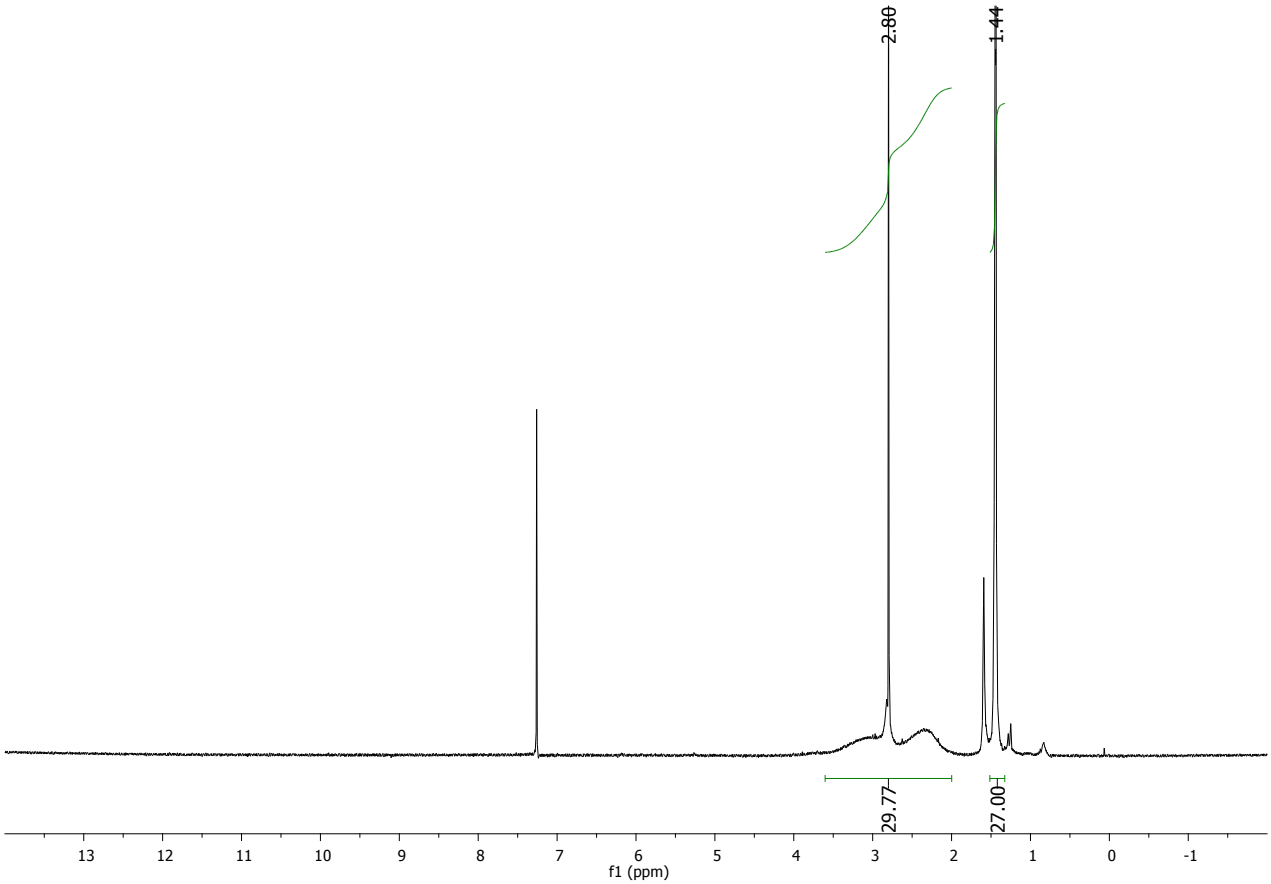


Figure S20. ^{1}H-NMR (400 MHz) of DOTA-OSU **15**

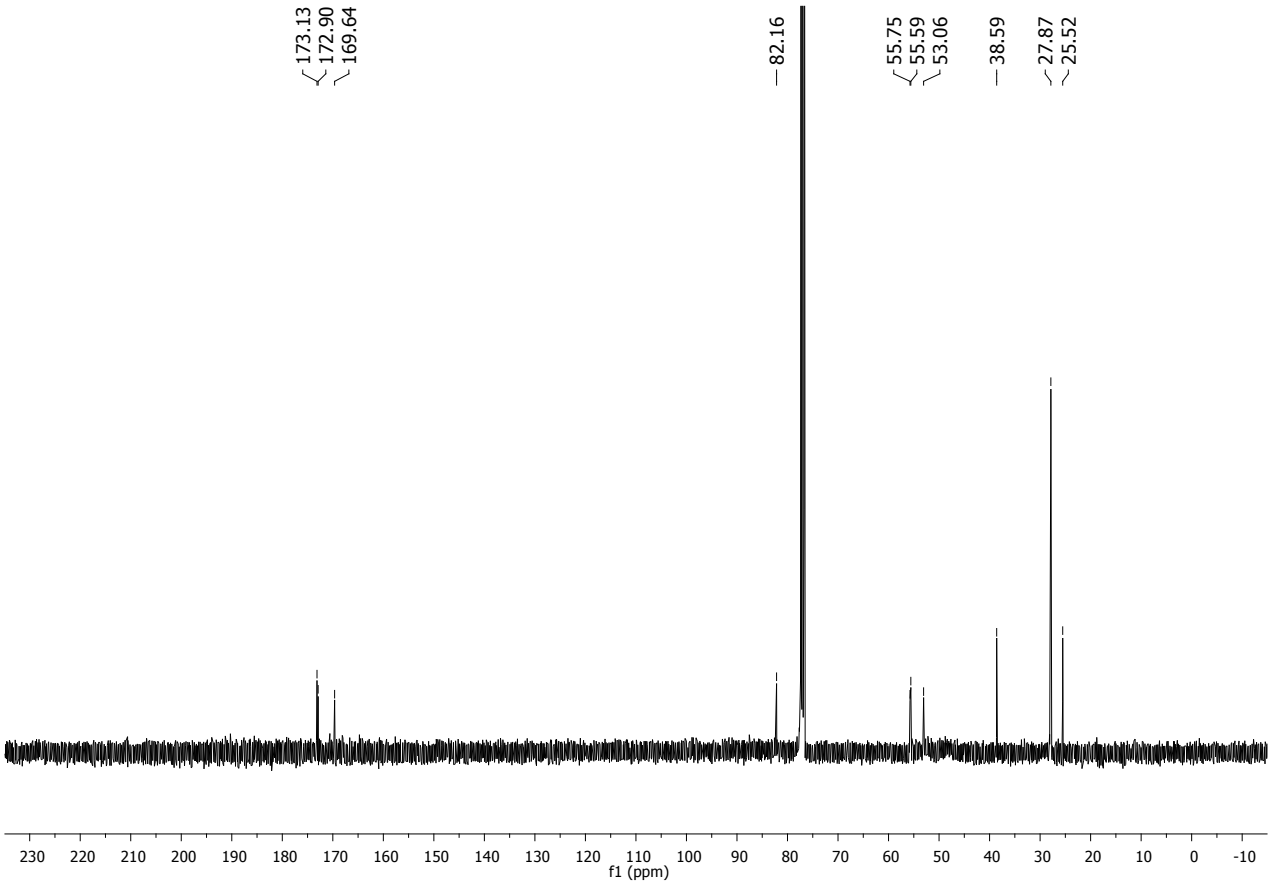


