Electronic Supplementary Material (ESI) for Toxicology Research.
This journal is © The Royal Society of Chemistry 2017

Supporting Information

Systematic toxicity investigation of graphene oxide: Evaluation of assay selection, cell type, exposure period and flake size

Valerie Gies[a] and Shan Zou[a,b]*

[a] Measurement Science and Standards, National Research Council Canada, 100 Sussex Drive, Ottawa, Ontario K1A 0R6, Canada; [b] Department of Chemistry, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario K1S 5B6, Canada.

*corresponding author, Email: shan.zou@nrc-cnrc.gc.ca.

Methods

Materials

Graphene oxide, 4 mg/mL, was purchased from Graphenea (Gipuzkoa, Spain) and diluted to 2 mg/mL with sterile water as the stock solution for further dilutions. Cell culture media such as Gibco™ high glucose Dullbecco's modified Eagle's medium (DMEM), Kaighn's modification of Ham's F-12 medium (FK12), Iscove's modified Dulbecco's medium (IMDM), heat-inactivated fetal bovine serum and 1X Penicillin-Streptomycin were all purchased from Life Technologies, NY, USA. WST-8, also known as Cell Counting Kit 8, was purchased from VistaLab Technologies, NY, USA. MTS was purchased from Abcam, CBG, UK. MTT was purchased as a solid from Sigma Aldrich, WI, USA. The 96 well and 24 well, flat bottom plates were obtained from Fisher Scientific, ON, Canada. The disposable cell counter slides were purchased from Logos Biosystems, Gyeonggi-do, South Korea.

Graphene oxide sample preparation

GO samples were prepared by probe sonicating 10 mL of 2 mg/mL GO in sterile water for a predetermined amount of time. The samples were sonicated in a 4°C water bath for intervals of up to 10 minutes and allowed to cool for 1 minute using a 130 W ultrasonic processor (Cole Parmer, QC, Canada). Figure S1 illustrates the different flake sizes of GO tuned by controlling the sonication energy delivered to the GO sample. Table S1 summarizes the sonication time at a power of 10 W for the three representative samples used to study the cytotoxicity. The probe/tip used for sonication was 6 mm in diameter. Other concentrations of GO were prepared by diluting the stock solution with sterile water.

Table S1: Sonication times and sonication energy for 10 mL of 2 mg/mL GO solution.

Sample	Sonication Time at 10 W (minutes)	Sonication Energy (MJ/g)
***s*-GO**	85	25
***m*-GO**	20	6.0
***l*-GO**	0.5	0.015

DLS measurements

DLS measurements of the varying sizes of GO samples were performed on the Zetasizer Nano ZS particle size analyser (Malvern Instruments, Worc, UK) in a semi-micro polystyrene disposable cuvette (VWR, PA, USA). Samples for DLS were diluted to 2 µg/mL in MiliQ water. Measurements were performed at 25°C with 120 s of equilibration time. For each sample, three sets of ten runs were recorded. The measurement angle was set to 173° with 0.4 to 10000 nm as the detectable particle range. The instrument output Z_{avg} was taken to represent the intensity-weighted arithmetic-average equivalent sphere hydrodynamic diameter, which was used as the descriptor for the flake size of the GO.

AFM measurements

AFM height/topography images were recorded using MultiMode NanoScope V with PeakForce QNM mode (Bruker Nano Surfaces Division, Santa Barbara, CA, USA). ScanAsyst-Air probes with a typical spring constant of 0.4 N/m and a resonance frequency of 50–90 kHz were used. The peak force was always kept at the lowest stable imaging level of 200–500 pN. Samples for AFM were made by spin-coating diluted GO dispersions (40 µL, 0.05 mg/mL) onto freshly cleaved 1 cm by 1 cm squares of mica substrate at 3000 rpm for 45 seconds. For each condition, three samples were prepared and for each sample, three to five images were recorded at three to

five different locations on the mica surface, for a total of nine to fifteen images per sample. The scan ranges of the images were 10 µm by 10 µm or 20 µm by 20 µm.

