U.S. FOOD & DRUG ADMINISTRATION

Dispersion of Graphitic material for biological studies

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Disclaimer: The views expressed are of the presenter and should not be considered as the official position or policy of U.S. FDA

Disclaimer

- This presentation is on “graphene material” – not on pure graphene
- It is intended to bring out challenges working with graphene material for biological studies
- A large batch of “pristine graphene material” was obtained for Graphene Consortium studies by the Universities in Arkansas (UAMS, UALR, UAF & FDA/NCTR)

Summery of Characterization at NCTR/FDA

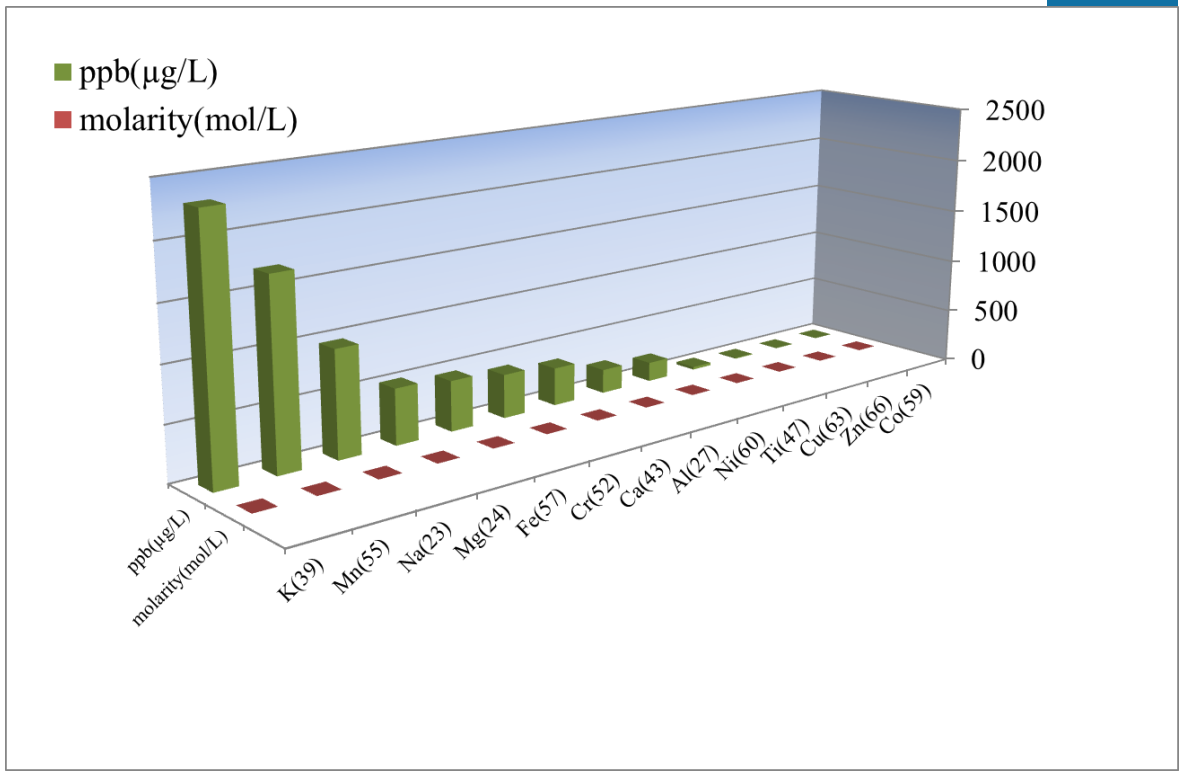
Type of analysis	Material Characterized	Result and overall conclusion
CHNO analysis	a)Graphene starting material b) Fn-graphene	Quantification of carbon, oxygen content; Conclusive
Dispersion of graphene	a) Graphene material b) Functionalized graphene	Significantly higher concentration and biologically compatible medium
Elemental analysis (ICP-MS)	a) Graphene starting material	Quantification of metal impurities in graphene; Conclusive
Raman spectroscopy	Graphene material	Characteristic bands confirmed composition
Scanning Electron Microscopy	Graphene material	Partially conclusive
Thermogravimetric analysis	Graphene material, Functionalized graphene	Overall morphology; functionalized inconclusive
UV-Vis Spectroscopy	Graphene material	Semiquantitative analysis
Low-voltage electron microscopy	Graphene material; graphene oxide	Particle data conclusive
Atomic force microscopy	Different dispersions on substrates	Conclusive flake size/structure/height information
Laser diffraction	Different dispersions of Graphene material; graphene oxide	Smaller size from microfluidization and larger from sonication

CHNO analysis of graphene material and functionalized graphene

Sample Name	Carbon Content (%)	Hydrogen Content (%)	Nitrogen Content (%)	Oxygen Content (%)	Carbon : Oxygen
Graphene Starting material	94.17	0.97	< 0.5	< 0.5	0.003985
Functionalized graphene (Fn-Gr)	87.46	1.16	< 0.5	6.91	0.059257

Quantitative metal impurity analysis of graphene material by ICP-MS

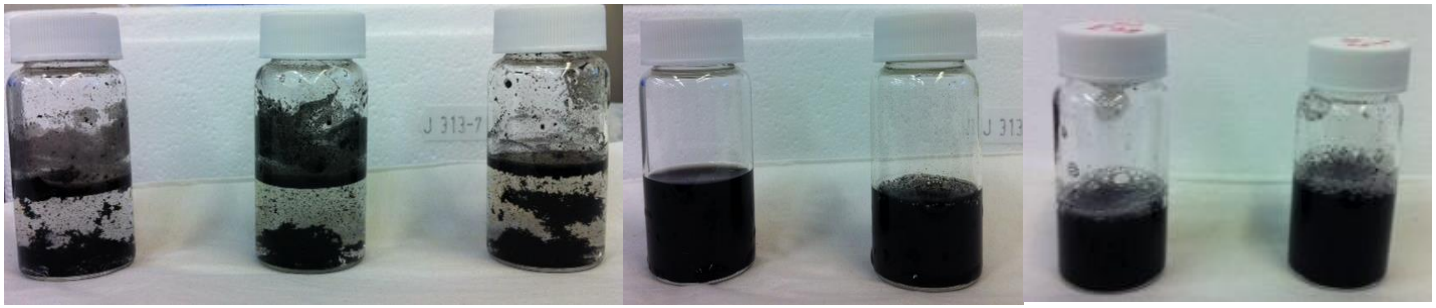
Element	ppb(µg/kg)	molarity(mol/L)
Co(59)	0.78555	1.334E-08
Zn(66)	2.58082	2.948E-08
Cu(63)	7.92265	1.247E-07
Ti(47)	24.7937	0.000000518
Ni(60)	178.536	0.000003042
Al(27)	221.021	0.000008192
Ca(43)	348.967	0.000008707
Cr(52)	401.606	0.000007724
Fe(57)	460.808	0.000008252
Mg(24)	509.979	0.00002098
Na(23)	967.294	0.00004207
Mn(55)	1693.44	0.00003082
K(39)	2301	5.89E-05



- Among 13 elements , K, Mn, Na, Mg, Fe, Cr and Ca were detected at higher level compared to others.
- Concentrations of these impurities are in ppb level except K and Mn.
- Other toxic element e.g. Pb, As, Se, Cd concentrations are insignificant or at matrix blank level.

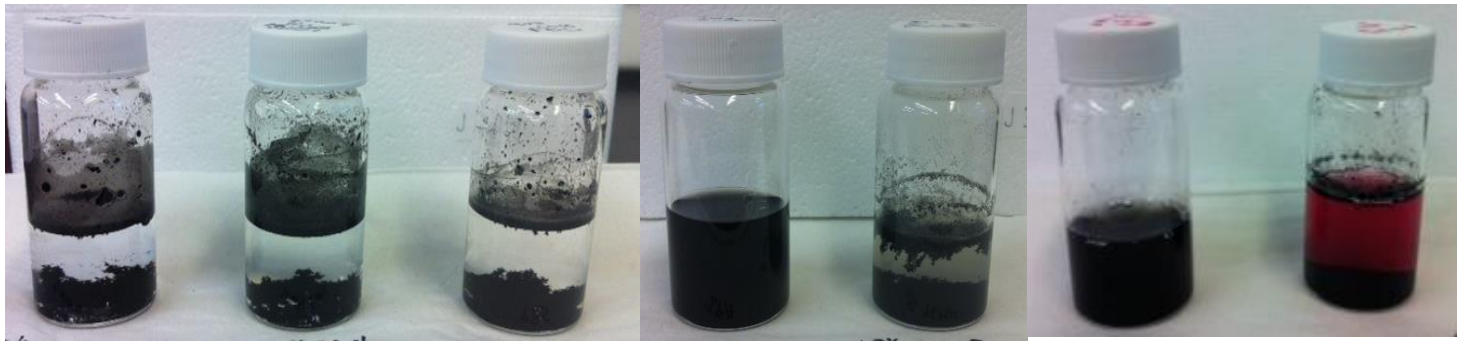
Physical Stability of Graphene material in different organic solvents and aqueous buffers

0 hours



Saline 10mM NaCl PBS IPA 10%NMP 2% BSA Growth medium

72 hours



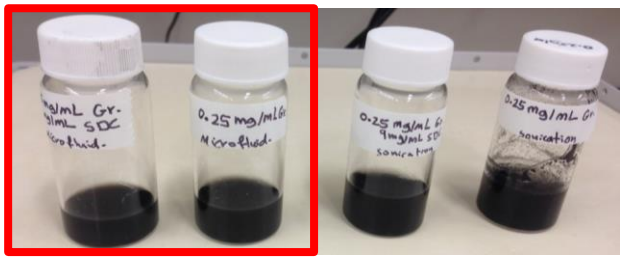
Dispersibility and Physical stability

- Most of the organic solvents were toxic to biological system
- In order to avoid toxicity different concentration of BSA solution was used to disperse
- 10%, 5% and 2% and 0.5% of BSA solution was used to disperse graphene.

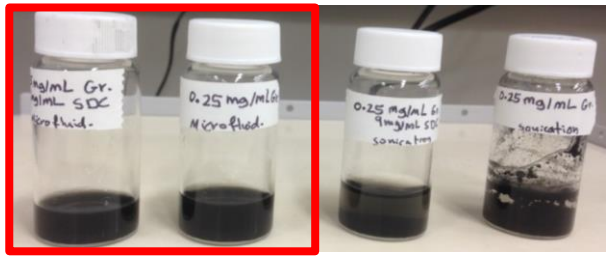
- In this dispersion weight ratio of BSA to graphene is 5:1.

- Concentration of graphene in the dispersion is 1 mg/mL

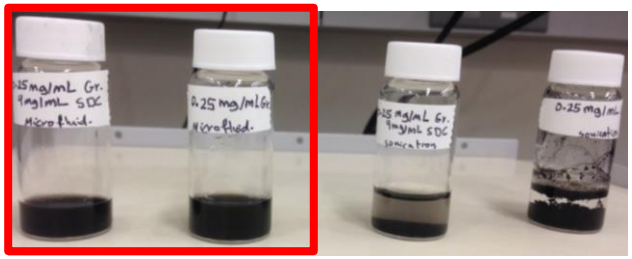
Graphene suspended in 0.5% of BSA solution, concentration of graphene 1mg/mL



Day 0



Day 1



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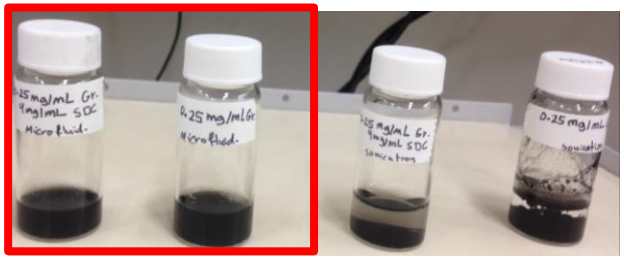
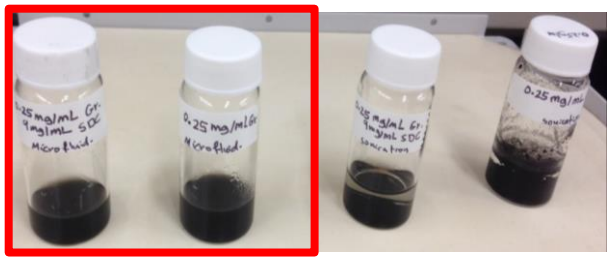
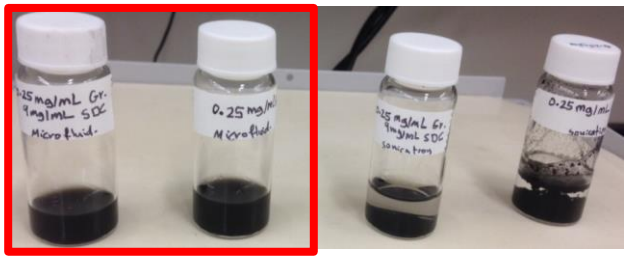


Day 4



Day 8



Day 9

Representative images showing the stability of graphene material (Gr.) dispersions by microfluidization and sonication methods for 9 days, Gr. dispersed in injectable water with and without 9 mg/ml SDC (sodium deoxycholate) by microfluidization and sonication methods. There is no difference in the stability of microfluidized Gr. dispersions with and without SDC (red rectangle)

Day 5

Injectable water

Distilled water

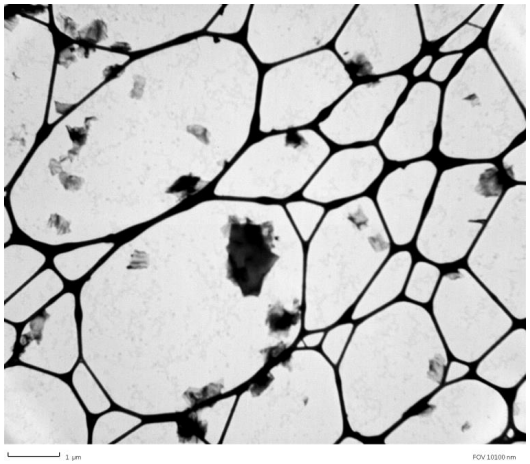
Tap water

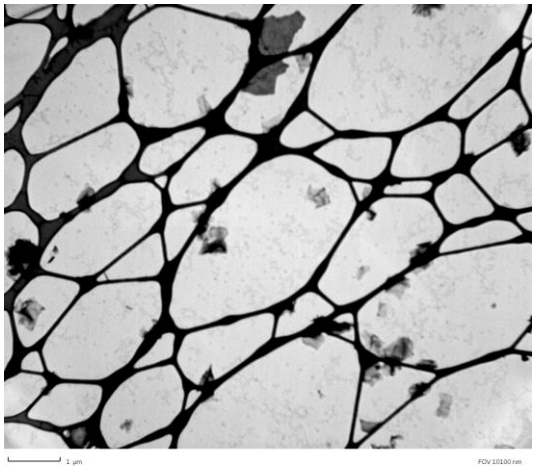
Deionized water

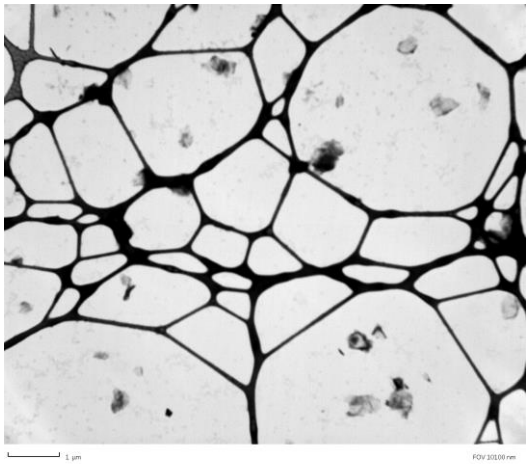
TF 7-3 Representative images showing the stability of graphene dispersions in injectable water, distilled water, tap water and deionized water by microfluidization and sonication methods. Graphene dispersion (red rectangle) by mirofluidization in injectable water is the most stable dispersion.

