Enzymatic 'stripping' and degradation of PEGylated carbon nanotubes

SUPPLEMENTARY INFORMATION

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1. Fabrication of 6-Aminofluorescein linked ox-SWCNTs.

Three milligrams of oven-dried ox-SWCNTs were sonicated for 1 h with 25 μL of 1 M *N*, *N'*-dicyclohexylcarbodiimide and 25 μL of 1M diisopropylethylamine. Next, 1 mg of fluoresceinamine (Sigma-Aldrich) was added and the amidation reaction was carried out for 48 h. Finally, free compounds were washed away by filtering the mixture through 100kDa-cut off filters.

2. Fabrication of PEG-coated (cPEG-SWCNTs).

Oven-dried pristine SWCNTs (Carbon Solutions, Inc.) were mixed in a 1:5 weight ratio with 1, 2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene-glycol)-2000] (Avanti Polar Lipids, Inc.) in PBS and sonicated for 6 h. Next, short hydrophilic amino-terminated PEG-coated SWCNTs were fractionated by stepwise centrifugation and washed from free phospholipids through 100kDa-cut off filters. Finally, 2kDa cPEG-SWCNT-m and 2kDa cPEG-SWCNT-488 were obtained by incubating amino-terminated PEG-coated SWCNTs with NHS-mPEO and NHS-DyLight 488 (Thermo Scientific), respectively.

3. Fabrication of PEG-functionalized SWCNTs (fPEG-SWCNTs).

Three milligrams of oven-dried ox-SWCNTs (Carbon Solutions) were mixed with α-amino-ω-Boc-amino linear 2, 5, or, 10kDa PEG (Rapp Polymere GmbH) in 10 mL of dichloromethane, 125 μL of 1 M *N,N'*-dicyclohexylcarbodiimide and 60 mg of 4-dimethylaminopyridine (Sigma-Aldrich). The amidation reaction was carried out for 48 h. Boc groups were unprotected by using a trifluoroacetic acid solution 1:1 (v/v) in dichloromethane. Next, short amino-terminated PEG-functionalized SWCNTs were fractionated by stepwise centrifugation and residual reagents were removed using 100kDa-cutoff filters. fPEG-SWCNT-m and fPEG-SWCNT-488 were obtained by incubating amino-terminated fPEG-SWCNTs with NHS-mPEO and NHS-DYLight 488, respectively.

4. Atomic Force Microscopy (AFM).

Mica substrates (Ted Pella) were spotted with a few microliters of nanotube solutions. After being rinsed in Milli-Q H_2O, mica substrates were dried under a gentle nitrogen stream and scanned through a 5500 AFM (Agilent Technologies, Inc.). Twenty AFM longitudinal cross-

section curves for each type of PEG-modified SWCNTs were recorded and used to characterize the conformation of PEG chains on PEG-modified SWCNTs.

5. Dynamic Light Scattering (DLS).

Surface charge of CNTs in phosphate buffer solution was obtained at 25°C using a Zetasizer NanoZS (Malvern).

4. *In vitro* biodegradation of CNTs by Raman and UV-Vis-NIR spectroscopy.

Lyophilized purified native human myeloperoxidase (MPO) was purchased from Athens Research and Technology, Inc. (Athens, GA). Hydrogen peroxide (30%, H_2O_2), 0.1 M phosphate buffer, sodium chloride (NaCl), and diethylenetriaminepentaacetic acid (DTPA) were obtained from Sigma Aldrich. DTPA was added to 0.1 M phosphate buffer solution at a final concentration of 50 μM. Next, stock solutions of H_2O_2 (50 mM) and NaCl (5 M) were prepared utilizing nanopure water as the solvent. The as-received lyophilized MPO was reconstituted in 133 μL of nanopure water for a final concentration of ~5 μM. Fifteen micrograms of a given nanotube were incubated in 250 μL of 0.1 M phosphate buffer / 50 μM DTPA that contained 140 mM NaCl and 100 nM MPO in sets of six. Employing an Epoch microplate spectrophotometer (BioTek, Winooski, VT), an initial (day 0, 0 h) absorbance value was measured at 999 nm. Every hour, H_2O_2 was apportioned at a final concentration of 200 μM for a total of 7 additions. This procedure (*i.e.* the addition of MPO and H_2O_2) was repeated on days 1-7. Between additions, the samples were stored at 37°C in an incubator (Thermo Scientific). At time points day 1 (24 h), 2 (48 h), 3 (72 h), 4 (96 h), 5 (120 h), 6 (144 h), and 7 (168 h), the Epoch microplate spectrophotometer was utilized to measure the absorbance value at 999 nm. Relative changes in absorbance with time for ox-SWCNT, 2kDa cPEG-SWCNT and 2, 5 and 10kDa fPEG-SWCNT were plotted with standard deviation error bars representing a sample size (n) of six. One-way ANOVA was performed for measuring the statistical significance of the degradation by comparing relative changes in the S_{22} absorption spectra of day 7 *versus* day 0. In addition, samples were collected on day 0 and 7 and Raman spectroscopy was performed using a Horiba XploRA ONE Raman microscope equipped with a 638 nm laser excitation and an Olympus MPLN objective with an NA of .90, integration time 1 s and 100x magnification. Three spectra with 20 accumulations were obtained for each sample and D-band (~1350 nm) and G-band

(~1598 nm) intensities were collected per measurement. From the results, the D:G ratio was obtained and averaged with standard error (S.E.).

