Synthesis, Pharmacokinetics, and Biological Use of Lysine-modified Single-Walled Carbon Nanotubes

J. Justin Mulvey[1,4] **Evan N. Feinberg**[1,5]**, Simone Alidori**[1]**, Michael R. McDevitt**[3]**, Daniel A. Heller**[1]**, David A. Scheinberg***[1,2]

[1]Molecular Pharmacology and Chemistry Program, Departments of [2]Medicine and [3]Radiology, [4]Tri-Institutional MD-PhD Program, New York, New York 10021

[5]Department of Applied Physics, Yale University, New Haven, CT 06511

Figure S1: TEM and SEM images of unmodified and H-Lys(Boc)-OH modified SWCNTs. Note, the length of a 50nm diameter bundle of SWCNTs in the right TEM image does not reflect the length of any individual tube. White dots on this TEM are grid imperfections allowing blanching from transmission of electron beams.

(**below**) TEM image of unmodified, bundled SWCNTs prepared by dispersing tubes in DMF then drying a dilute sample on a carbon coated TEM grid. The bundles are similar in girth to those observed by Maruyama in *Chemical Physics Letters*, *Volume 360, Issues 3–4, 10 July 2002, Pages 229-234, Figure 2A*
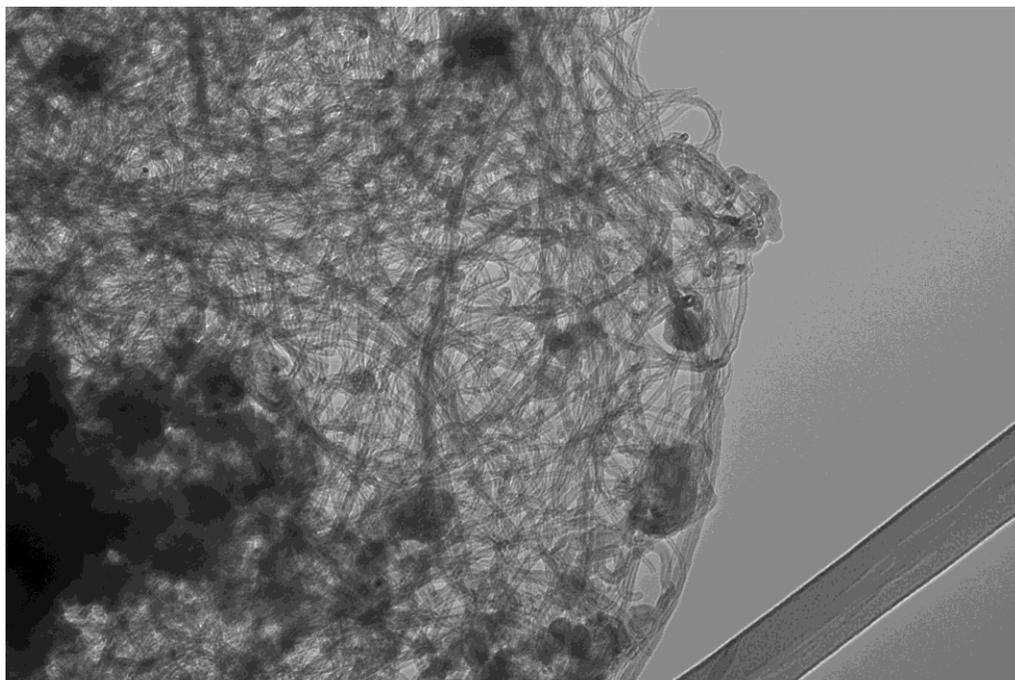

```
comocat 3 100kx.tif
Print Mag:   113000x @ 7.0 in            100 nm
15:33:11 11/11/2013                      Direct Mag: 100000x
```

(**left**) TEM image of soluble, functionalized SWCNT-Lys-BOC made by diluting and drying very low concentration, soluble SWCNT-Lys-BOC on a carbon coated TEM grid in distilled water. Bar is 500nm. Overcast circles are grid imperfections.
(**right**) SEM image acquired after lyophilizing SWCNT-Lys-BOC into a pellet which was imaged directly
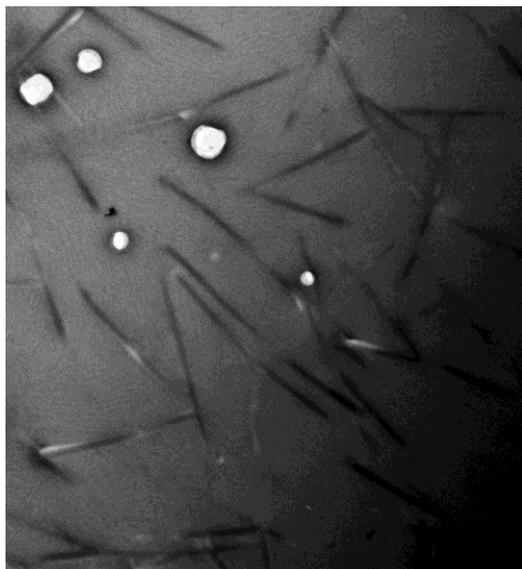

```
JJM 8-2-12 unemitted reverse dialysis lysine tubes -4.tif
Print Mag: 30300x @ 7.0 in            500 nm
                                      Direct Mag: 30000x
```
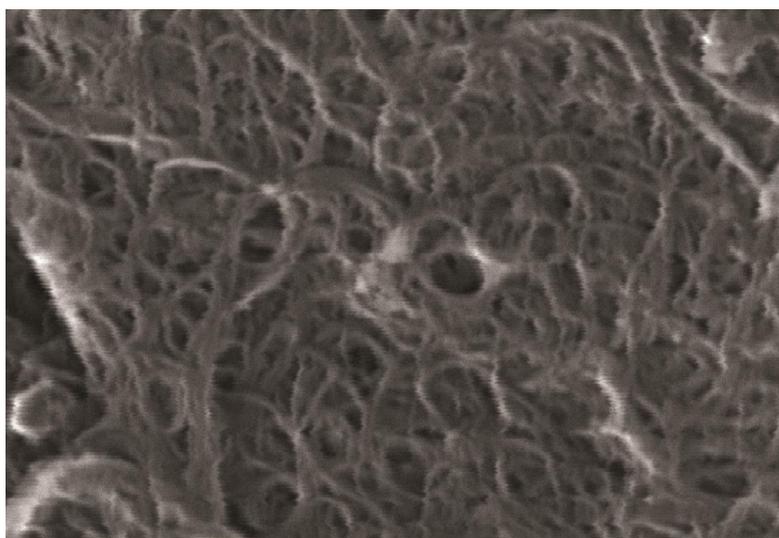


```
100 nm*    Mag = 319.26 K X EHT = 10.00 kV   Signal A = InLens        File Name = 319 swcnt thicket 03.tif
           Reference Mag = Out Dev.      WD = 8.3 mm      Width = 939.7 nm        Date :11 Nov 2013
```

Figure S2: Of note in both figures, solvated or not, the bundled nanotubes are large enough to be contained by a 10,000 Molecular Weight Cut-Off membrane. A) Photograph of water soluble, lysine modified SWCNTs undergoing dialysis in a 10,000 MWCO Slidalyzer Dialysis Cassette at pH 7. B) The same sample after adjustment to pH 12. Precipitated SWCNT shown as black granular material.

A: Solvated, lysine modified SWCNTs (2) at pH 7   **B: Crashed-out, lysine modified SWNTs (2) at pH 12**
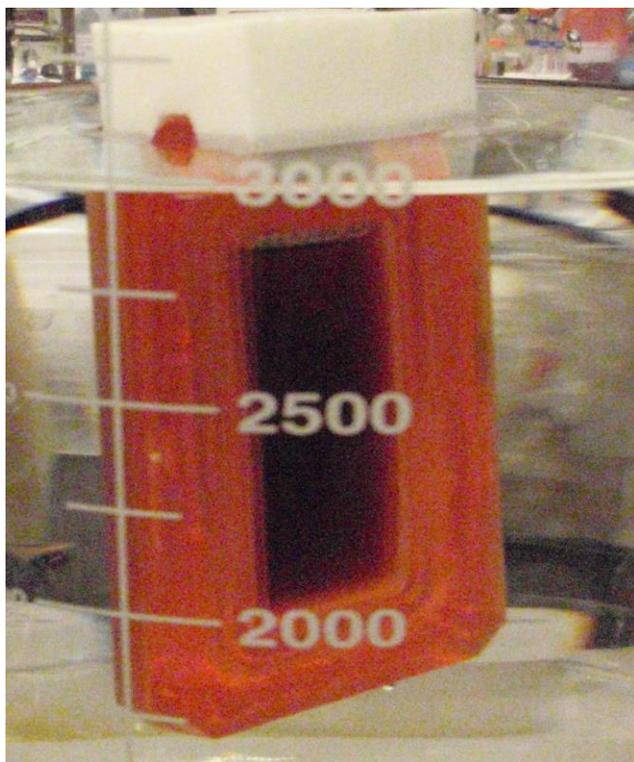
Figure S3:   Concentration curve derivation from 600nm absorption and HPLC trace of SWCNT-Lys-NH$_2$

Figure S3A: UV/Vis Absorption Spectrum of (**1**) showing characteristic SWCNT Absorbance slope
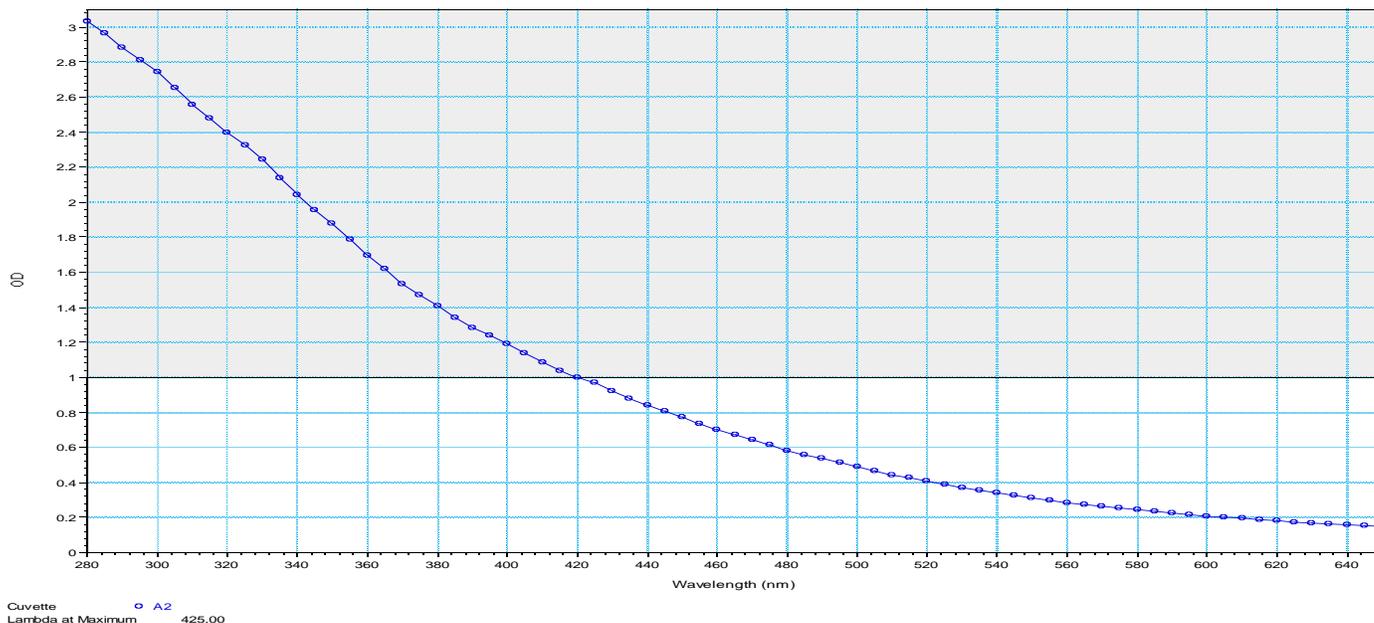
Figure *S3B*: UV/Vis Absorption Spectrum of SWCNT-Lys-NH$_2$,(**2**) showing characteristic SWCNT Absorbance slope