Figure S21. ^{13}C-NMR (100 MHz) of DOTA-OSU **15**

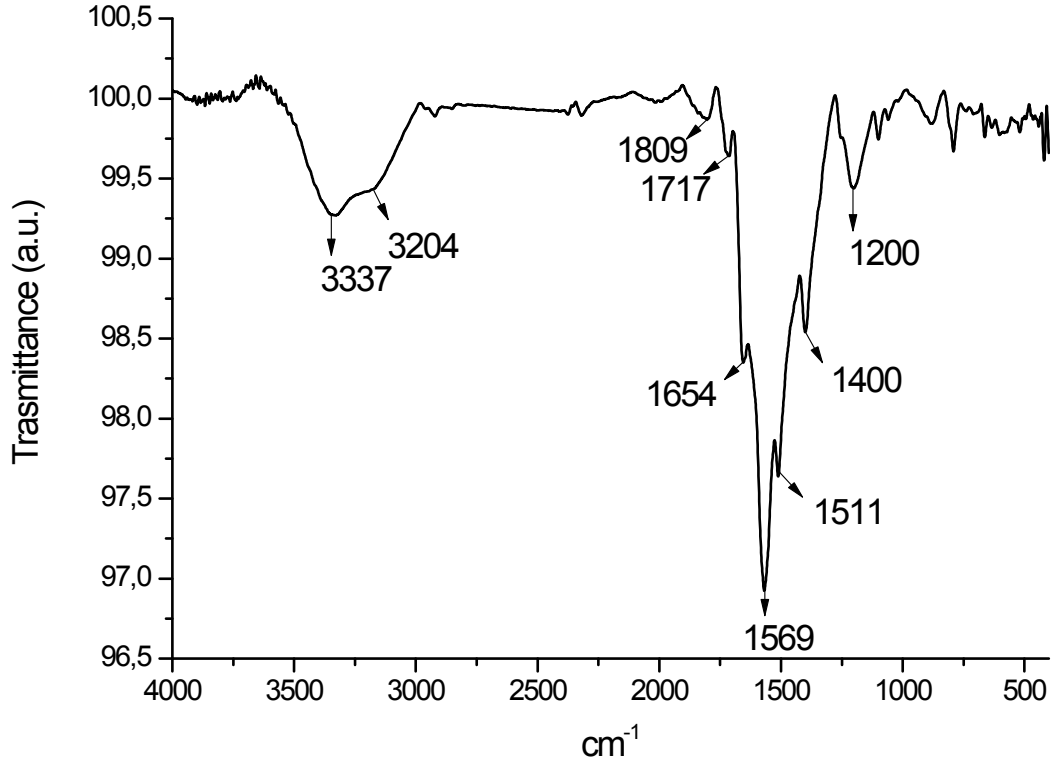