Microfluidized Graphene

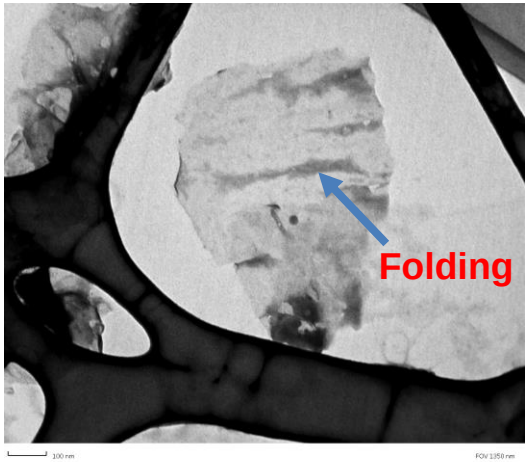
Low Magnification, FOV 10 μm

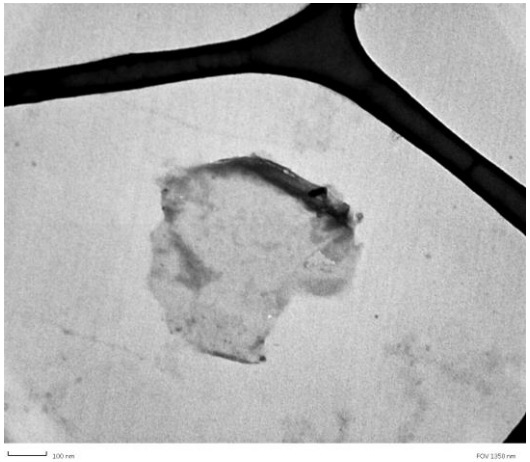


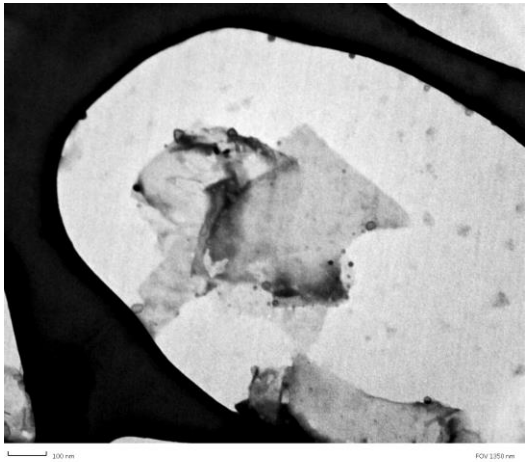




High Magnification, FOV 1.3 μm



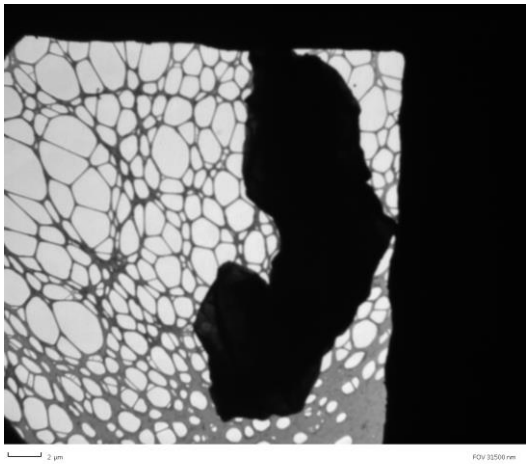
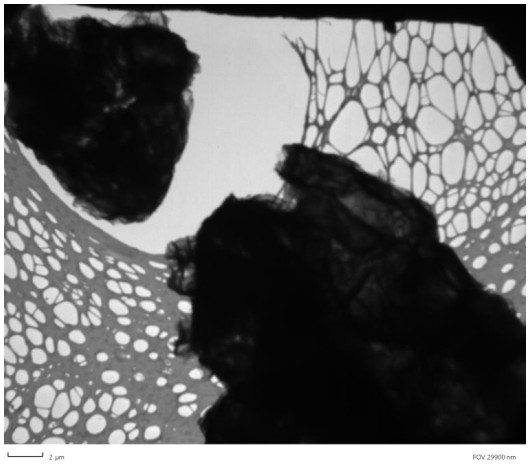
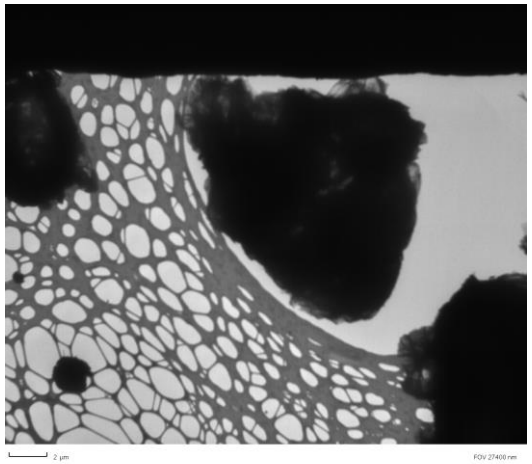




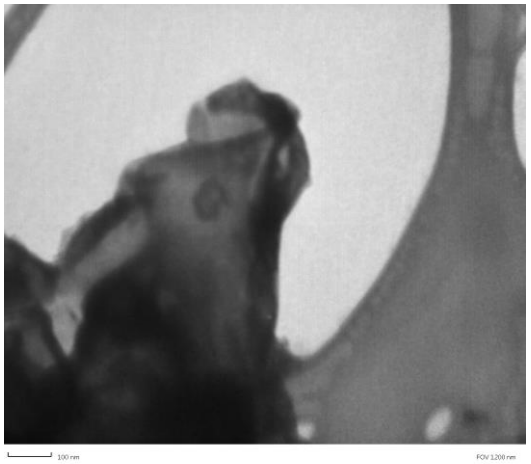
- Microfluidized graphene sample were well dispersed with lateral size of the flakes in sub-micrometer range
- Folding in the flakes were consistently observed

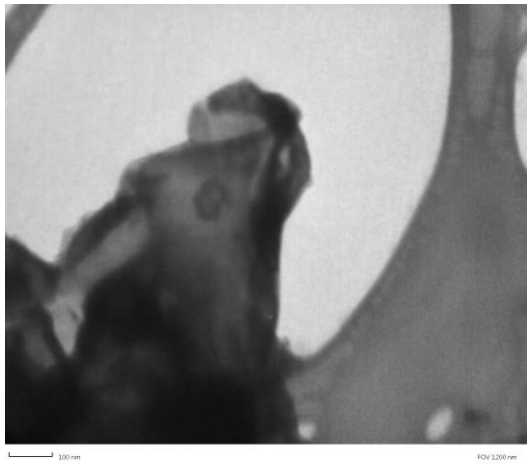
Sonicated Graphene

Low Magnification, FOV 30 μm

High Magnification, FOV 1.3 μm

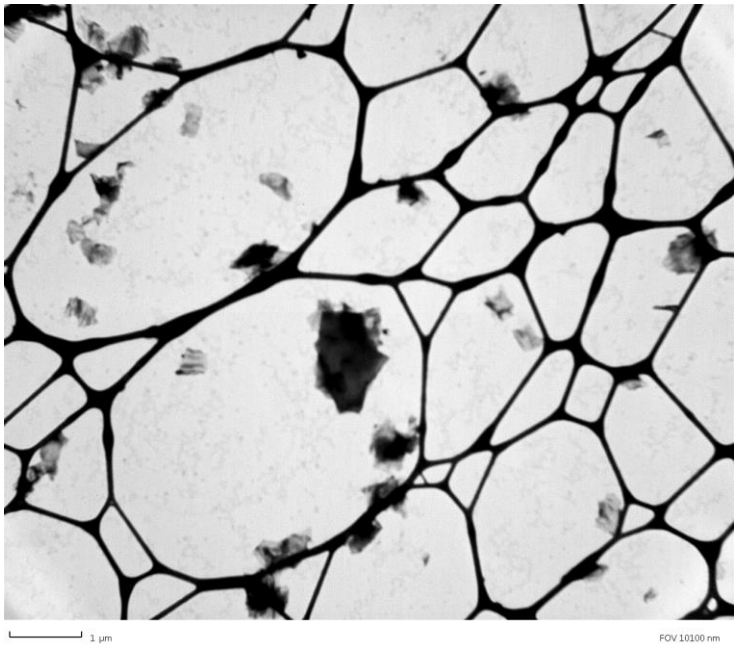




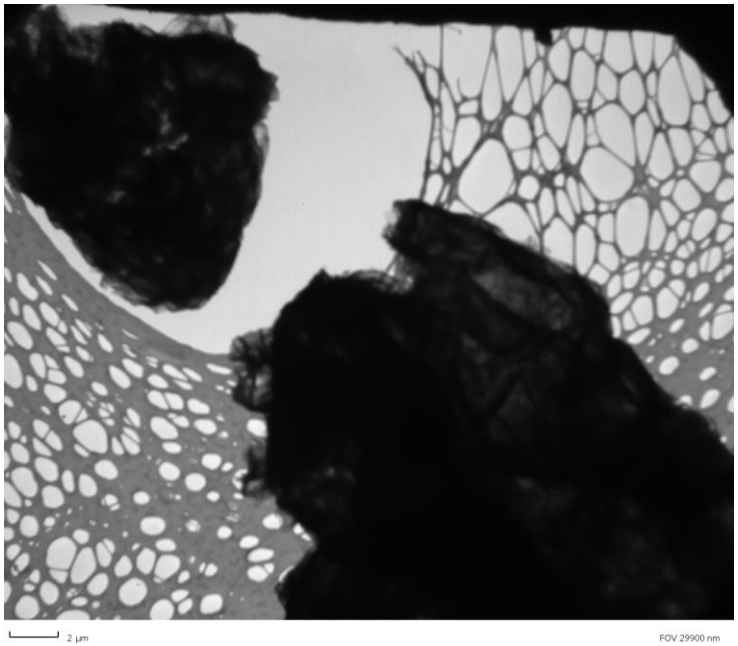
- Graphene sheets prepared using sonication method show large agglomerates, small flakes like the microfluidized samples were rarely observed

Comparison of Microfluidized and Sonicated Graphene

Microfluidized, FOV 10 μm

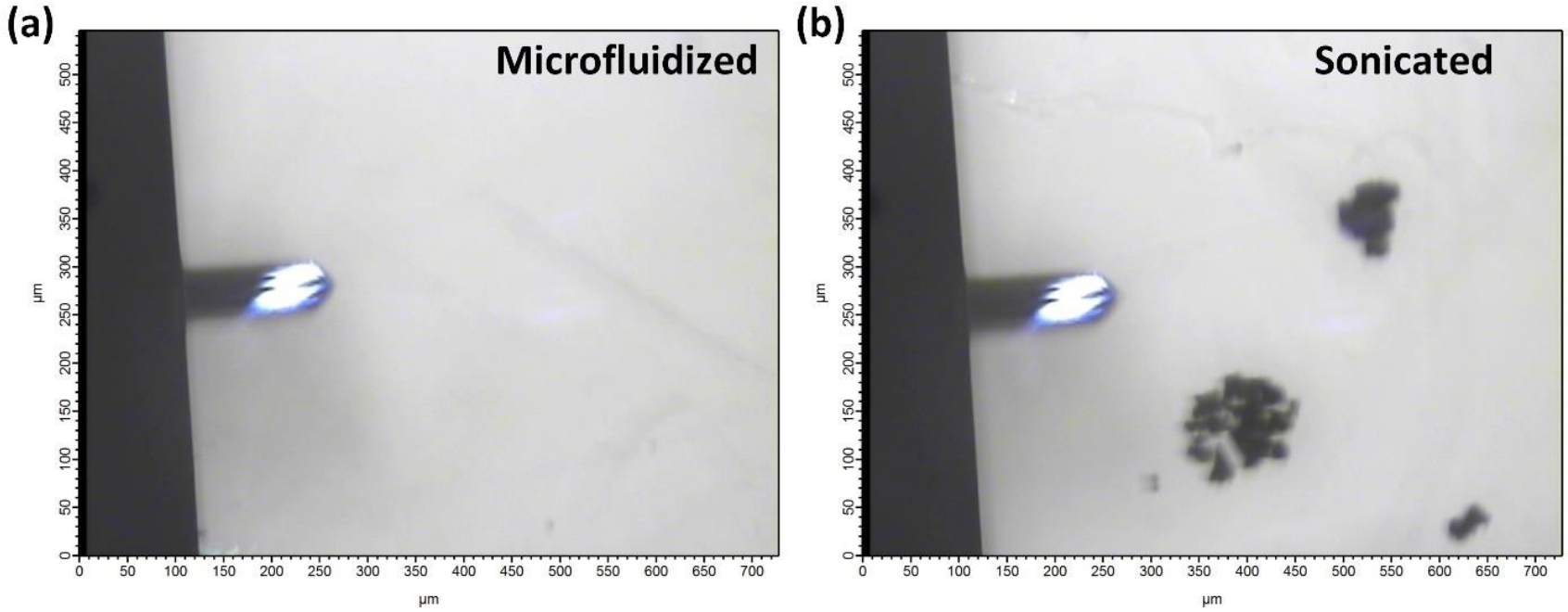


Sonicated, FOV 10 μm



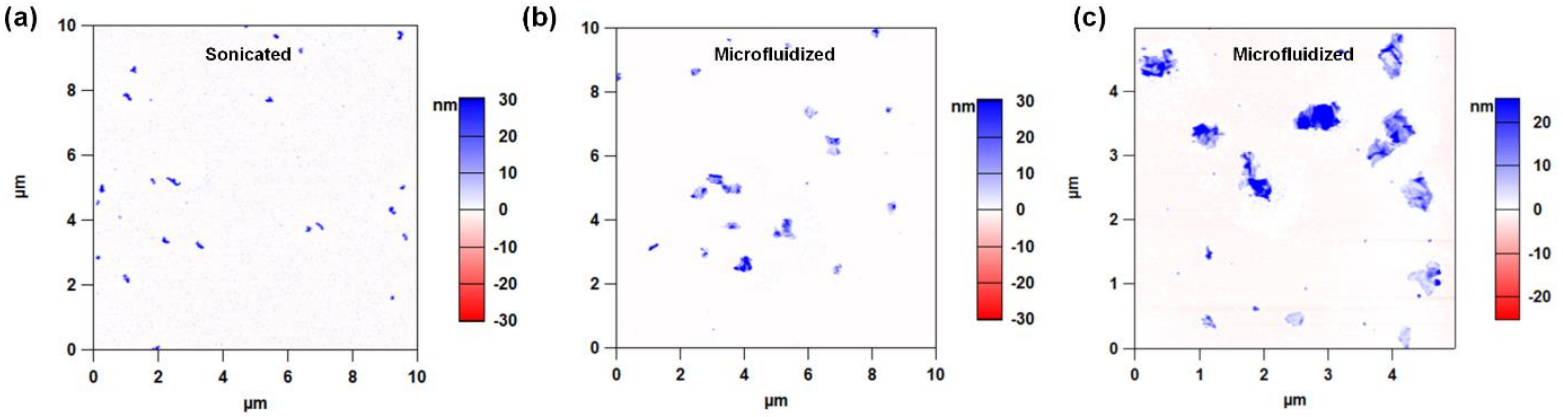
- Graphene sheets prepared with microfluidization method yielded smaller sized flakes compared to sonication method
- This observation is consistent with the higher stability of the flakes in suspension for the microfluidized sample