6. Transmission Electron Microscopy (TEM).

Fifteen micrograms of pristine SWCNTs, ox-SWCNTs, 2kDa cPEG-SWCNTs, 2, 5 and 10kDa fPEG-SWCNTs and were incubated in the presence of 50 μM metal chelator DTPA in 0.1 M phosphate buffer, in 250 μL solution containing 140 mM NaCl and 100 nM MPO. Every hour, H_2O_2 was apportioned at a final concentration of 200 μM for a total of 7 additions. Samples were diluted 1 : 100 with nanopure water. Seven microliters of the suspended sample were placed on a lacey carbon grid (Pacific-Grid Tech) and permitted to dry in ambient conditions for overnight prior to TEM imaging (FEI Morgagni, 80 keV).

7. Isolation of primary human neutrophils.

Isolation and culture of peripheral blood neutrophils from buffy coats obtained from healthy adult blood donors at the Karolinska University Hospital Blood Bank was performed using previously established protocols.[1] Briefly, neutrophils were isolated from heparinized blood by a method of dextran sedimentation and density gradient centrifugation. Residual erythrocytes were removed by hypotonic lysis. Isolated human neutrophils (1×10^6 cells/mL) were cultured in RPMI-1640 cell culture medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO_2.

8. Sterility assessment of CNTs.

To assess the sterility of the SWCNT samples, 1 μg of each CNT suspension was spread on sterile LB Agar plates prepared from tablets containing enzymatic digest of casein 9.14 g/L, yeast extract 4.57 g/L low sodium, sodium chloride 4.57 g/L and bacteriological agar 13.72 g/L (from Sigma Aldrich). The plates with CNTs were then incubated at 37°C for 24 h. No contamination was observed (data not shown).

9. *Ex vivo* biodegradation of CNTs assessed by Raman spectroscopy.

Human neutrophils were isolated and cultured as described above. For biodegradation studies, neutrophils were exposed to 10 µg/mL of pristine SWCNTs, ox-SWCNTs, 2kDa cPEG-SWCNTs and 2, 5 and 10kDa fPEG-SWCNTs, respectively and co-stimulated with 10 nM of formyl-methionyl-leucyl-phenylalanine (fMLP) and 5 µg/mL of CB, for the indicated time-points (1, 4 and 8 h). Then, neutrophils were washed with sterile luke-warm PBS and centrifuged at 1200 rpm. The cell pellet was collected and resuspended in 1 mL of PBS. The cells were disrupted using probe based ultrasonication for a period of 1 min at 50% power. Cell lysate suspensions containing the SWCNTs were drop-casted onto a quartz slide and dried; biodegradation analysis was performed by Raman spectroscopy as previously described[3] using a WITec alpha300 system in combination with a 532 nm laser for excitation and a 100x objective with an NA of 0.95, integration time 0.5 s and 600x magnification. Six random spectra from different positions of the quartz slide were recorded for each sample and time point, and averaged. One way ANOVA followed by Tukey post-hoc test was performed to calculate the statistical significance.

10. Confocal microscopy of cellular uptake of CNTs.

Human neutrophils were isolated from buffy coat using the methodology described above. Freshly isolated neutrophils (5 x 10^5 cells/mL) were plated on sterilized glass cover-slip coated with Poly-L-Lysine (0.01% solution) in cell culture media. The cells were pre-incubated for 1 h and then exposed to 2.5 µg/mL of ox-SWCNT-488, 2, 5 and 10kDa fPEG-SWCNT-488 and 2kDa cPEG-SWCNT-488, co-stimulated with 10 nM of fMLP and 5 µg/mL of cytochalasin B for time periods of 1, 3 and 6 h. For the confocal microscopy analysis, neutrophils were washed with luke-warm PBS and fixed on the cover-slip using 4% formaldehyde for 1 h. Fixed neutrophils were mounted on a clean glass slide using Vectashield mounting medium with DAPI nuclear stain. Fluorescent images were taken in Z-Stack mode at 400x magnification using Zeiss LSM510 META laser scanning microscope and using laser lines at 405 and 488 nm for fluorochrome excitation.