Figure S22. FT-IR spectrum of CNT-Met adduct **3.**

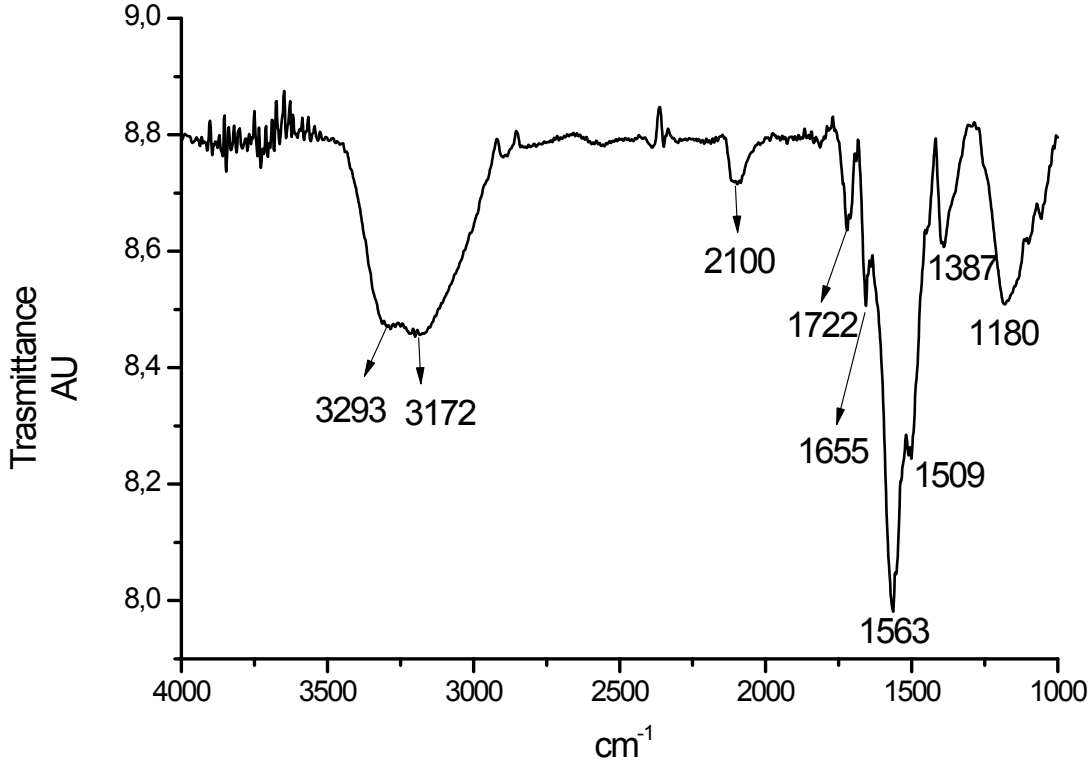


Figure S23. FT-IR of CNT-Met-N_3 **7**.