AFM Video Camera Image: Graphene

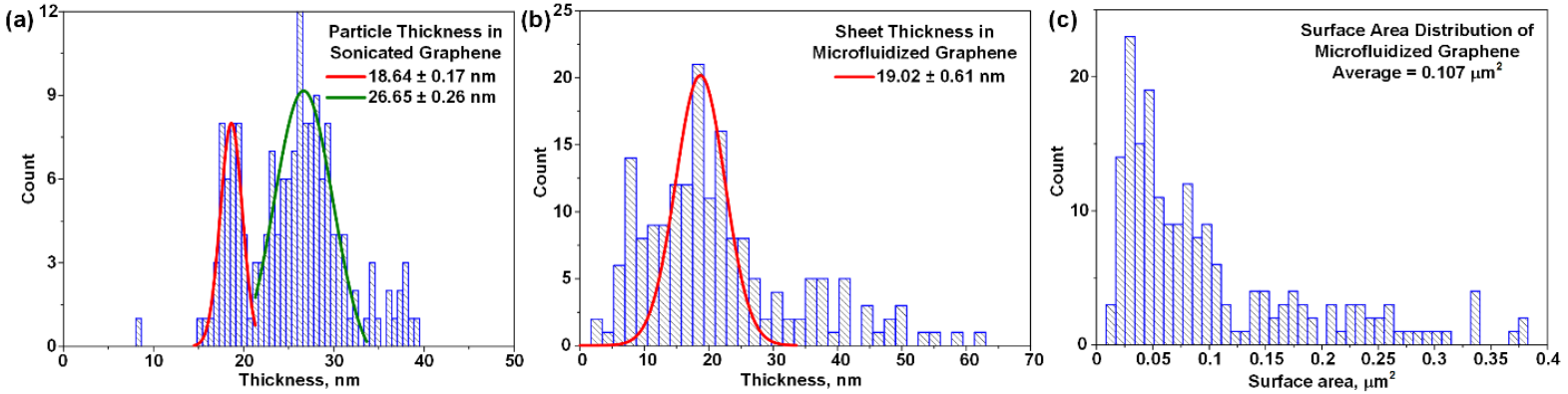


- Microfluidized graphene shows smooth surface indicating absence of large agglomerates
- Large agglomerates/aggregates of graphene was found in the sonicated graphene surface
- Similar trend in the video camera image was observed with graphene oxide

AFM Height Images of Graphene material



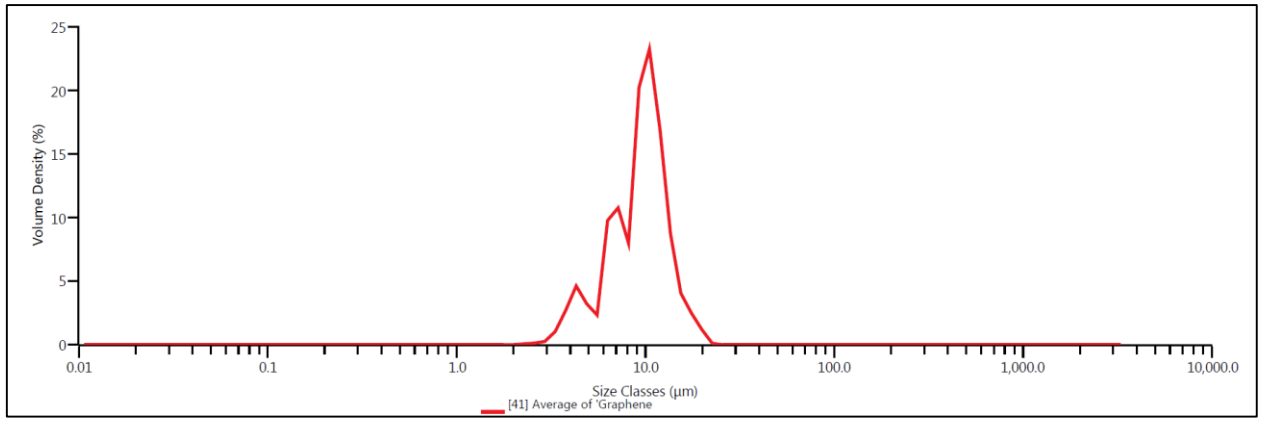
AFM Height Image Analysis: Graphene



- Along with the large agglomerates, nanoparticles were observed in the sonicated graphene surface (Figure a in both images and thickness analysis)
- The mean flake thickness was measured to be 19 nm, whereas the mean surface area was 0.11 μm^2
- Flakes in the sonicated samples were not imaged due to larger dimensions

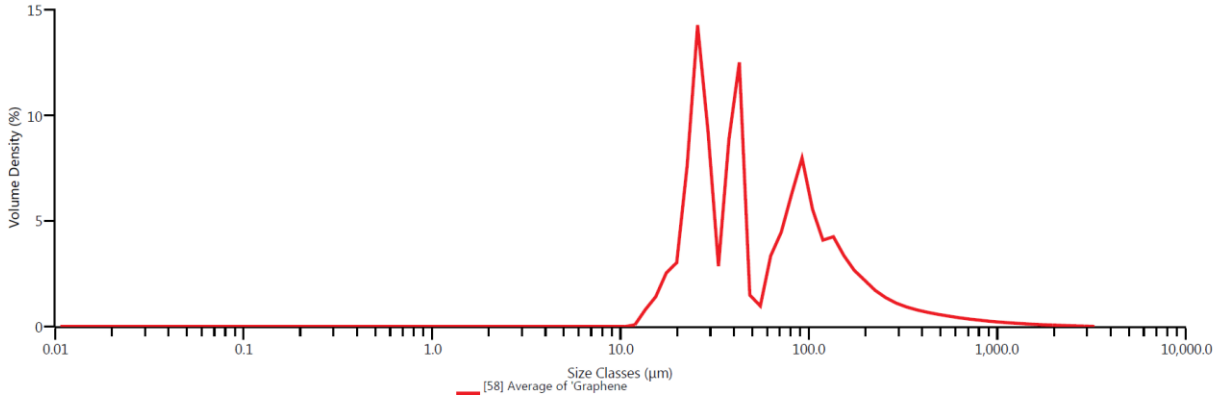
Preliminary Laser diffraction data (too be optimized) for size distribution

Concentration	0.0006 %
Span	0.860
Uniformity	0.258
Specific Surface Area	715.6 m^2/kg
D [3,2]	8.38 µm
D [4,3]	9.59 µm
Dv (10)	5.16 µm
Dv (50)	9.69 µm
Dv (90)	13.5 µm



Histogram of Microfluidized graphene in injectable water

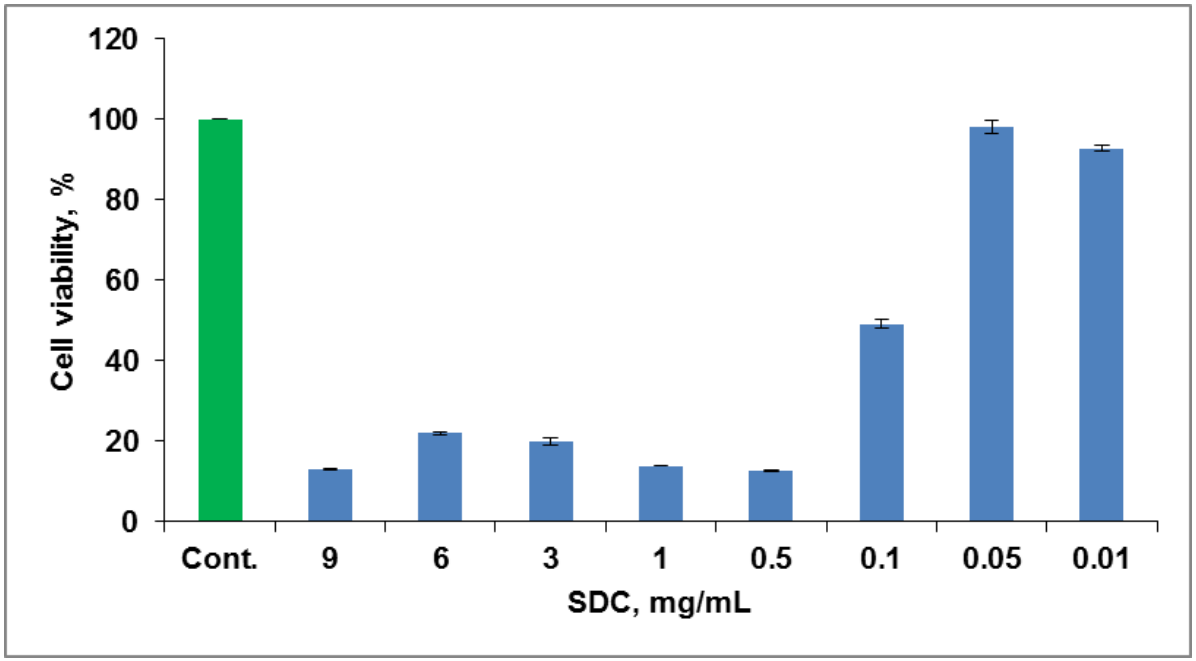
Concentration	0.0026 %
Span	3.762
Uniformity	1.585
Specific Surface Area	140.1 m^2/kg
D [3,2]	42.8 µm
D [4,3]	99.0 µm
Dv (10)	22.9 µm
Dv (50)	43.8 µm
Dv (90)	188 µm



Histogram of Sonicated graphene in injectable water

Sonicated graphene material are much larger compared to dispersions prepared by microfluidization

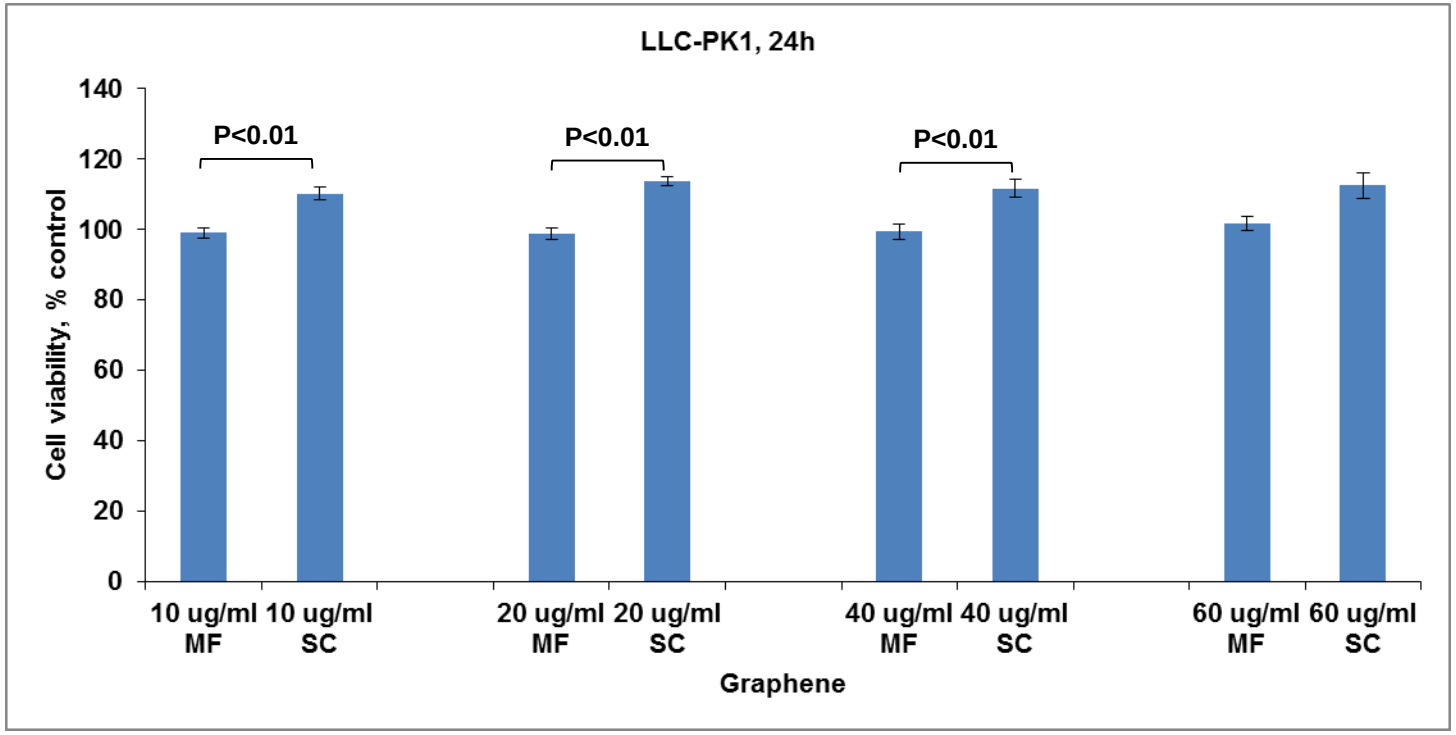
Cell viability assay – Sodium deoxycholate



Cell viability test (MTS) for HepG2 cells treated with different concentrations of sodium deoxycholate (SDC) for 24 h, non treated cells were used as control. The SDC is cytotoxic at 9 mg/ml which is the optimal concentration for more stable dispersions.

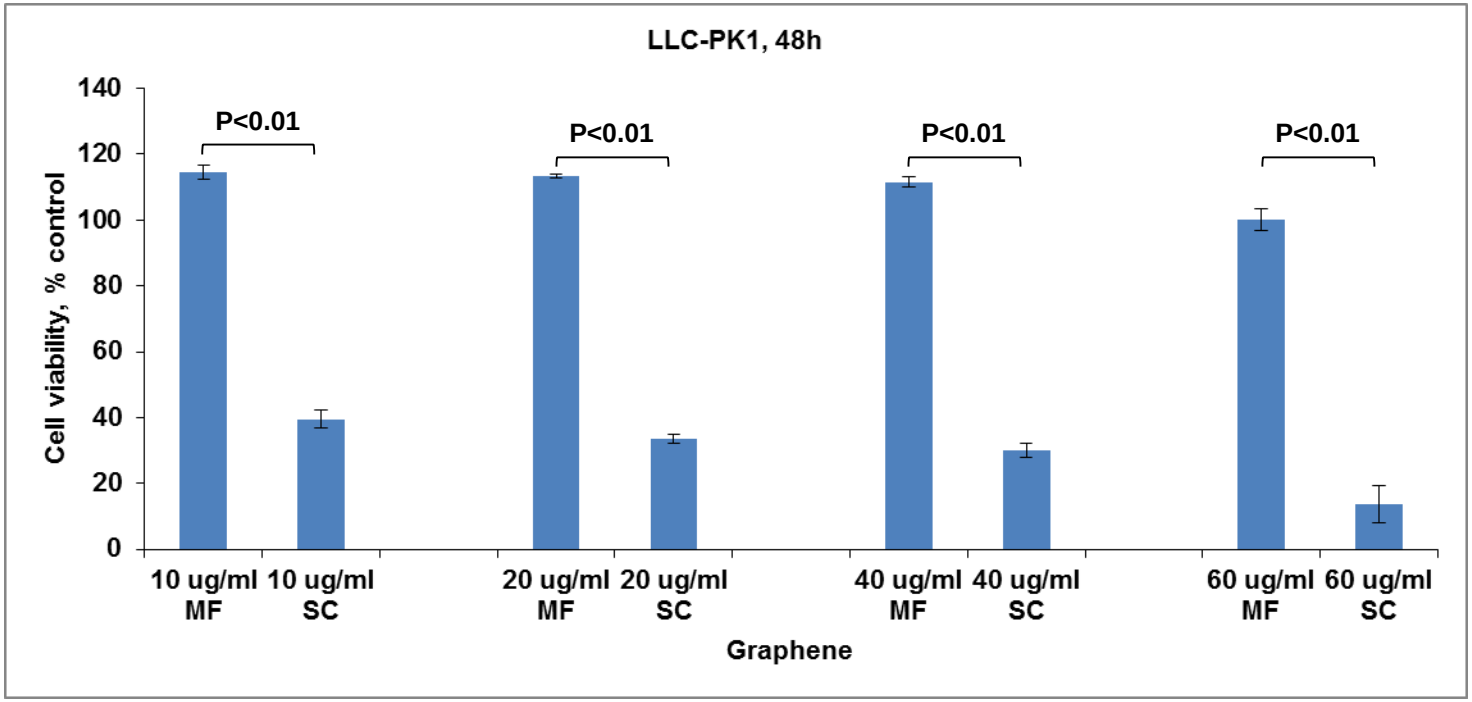
The cytotoxicity of SDC limited its use although it was one of the best dispersion for biological experiments.