AFM images were analyzed using the open source softwares ImageJ and Gwyddion. On ImageJ, the images were converted to 8-bit and the threshold was set to effectively select the isolated GO flakes. Flakes that touched the edges of the images or were overlayed with other flakes were not considered. Images with a large area were taken to reduce the amount of bias this exclusion criteria presented. Any feature in the image that was less than 4 pixels was taken to be background noise and was excluded. Using the 'measure Feret's diameter' operation, the Feret's diameter was measured for all of the flakes. From there, the known ratio of pixels to nm was used to determine the diameter of the flakes. When selecting a threshold that isolated flakes from the background was not feasable, manual scaling selecting the longest lateral distance on a flake was performed uisng the Gwyddion software. A known ratio of pixels to nm was then used to determine the diameter of the flakes. This manual scaling only applies to the *l*-GO samples in this work.

Cell culture specifics

NIH 3T3, RAW 246.7, NB4 and U87 cells were cultured in Gibco™ DMEM supplemented with 10% Gibco™ heat-inactivated fetal bovine serum (FBS) and Gibco™ 1X Penicillin-Streptomycin in an incubator at 37°C with 5% CO_2 and a high relative humidity. A549 cells were cultured under the same conditions in Gibco™ FK12 culture medium and HL60 in Gibco™ IMDM.

Cell viabilities

For the three formazan based assays, WST-8, MTS and MTT measurements were performed in a 96 well, flat bottom plate and the cells were seeded to a density of 1.5-2.5 $\times 10^4$ cells/well,

depending on the cell line. The cells were treated with 5, 10, 20, 30, 50, 75, 100 and 200 μg/mL of GO. For WST-8 and MTS, a blank (identical to the sample without cells) was prepared for every concentration of GO. Preparing a blank for each concentration of GO was essential since the absorbance of GO was significant (up to half of the total absorbance) at the wave lengths studied. Control samples were prepared with water in place of GO solution for all three assays as well as control blanks, which were cell free media with the same volume of water as the control sample. To minimize the impact of the different rates of vaporization of the interior vs exterior wells on the plate, the outer wells were not used to collect data points. The cells were incubated for 24, 48 and 96 h. Following the exposure period, WST-8 and MTS reagent was added to all respective wells. For the MTT assay, the media was first removed, the cells were washed twice with PBS and fresh media was added before the addition of the reagent. After 4 h of incubation with the assay reagent, the optical densities of the WST-8 and MTS treated plates were measured at 450 and 490 nm respectively. For the MTT treated plates, all but 25 uL of solution was removed from the wells and 50 uL of DMSO was added and mixed in each well. The plate was incubated for another 10 minutes and the optical density was recorded at 540 nm. Optical densities were recorded on the FLUOstar Omega microplate reader (BMG Labtech, ON, Canada).

To determine the percent viability of the cells after GO treatments for the WST-8 and MTS assays, the following equation was used:

$$\%\ viability = \frac{OD_{sample\ n} - OD_{sample\ blank\ n}}{OD_{control} - OD_{control\ blank}} \times 100\% \qquad \text{(S1)}$$

where $OD_{sample\ n}$ is the optical denisty of the cell containing sample with n μg/mL GO treatments, $OD_{sample\ blank\ n}$ is the optical density of the same n μg/mL GO without cells present, $OD_{control}$ is the

optical denisty of the untreated cells and $OD_{control\ blank}$ is the optical density of the media alone. The same equation was used for the MTT assay but the sample blank term ($OD_{sample\ blank\ n}$) was removed since the influence of the GO absorption should be removed when the cells were washed.

For the TB stain assay, cells were exposed to the same series of concentrations of GO in a 24 well plate. Cells treated with the same volume of water as GO were taken as the control. The cells were seeded to a density of 7.5-10 $\times 10^4$cells/well. The cells were exposed to GO for 24 h. The media containing the GO was then removed and the cells were washed with PBS two times. The cells were suspended with 1X trypsin in PBS + 53mM EDTA for ten minutes. Media was added to deactivate the suspending solution. A 1:1 cell solution:trypan blue solution was agitated in replicates of two for each concentration. Using the Luna Automated Cell Counter (Logos Biosystems, Gyeonggi-do, South Korea) the number of viable cells were recorded. This experiment was performed twice.

Additional control experiment were performed on the NIH 3T3 and NB4 cell lines to investigate possible impurities present in the Graphenea GO as well as to determine if contamination due to probe sonication, performed using a sterile titanium probe, contribututed to the apparent cytotoxicity of GO. Samples of as produced GO and *s*-GO were centrifuged for 1 h at 15000 rpm, the supernatant was collected and the process was repeated two more times for a total of 3 h of centrifugation. The supernatant was diluted as if it were the 200 µg/mL GO sample and then used to treat cells. Identical experimental conditions were used to study the toxicity of a sample of sterile water that was probe sonicated for 85 minutes at ten watts.

