High Throughput Screening of Nanomaterials: The Arkansas Research Consortium in Nanotoxicity, a partnership between Arkansas and the FDA

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ABSTRACT

Arkansas and the FDA have executed a Memorandum of Understanding with the US Food and Drug Administration to establish the Arkansas Research Consortium in Nanotoxicity (ARCN) as a collaboration between state universities and the FDA National Center of Toxicological Research in Jefferson, AR. This research effort is coordinated by the Arkansas Research Alliance and directed by a scientists with expertise in synthesis, characterization, detection and toxicity testing in *in vitro*, *in vivo* and environmental models. Research projects are distributed in laboratories at 5 state Universities, the University of Arkansas, University of Arkansas at Pine Bluff, University of Arkansas at Little Rock, the University of Arkansas for Medical Sciences and Arkansas State University. This group has selected carbon-based graphenes as a model for developing high-throughput, rapid screening procedures that are broadly applicable to a variety of existing and yet-to-be developed nanomaterials.

Keywords: *graphene, characterization, atomic force microscopy, photoacoustic imaging, biological assay*

1. ARCN RESEARCH

Arkansas researchers provide unique technological resources for an advanced nanotechnology-oriented research. Capabilities range from nanomaterial synthesis, characterization, and toxicity testing to broad applications in industry, agriculture, biology and medicine. Among world recognized achievements made by researchers in AR are photothermal nanodrugs,[1] in vivo blood nanotests,[2] in vivo killing circuiting tumor cells and infections,[3] or eradication of insects harmful to humans or the environment.(e.g., mosquitoes)[4]. The Arkansas nano-related resources include cutting-edge analytical chemistry and nano-imaging tools in Nanotechnology-oriented Centers located at University of Arkansas at Fayetteville (UA) at Little Rock (UALR), advanced nanotoxicity assays in the National Center for Toxicological Research (NCTR) in Jefferson, AR, excellent biological, medical and research infrastructure at the University of Arkansas for Medical Science (UAMS), and facilitates for environmental studies at Arkansas State University and the University of Arkansas at Pine Bluff.

Specific research projects are funded by a contract with the FDA and include synthesizing graphenic nanomaterials, evaluating a variety of methods to characterize graphene-based materials and detect this class of materials in biological and environmental matrices. Funds contributed by the State of Arkansas are being invested in research focused on initial efforts to understand the mechanisms by which graphene-based nanomaterials may interact with *in vitro* systems. Future work will be directed toward further cellular and molecular mechanisms based on in vitro and in vivo models. A significant effort understand potential environmental impacts is also planned.

Graphene with its novel electrical, electronic, optical, structural or mechanic characteristics has found a plethora of applications in a number of scientific areas, ranging from electronics to materials science and nanomedicine. More recently, this material has proven to be one of the foundation block for developing a variety of biological and medicine related applications, ranging from drug/gene delivery to thermal ablation of cancer cells. Given its large number of applications, it is absolutely essential to understand how its characteristics interact with biological systems, including cells, tissues, organs, and extending to as plants, aquatic and terrestrial flora and the environment. Thus, while our goal is to develop characterization "on-demand" of any new nanoscale material, we have selected graphene as our test case. To accurately assess and elucidate mechanisms responsible for any of these impacts, a number of characteristics of this material that could impact its interactions with biological systems must be well defined and understood. These include size, chemical composition and surface functionalization, dispersion, electrical properties, chemical and physical stability (e.g. oxidation and aggregation, respectively) and propensity for agglomeration and dispersion. Careful characterization will allow us to select graphene samples whose characteristics are known and consistent. This is critical for studies focused on understanding interactions of the nanomaterials with biological and environmental systems and for determining the mechanisms underlying those interactions. Most importantly, it will allow us to produce reliable, reproducible data related to the toxicity of graphene and graphene-based nanomaterials. Thorough characterization opens the doors to methods to

image, track, and quantify the concentration of nanoparticles and their biological activity. The chemical and biological activity of graphene nanoparticles will depend critically on size, shape, charge, and surroundings. This dependence is due to an increased role of the nature of the surface in the activity as the size of the nanoparticle is decreased. As a result, it is necessary to determine those characteristics so that the physical characteristics of graphene nanoparticles can be correlated with its bioactivity.