Cell viability at 24 hrs exposure – no difference between MF & SC



Measurement of cytotoxicity of graphene dispersed by microfluidization and sonication methods in LLC-PK1cell line by MTS assay after 24h exposure

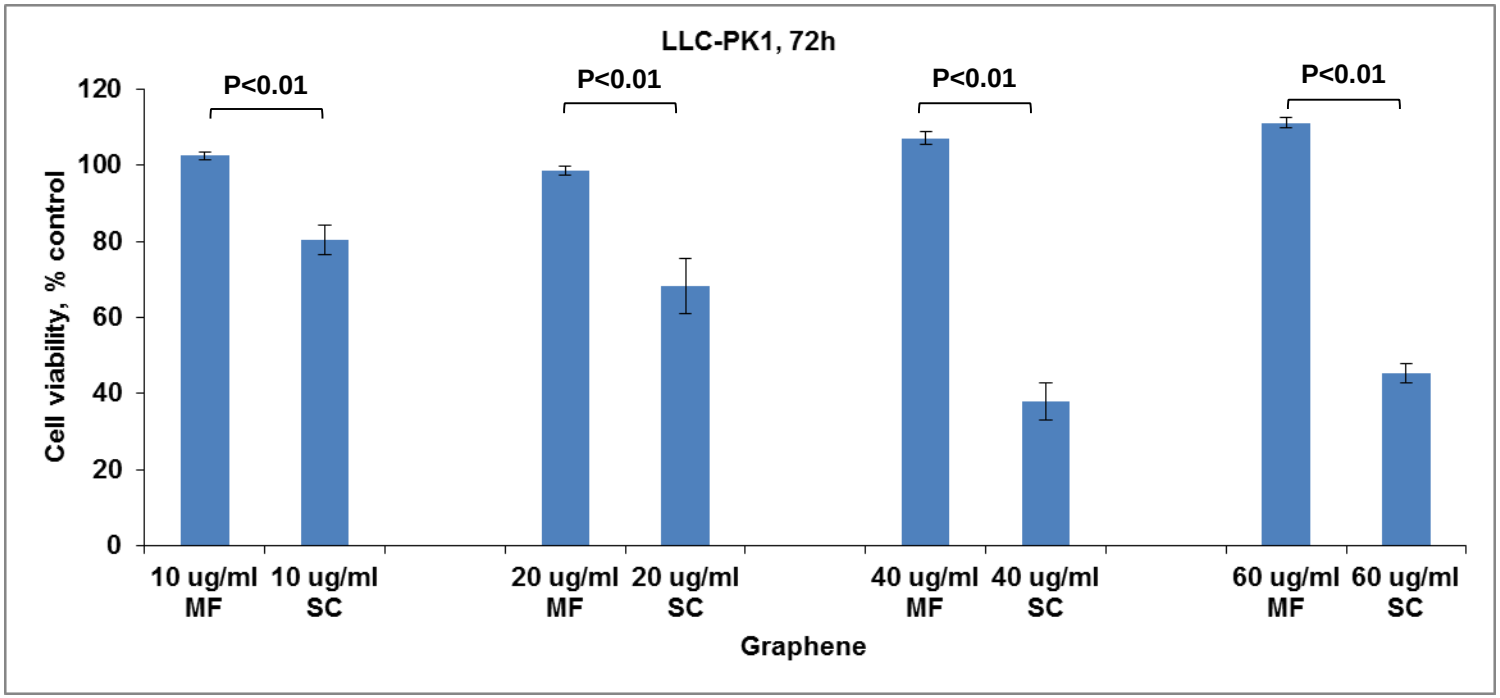
Differences in cytotoxicity were observed between microfluidized and sonicated Graphene material (48 h and 72 h exposure)



MF: microfluidized
SC: sonicated

Measurement of cytotoxicity of graphene dispersed by microfluidization and sonication methods in LLC-PK1cell line by MTS assay after 48h exposure

Differences in cytotoxicity were observed between microfluidized and sonicated Graphene material



MF: microfluidized
SC: sonicated

Measurement of cytotoxicity of graphene dispersed by microfluidization and sonication methods in LLC-PK1cell line by MTS assay after 72h exposure

Red: untreated
Green: H2O treated control
Blue: 60 ug/ml Gr. MF
Pink: 60 ug/ml Gr.SC
Turquoise: 40ug/ml Gr.MF
Purple: 40 ug/ml Gr.SC
Gray: 20 ug/ml Gr.MF
Dark green: 20 ug/ml Gr.SC
Orange: 10 ug/ml Gr.MF
Sky blue: 10 ug/ml Gr.SC

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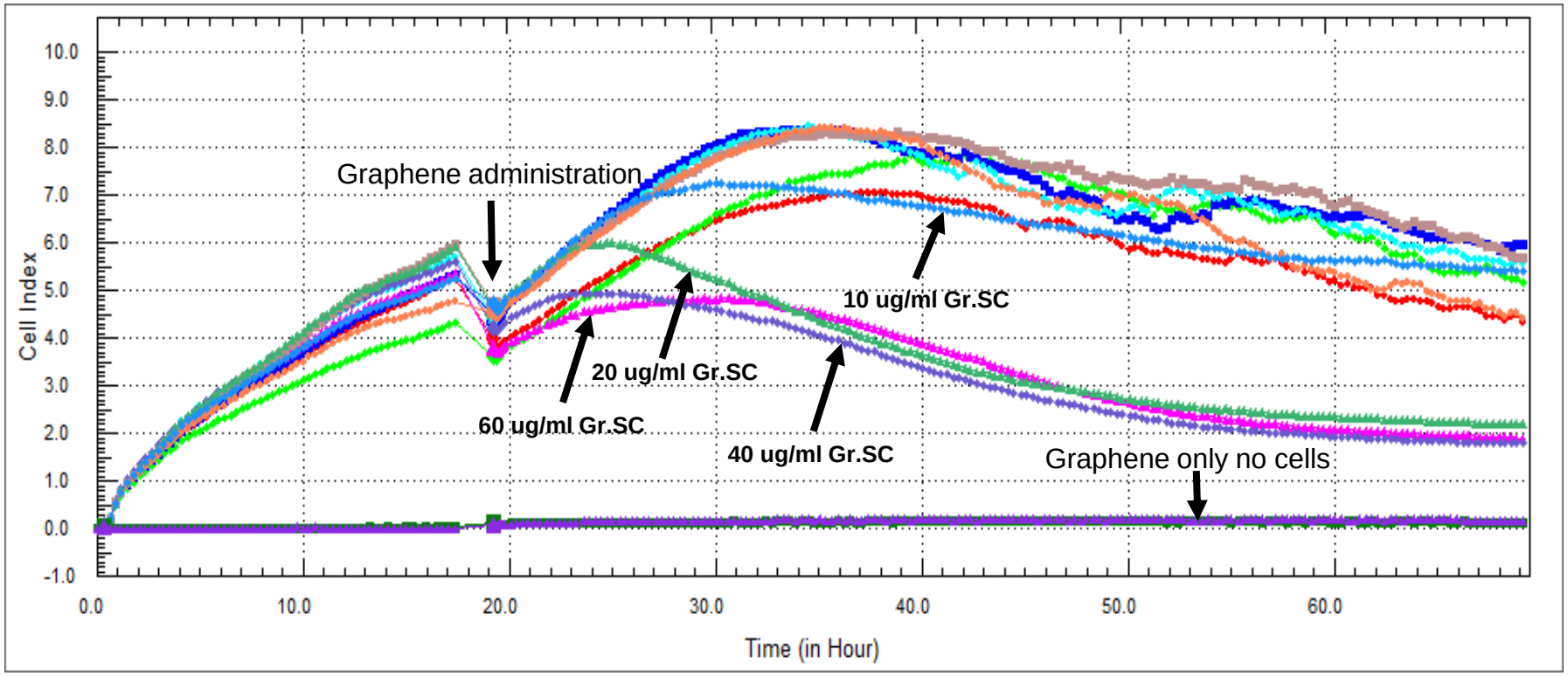
Real-time cell analysis (RTCA) data on cell viability



Label-free, real-time cell analysis (RTCA) data, LLC-PK1 treated with 20, 40 and 60 ug/ml graphene (Gr.) dispersions prepared by microfluidization (MF) and sonication (SC) methods cell. Cell proliferation and cell death were continuously monitored using xCELLigence RTCA DP instrument for **48h after treatment.**

Graphene dispersion conclusion

- Graphene dispersed by microfluidization method in injectable water was more stable than graphene dispersed in injectable water by sonication method.
- Graphene dispersed by microfluidaization in injectable water was more stable than graphene dispersed by distilled water, deionized water and tap water.
- SDC with microfluidization improve dispersion of graphene in water and PBS. The SDC was cytotoxic at the optimal concentration required to get stable dispersions of graphene.
- BSA with microfluidization did not improve graphene dispersion.
- There was no difference in the stability of graphene dispersions in injectable water with microfluidization compared to those in injectable water with SDC with microfluidization.

Graphene toxicity studies summary and conclusion

1- Measuring cytotoxity of graphene by MTS assay (CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay):

- Microfluidized graphene is not cytotoxic to the LLC-PK1 in the tested concentrations (10, 20, 40 and 60 ug/ml) 24, 48 and 72h after exposure.
- Sonicated graphene is cytotoxic to the LLC-PK1 in the tested concentrations (10, 20, 40 and 60 ug/ml) 48 and 72h after exposure.

2- Measuring cytotoxicity of graphene by RTCA:

- Sonicated graphene is cytotoxic to LLC-PK1 in the tested concentrations (10, 20, 40 and 60 ug/ml) 48h after exposure while microfluidized graphene is not cytotoxic to LLC-PK1 cells in the tested concentrations (10, 20, 40 and 60 ug/ml) 24, 48, 72h after exposure.

3- Measuring cytotoxicity of graphene by TUNEL assay (Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling Assay):

- Microfluidized graphene is not cytotoxic to the LLC-PK1 in the tested concentrations (10, 20, 40 and 60 ug/ml) 24, 48 and 72h after exposure.
- Sonicated graphene is cytotoxic to the LLC-PK1 in the tested concentrations (10, 20, 40 and 60 ug/ml) 24, 48 and 72h after exposure.
- The above results confirm that microfluidized graphene is not cytotoxic while sonicated graphene is cytotoxic to the LLC-PK1.

General conclusion

- Microfluidized graphene material has better dispersion and stability compared to sonicated material
- Larger flake sizes/agglomerates observed for sonicated graphene material vs smaller flake sizes for microfluidized material
- The methods (microfluidization vs sonication) of preparation play an important role in the resultant toxicity of graphene.
- Graphene dispersions prepared by sonication methods are cytotoxic to LLC-PK1 while graphene dispersions prepared by microfluidization methods are not cytotoxic.

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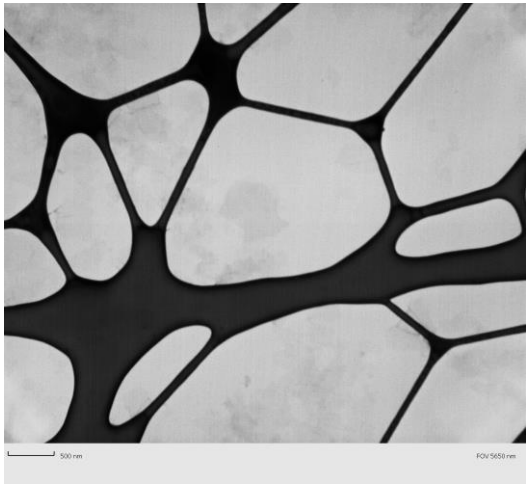
Sample Preparation for LVEM 25

- Microfluidized and sonicated graphene and graphene oxide has been imaged in this work using LVEM 25
- The samples were diluted to 100 ppm for graphene, whereas the samples were diluted to 10 ppm for the graphene oxide sample due to its higher degree of miniaturization
- 15 μl of the diluted suspension was deposited on a thin carbon coated (3 nm coating) holey carbon grid
- The samples were dried overnight followed by one hour of vacuum drying at 50°C
- The grids were then imaged using the LVEM 25

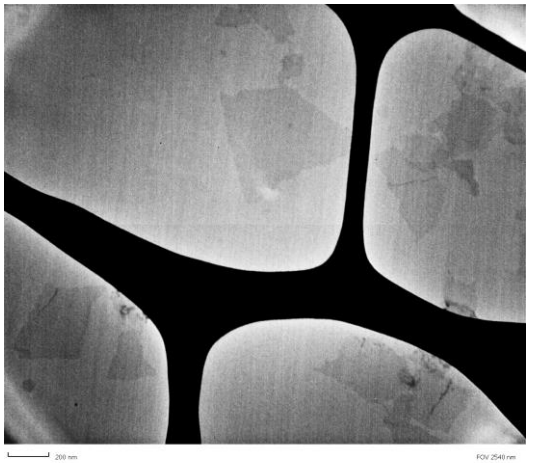
Graphene Oxide Microfluidized

FOV 5.6 μm

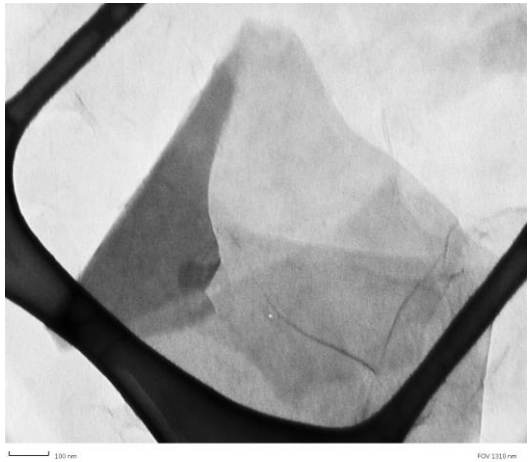


FOV 5.6 μm

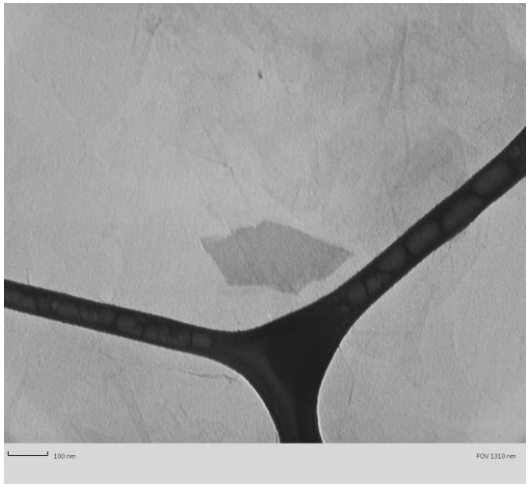
FOV 2.5 μm



FOV 1.3 μm



FOV 1.3 μm



FOV 1.3 μm

- Graphene oxide prepared using microfluidized method shows highly miniaturized flakes with monolayer of graphene
- Sheet thickness has been measured using AFM

Sample Preparation for AFM

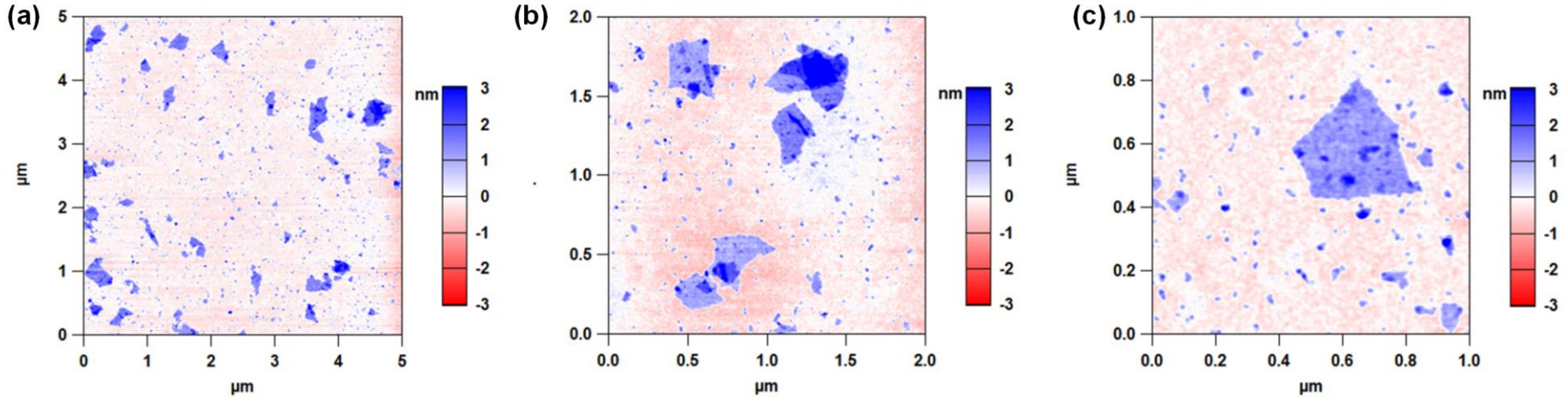
1. The samples were diluted in miliQ water to 10 ppm.