Stability of GO in Media

Three samples were prepared, 50 µg/*mL* of *s*-GO and *l*-GO, and 200 µg/mL of *s*-GO were prepared in phenol red-free DMEM ('clear'-DMEM shortened to C-DMEM) with 10% FBS and 1X Penicillin-Streptomycin The transmission of the sample was measured over the course of 24 hours at 37°C using the Turbiscan™ Lab Stability Analyzer (Formulaction SA, France).

Assay interference evaluation

Samples were prepared identically to the cell viability assays with varying concentrations of GO and a constant amount of assay reagent. The samples were allowed to incubate for 4 h at 37°C as they would be when performing the viability assays. In the case of the MTT, the MTT treated samples were centrifuged, the supernatant was removed and replaced with DMSO. The DMSO treated sample was agitated and incubated for 10 minutes, re-centrifuged and the supernatant was collected. The absorption spectra of all three samples (the WST-8 with *n* µg/mL GO, MTS with *n* µg/mL GO and the DMSO which would have solubilized any formazan produced) were measured on the Cary 5000 UV-Vis-NIR spectrometer (Agilent, CA, USA).

Bright field imaging

Cells were seeded in a 24 well plate to a density of 7.5-10 $\times 10^4$cells/well with 0, 5, 10, 20, 30, 50, 75, 100 and 200 µg/mL of GO. Cells treated with the same volume water as GO were taken as the control. The cells were exposed to GO for 24 h after which the media containing the GO was removed and the cells were washed with PBS two times. The images were recorded on the Olympus LX81-DSU Microscope (Olympus America INC, NY, USA) using the CoolSNAP ES Camera (Photometrics, AZ, USA) and X-Cite Series 12OPC excitation source (Excelitas

Technologies, QC, Canada) with a 20X objective (Olympus America INC, NY, USA). The binning was set to 1 and the exposure time was 50 ms. At least five images were taken for each concentration of GO treated samples at approximately the centres of the wells.