Cuvette   o A4
Lambda at Maximum   405.00
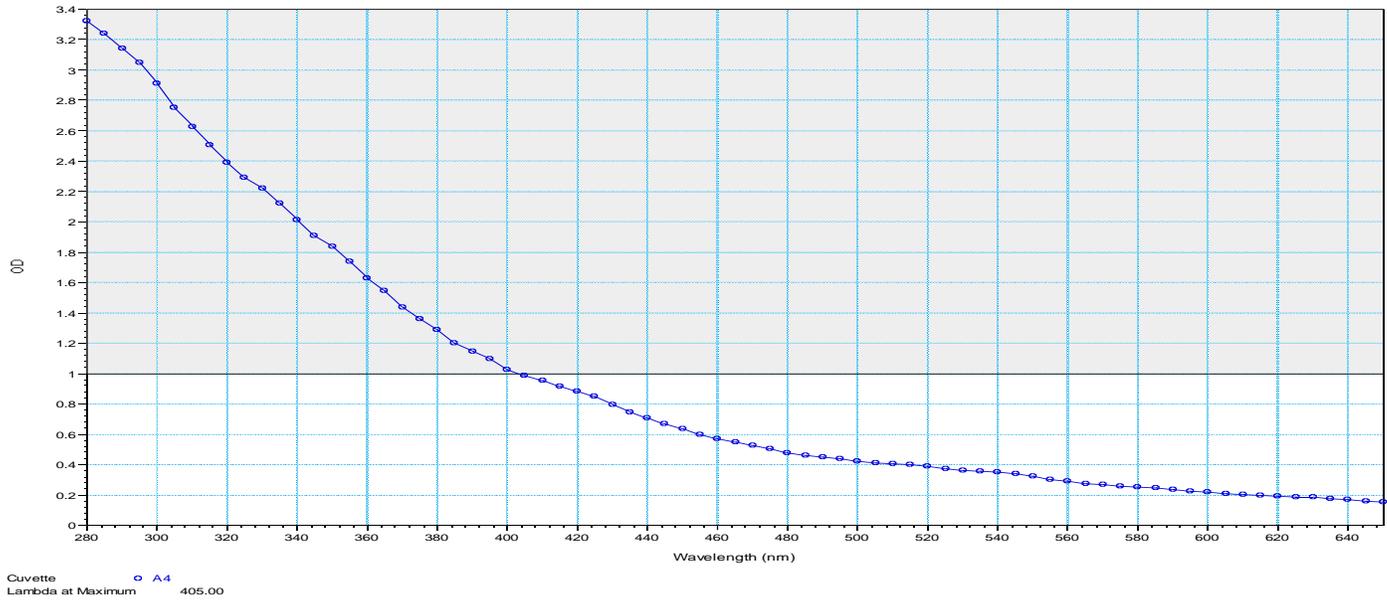
Figure *S3C*: SWCNT-Lys-NH$_2$,(**2**) 600nm absorbance versus concentration curve with derived equation.

$$A600 = 0.1234 (xg/L) + 0.0188$$
$$R^2 = 0.9996$$
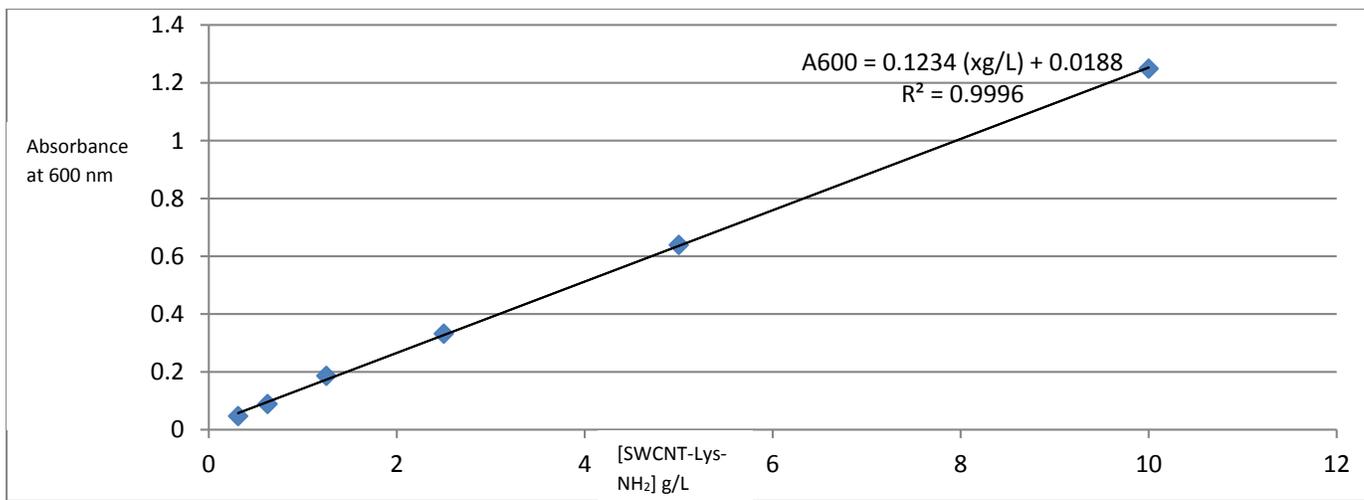
Figure *S3D*: SWCNT-Lys-NH$_2$,(**2**) C18 HPLC trace showing single, broad peak in 20-100% gradient of acetonitrile over 50 minutes. This also corroborates the signature slope of modified CNT, and modification enhancements 230-260.
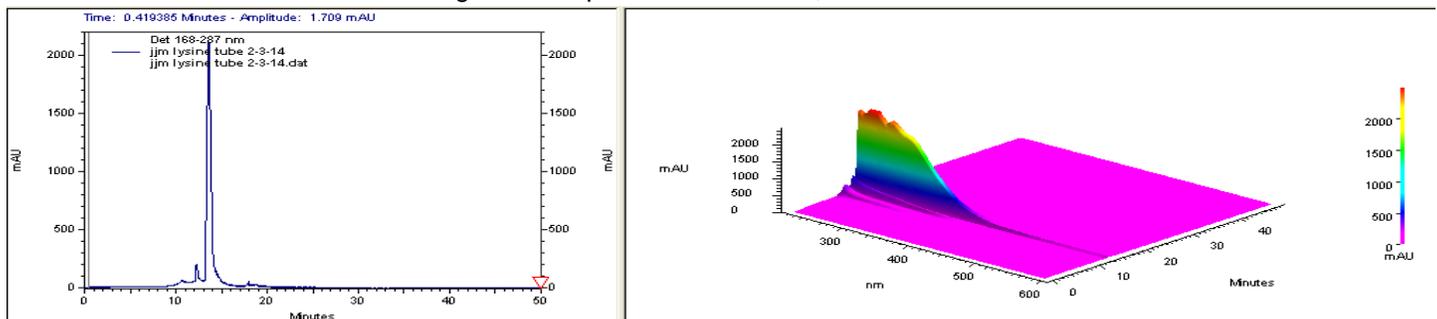
Figure S4: Kaiser/Sarin assay and calculation of degree of amine modification

Glycine Standard Curve for Kaiser Assay

Absorbance @ 570 nm

[Glycine] in Mol/L

$y = 917x + 0.0703$
$R^2 = 0.9918$

◆ Glycine
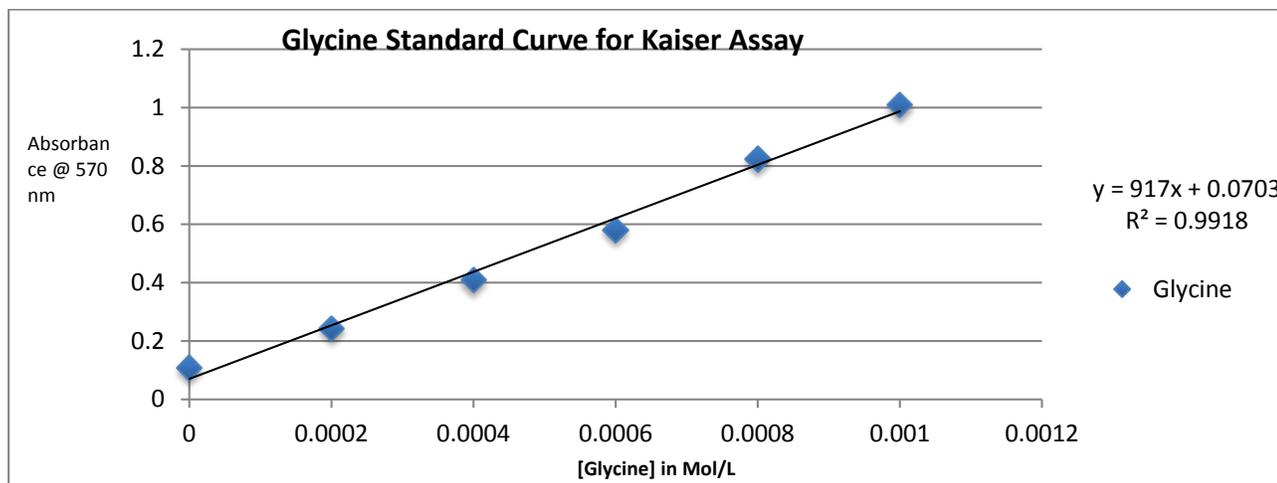
Derived Amine to Absorbance Equation:

A570 = [Molarity of Amines] * 917 + 0.0703

H-Lys(Boc)-OH Generated Product (**2**):

[Concentration of Analyte] = 0.9 g/L   (massed and diluted)

A570 = 0.635

[NH2] = 6.16E-4 M

[Carbon] = 0.9 g/L / (12 g /mol) = 0.075 M

→ 1 Amine / 121.8 Carbons

Prato Reaction Product (**1**):

[Concentration of Analyte] = 0.68 g/L

A570 = 0.375

[NH2] = 3.32E-4 M

[Carbon] = 0.68 g/L / (12 g/mol) = 0.057M

→ 1 Amine / 171.7 Carbons

Derived Ratio:

170.1/121.8 = 1.41

This analysis was completed twice on separately prepared samples, the second analysis yielding a similar ratio of 1.39.

One possible explanation for the increased functionalization is that the non-polar, carbon lysine side-chain abstains from stabilizing interactions with the ylide during the reaction as a more polar, PEGylated adduct might. Another possible explanation is that without an additional hyperconjugating alkanyl adduct to the ylide nitrogen as seen in the Prato Reaction, the ylide in this reaction would be less stabilized and more reactive. A third, or contributing possibility is that unreacted fullerenes are more quickly exposed to reagents as the more DMF-soluble product on the surface of visible clusters is expediently removed into solution.

Figure S5: Solid state FTIR spectra of: **A)** L-lysine. **B)** H-Lys(Boc)-OH (Starting Material.) **C)** Modified SWCNTs. **D)** Modified C60. **E)** *Unpurified* Sigma Aldrich SWCNTs as provided. **F)** *Unpurified* Sigma Aldrich C60 as provided.
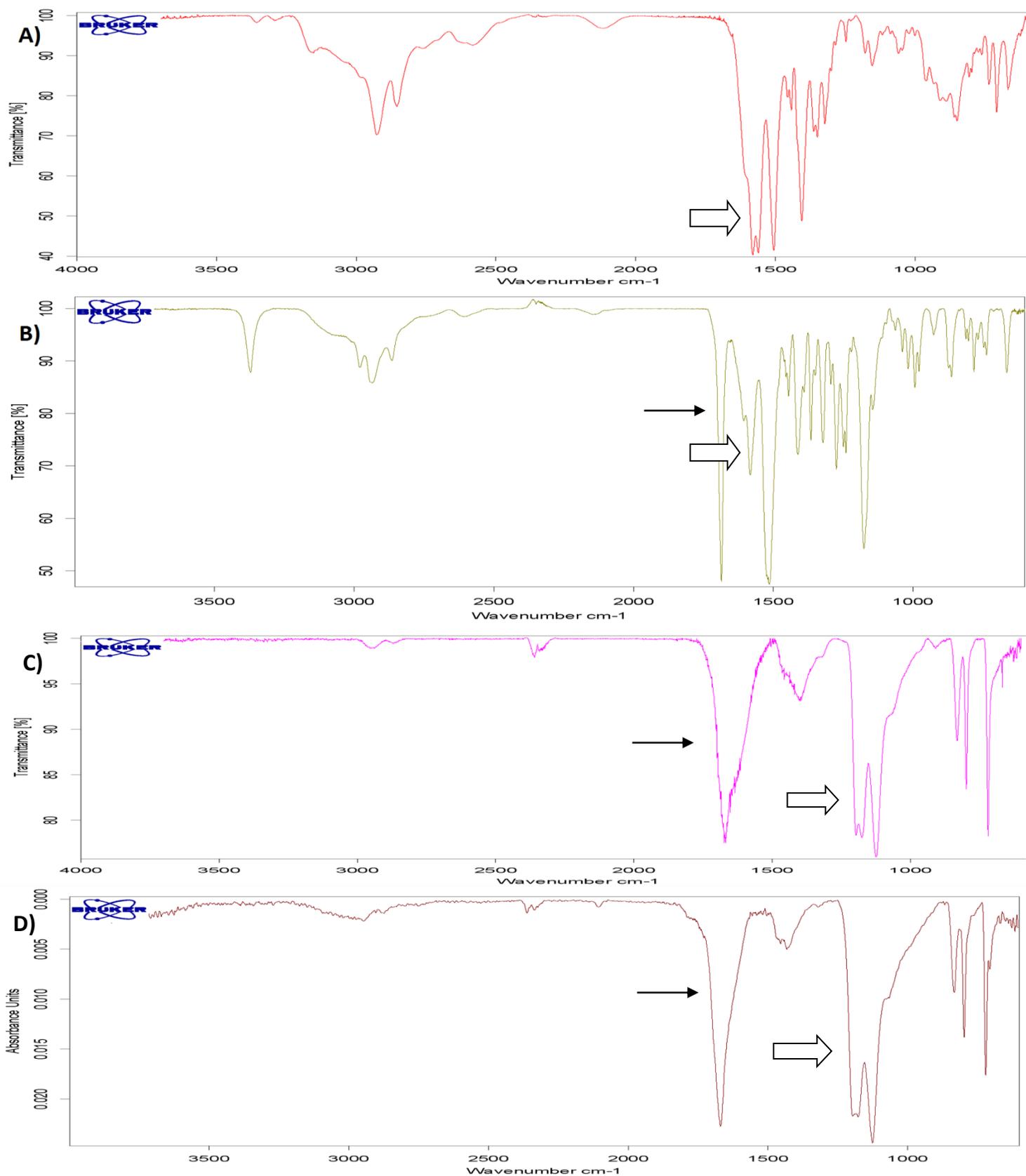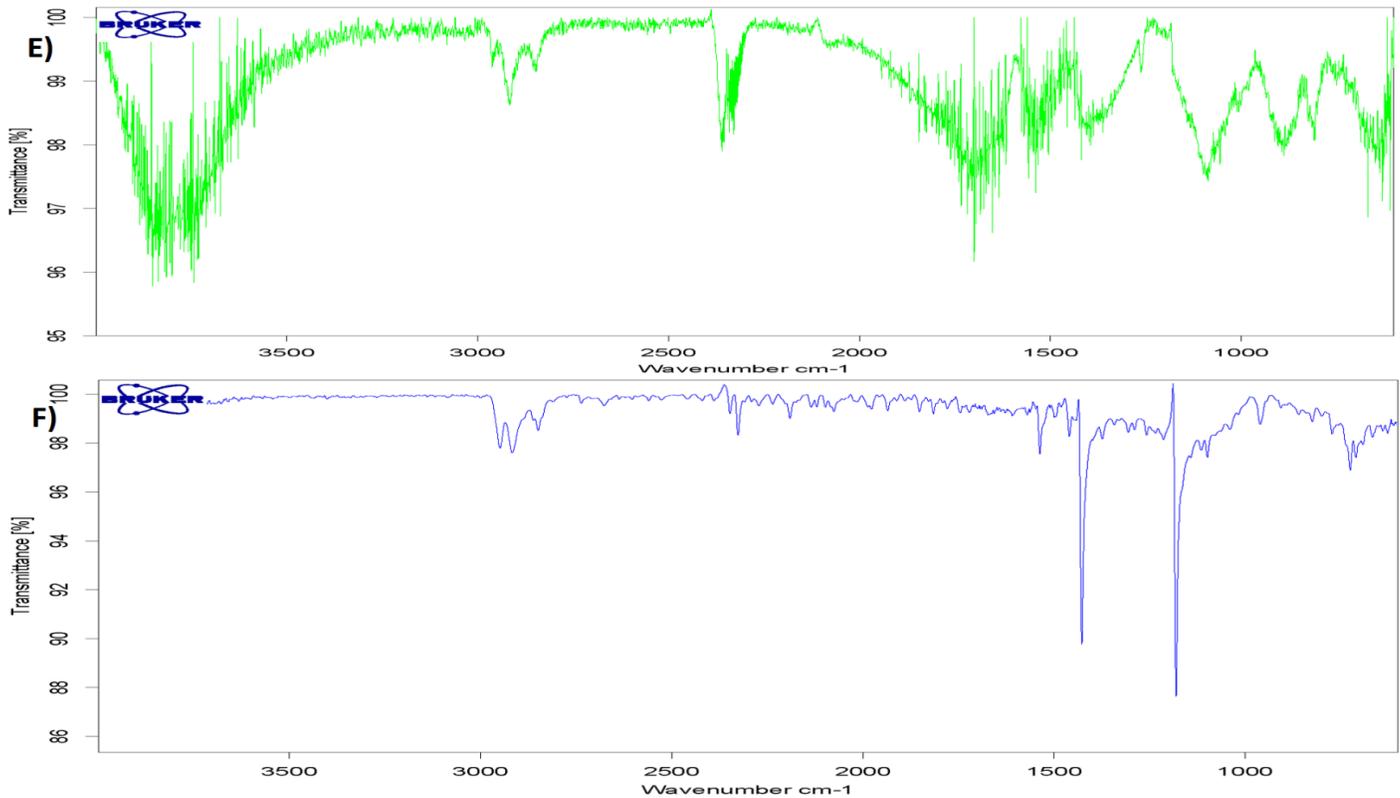
A) Shows a characteristic side chain amine N-H wag triad of peaks around 700 cm^{-1}. At 1400 cm^{-1} there is an N-H bend from the amino-acid amine (no longer present in (C)), and three peaks at 1500-1600 cm^{-1} representing the N-H bend from the primary side chain amine. Just below 3000 cm^{-1} are SP3 C-H stretches. The shelf beneath them extending to 3400 cm^{-1} encompasses the primary N-H stretches.