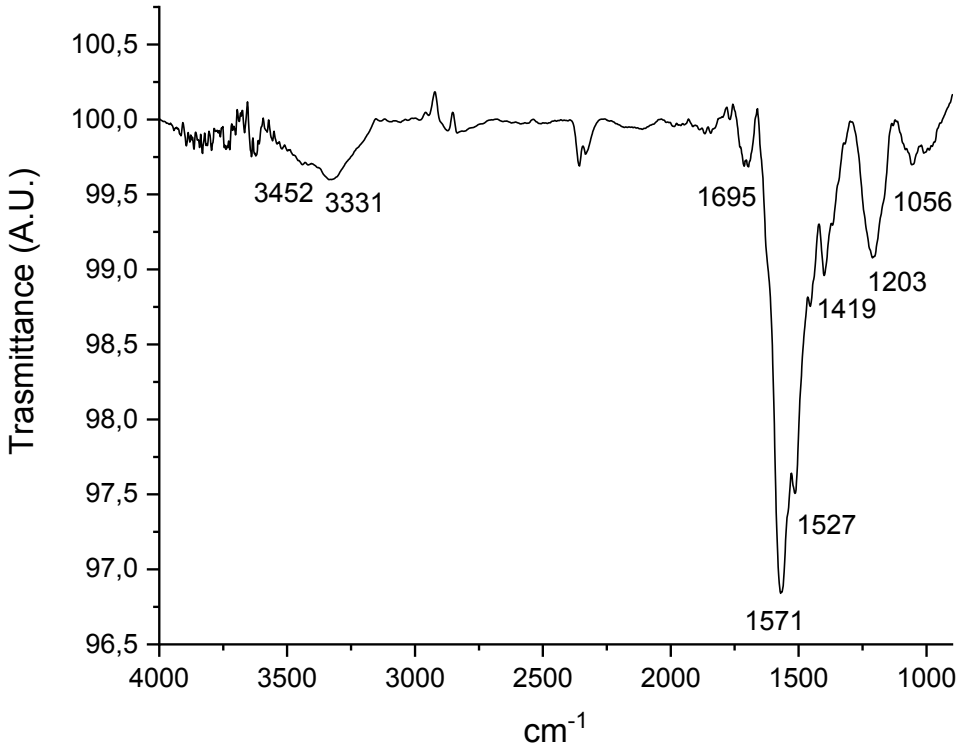


Figure S24. FT-IR of CNT-Met-Bio-NHBoc **10**.

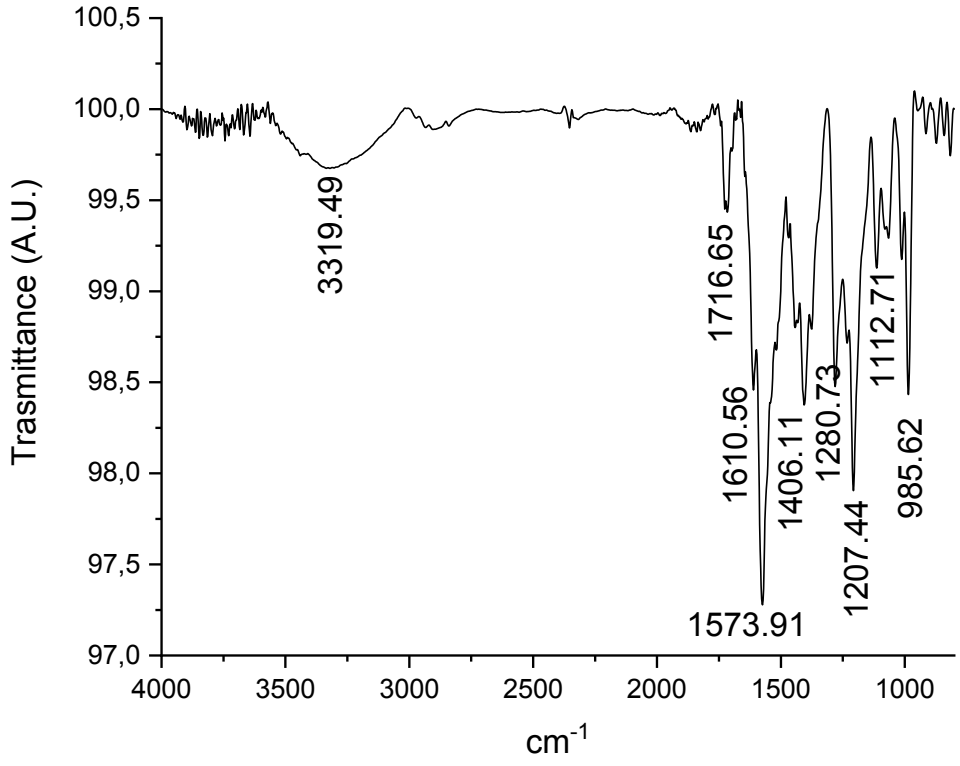


Figure S25. Figure S24. FT-IR of DDS **12**.

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