2. Freshly cleaved mica substrate is mounted on a glass substrate using double sided tape. The glass slide was then placed on a hot plate at 100°C.

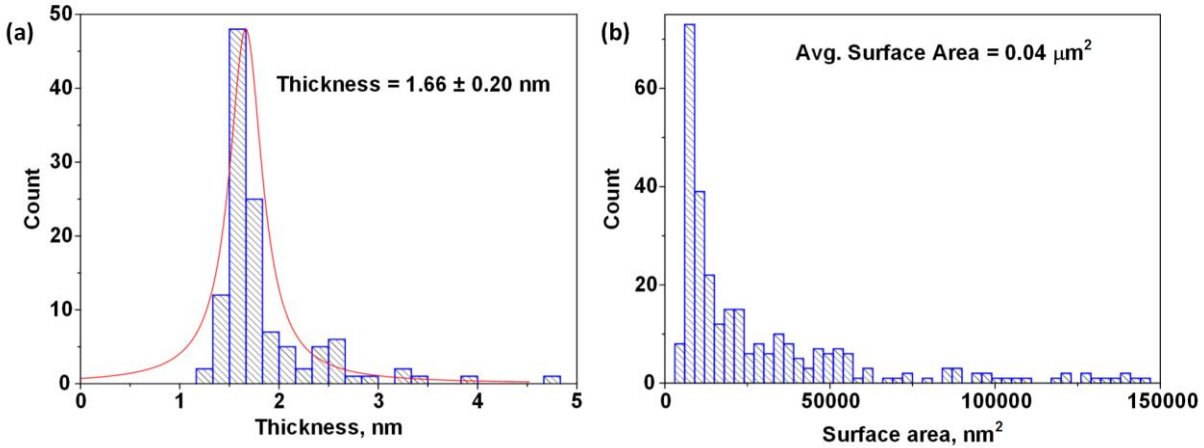
3. After heating the slide for 5 minutes, 30 μl of 10 ppm graphene suspension was deposited on to mica for fast drying. After 30 seconds of drying (With no visible water present), the slide was further dried in vacuum oven at 60°C overnight.

4. This method is used to minimize agglomeration of graphene flakes on the sides of the mica disc.

AFM Height Images of Microfluidized Graphene Oxide

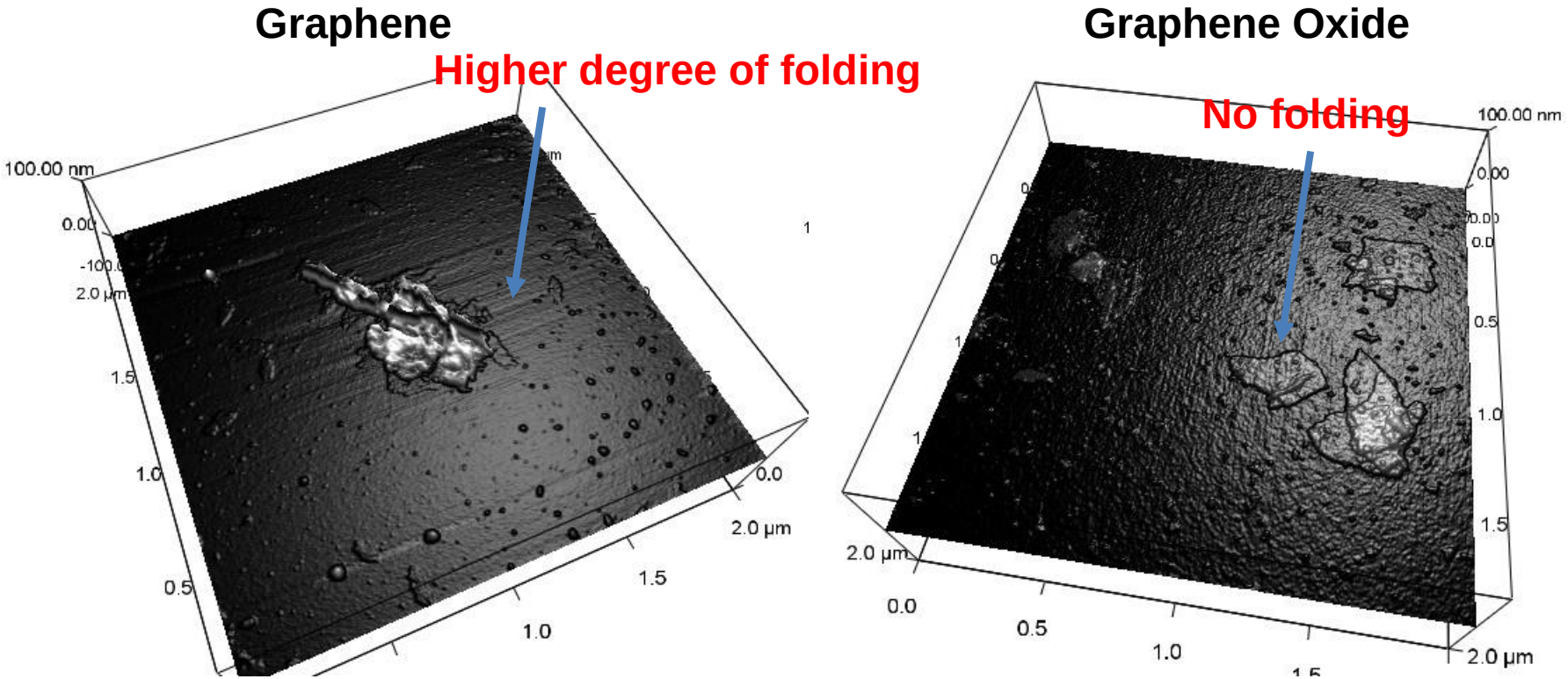


AFM Height Image Analysis: Graphene Oxide



- Average thickness of microfluidized graphene oxide sheets were 1.66 nm, indicating presence of mostly monolayer and bilayer. Average surface area was 0.04 μm^2 that is significantly smaller than the microfluidized graphene
- Sonicated samples were not imaged in AFM due to large dimensions

Comparing Sheet Folding in Microfluidized Graphene and Graphene Oxide



Both images are displayed in same Z-range (100 nm)

- Comparison of 3D morphology of the flakes clearly shows higher degree of sheet folding on the surface for microfluidized graphene compared to graphene oxide

Conclusions

- Microfluidization method of preparation of graphene and graphene oxide samples yielded highly miniaturized sheets
- Sonicated samples primarily contain >10 μm size thick sheets
- Analysis of the sonicated samples showed presence of nanoparticles with an average diameter of ~20nm for the graphene sample
- Mean thickness of graphene oxide was 1.66 nm that indicates the majority of sample contains one or two layers of sheets
- 3D morphology analysis suggests higher degree of folding on mica substrate for microfluidized graphene compared to graphene oxide.

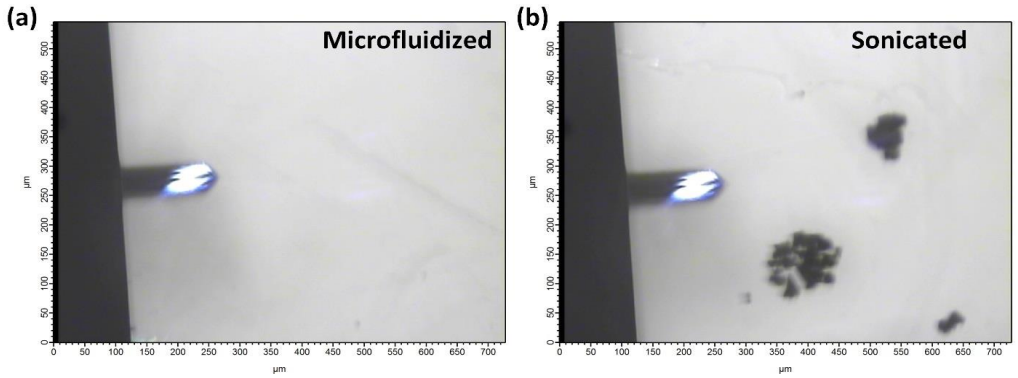


Figure 1. Graphene sample surface visualized through the AFM video camera. Representative sample areas are shown for (a) microfluidized and (b) sonicated graphene sample. The surface distribution of graphene looked significantly different when observed through the AFM video camera image.

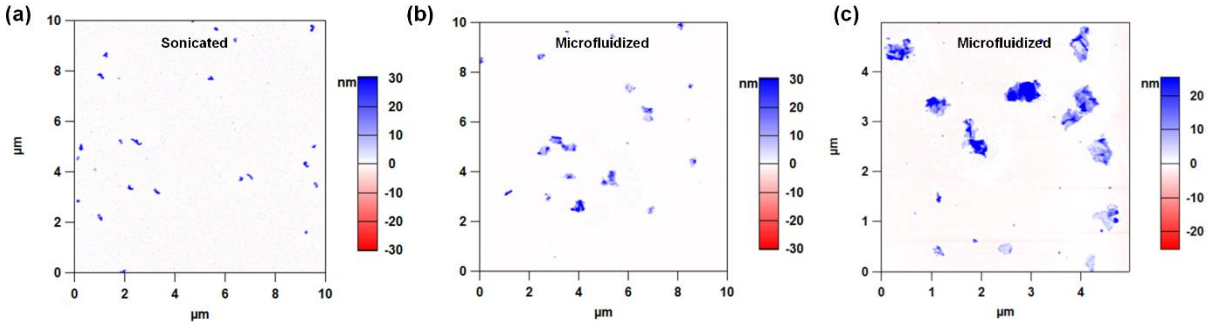


Figure 2. (a) Representative morphology of sonicated graphene surface. (a) Microfluidized graphene sheets visualized using AFM. (c) A zoomed in image of the same sample with microfluidized graphene.

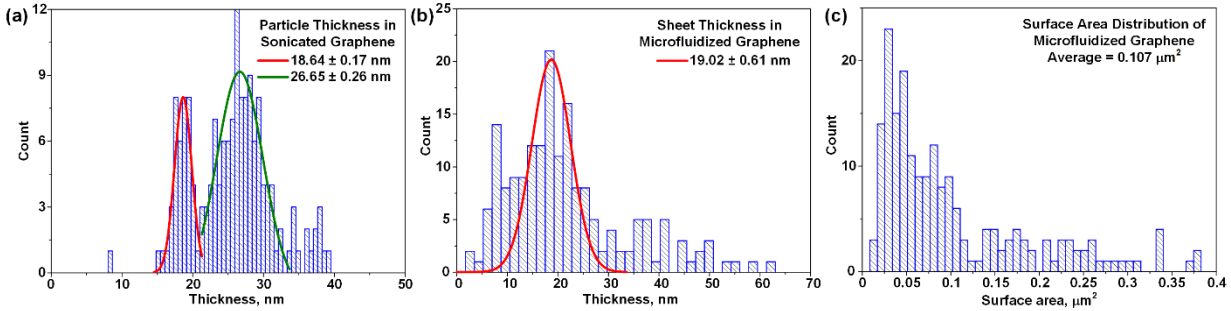


Figure 3. Thickness and surface area of graphene was measured using AFM height images. (a) Thickness of the particles found in the sonicated graphene sample. (b) Thickness of graphene sheets in the microfluidized sample. (c) Surface area distribution of microfluidized graphene sheets measured in imageJ analysis. The size analysis confirms evenly distributed size of microfluidized graphene sheets, while the sonicated graphene consists of large chunks of materials and small particles present throughout the sample.

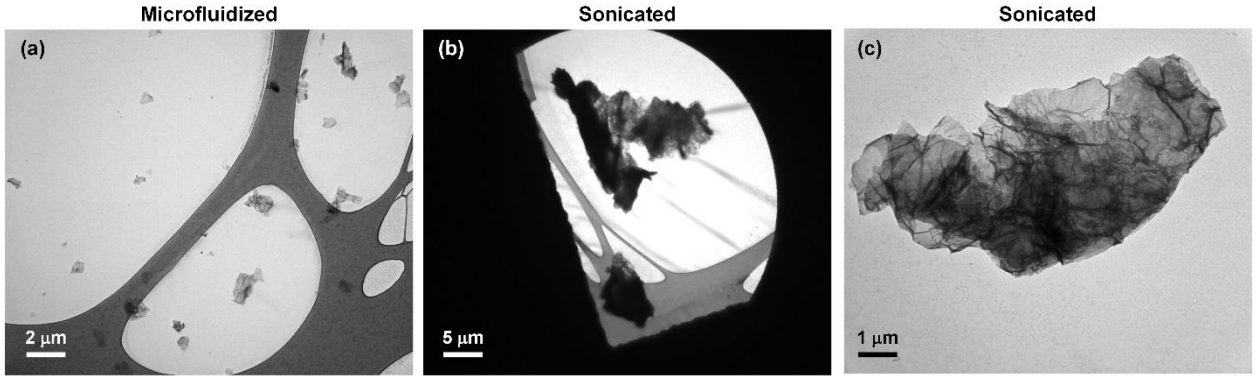
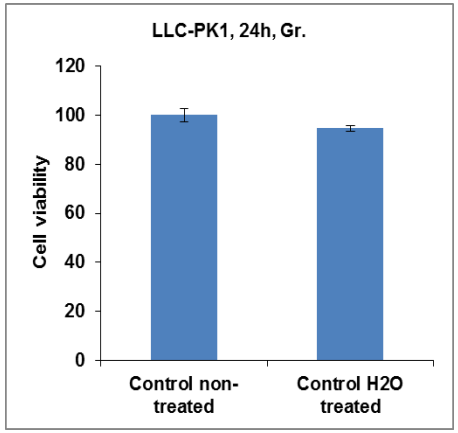
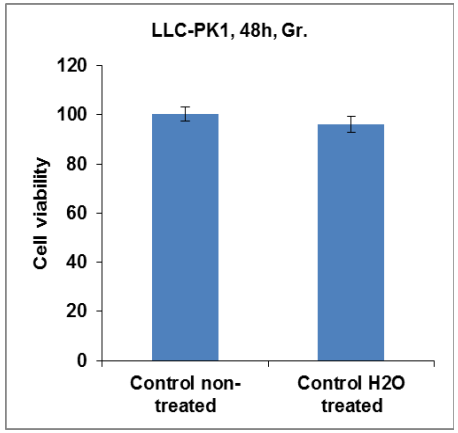
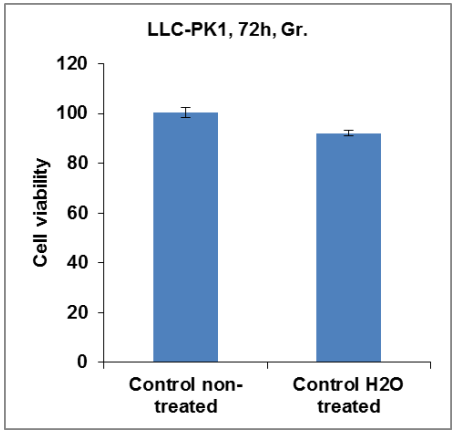


Figure 4. Representative LVEM 25 image of (a) microfluidized, (b) sonicated graphene. (c) Zoomed in image of the sonicated graphene flakes. The microfluidized version of graphene flakes has similar nano-dimensional sheets whereas the sonicated sample contained large size moieties. Figure 4c shows representative image of a large graphene flake from the sonicated samples, whereas small moieties were seldom found in the sample grid. Moreover, the background in this sample was thicker compared to the microfluidized version.