11. **Thermogravimetric Analysis (TGA).**

Defunctionalization studies were done by treating 1 mg of 5kDa fPEG-SWCNTs with 1 U/500 µl of neutrophil elastase (NE) (purchased from Sigma) for a period of 6 h and 6 days (144 h). As a

control, equal weights of NE treated ox-SWCNTs were included in parallel to the 5kDa fPEG-SWCNTs. For the 6 h treatment, 1 U of NE (600 µg in total) was added to all the PEG- and ox-SWCNTs samples at once and for the longer treatment (6 days), 100 µg of NE was added every 24 h. Addition of NE to the samples was done under sterile conditions and the samples were incubated at 37°C. After exposure completion , samples were collected and stored at 4°C. TGA was used to analyze changes in the density of the PEG chains on the surface of the SWCNTs. Before the TGA analysis, NE treated PEG-SWCNT were purified by centrifugation a minimum of 5 times using Amicon ultra centrifugal filter units (100 kDa) to remove the free and detached PEG chains. 5kDa PEG chains were used as a control in the study. TGA was performed using TGA Q500, TA Instruments (Sollentuna, Sweden) from ambient temperature to 700^{o}C in a nitrogen atmosphere (20 ml/min).

References

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SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1: Characterization of ox-SWCNTs and PEG-modified SWCNTs. AFM image of 2kDa cPEG-SWCNT (scan size 2.5 × 2.5 µm), longitudinal cross-sections and values of h_m for the nanotubes into the yellow dotted circles. The yellow arrow indicates a flat ~1 nm-tall portion that has been interpreted as a portion of SWCNT sidewall devoid of PEG coverage. Scale bar 300 nm.

Supplementary Figure 2: MPO-driven *in vitro* degradation of SWCNTs determined by Raman spectroscopy. Raman spectroscopic (Raman shift Rel cm^{-1}) analysis of MPO + NaCl assisted degradation of **A.** pristine SWCNTs, **B.** ox-SWCNTs, **C.** 2kDa cPEG-SWCNTs, **D.** 2kDa fPEG-SWCNTs, **E.** 5kDa fPEG-SWCNTs, **F.** 10kDa fPEG-SWCNTs. 15 µg/ml of individual SWCNTs were treated with 100 nM/day of recombinant MPO, 140 mM of NaCl and 200 µM of H_2O_2/h in the presence of the metal chelator, DTPA (50 µM). Measurements of biodegradation were performed on 0, 4 and 7 days by Raman spectroscopy.

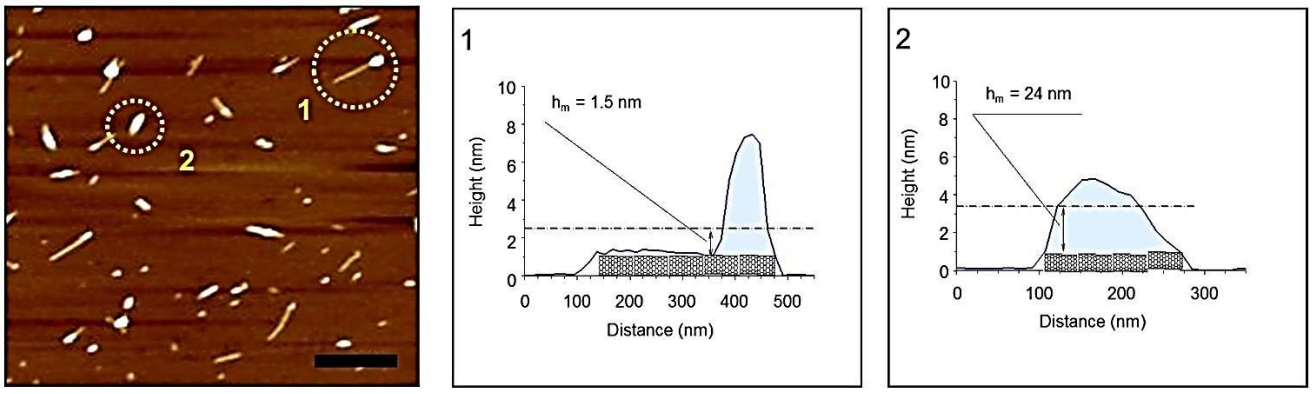
Supplementary Figure 3: Time-dependent *in vitro* degradation of SWCNTs determined by UV-Vis-NIR spectroscopy. MPO-driven degradation of 15 µg/ml of ox-SWCNTs, 2kDa cPEG-SWCNTs and 2, 5 and 10kDa fPEG-SWCNTs using 100 nM/day of recombinant MPO, 140 mM of NaCl and 200 µM of H_2O_2 per h in the presence of the chelator, DTPA (50 µM). Measurements were performed every day for 7 days by UV-Vis-NIR spectroscopy using the value of absorption spectra at 999 nm. *p* values * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001, by One-way ANOVA followed by Tukey post-hoc test.