B) Very Similar to (A) with identifying carbonyl at 1650 cm^{-1} marked by the black arrow.

C) The energy shift of the triad of peaks marked by the block arrow showing a different chemical environment is supportive of functionalization. The strong carbonyl peak is still present giving convincing evidence that the BOC protecting group is still attached and broadened by underlying C=C stretches. In between these two aforementioned peaks is another cluster of C=C stretches found in (E) and in (F). Since nanotubes range in chirality and length and degree of imperfection compared to more homogeneous C60, the difference between the spectra and (E) and (F) is evident. The characteristic side chain amine N-H wag triad of the lysine adduct arises around 700 cm^{-1}, correlating directly with the spectrum of the amino acid (A). At 1200 cm^{-1} is the bend of the primary amine, also seen in the spectrum of the free amino acid, significantly shifted after the addition reaction. Just below 3000 cm^{-1} are the SP3 C-H stretches of the adduct. These should not be attributed to those peaks shown in (E) as peaks from (E) are attributed to the presence of amorphous carbon species cleared in purifications.

D) C60 adducted with BOC-protected lysine (BOC not removed). Very similar to (C).

E) Depicts significant C=C peaks at 1700 cm^{-1} and 1535 cm^{-1} with a wide variety of discrete energy levels as well as residual CO2 at 2400 cm^{-1}, and amorphous carbon impurities seen in the relatively large amount of SP3 C-H stretches. These SP3 C-H stretches just below 3000 cm^{-1} as well as the shelf below 1200 cm^{-1} are reduced by removal of amorphous carbon and tubes below 25nm in length respectively.[1]

F) Two discrete C=C stretches are seen between 1500 cm^{-1} and 1000 cm^{-1} that accord with literature. Far IR shelf at 650 cm^{-1} is representative of fullerenes with size smaller than 25 nanometers on a breathing axis; it is also a sharper peak than seen with the SWCNTs because C60 is of a discrete size. It should be noted that the literature suggests two identifying fingerprint peaks below the end-range of this FTIR instrument (600 cm^{-1}). Again, SP3 C-H stretches were consistently present *out of the bottle* and are believed to be amorphous carbon in unpurified starting material.

Figure S6: NMR Spectra and Analyses

Figure S6A: Starting H-Lys(Boc)-OH H-NMR (δ in ppm) in D_2O

H-NMR: δ Multiplet=1.44; δ Singlet=1.47; δ Pentet=1.56; δ Multiplet=1.90; δ triplet=3.12; δ Triplet=1.56; δ Triplet=1.76
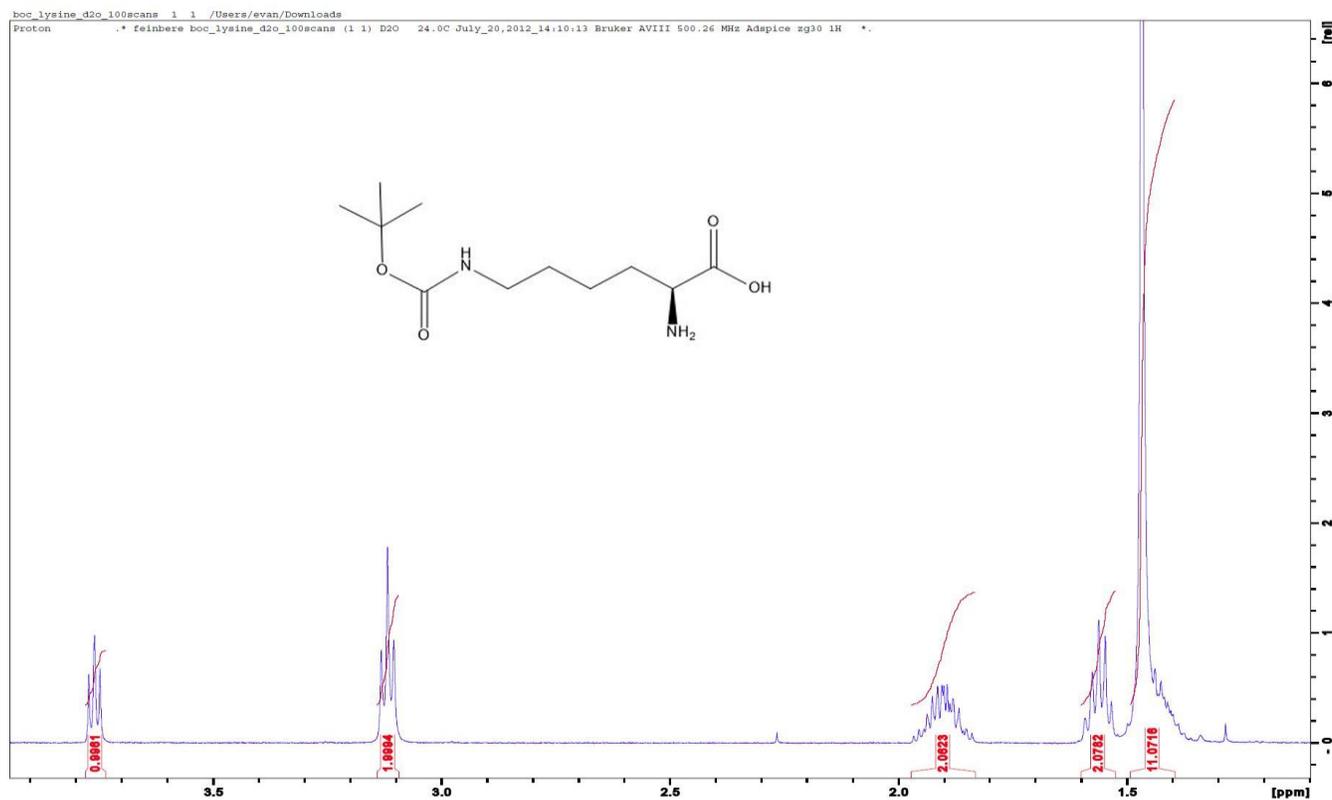
Figure S6B: Starting H-Lys(Boc)-OH C13-NMR (δ in ppm) in D_2O: δ = 21.59; δ = 27.61; δ = 28.55; δ = 30.06; δ = 39.51; δ = 54.51; δ = 80.83; δ = 158.33; δ = 174.76. Two carbonyl peaks are seen downfield of 150ppm
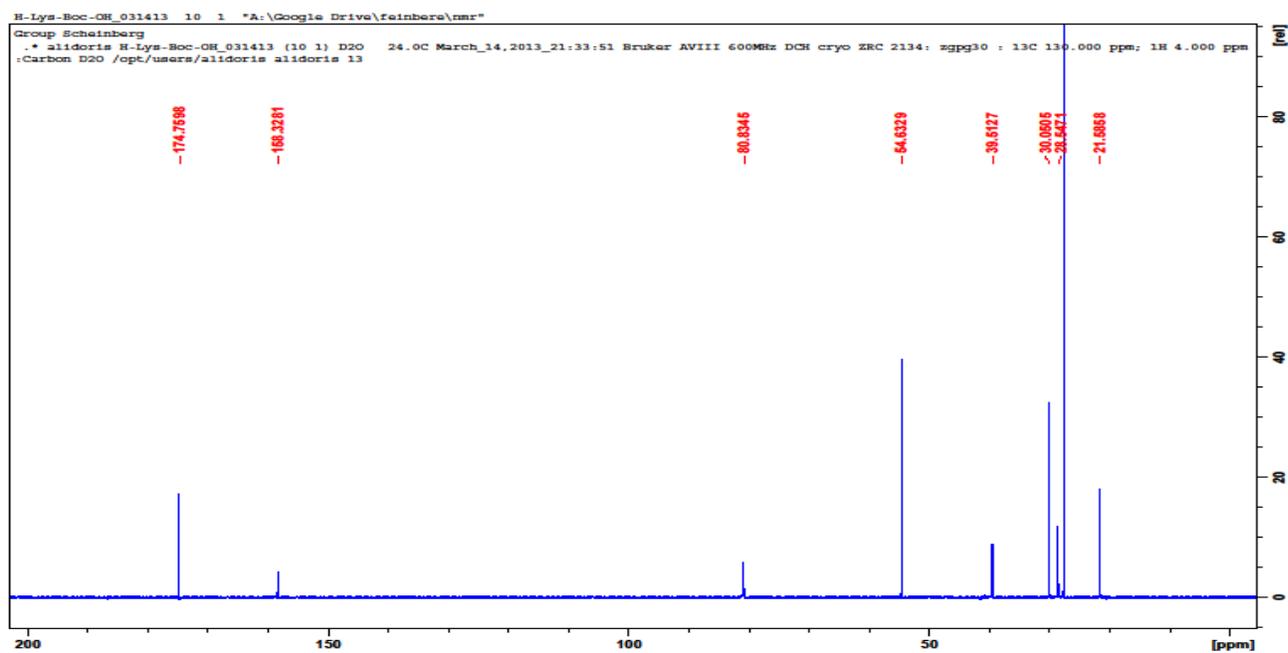
Figure S6C [Three different zooms of a single spectrum i, ii, iii]: BOC-protected pyrrolidine adduct on C60 H-NMR (δ in ppm) in D_2O

Lysine modified C60 fullerenes were moderately soluble in D_2O, slightly soluble in deuterated methanol, sparingly soluble in deuterated acetonitrile, and insoluble in deuterated chloroform, benzene and DMSO. NMR spectra of SWCNT were similar, but less resolved.