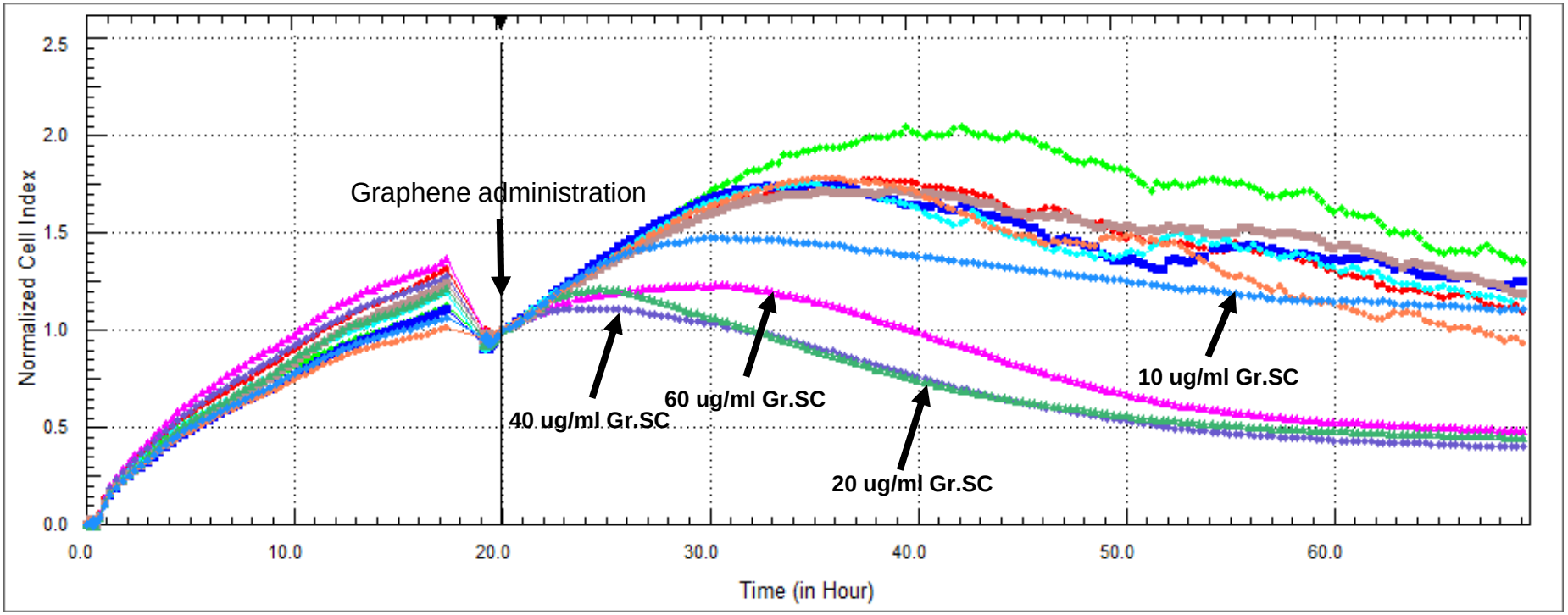




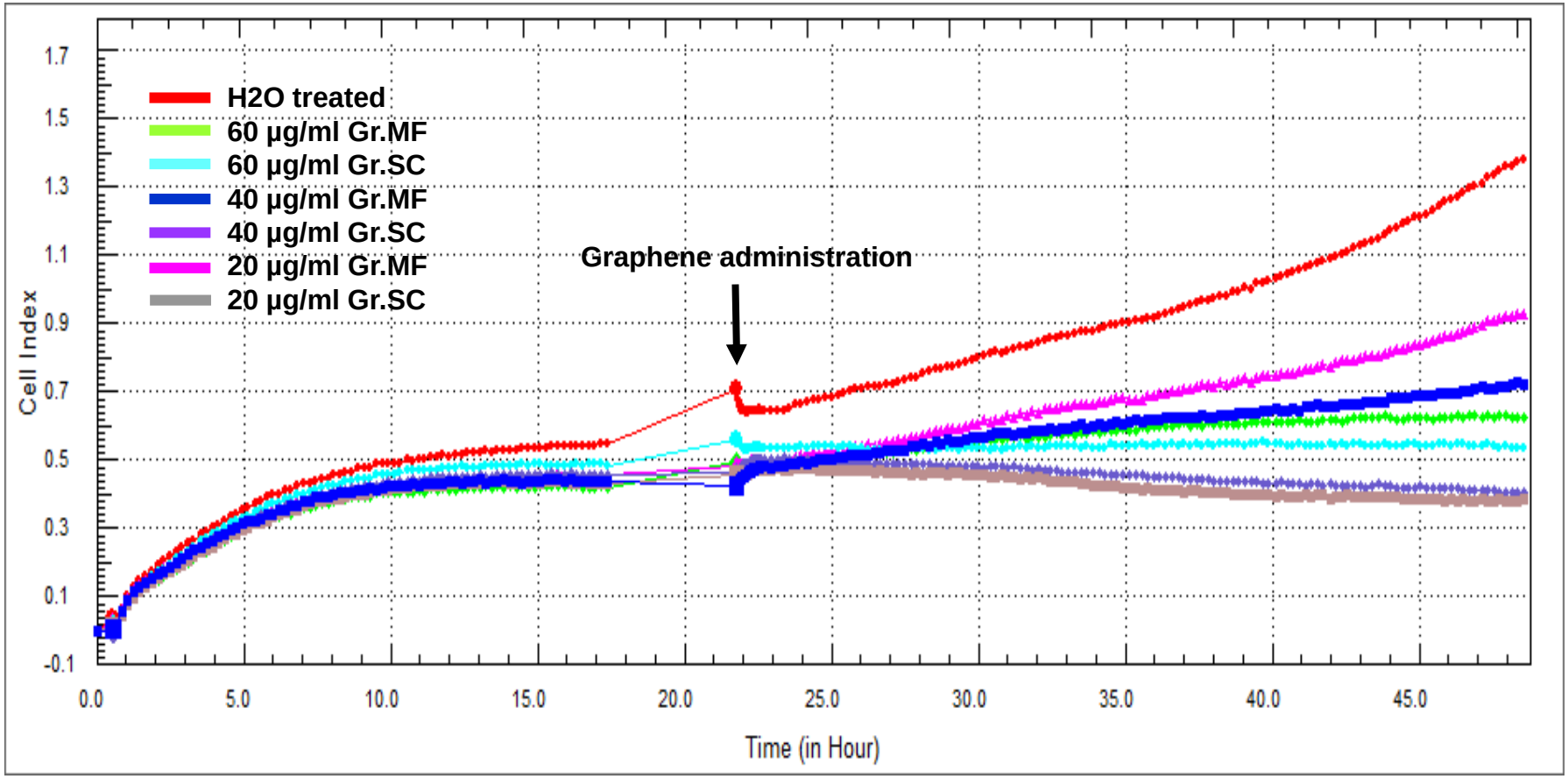


TF 8-6, 8-7, 8-8 Comparison of the cell viability between LLC-PK1 cells treated with injectable water (same volume used in all the corresponding experiments) and untreated cells, the data were obtained from the same experiments Performed to measure the cell viability of microfluidized and sonicated graphene dispersions to show no difference in the cell viability between water treated and untreated samples

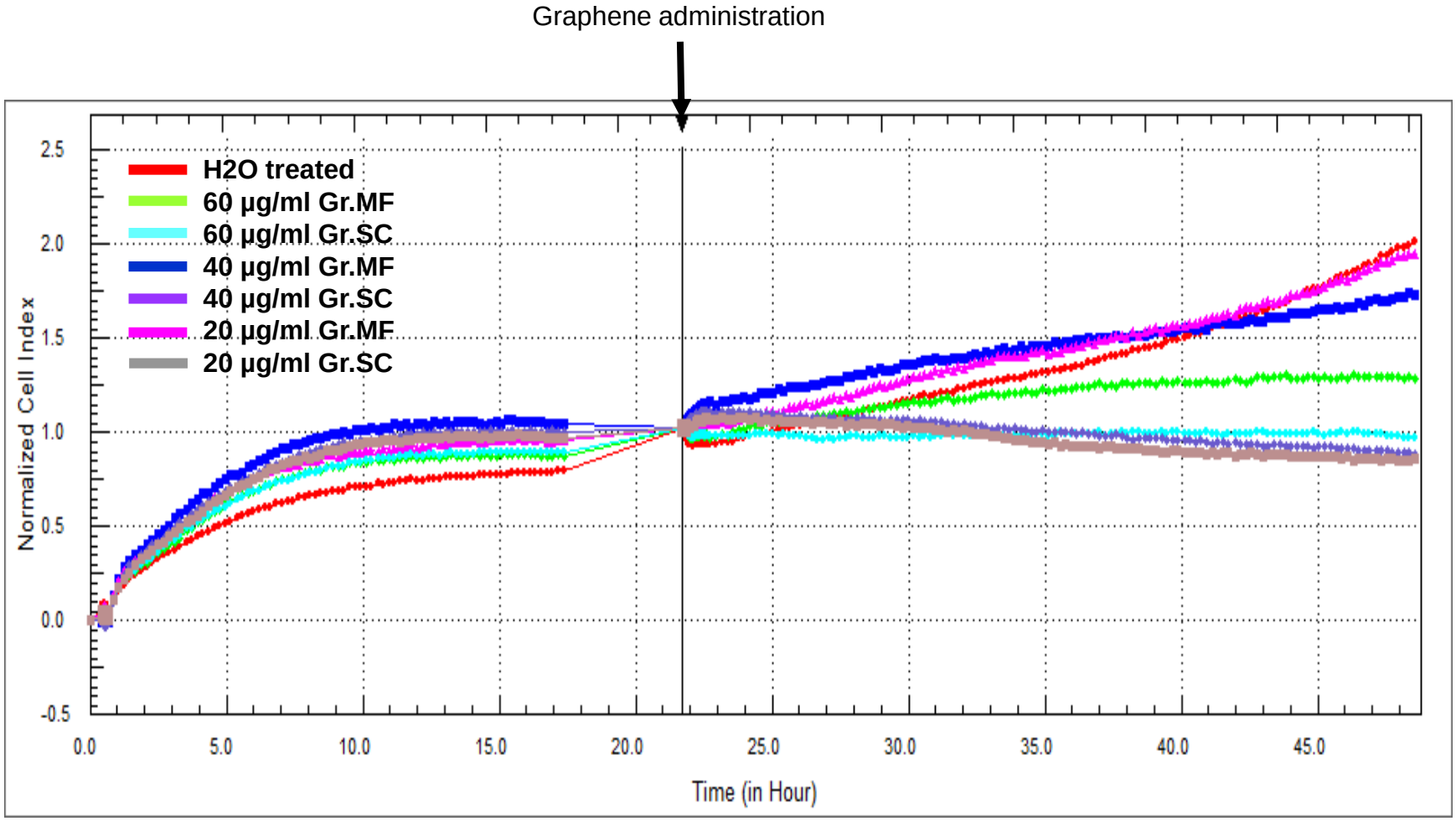
Red: nontreated
Green: H2O treated control
Blue: 60 ug/ml Gr. MF
Pink: 60 ug/ml Gr.Sc
Turquoise: 40ug/ml Gr.MF
Purple: 40 ug/ml Gr.SC
Gray: 20 ug/ml Gr.MF
Dark green: 20 ug/ml Gr.SC
Orange: 10 ug/ml Gr.MF
Sky blue: 10 ug/ml Gr.SC



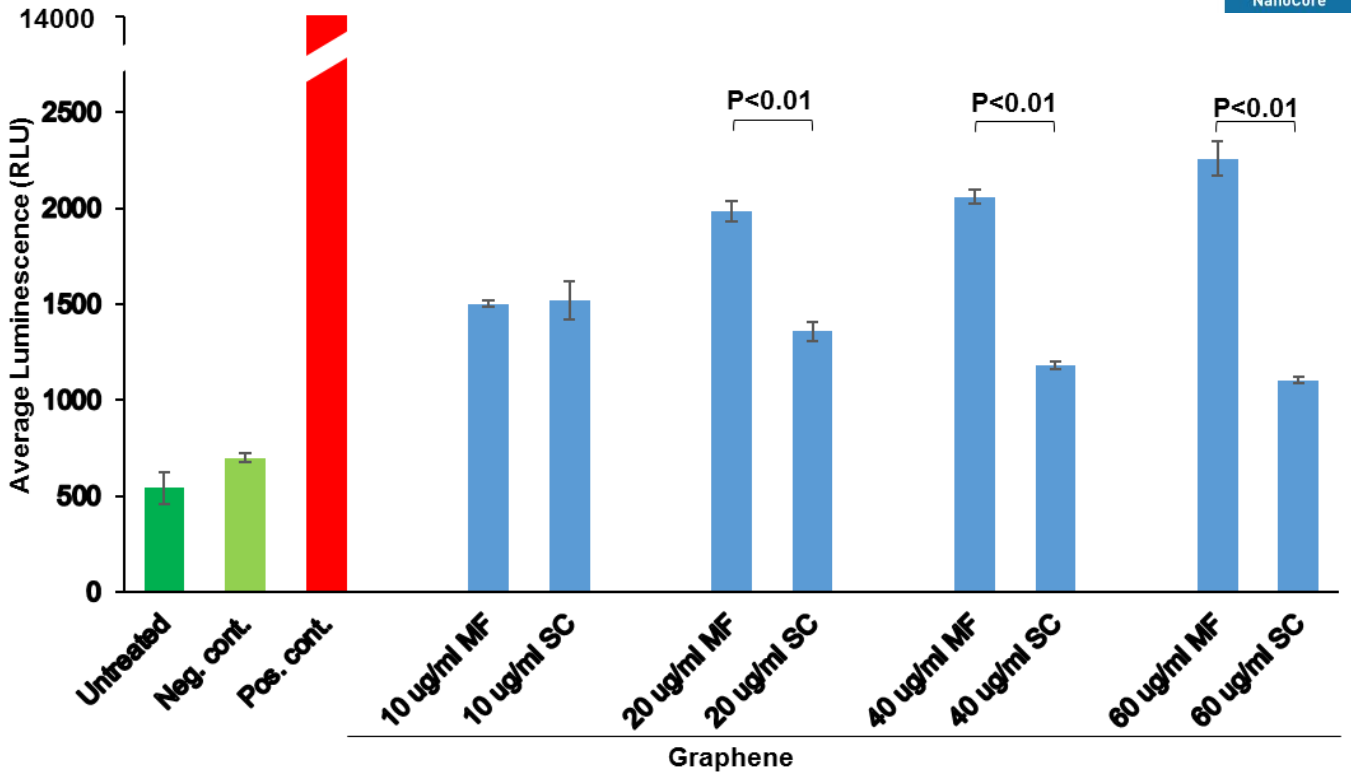
TF 4-7 Label-free, real-time cell analysis (RTCA) data, LLC-PK1 treated with 20, 40 and 60 ug/ml graphene (Gr.) dispersions prepared by microfluidization and sonication methods cell. Cell proliferation and cell death were continuously monitored using xCELLigence RTCA DP instrument for 48h after treatment.