Supplementary Figure 4: Transmission electron microscopy imaging of *in vitro* degradation of CNTs by recombinant MPO. 15 µg/ml of pristine SWCNTs (**a.** 0 day; **b.** 4 day), ox-SWCNTs (**c.** 0 day; **d.** 4 day), 2kDa cPEG-SWCNTs (**e.** 4 day), 2kDa fPEG-SWCNTs (**f.** 4 day); 5kDa fPEG-SWCNTs (**g.** 4 day) and 10kDa fPEG-SWCNTs (**h.** 4 day) incubated with 100 nM/day of recombinant MPO, 200 µM of H_2O_2 and 140 mM of NaCl per h, in the presence of 50 µM DTPA.

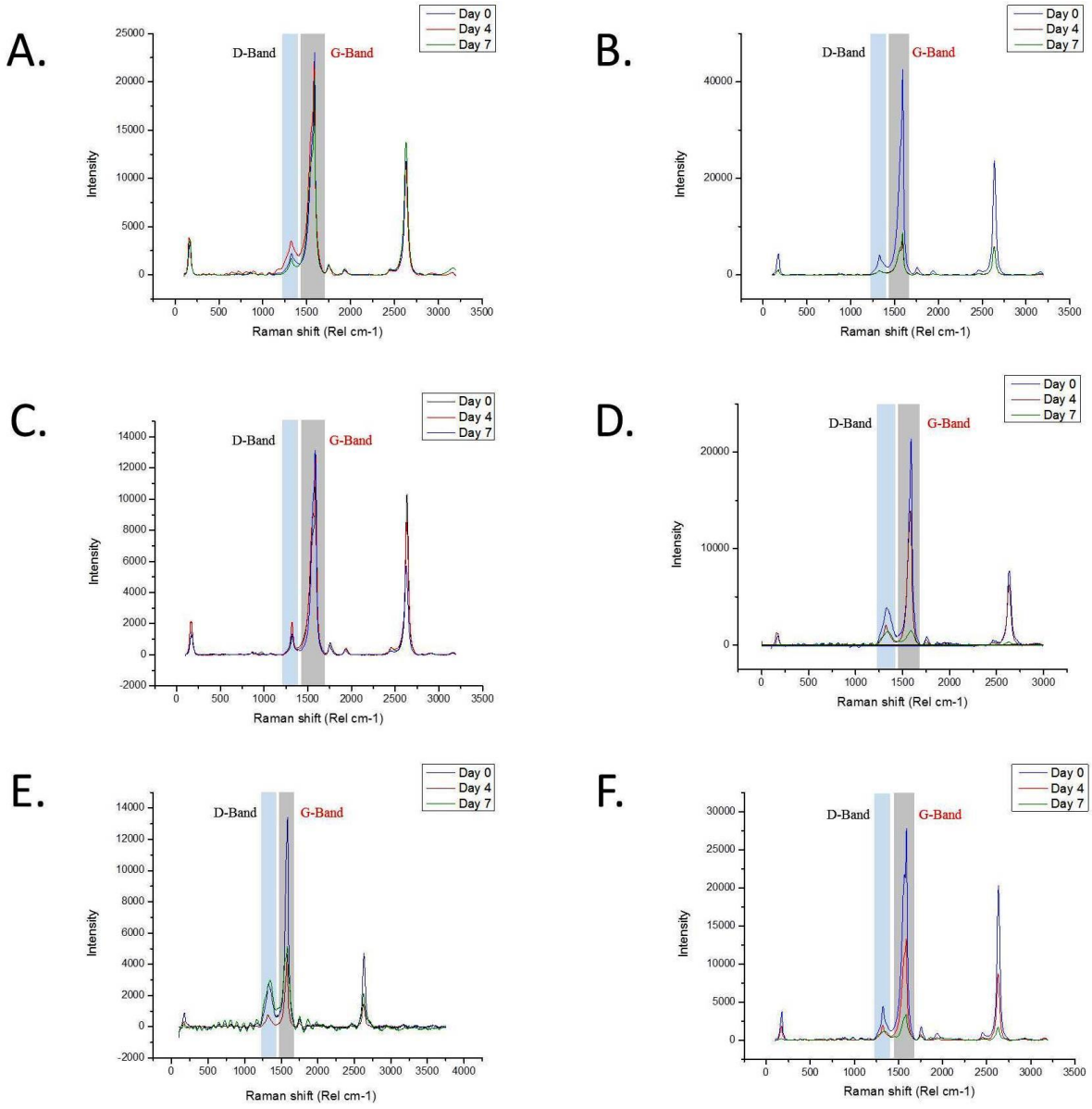
Supplementary Figure 5: *Ex vivo* biodegradation of SWCNTs by activated neutrophils assessed by Raman spectroscopy. Raman spectroscopic (Raman shift Rel cm^{-1}) analysis of biodegradation of **A.** pristine SWCNTs, **B.** ox-SWCNTs, **C.** 2kDa cPEG-SWCNT, **D.** 2kDa fPEG-SWCNT, **E.** 5kDa fPEG-SWCNT, and **F.** 10kDa fPEG-SWCNT following incubation with isolated human neutrophils (2 x 10^6 cells) for 1, 4 and 8 h, with stimulation with the agonists, fMLP (10 nM) and cytochalasin B (5 μg/mL).