We ascribe upshifting and downshifting observed with NMR spectra of C60 and SWCNT adducts to the global and individual ring currents induced in magnetic fields.[2,3]

δ Singlet = 1.93; δ Triplet =1.96; δ Singlet = 2.62; Singlet = 2.75; δ Leaning Pentet = 2.85; δ Leaning Pentet = 3.15; δ Triplet = 3.78; δ Quartet = 4.84; Singlet = 8.47

Assigned Proton Peaks; see [4].

1. Leaning pentets representing neighboring methylenes at the center of the lysine backbone, centered around δ 3.0
2. Amide hydrogen at δ 8.5 (far left side)
3. BOC Signature at δ 1.93
4. Solvent exchangable, diminished pyrrolidine amine at δ 2.75
5. BOC linked amide Peak at δ 8.47
6. Minute peaks at 7.45 were inconsistently present through repeated analysis and are proposed to represent the partial protonation of the pyrrolidine amine into a cation split by magnetically inequivalent adjacent protons.

S6Ci
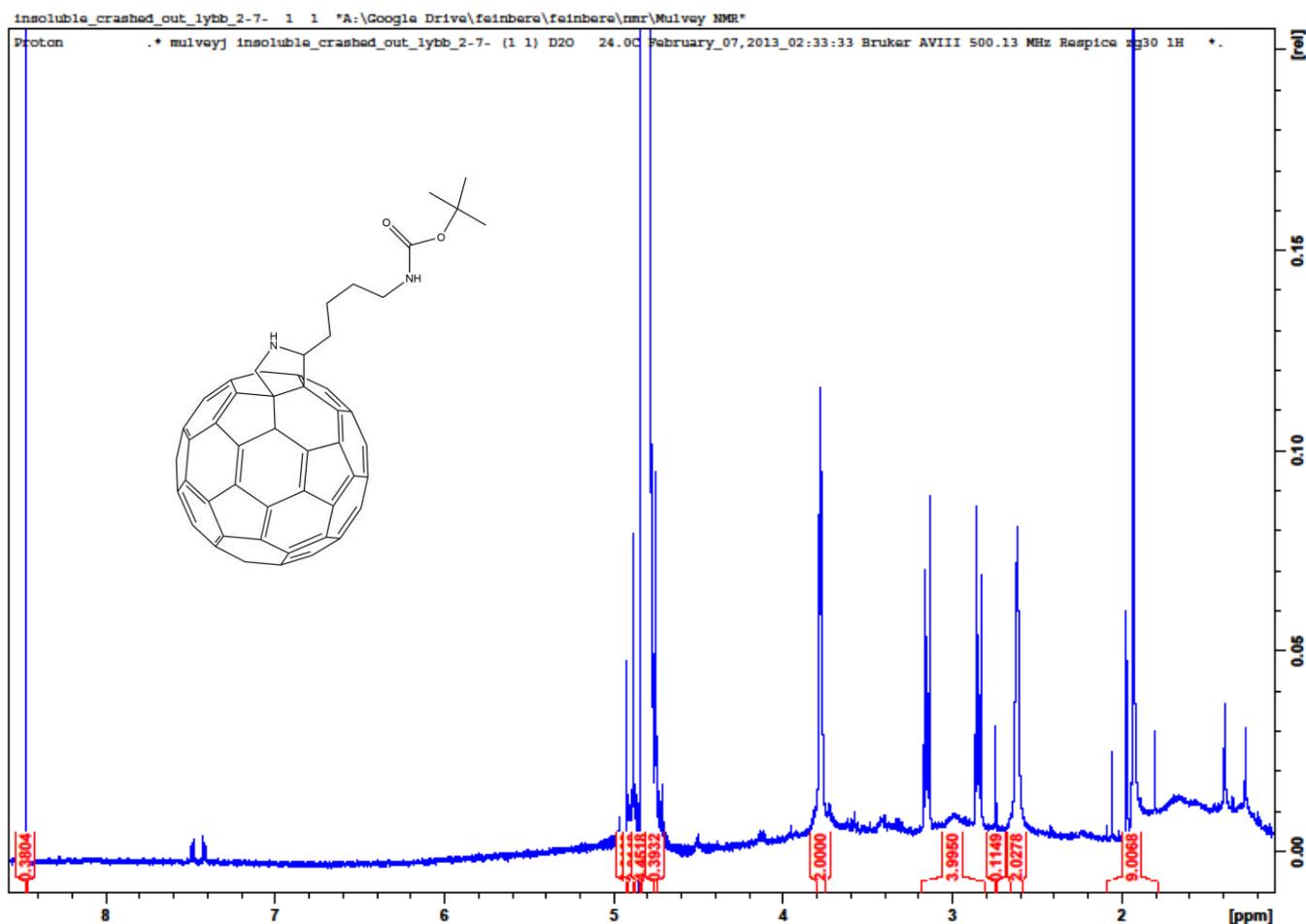
S6Cii
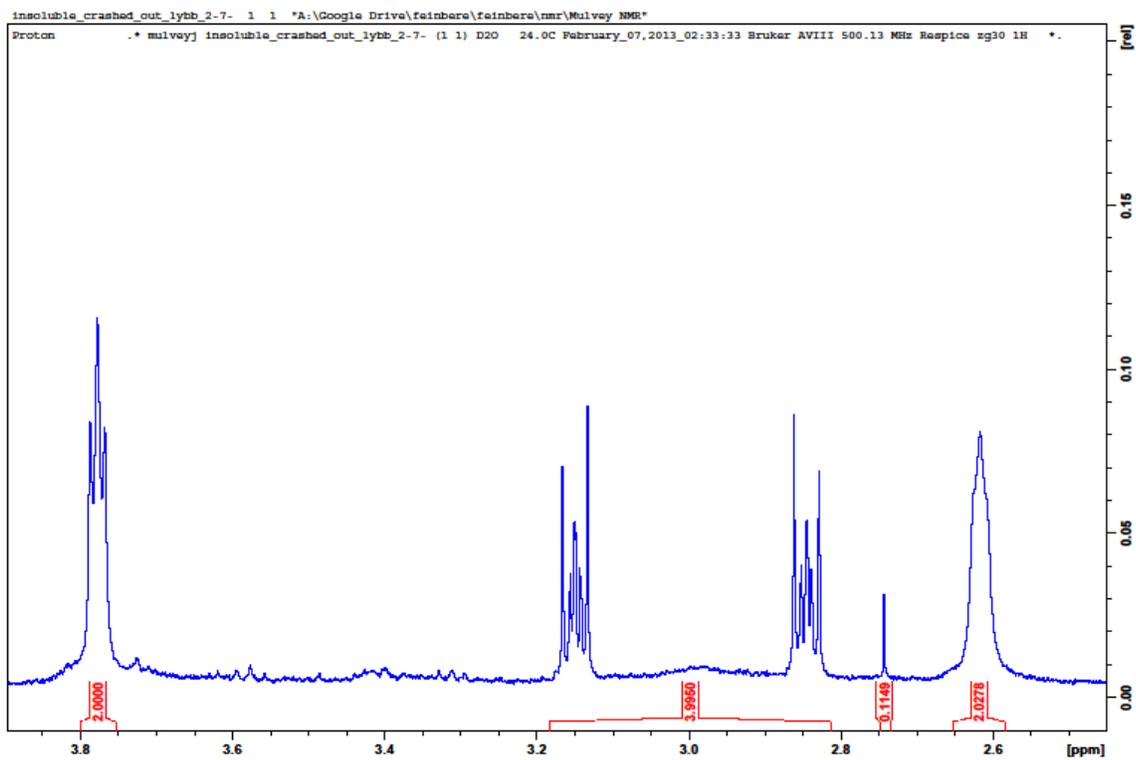
S6Ciii
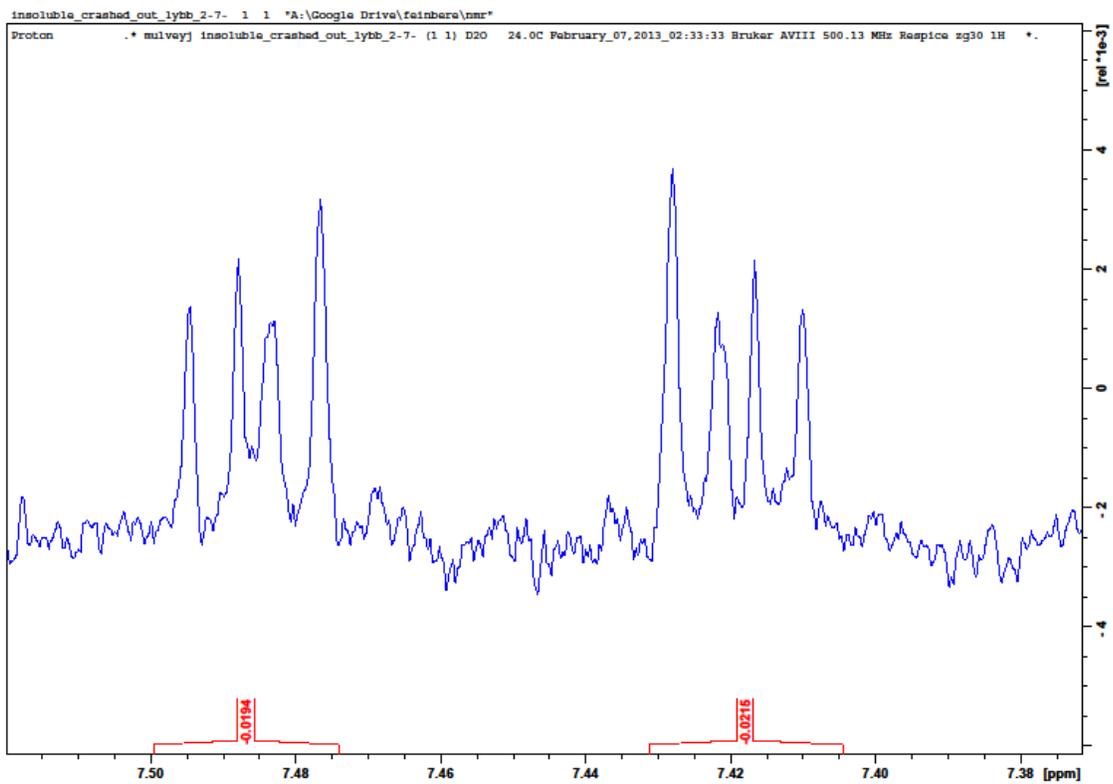
Figure S6D: BOC protected Pyrrolidine Adduct on C60 ; Carbon 13 NMR in D2O.

Peak assignments: BOC Carbonyl (177), fullerene peaks (166, 165, 158,157). Quartenary carbon on fullerene (121) Compare to S6b with loss of acid carbonyl (before reaction at 168), the addition of fullerene peaks, and the shifting of adduct chain peaks compared to starting material showing their change in environment due to sphere currents.
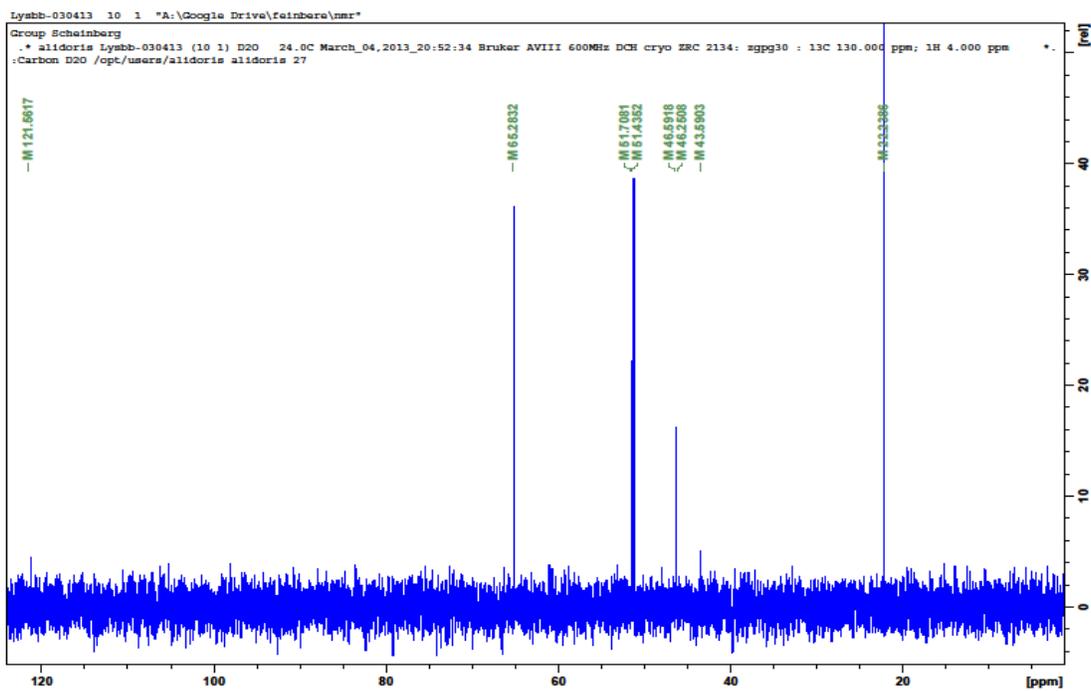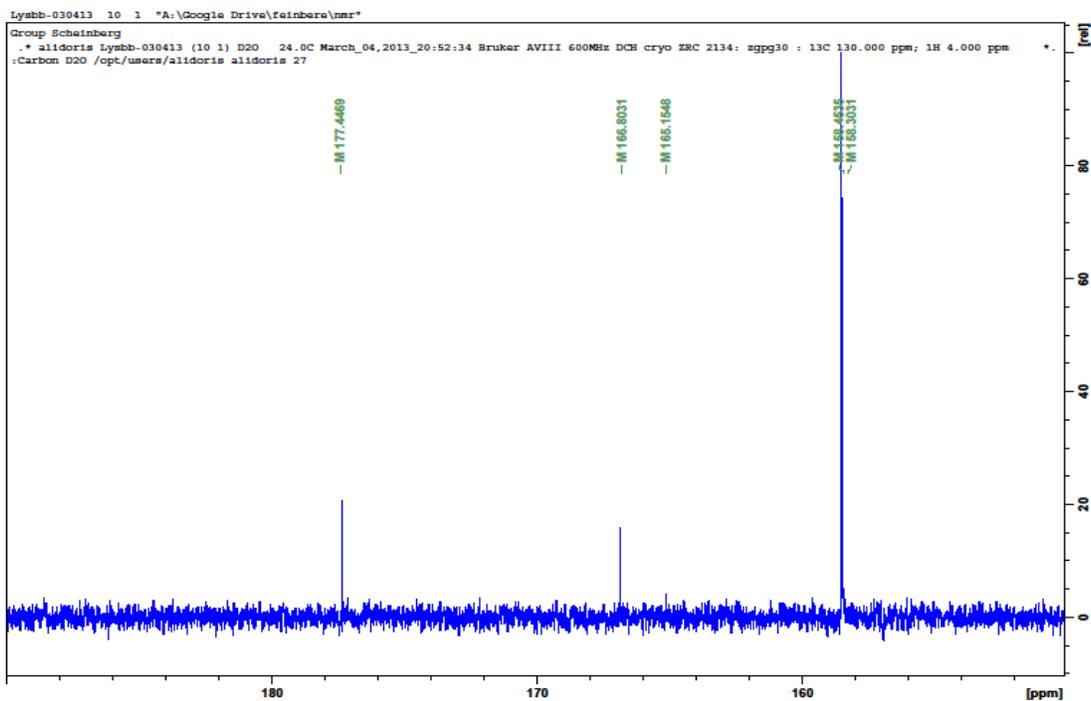
Figure S7: Mass Spectroscopy and Calculations for Adducted C60 Molecules