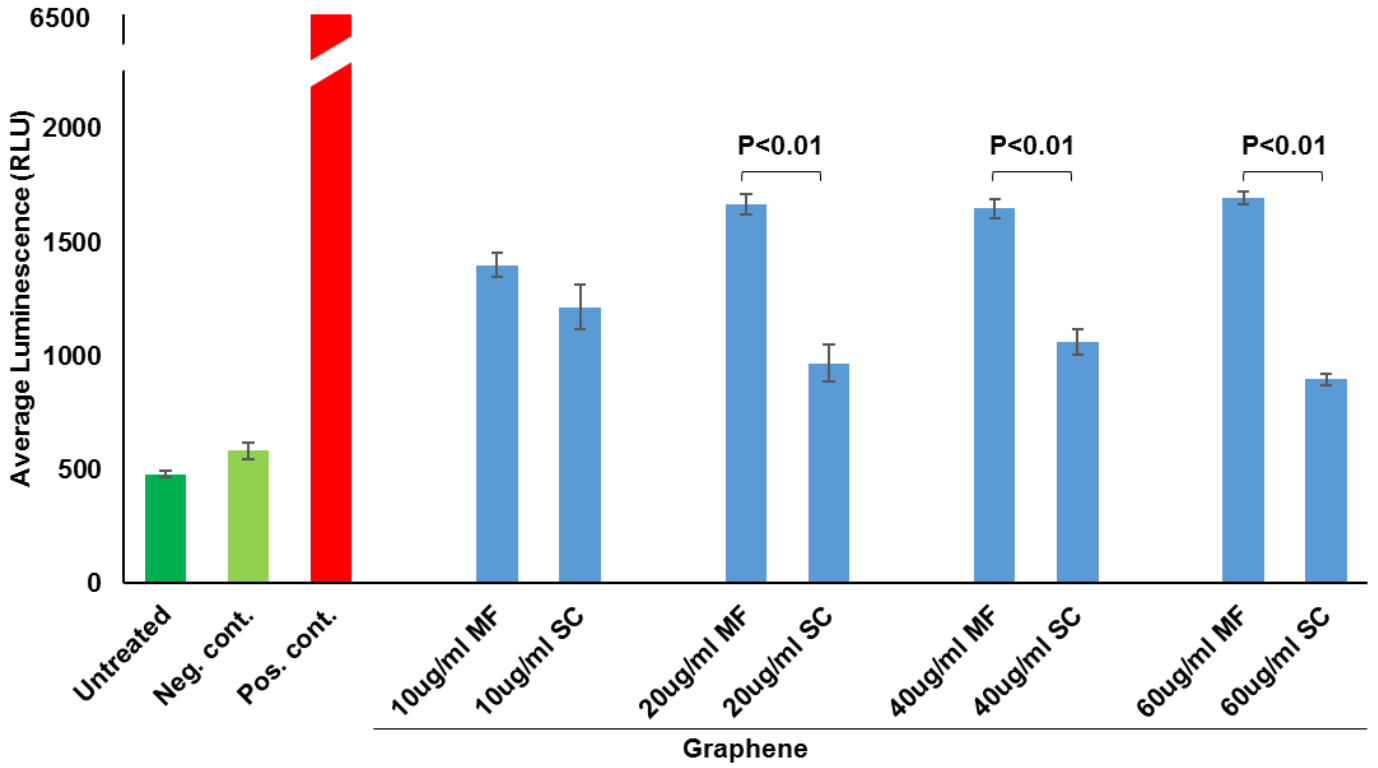
TF 4-7 Label-free, real-time cell analysis (RTCA) data, LLC-PK1 treated with 20, 40 and 60 ug/ml graphene (Gr.) dispersions prepared by microfluidization and sonication methods cell. Cell proliferation and cell death were continuously monitored using xCELLigence RTCA DP instrument for 24h after treatment.



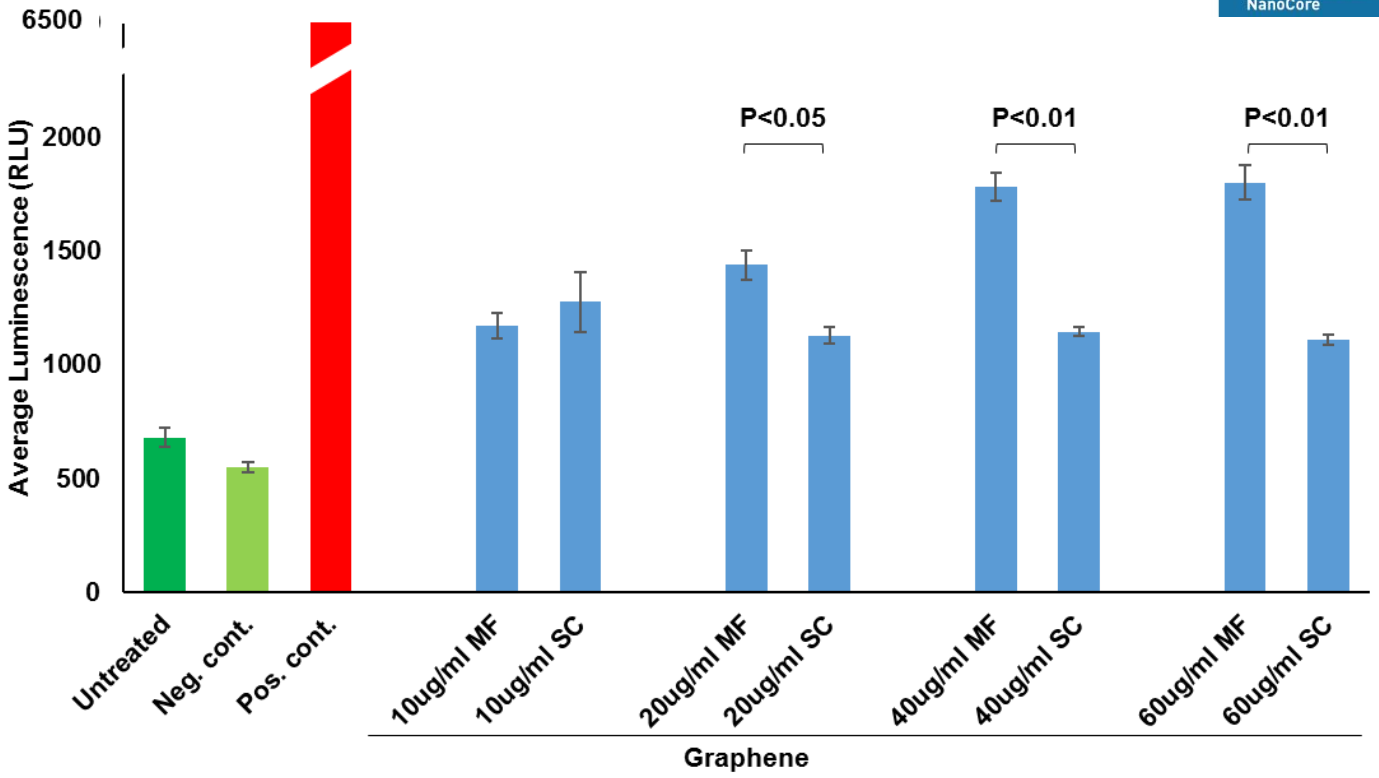
TF 4-7 Label-free, real-time cell analysis (RTCA) data, LLC-PK1 treated with 20, 40 and 60 ug/ml graphene (Gr.) dispersions prepared by microfluidization and sonication methods cell. Cell proliferation and cell death were continuously monitored using xCELLigence RTCA DP instrument for 24h after treatment.



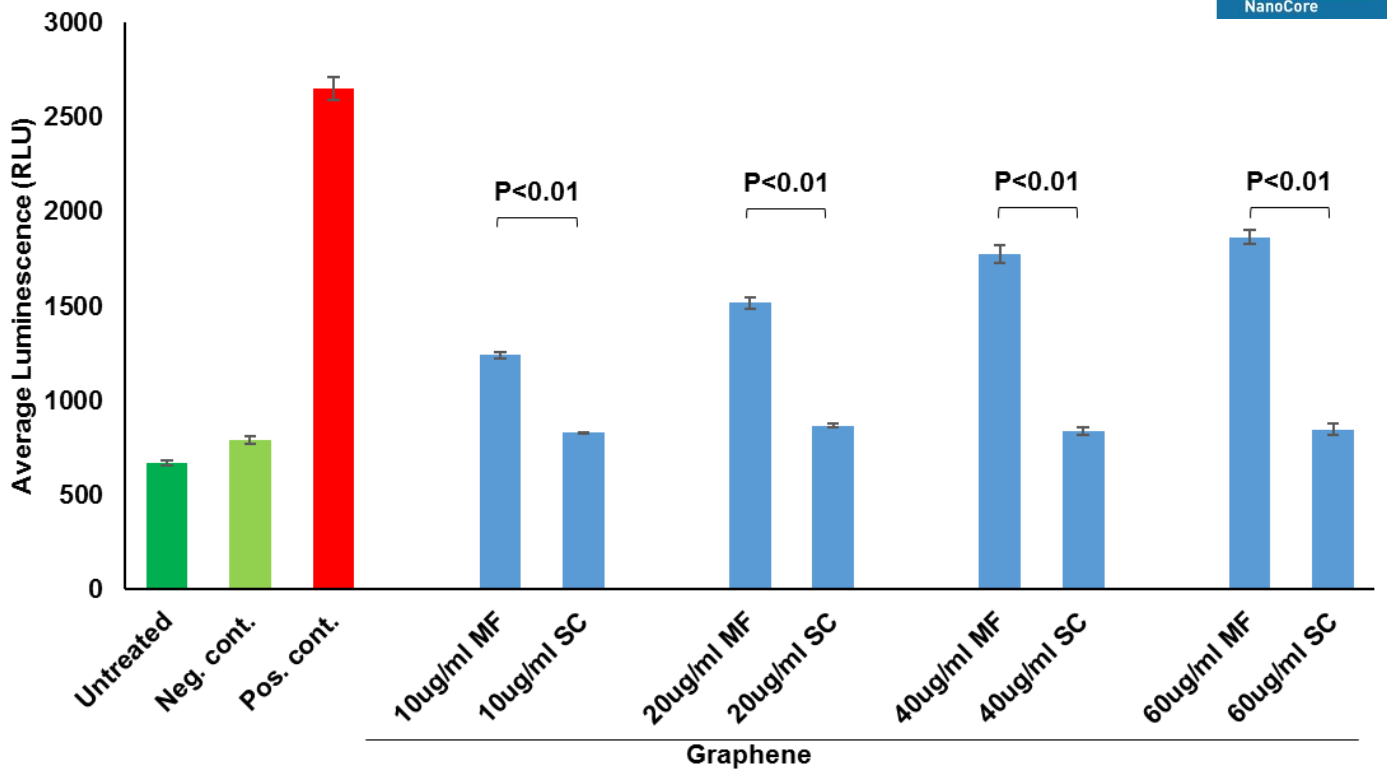
TF 9-2 ROS-Glo™ H2O2 Assay (Homogeneous Assay (Lytic)) signals from LLC-PK1cells exposed to graphene (microfluidized; MF and sonicated; SC) for 24h, cells treated with injectable H2O was used as a negative control; Neg. cont. and 50 uM menadione in medium was used as a positive control; Pos. cont.



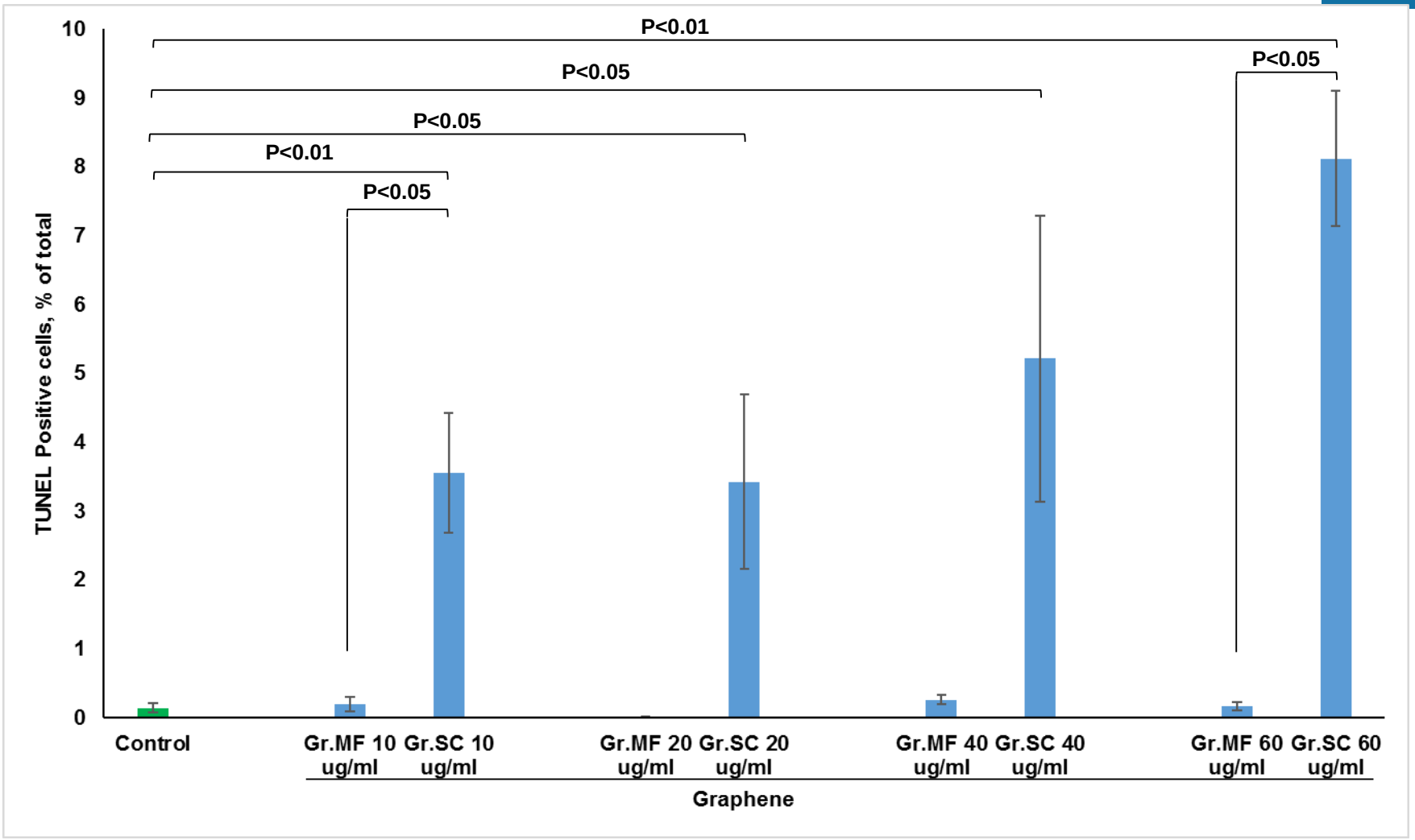
TF 9-1 ROS-Glo™ H2O2 Assay (Homogeneous Assay (Lytic)) signals from LLC-PK1cells exposed to graphene (microfluidized; MF and sonicated; SC) for 48h, cells treated with injectable H2O was used as a negative control; Neg. cont. and 50 uM menadione in medium was used as a positive control; Pos. cont.



TF 9-1 ROS-Glo™ H2O2 Assay (Homogeneous Assay (Lytic)) signals from LLC-PK1cells exposed to graphene (microfluidized; MF and sonicated; SC) for 72h, cells treated with injectable H2O was used as a negative control; Neg. cont. and 50 uM menadione in medium was used as a positive control; Pos. cont.



TF 9-2 ROS-Glo™ H2O2 Assay (Non-Lytic Assay) signals from LLC-PK1cells exposed to graphene (microfluidized; MF and sonicated; SC) for 48h, cells treated with injectable H2O was used as a negative control; Neg. cont. and 50 uM menadione in medium was used as a positive control; Pos. cont.