2. CHARACTERIZATION

Proper characterization of the test material prior to application to biological systems is key to understanding potential toxicity. Many organizations and agencies agree that a minimum characterization list should be developed to enable scientists to understand the test material that was tested, but also, to enable other scientists to mine data for structure-activity relationships and search for characterization features that are common to toxic (and non-toxic) nanomaterials. The characterization of the graphenic nanomaterials being conducted by the ARCN are focused on developing high-throughput assays that fulfill these minimum characterization requirements.

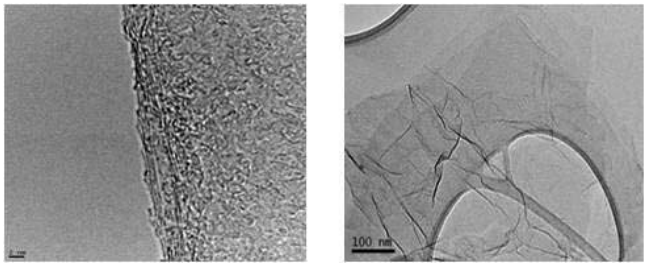

Figure 1. TEM images of graphene sample G-1. Apparent thickness ~1 nm.

At present, the main focus of our research is to carefully characterize the physical and chemical properties of commercially available graphenes. Three different types of graphitic materials with average thicknesses of 1-1.2 nm(G-1) , 10-20 nm (G-10) and 50-100 nm (G-50), were purchased from Angstron Material, Inc. These materials have been characterized using Electron microscopy (SEM and TEM), Thermogravimetrical Analysis, BET surface area analysis and Raman spectroscopy.

Before TEM analysis, all graphene samples were sonicated in distilled water in a bath sonicator without surfactant. The resulting suspensions were deposited on TEM grids. Figure 1 shows the TEM images of the G_1 sample. In this sample, the graphene flakes had a thickness of 1.5 nm and varied between 750 nm-1.1 μm in length. The G-1 sample is very "fluffy" and SEM images verified the presence of large transparent sheets. The G-10 sample was much more granular and SEM analysis showed the presence of large and thick sheets. The size of the flakes was approximately about 2 μm and the thickness ranged from 3.6-7.5 nm (Figure 2).

A mixture of large graphitic structures (several microns in size) present in the G-50 graphitic sample is shown in the SEM image in Figure 3.

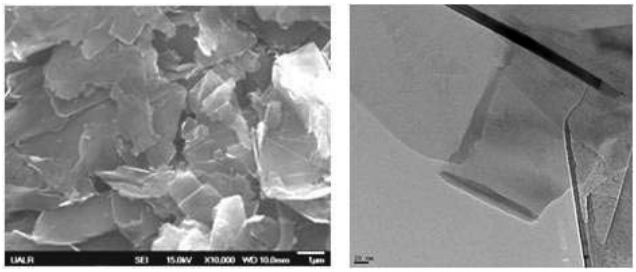

Figure 2. SEM and TEM images of G-10 graphene samples

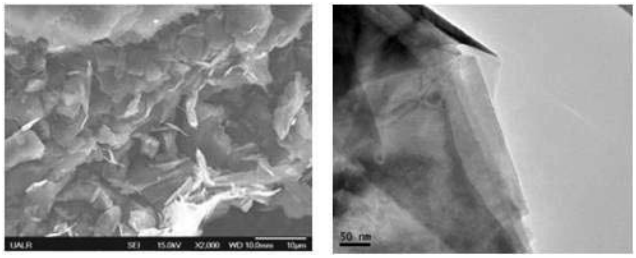

Figure 3. SEM and TEM analysis of the G-50 graphene

Elemental analyses (EDAX) of all the graphitic samples indicated the presence of about 97 wt.% carbon, about 2 wt.% oxygen and traces of a few impurities (data not shown).