Figure S7A: Deprotected Primary Amine Product (3) – 10DG, Dialysis purified, run in MeOH.
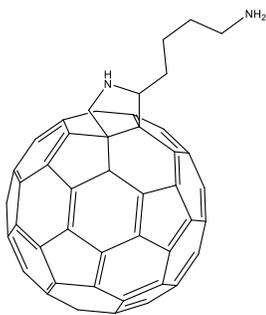
C = 12.011 x 66 = 792.73

N = 14.007 x 2 = 28.014

H = 1.008 x 14 = 14.11

Sum = 834.854

Ionizing proton = 1.008

methanol = 12.011 + 16.000 + 4(1.008) = 32.043

Free Primary Amine

(792.73 + 28.014 + 14.11 + 1.008 + 32.043) = 867.905 (theoretical)   Peak found 868.33

(792.73 + 28.014 + 14.11 + 2.016 +64.086)/2 = 450.478 (theoretical)   Peak found 448.19

The peak at 668.13 is thought to be an ionization fragment, or very speculatively the double ion of a 5-fold adduct with sodium. (720.66 + 114.19*5 + 23*2)/2 = 668.80 (theoretical)   Peak found 668.13
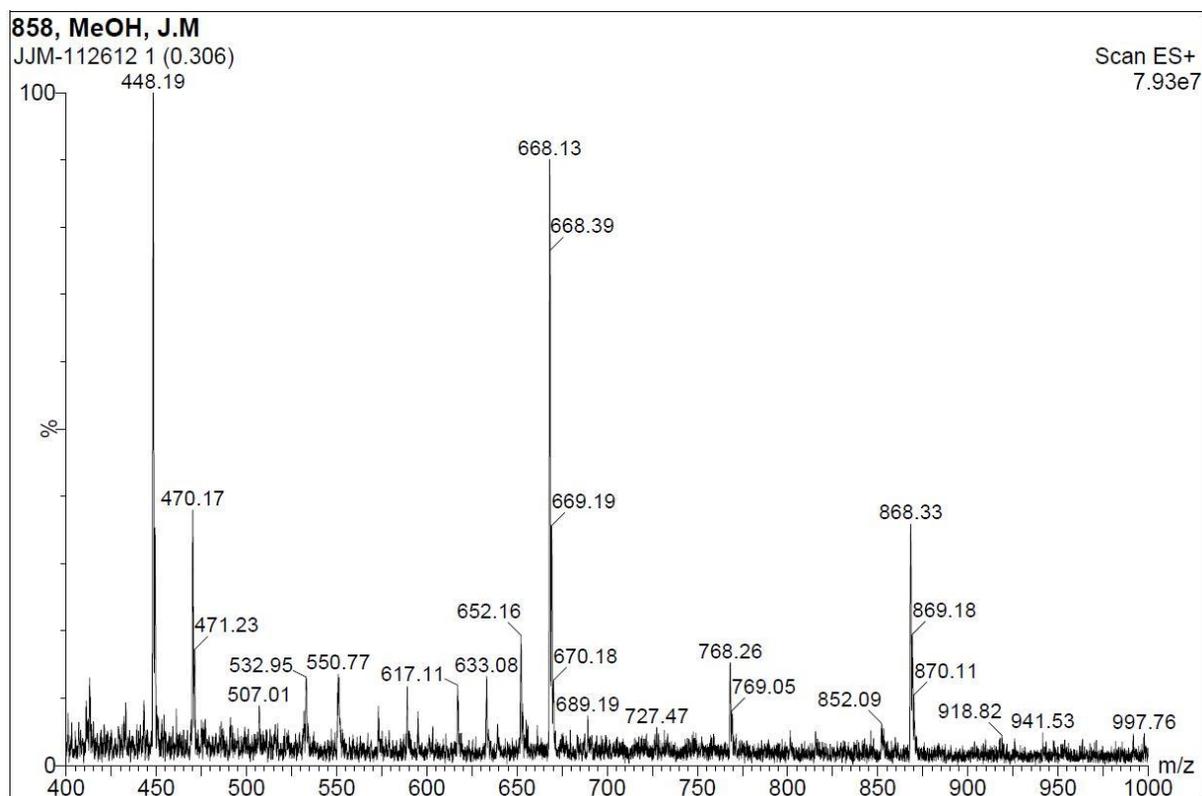
Figure 7B: BOC protected Primary Amine Product (4) run in acetonitrile
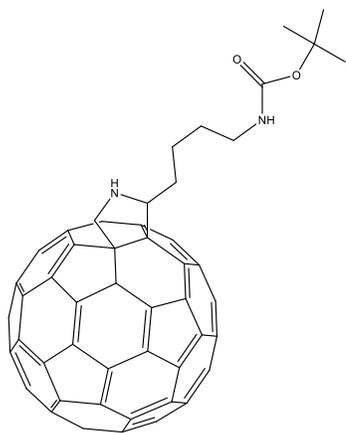
C = 12.011 x 71 = 840.770

N = 14.007 x 2 = 28.014

H = 1.008 x 22 = 22.176

O = 15.999 x 2 = 31.998

Sum = 934.969

Sodium = 23

(934.969 + 23) /2 = 958.977/2 = 478.98 (Theoretical) Peak found 479.11
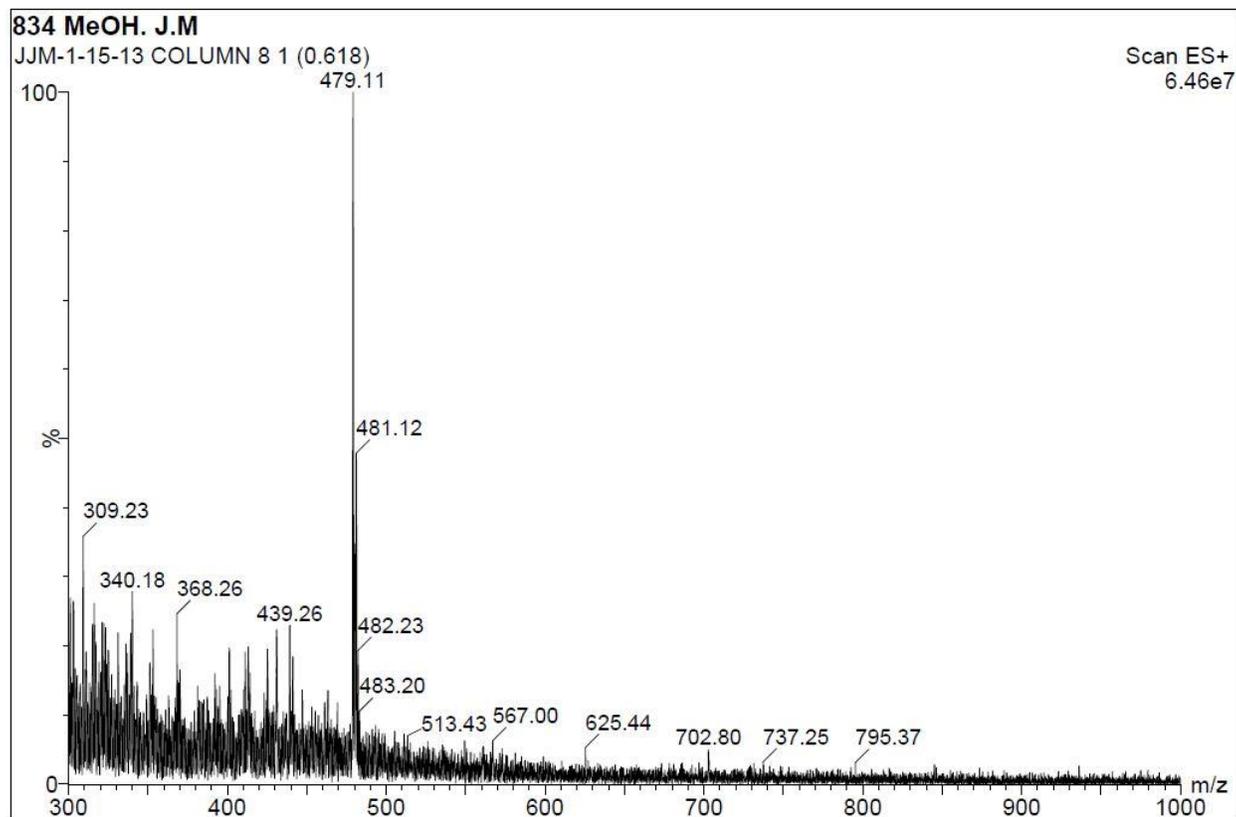
Figure S8: Degradation of amino acid used in Figure 1a after exposure to room temperature and laboratory light showing PEG fragmentation.