Methods

Modification of GO flake size

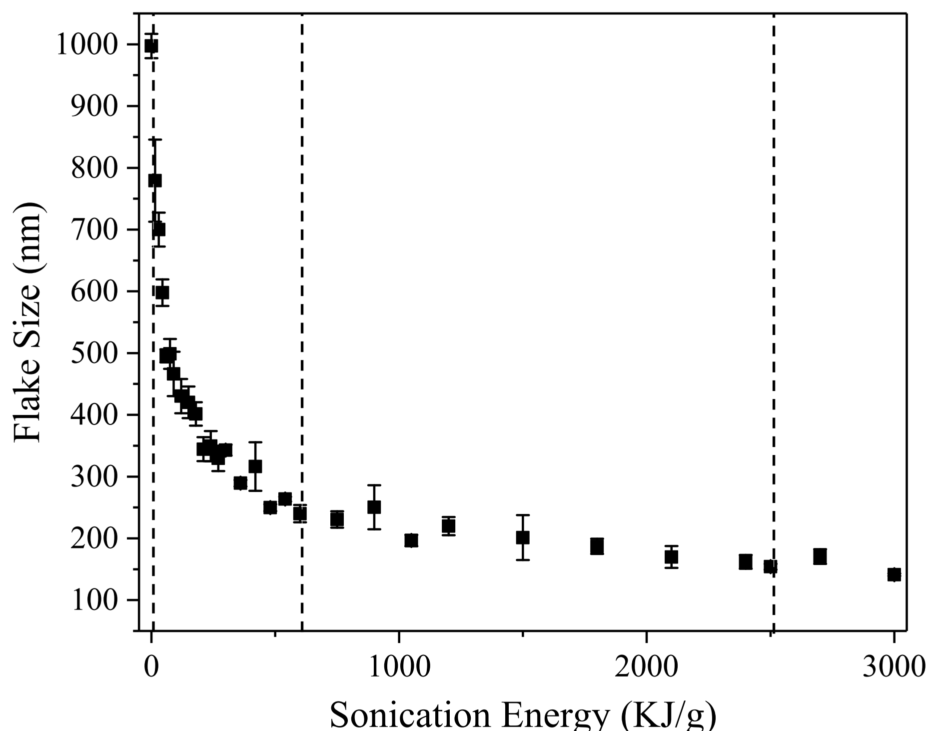
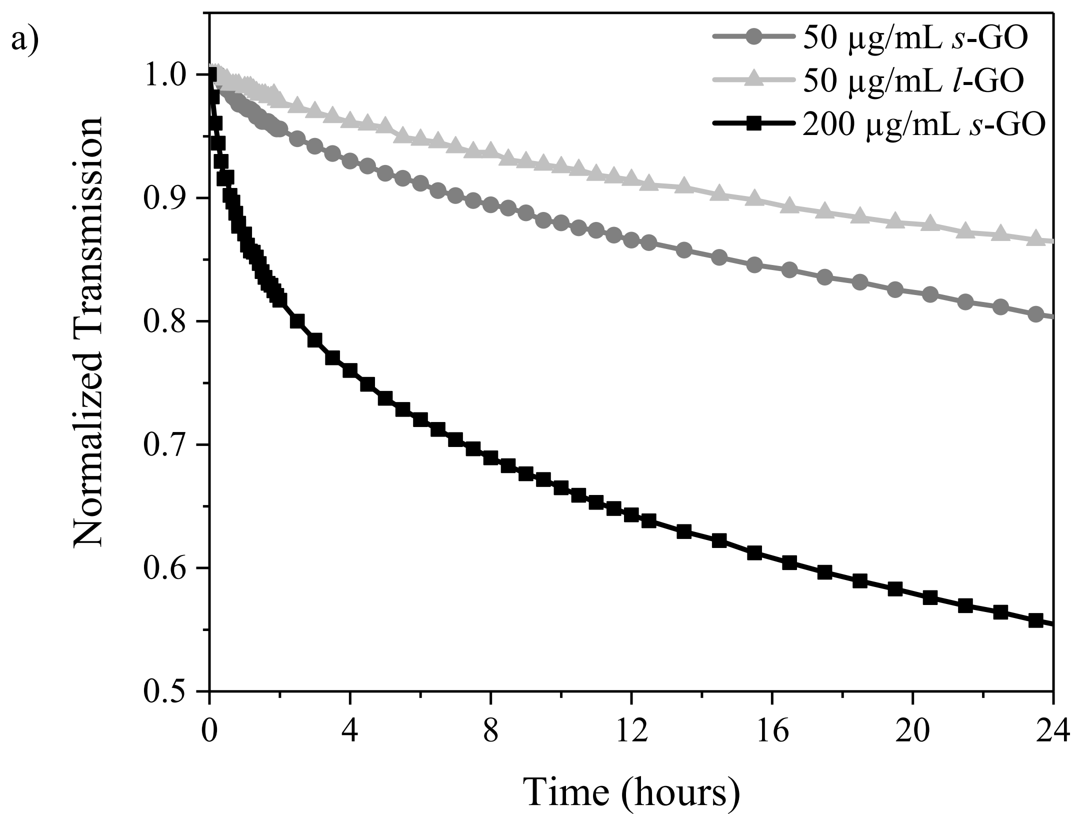


Figure S1: Change in GO flake size with increased sonication energy determined by DLS. A 2 mg/mL solution of GO was sonicated using a 6 mm probe for set increments of time at 10W. Time and power output were then used to calculate energy input. The solution was sonicated at 4 ^{o}C to prevent the sample from boiling during sonication. Aliquots were taken after each sonication interval, diluted to 2 µg/mL and DLS measurements were taken in triplicate. Error bars represent the standard error between the three triplicate measurements. The error on the energy reported is up to 10% of the total energy. Dashed lines represent the sonication energies used in this work to produce the three sizes of GO.

Probe sonication was used to obtain different sizes of GO flakes, the relationship between sonication energy and flake size is shown in Figure S1. The change in flake size as a response to sonication follows a power law. Fitting the curve to a power function is described elsewhere.[1] The curve displayed in Figure S1 further illustrates the reason behind the selection of the three sizes of GO used in this work: 1000, 250 and 150 nm. The 1000 nm size selected is representative of the as received size of the Graphenea GO. The 150 nm size is representative of the smallest size reasonably attainable with sonication; it is clear that the change in size plateaus with increasing energies to approximately 150 nm. 250 nm represents the area where the change in size in response to change in sonication energy is closest to -1, an area moderate to the initially large change in response to change in sonication and the plateau of the change.