Supplementary Figure 6: Uptake of oxidized and PEGylated SWCNTs by human neutrophils at 3 h. Confocal images of neutrophil uptake of 2.5 μg/ml PEG-SWCNT after 3 h exposure, **a.** negative control; **b.** 2kDa Poly(ethylene glycol) functionalized single walled carbon nanotubes (fPEG-SWCNT); **c.** 5kDa fPEG-SWCNT; **d.** 10kDa fPEG-SWCNT; **e.** 2kDa Poly(ethylene glycol) coated SWCNT; **f.** oxidized SWCNT. White arrows show the location of SWCNTs.

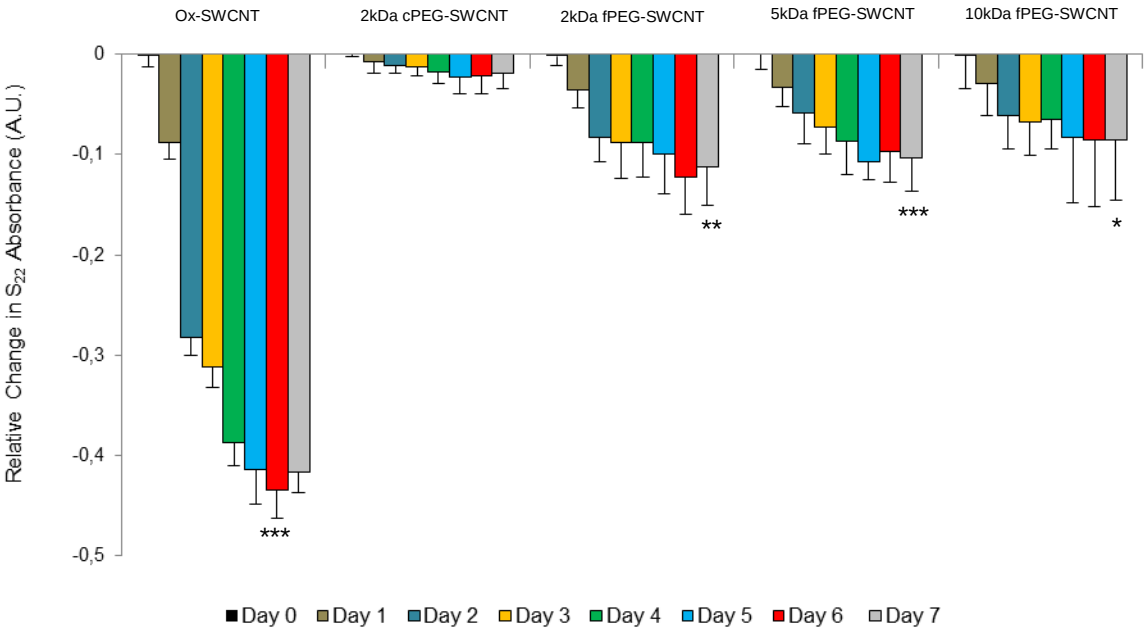
Supplementary Figure 7: Uptake of oxidized and PEGylated SWCNTs by human origin neutrophils at 6 h. Confocal images of neutrophil uptake of 2.5 μg/ml PEG-SWCNT after 6 h exposure, **a.** negative control; **b.** 2kDa Poly(ethylene glycol) functionalized single walled carbon nanotubes (fPEG-SWCNT); **c.** 5kDa fPEG-SWCNT; **d.** 10kDa fPEG-SWCNT; **e.** 2kDa Poly(ethylene glycol) coated SWCNT; **f.** oxidized SWCNT. White arrows show the location of SWCNTs.