Figure S8a: Initial NMR sample taken in DMSO
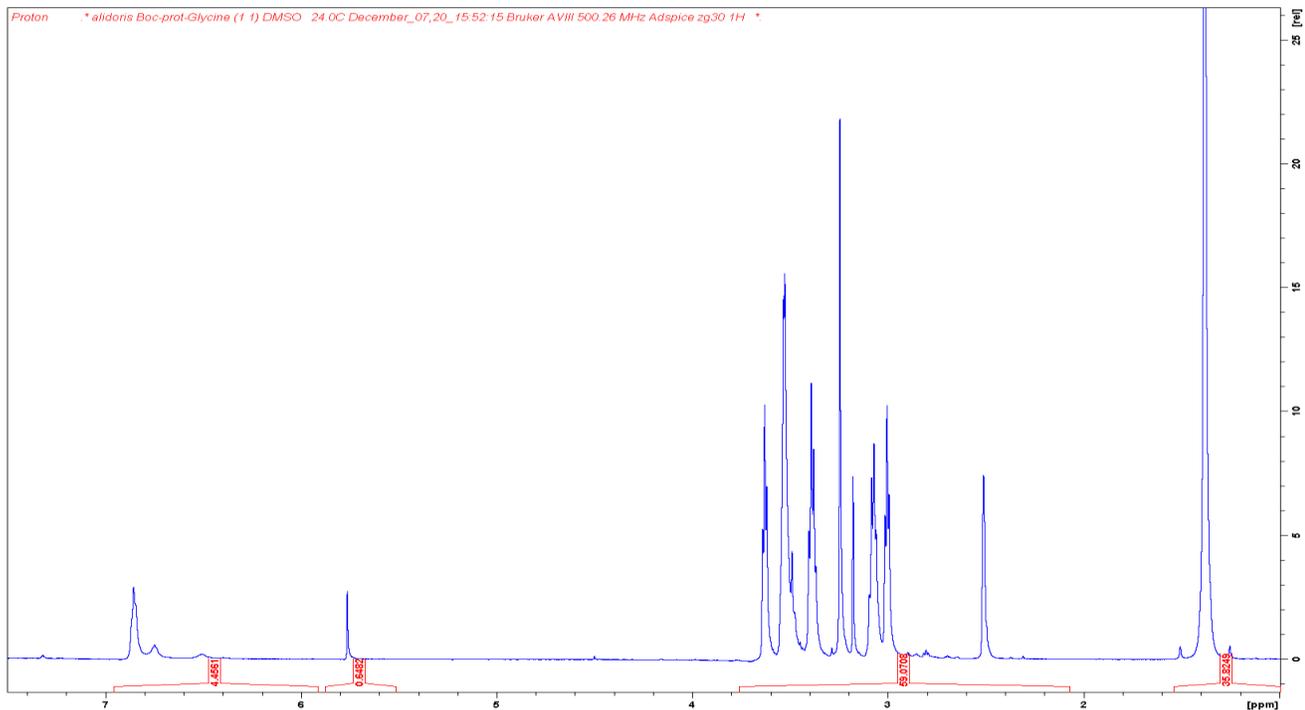
Figure S8b: Identical NMR spectrum in DMSO of **S8a** NMR tube contents left at ambient light and temperature for 1 month. Additional peaks, including a broad peak δ4.65 suggestive of PEG decomposition. According to recent literature, this peak is representative of 1,2 Ethanediol in DMSO[5], the bi-product of such fragmentation.
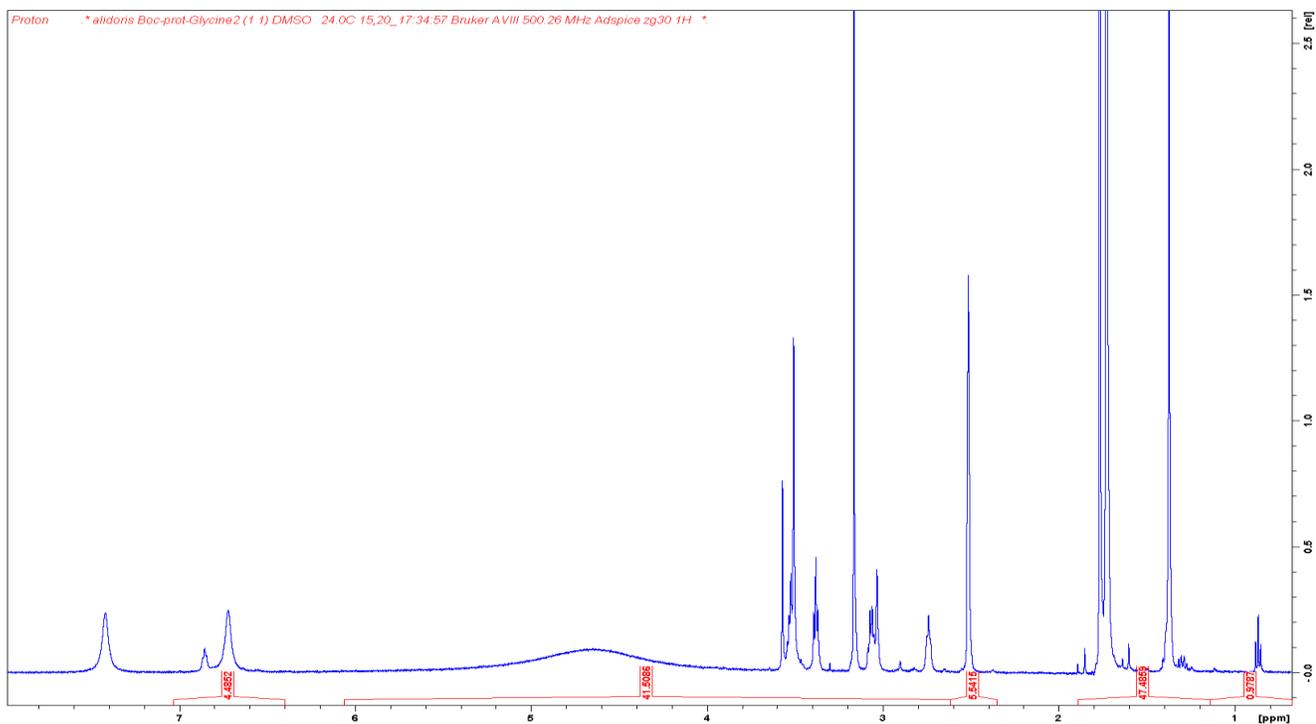
Figure S9: RAMAN spectra of: **A)** quartz glass background **B)** stock SWCNTs **C)** BOC-Lysine Modified SWCNTs

Absolute intensity of peaks between spectra varies with different LASER aperture and scan number settings for clearest image and should not be compared. The intensity ratios of peaks of interest to same-spectrum background peaks in the first three spectra provide a basis of allocating intensity to peaks in S9d. For instance, despite their initial presentation, peaks near 1500 cm^{-1} in S9c are at a comparable magnitude to background peaks in S9c indicating that the lysine peaks contribute only scantly to the dband of the modified nanotubes. The area related to the D band is a populous site in this study with weak, broad signals even appearing in background scans. Comparing the "D band" in S9d to background peaks in that spectrum support it to be predominantly a SWCNT-derived D band, with minor contributions from background, and both starting materials. Green dots mark background peaks. There is a reproducible luminescence for plated, water-soluble material (S9c and S9d) between 1000 and 2000 cm^{-1} due to heightened dispersion (ref 31) that has not been altered or removed as baseline.

A) Quartz glass Raman spectrum (background)
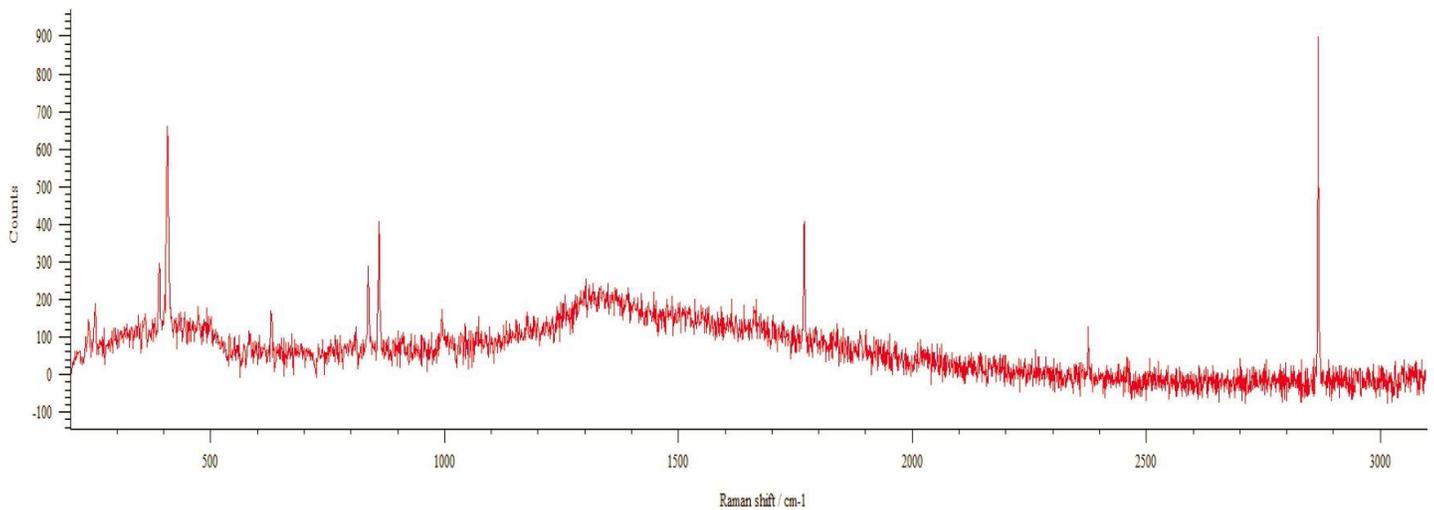
B) Stock SWCNTs Raman spectrum. Cosmic Ray (not reproduced) around 980 cm^{-1} was not removed from spectrum. Single peak at ~2880 cm^{-1}, two peaks at ~850 cm^{-1}, and a single peak at ~410 cm^{-1} represent background peaks.
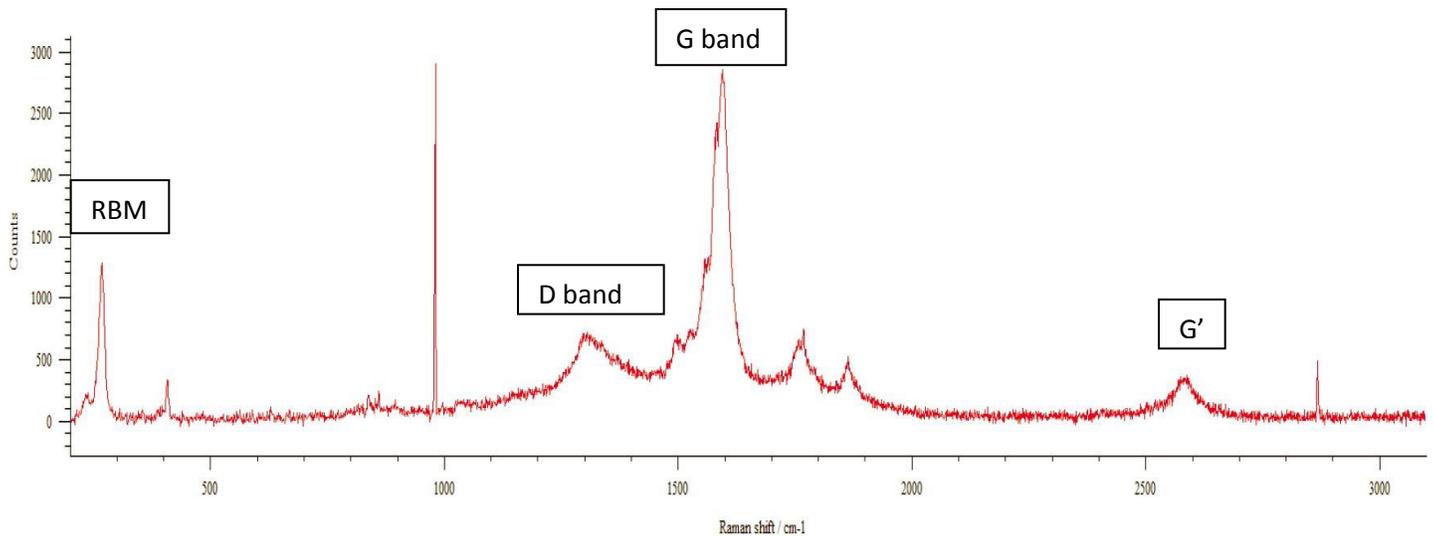
C) BOC-Lysine Modified SWCNTs. Single peak at ~2880 cm⁻¹, single peak at ~2390 cm⁻¹, and a sin G' eak ~410 cm⁻¹ represent background peaks. D band presumed enhanced by BOC'd lysine adduct as well as tube modification.
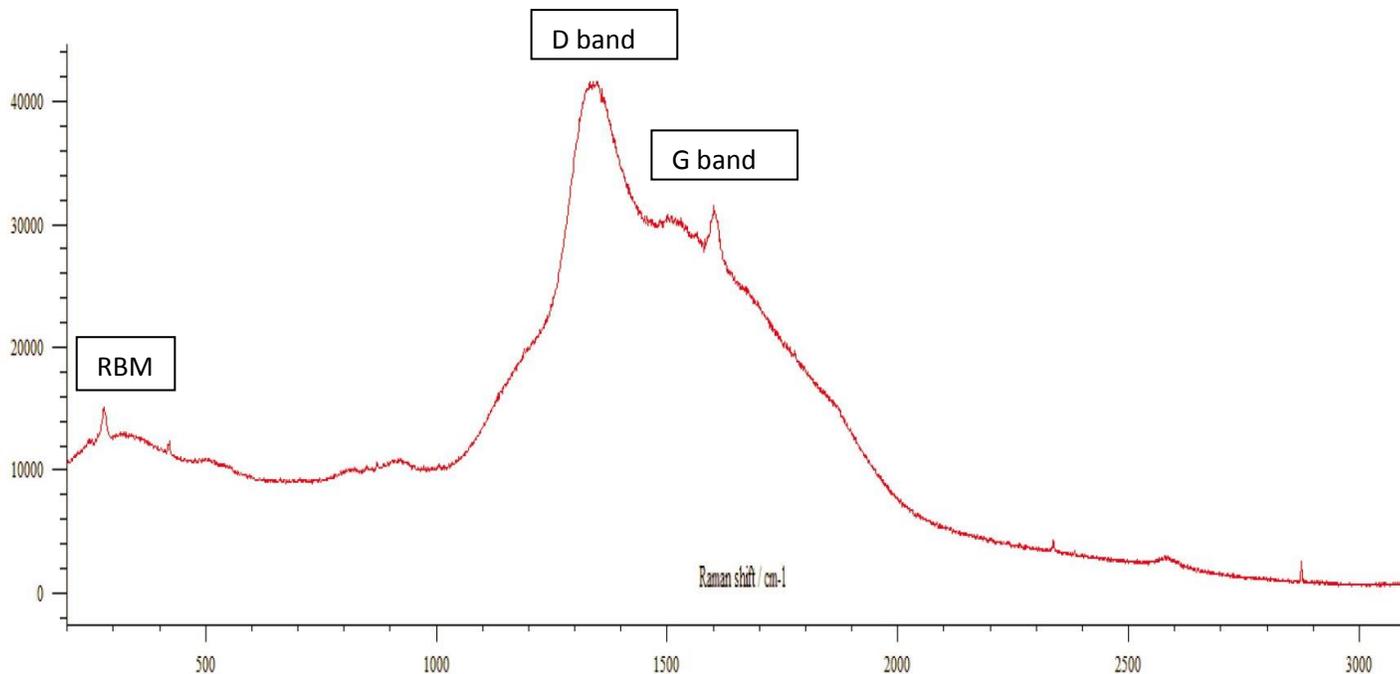
Figure S10: HPLC Analysis of SWCNT-Lys-DOTA(^{111}In),(**5**)