Stability of GO



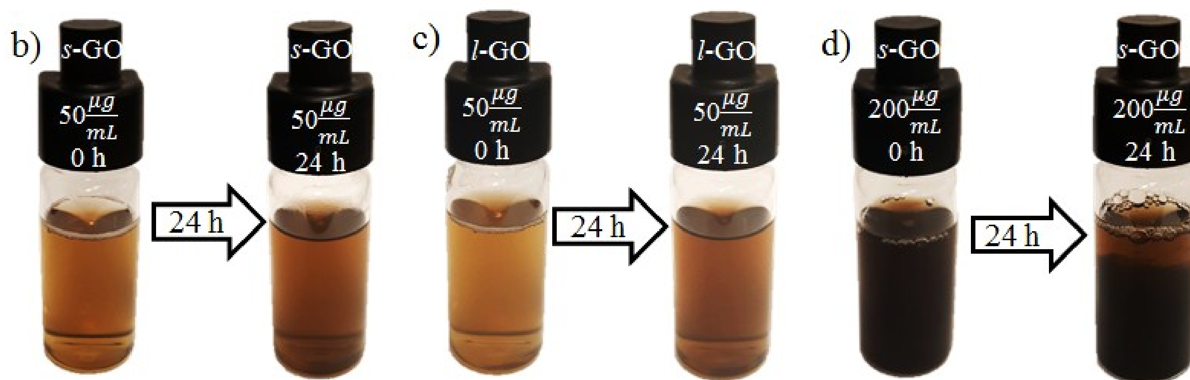


Figure S2: a) Normalized transmission of 50 μg/mL *s*-GO and *l*-GO and 200 μg/mL *s*-GO in C-DMEM over 24 h. Photos of 50 μg/mL *s*-GO (b), 50 μg/mL *l*-GO (c) and 200 μg/mL *s*-GO (d) at 0 h and 24 h post GO addition to C-DMEM.

As with many nanomaterials, it was expected for the graphene oxide to aggregate in cell media due to the high ionic content and neutral pH.[2] Serum is known to interact with the surface of GO which may influence the stability of the flakes in solution.[3] The stability of *s*-GO was observed to be low at 200 µg/mL in cell culture media: *s*-GO clearly aggregated and settled over the course of the 24 h incubation period and the transmission decreased by 45% as shown in Figure S2. GO was observed to not settle at all for the 50 µg/mL incubation period, though aggregation likely occurred to some extent as observed by the decrease in the transmission of the samples. The transmission of the 50 µg/mL *s*-GO and *l*-GO samples were observed to decrease by 20 and 15% respectively. Concerns regarding possible variances in toxicity due to size as a result of different rates of aggregation may be minimized since the different flake sizes have similar dispersion stabilities.