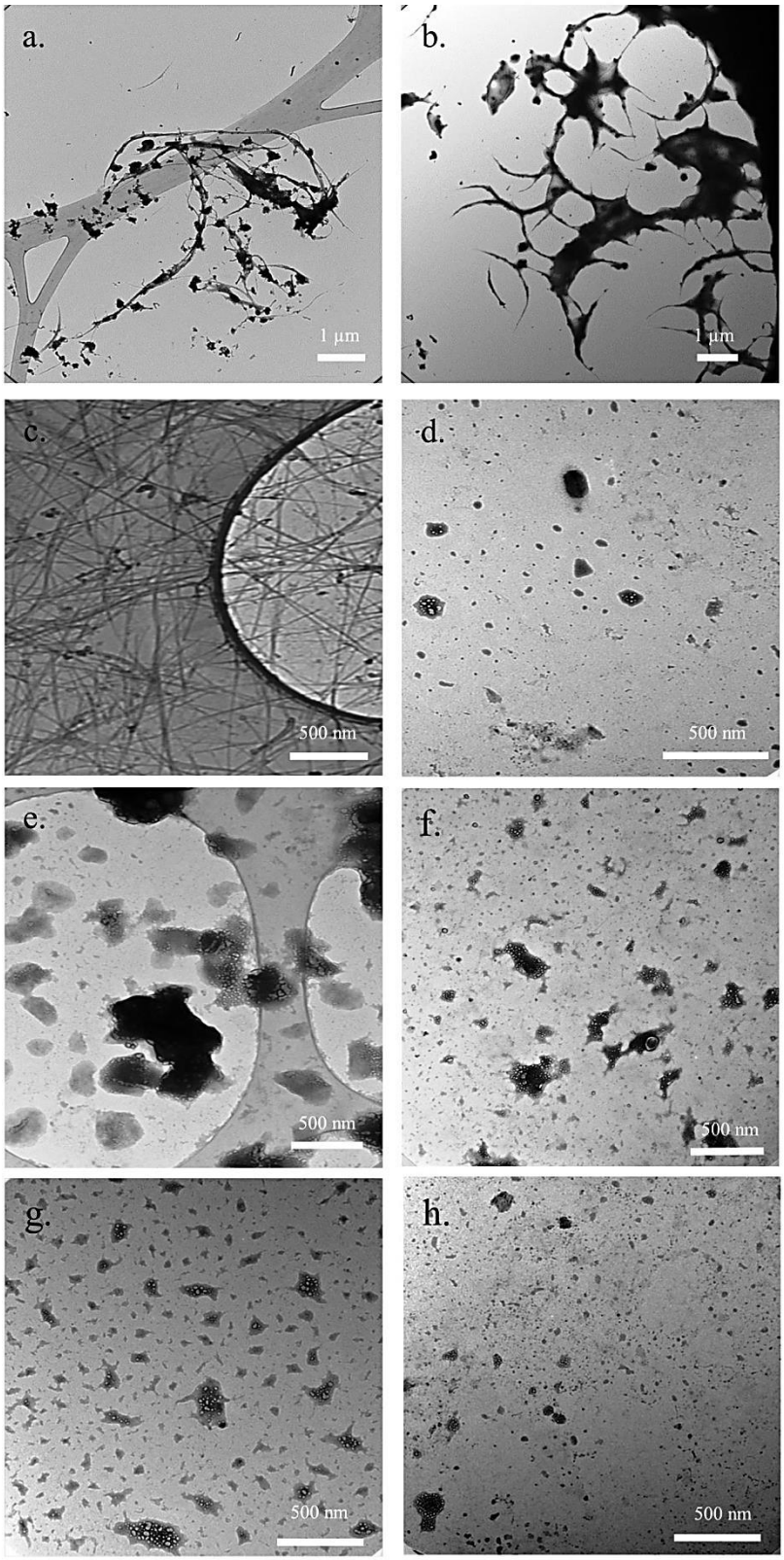
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