TF 8-9 TUNEL assay for LLC-Pk1 cells exposed to Gr. (microfluidized;MF and sonicated; SC) for 24h, cells treated with injectable water were used as negative control

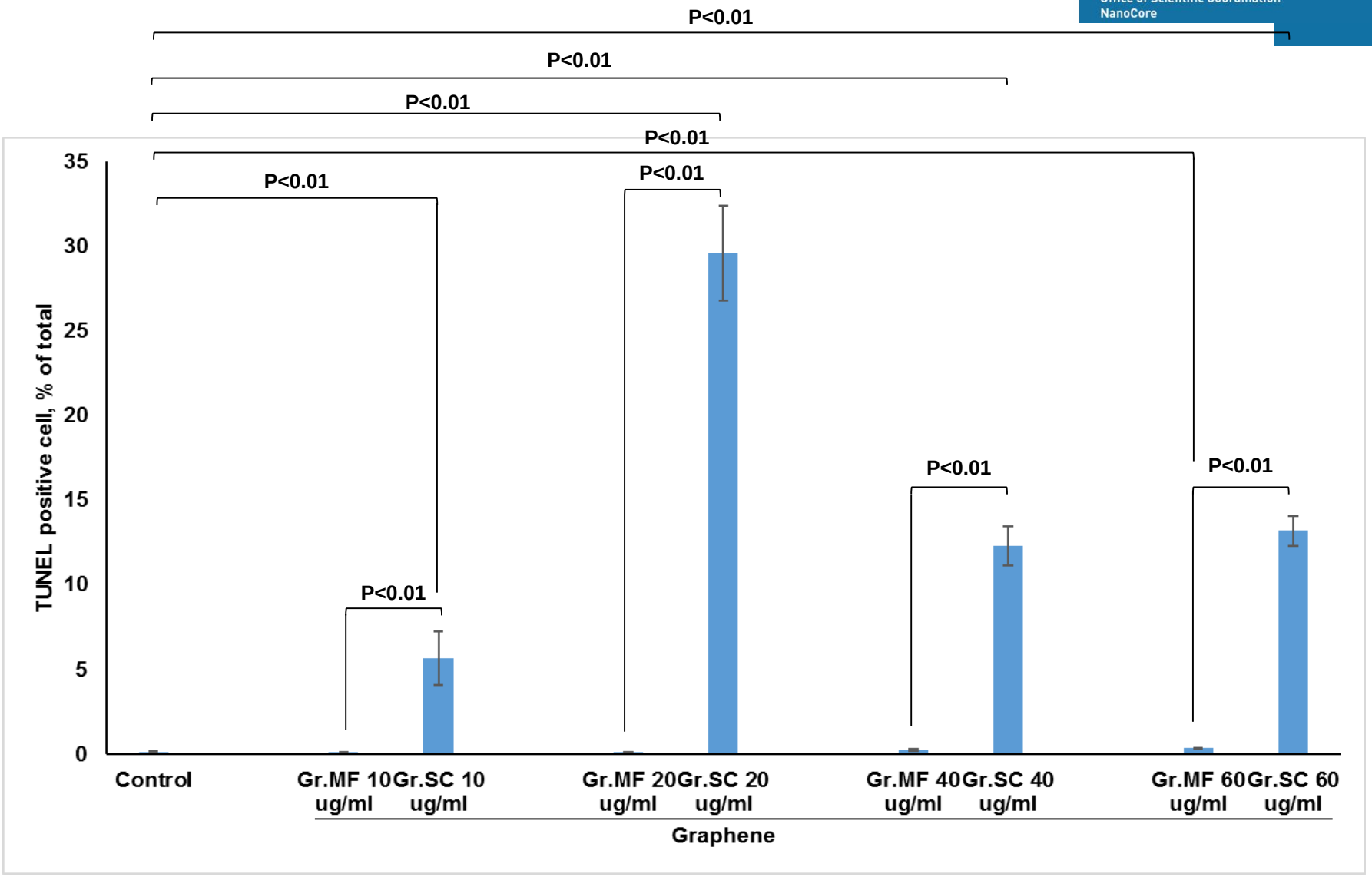
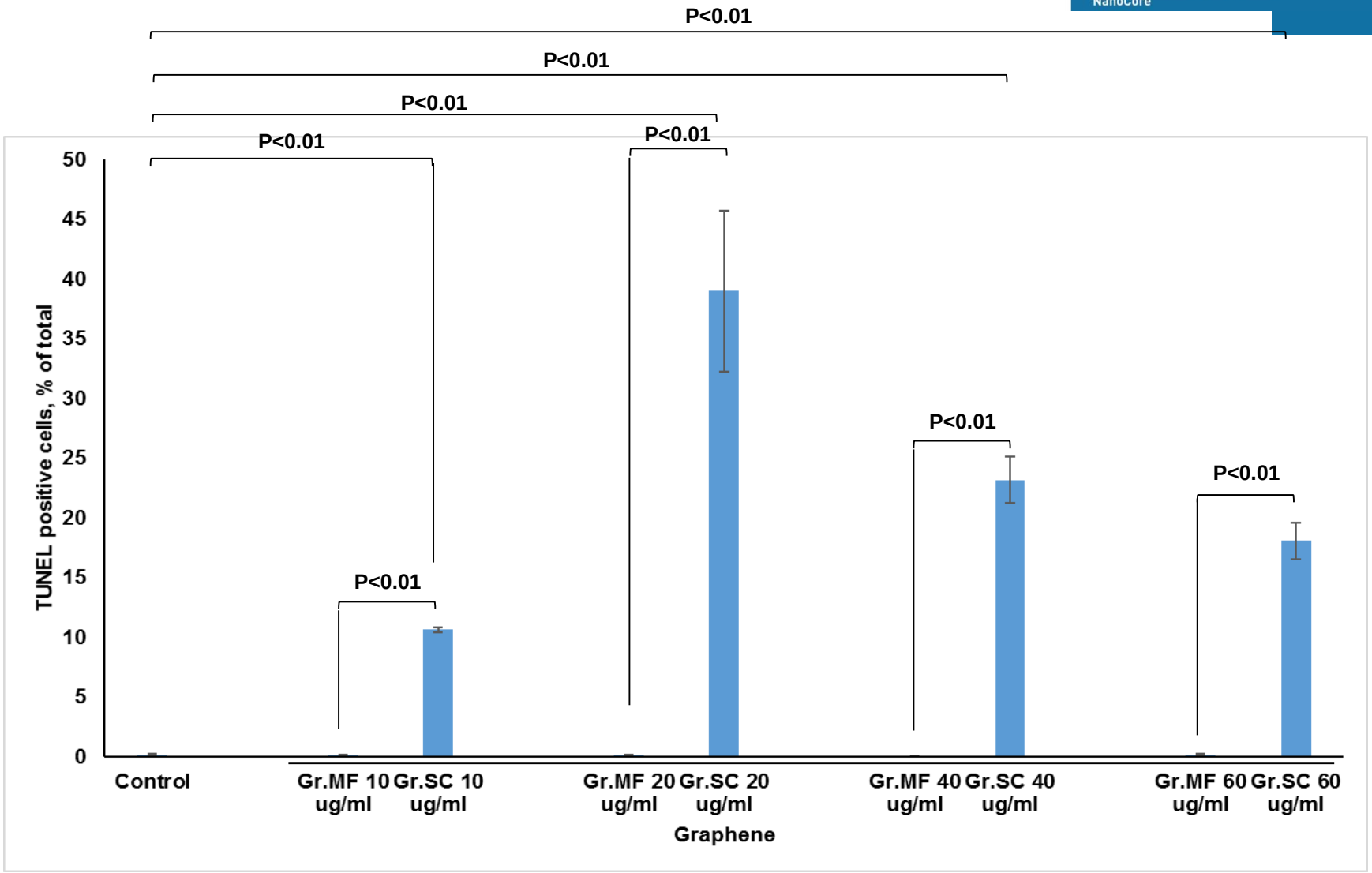
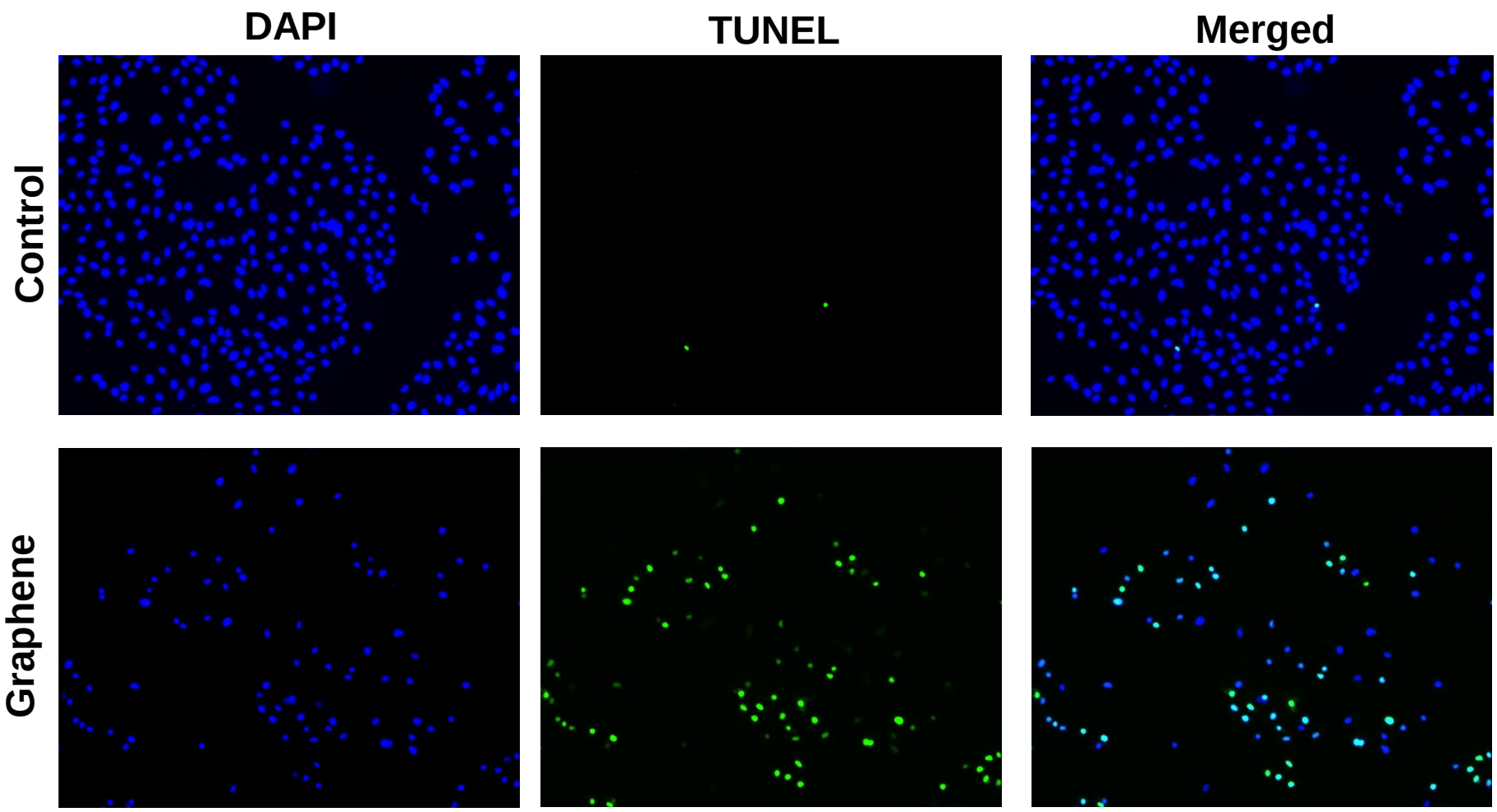


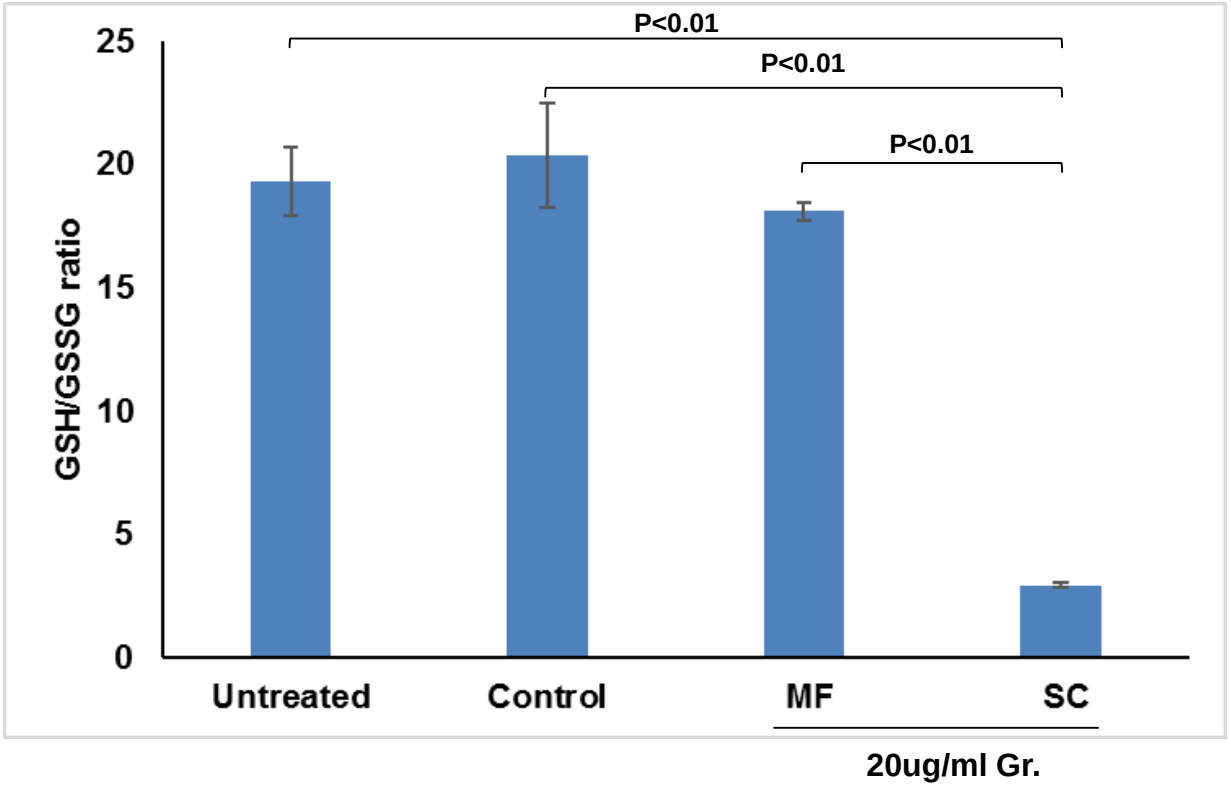
Figure 3 TUNEL assay for LLC-Pk1 cells exposed to Gr. (microfluidized;MF and sonicated; SC) for 48h, cells treated with injectable water were used as negative control



TF 8-9 TUNEL assay for LLC-Pk1 cells exposed to Gr. (microfluidized;MF and sonicated; SC) for 72h, cells treated with injectable water were used as negative control



TF 8-9 Representative images for LLC-PK1 treated with graphene dispersion prepared by sonication method and control cells (treated with injectable water) stained with TUNEL and DAPI, 100X magnification



TF10-1 Reduced glutathione/Oxidized glutathione (GSH/GSSG) ratio in LLC-PK1 exposed to graphene (Gr.) dispersions prepared by sonication (SC) and microfluidization (MF) methods for 24h by using GSH/GSSG-Glo™ Assay

Graphene toxicity mechanistic studies

1-Measuring the hydrogen peroxide (H_2O_2) by ROS-Glo™ H_2O_2 Assay:

- H_2O_2 generated from the LLC-PK1 cell culture treated with microfluidized graphene is significantly more than sonicated graphene in the treated concentrations (20, 40 and 60 ug/ml) 24, 48 and 72h after exposure.

2- Measuring reduced to oxidized glutathione (GSH/GSSG) ratio:

- The GSH/GSSG ratio of LLC-PK1 cells exposed to 20 ug/ml sonicated graphene for 24h is significantly lower than cells exposed to microfluidized graphene, control (H_2O treated or vehicle treated) and untreated cell groups.
- There is no difference in the GSH/GSSG ratio in cells exposed to 20 ug/ml microfluidized graphene compared to control and untreated groups.