A) Urine Alone. (As seen in D and E, some mouse urine peaks are variable)
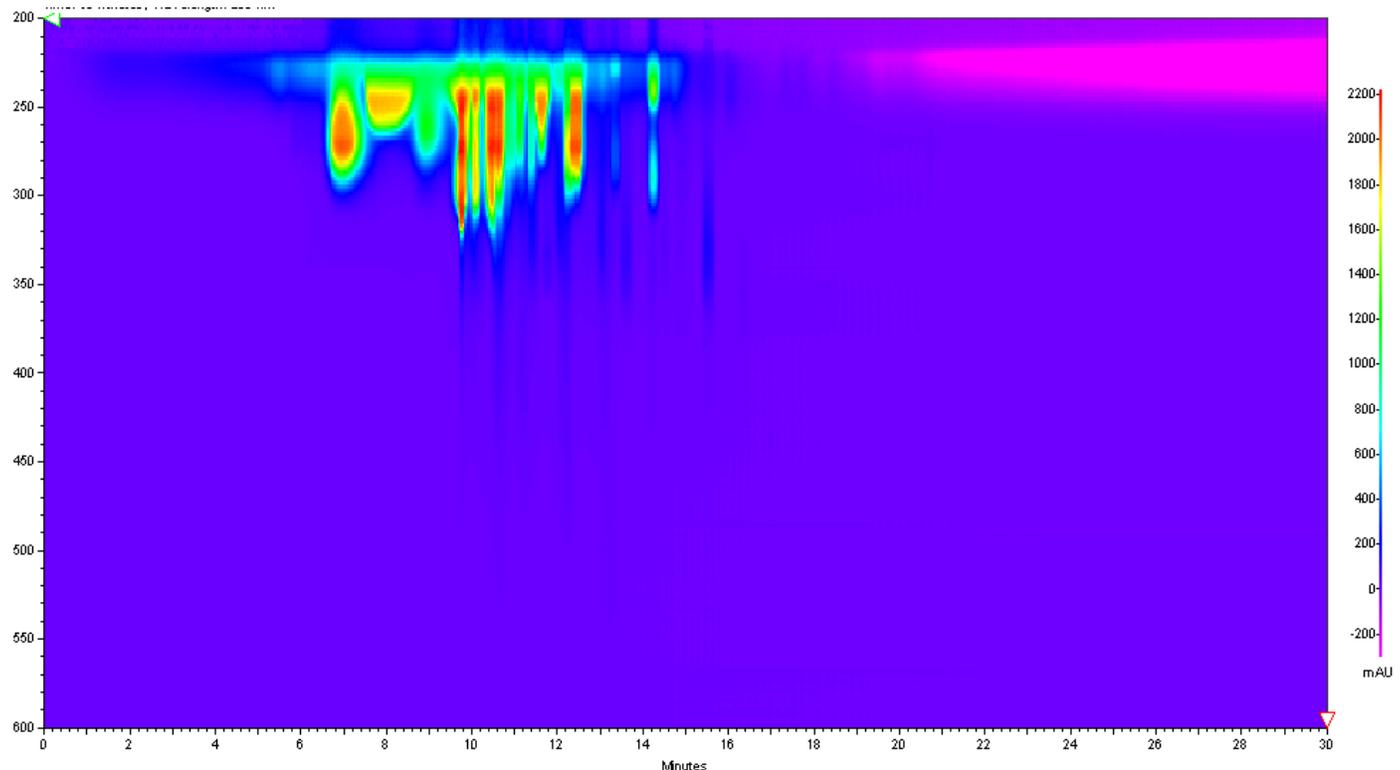
B) SWCNT-Lys-DOTA(^{111}In), (**5**) after 10DG purification, UV and radio trace of sample Please note that the radiodectector and UV detector are slightly offset depending on sequence of the HPLC modules
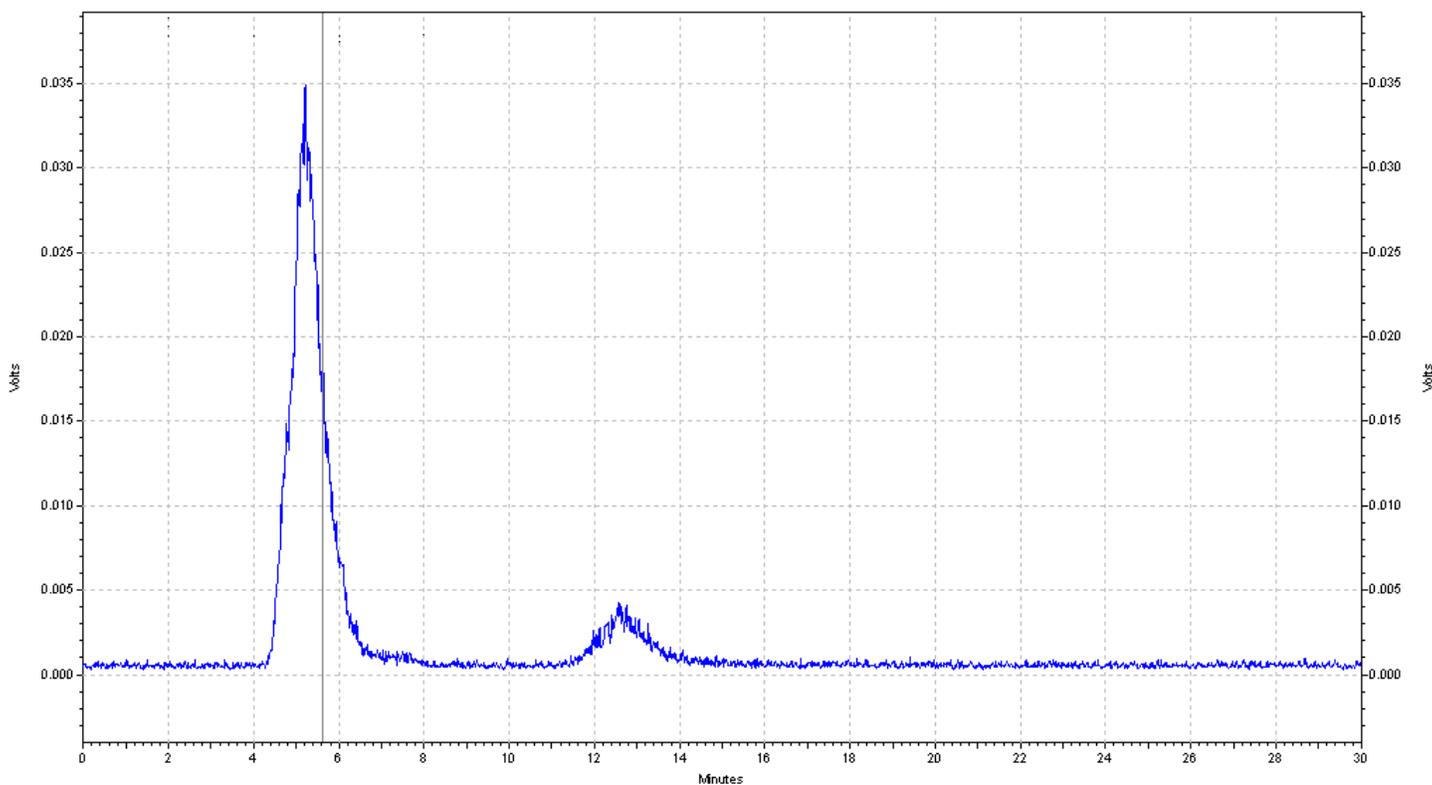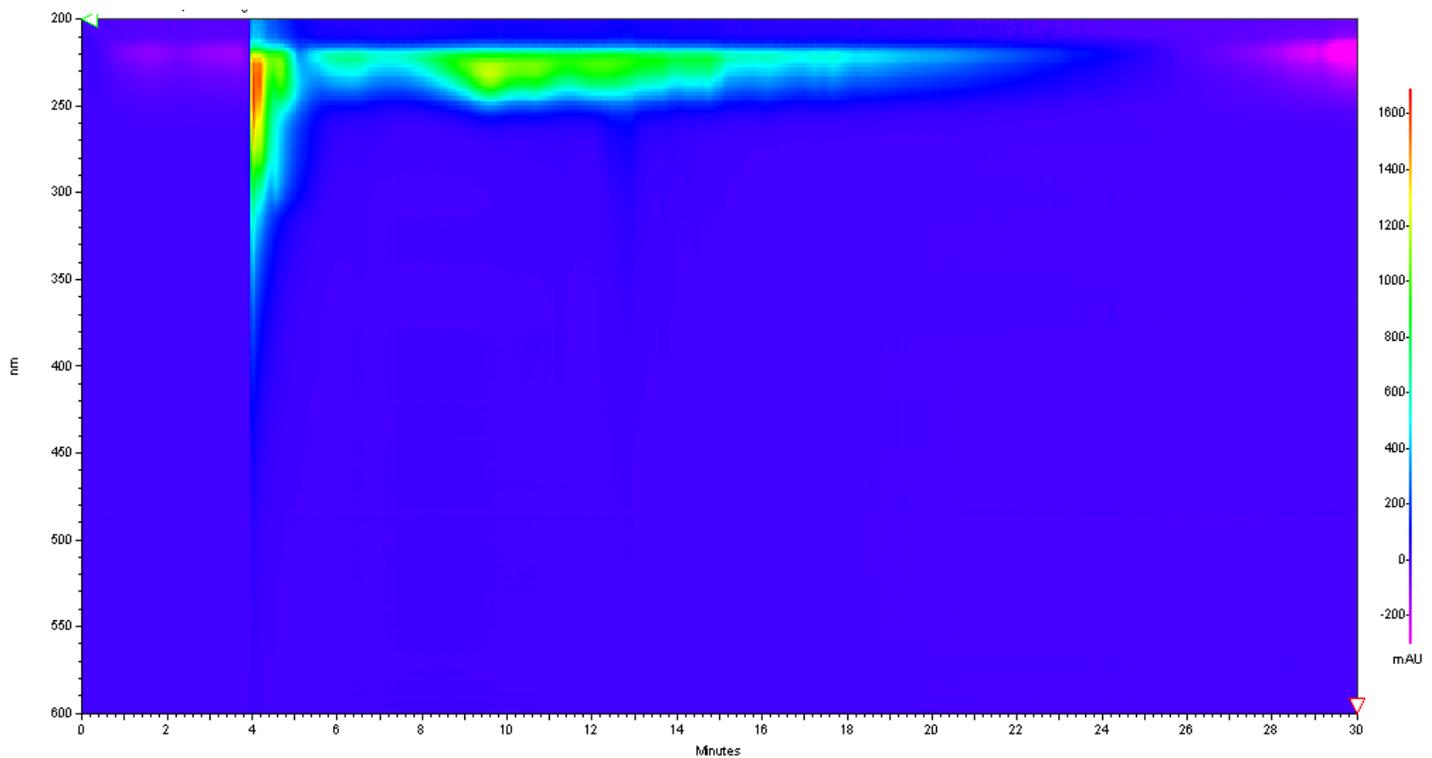
C) DOTA-SCN (unattached chelator) alone (multiple peaks represent different SCN group hydrolysis states)
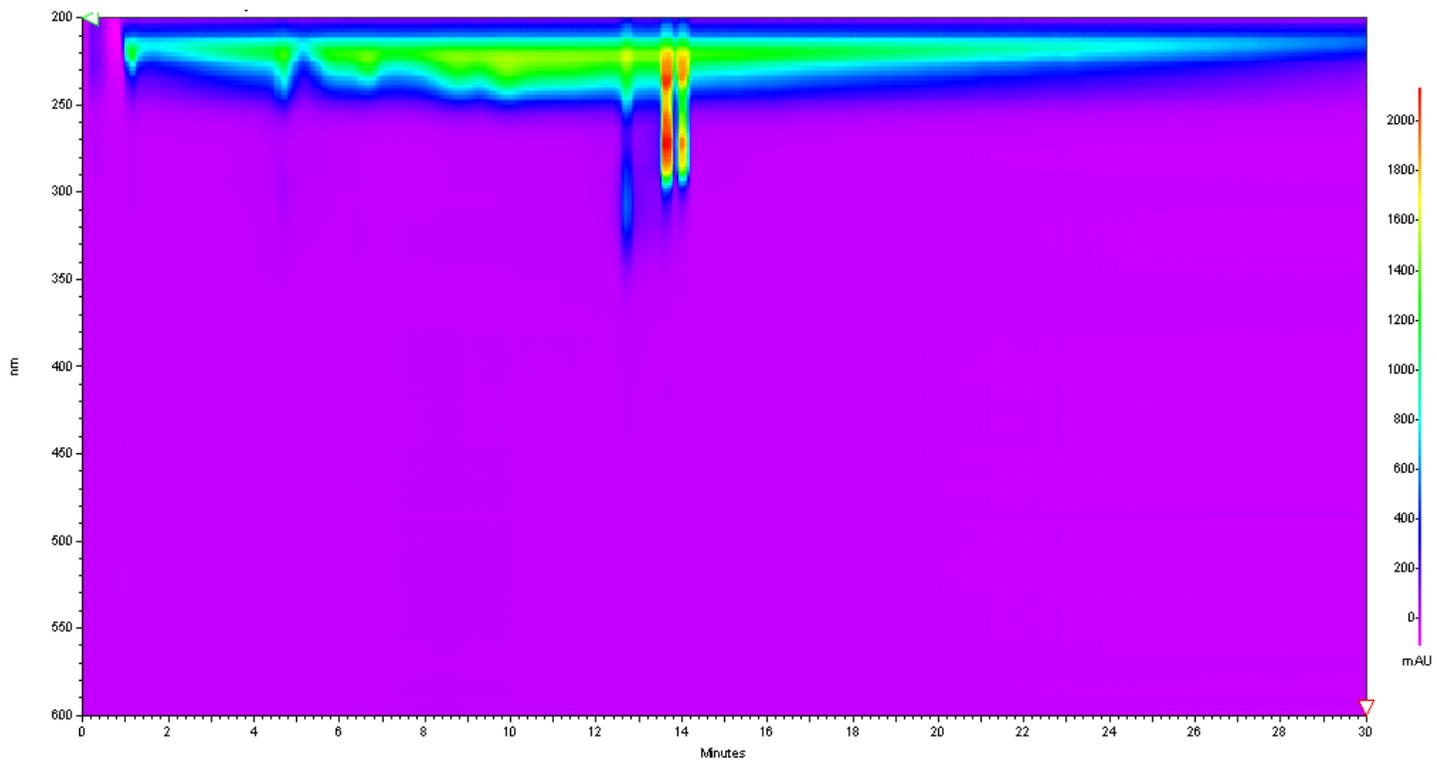
D) Unlabelled (**5**) mixed with pre-injection mouse urine
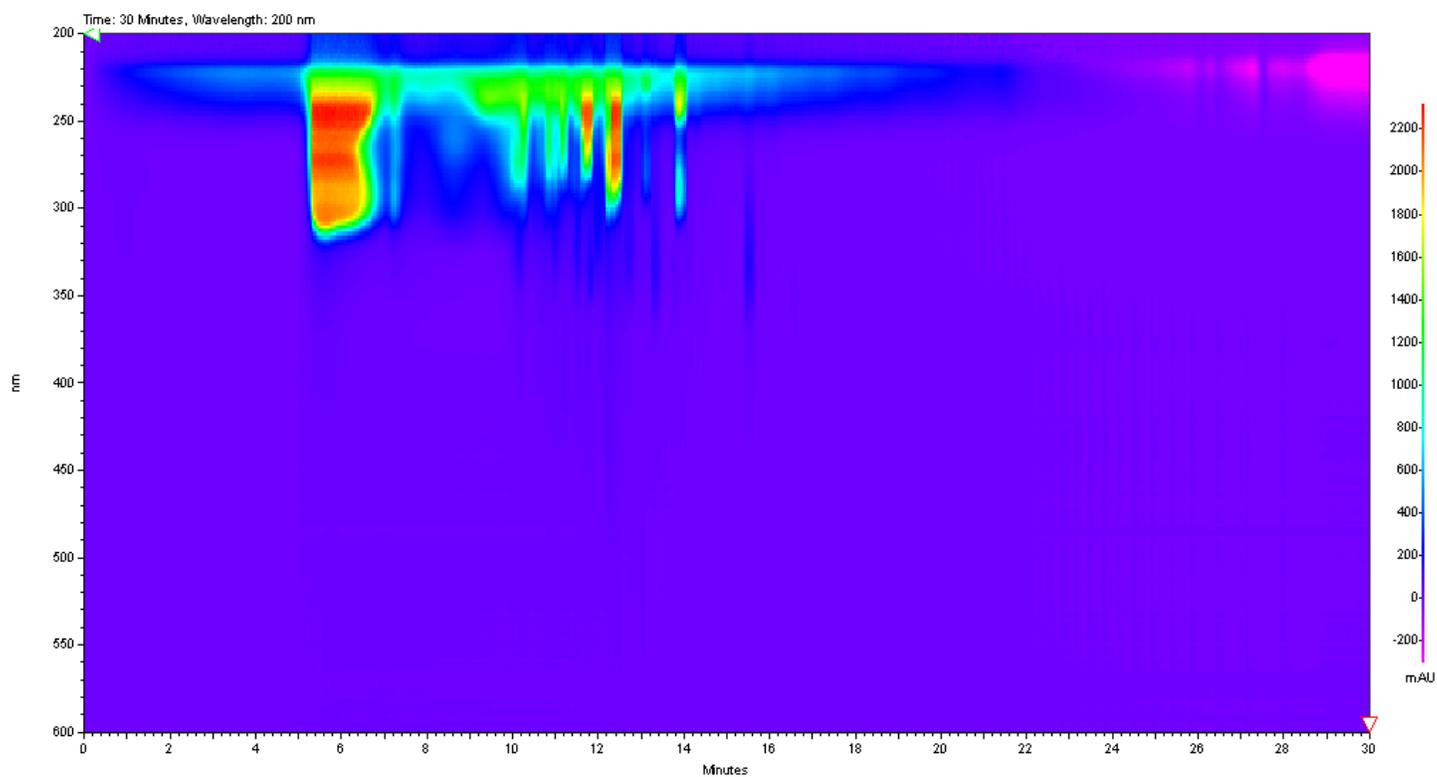
E) Vacuum Concentrated mouse IP injected (**5**) collected in urine of the mouse. UV and radio traces. Radioactivity