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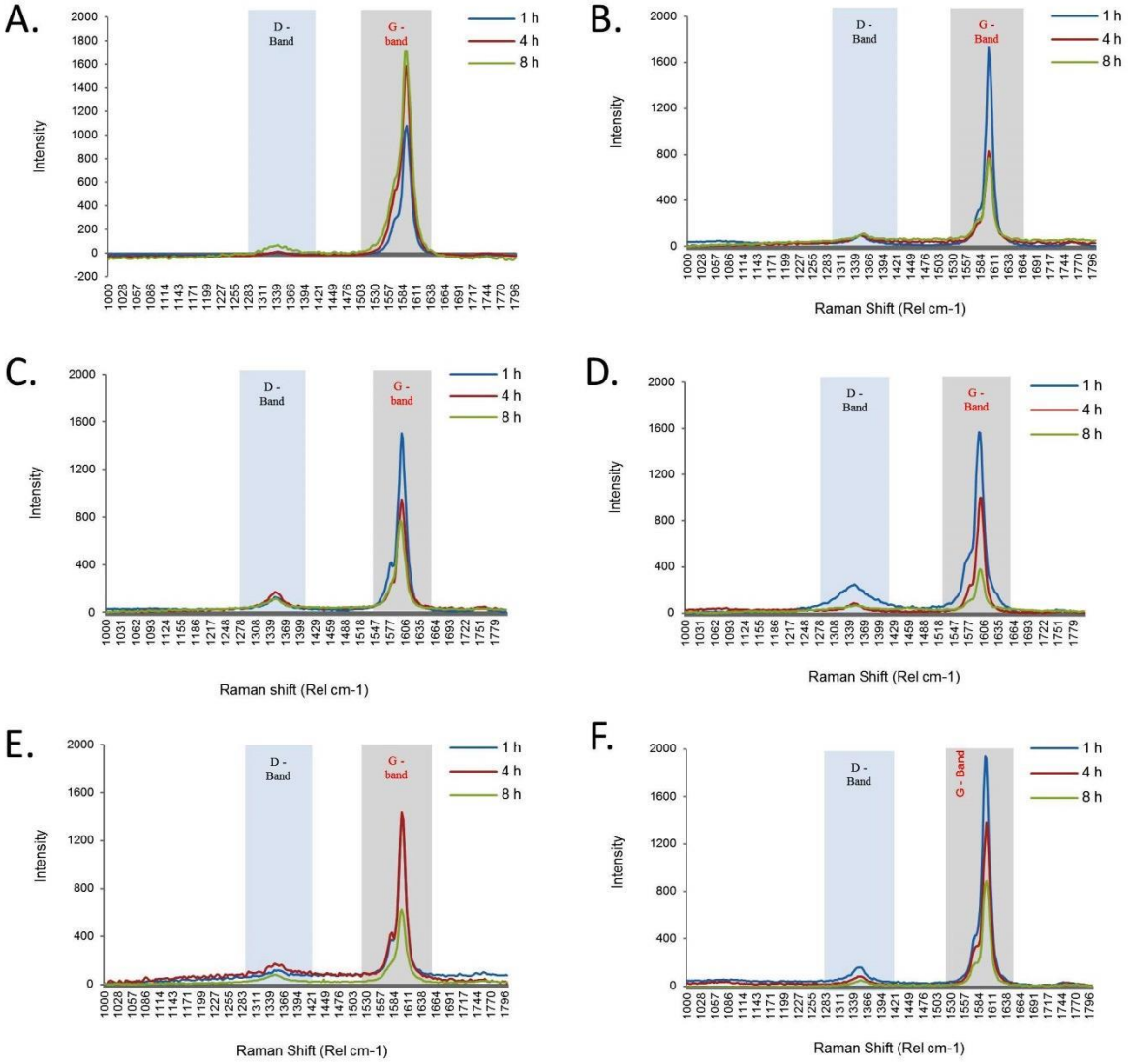
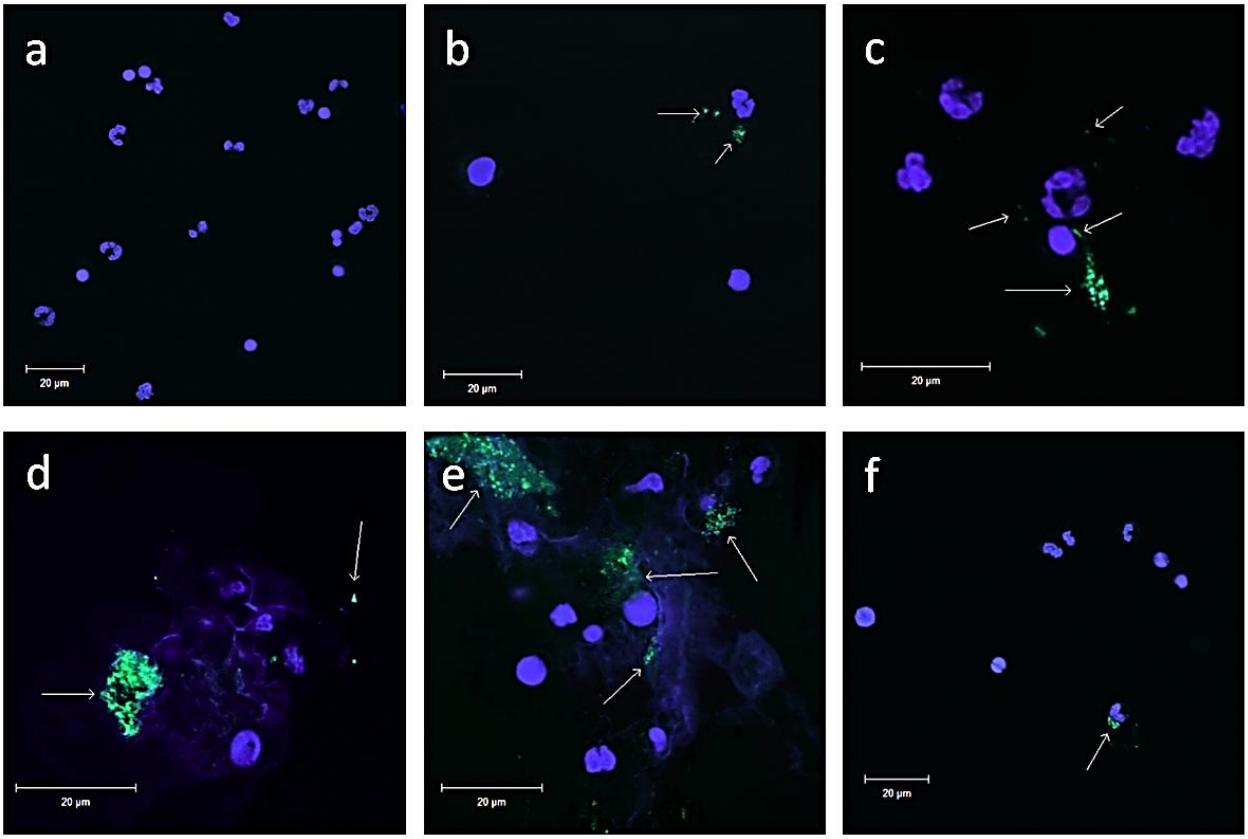