Assay interference evaluation

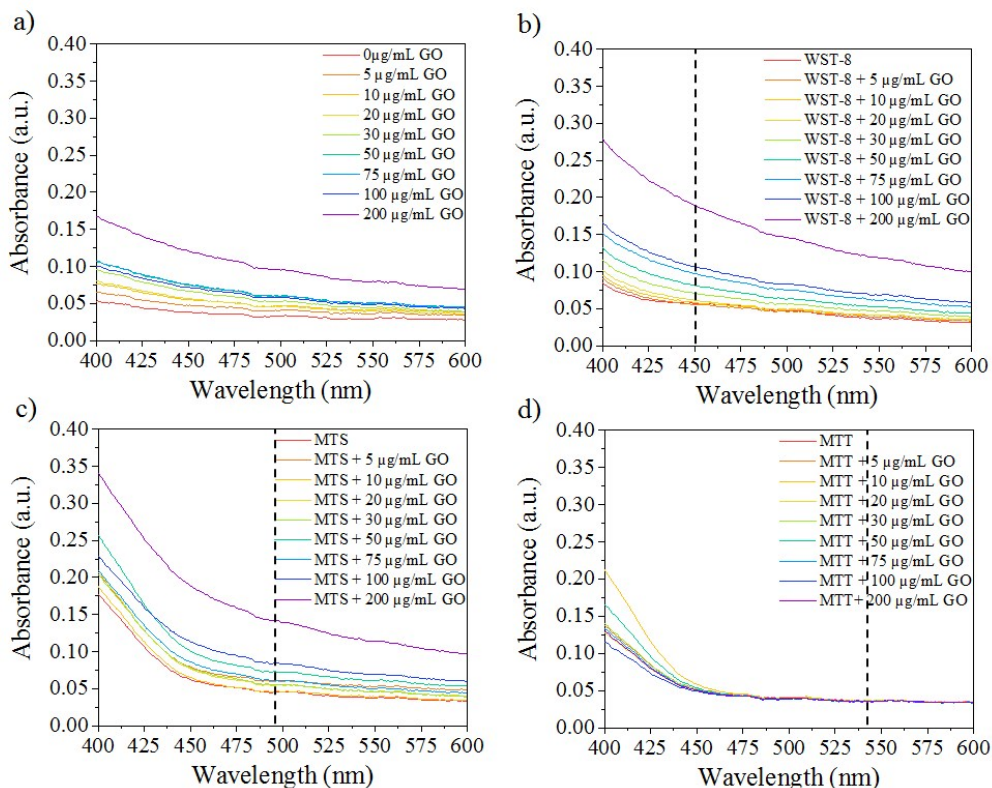


Figure S3: Absorption spectra of 0, 5, 10, 20, 30, 50, 75, 100 and 200 µg/mL of *s*-GO in a) C-DMEM b) C-DMEM with the WST-8 reagent and c) C-DMEM with the MTS reagent. d) Absorption spectra of DMSO and DMSO soluble reagents present in C-DMEM treated with 0, 5, 10, 20, 30, 50, 75, 100 and 200 µg/mL of *s*-GO and the MTT reagent. A dashed black line is added to each plot at the wavelength where a peak were to be observed if a reaction between the assay reagent and the assay were to occur.

A downside to formazan based viabilities is the possibility of external, non-cellular factors reducing the assay reagent to the colored formazan, resulting in falsely high viabilities. This was observed to occur with nanomaterials such as boron nitride nanotubes and carbon nanotubes.[4,5] An interaction between GO and the MTT assay was previously observed.[6] Before using a formazan based assay to study the cytotoxicity of nanomaterials, it is important to consider the

possibility of a reaction between the formazan and the material being studied, in this case graphene oxide.

Figure S3 illustrates that no reaction between *s*-GO and WST-8, MTS or MTT occurred; a very distinct peak would have appeared at the indicated black dashed line if a reaction were to have occurred. Measurements were performed in phenol red-free DMEM so the phenol red peak could not obscure or interfere with any potential formazan peaks. It was observed to be insufficient to take a single absorption measurement at the wavelength used to measure optical density specific to the assay because of the effects of scattering. Scattering is evaluated for the WST-8 and MTS GO containing samples when compared to strict GO. This is potentially due to physisorption of the dyes onto the surface of the material. This change in scattering was accounted for in the viability measurements by use of sample blanks at every concentration of GO. Only considering the appropriateness of the assay based on whether or not it reacts with the assay reagent, all three formazan based assay were deemed acceptable to measure the toxicity of GO.

References

1. B. R. Coleman, T. Knight, V. Gies, Z. J. Jakubek and S. Zou, Manipulation and quantification of graphene oxide flake size: Photoluminescence and cytotoxicity, *ACS Appl. Mater. Interfaces*, 2017, **9**, 28911-28921..
2. J. M. Zook, R. I. MacCuspie, L. E. Locascio, M. W. Halter and J. T. Elliott, Stable nanoparticle aggregates/agglomerates of different sizes and the effect of their size on hemolytic cytotoxicity. *Nanotoxicology,* 2011, **5,** 517-530.
3. W. Hu, C. Peng, M. Lv, X. Li, Y. Zhang, N. Chen, C. Fan and Q. Huang, Protein corona-mediated mitigation of cytotoxicity of graphene oxide. *ACS Nano,* 5**,** 3693-3700 (2011).
4. L. Belyanskaya, P. Manser, P. Spohn, A. Bruinink and P. Wick, The reliability and limits of the MTT reduction assay for carbon nanotubes–cell interaction, *Carbon*, 2007, **45**, 2643-2648.
5. G. Ciofani, S. Danti, D. D'Alessandro, S. Moscato and A. Menciassi, Assessing cytotoxicity of boron nitride nanotubes: Interference with the MTT assay, *Biochem. Biophys. Res. Commun.*, 2010, **394**, 405-411.
6. K.-H. Liao, Y.-S. Lin, C. W. Macosko and C. L. Haynes, Cytotoxicity of graphene oxide and graphene in human erythrocytes and skin fibroblasts, *ACS Appl. Mater. Interfaces*, 2011, **3**, 2607-2615.