detected after urine vacuum concentration is 33.3 times greater on (**5**) than detached DOTA. This is 97% and representative of the pre-injected radiochemical purity of the sample.
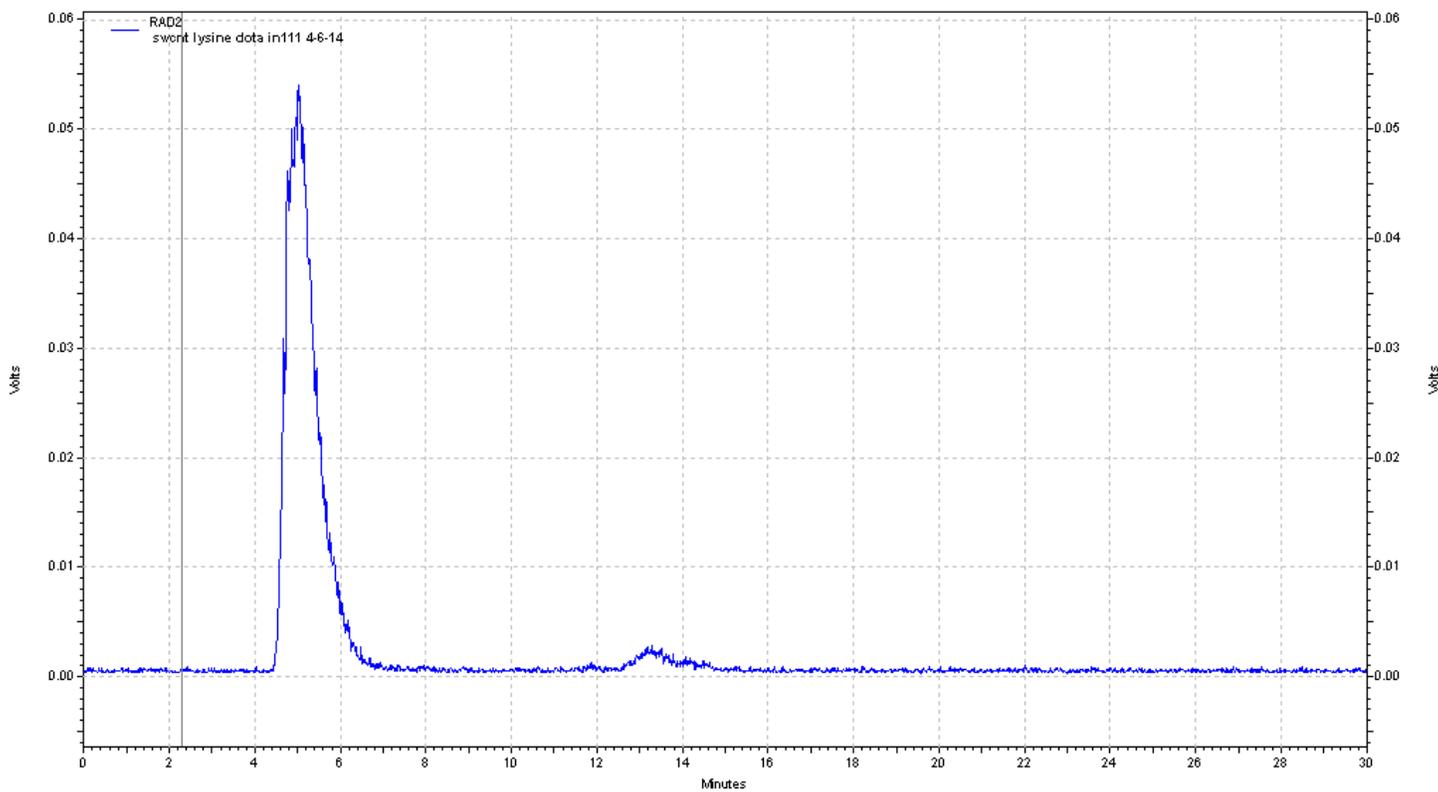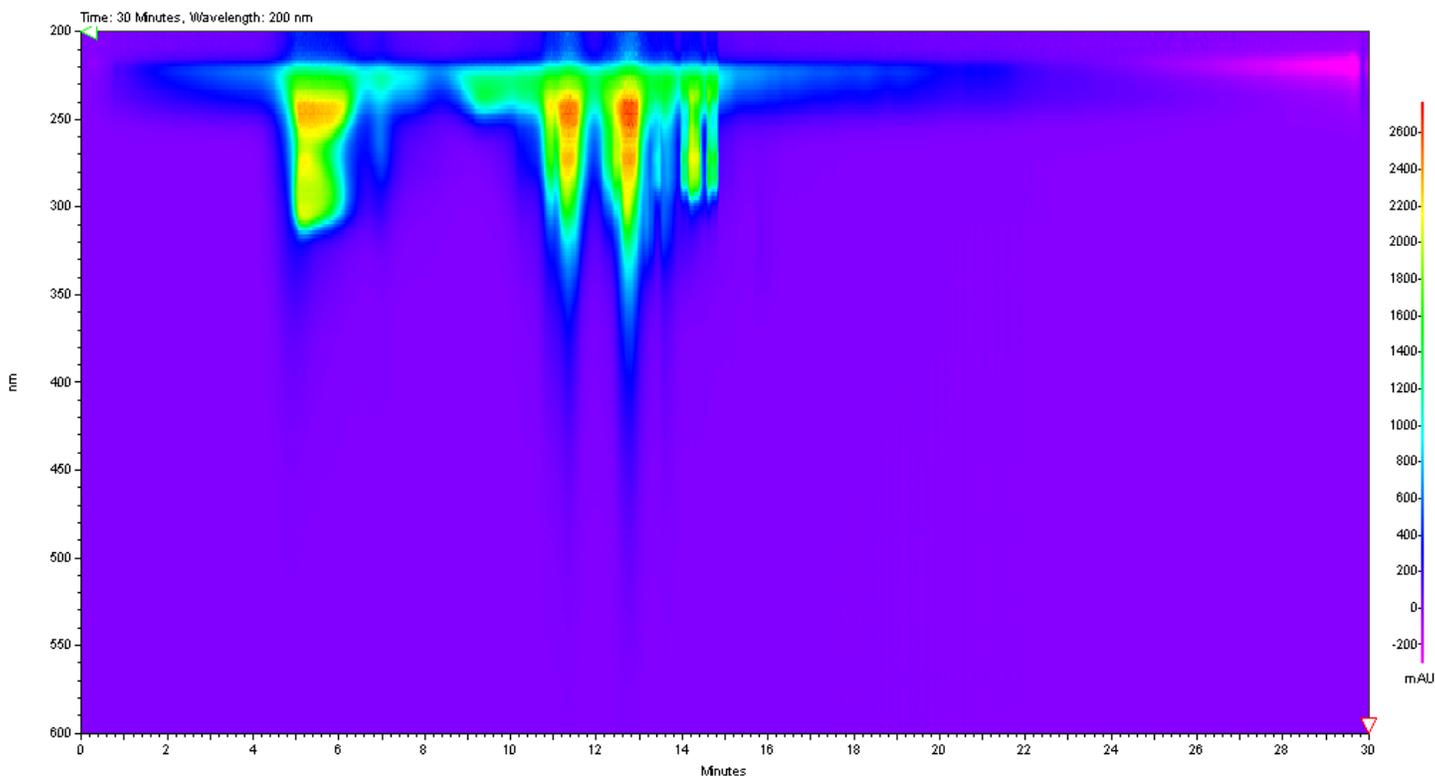
Supporting Information Literature Cited

1 K. Sbai, A. Rahmani, H. Chadli, J.-L. Bantignies, P. Hermet, J.-L. Sauvajol, *J. Phys. Chem. B* **2006**, *110*, 12388–12393.

2 M. P. Johansson, J. Jusélius, D. Sundholm, *Angew. Chemie Int. Ed.* **2005**, *44*, 1843–1846.

3 D. Sebastiani, K. N. Kudin, *ACS Nano* **2008**, *2*, 661–668.

4 M. A. Lebedeva, T. W. Chamberlain, M. Schröder, A. N. Khlobystov, *Tetrahedron* **2012**, *68*, 4976–4985.

5 J. S. Lomas, *Magn. Reson. Chem.* **2013**, *51*, 32–41.