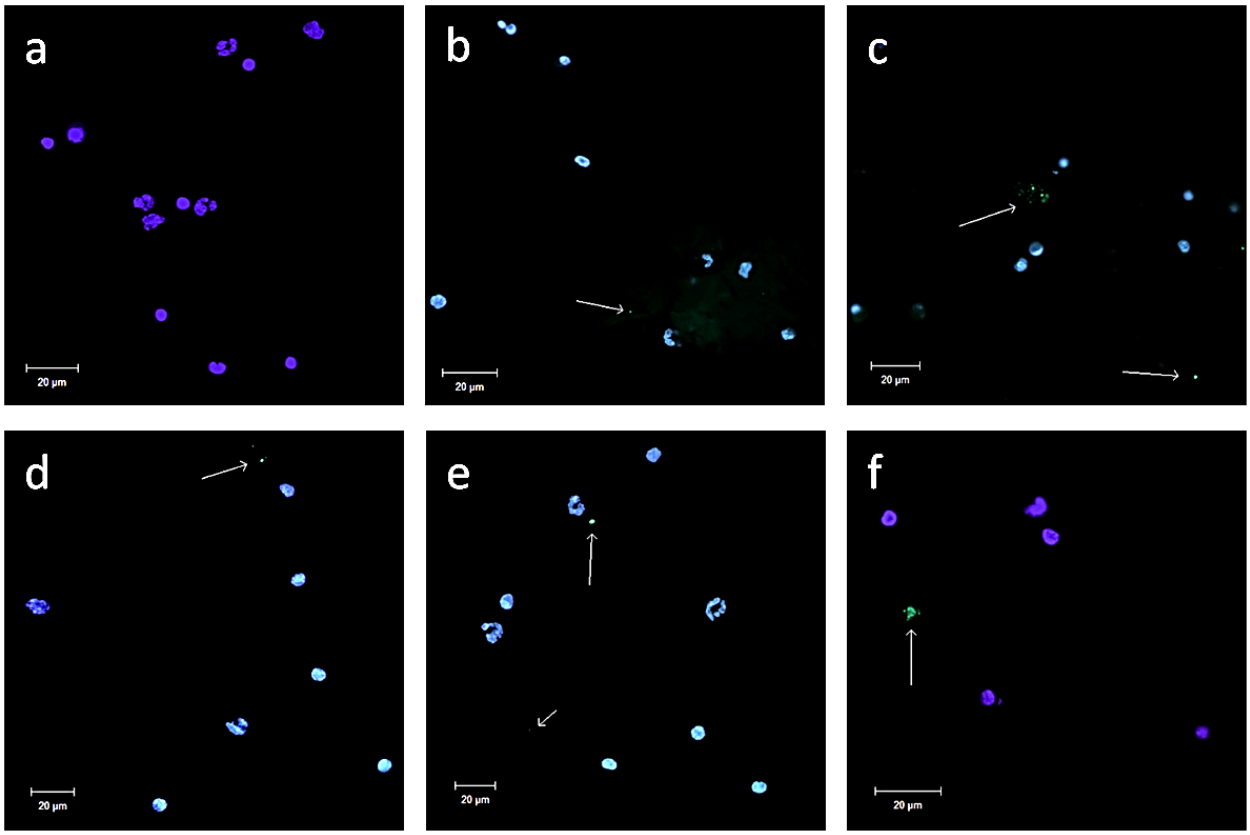


Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 7