To determine the atomic scale morphological characteristics we are also using high-resolution scanning tunneling microscopy (STM), ultra-fast scanning atomic force microscopy (AFM) to characterize these graphenes). STM will allow us to have atomic scale image of the graphene surface, AFM will give us a near atomic view over much larger areas. TEM will be used to image the stacking geometry. The top portion of Figure 4 shows an STM image of GaAs that clearly shows the construction of Ga and As atoms. The bottom portion of Figure 4 shows a high resolution TEM image of graphene along with the diffraction pattern that confirms it is a single layer of carbon. STM, AFM, and TEM images of graphene will play a significant role in determining the size, shape, and surface structure of graphene nanoparticles needed to understand and correlate with bioactivity. Other methods currently being employed to characterize graphene include: X-ray photoluminescence spectroscopy (XPS) which will provide detail on the composition of the graphene, such as oxidation or impurities; Magnetic properties determined using SQUID measurements for potential tagging. The SQUID measurements may prove to be a key quantitative tool to determine the can prove helpful in determining the amount of graphene consumed by cells; Electrical properties using Hall measurements to potentially use the effect on the conductivity of a fluid to quantify concentration. Raman and x-ray diffraction spectroscopy to support conclusions on the thickness of the graphene films (1 to 5 monolayers). For example, the ratio

and amplitude of the 2D to G Raman bands can determine the number of layers; and Terahertz spectrum in solution to potentially determine concentration.

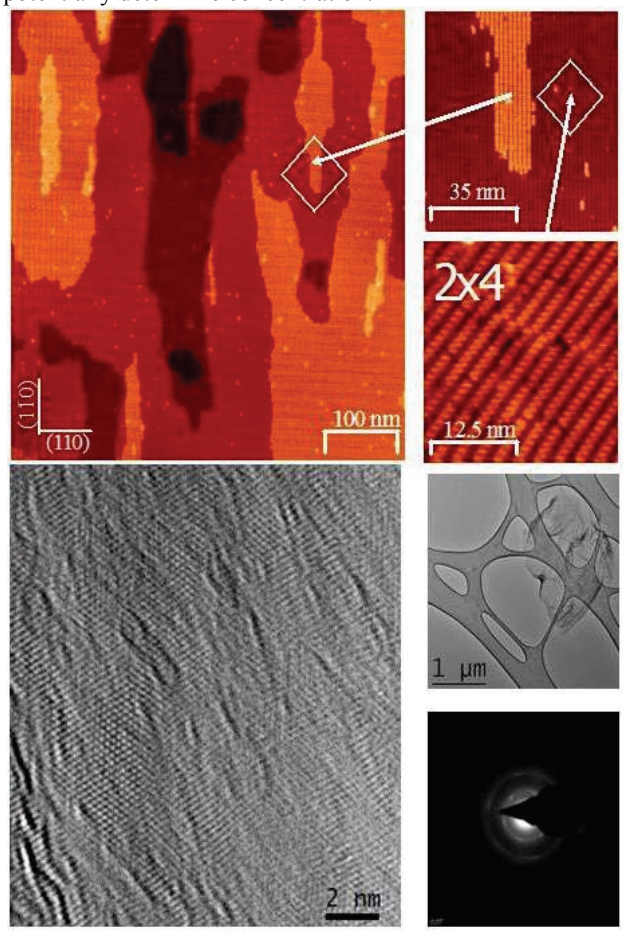

Figure 4. (top) STM image showing the GaAs reconstruction of surface atoms. Each change in color in the image is a change in one monolayer indicating the significance of STM images. (bottom) TEM image of graphene. The image in the lower right shows a diffraction pattern with six fold symmetry confirming there is a single layer of carbon.

3. REAL-TIME PHOTOACOUSTIC AND PHOTOTOHERMAL MONITORING OF GRAPHENE IN SINGLE CELLS.

Despite dramatic progress in the development of imaging tools, real-time monitoring pf interaction of nanomaterials, especially low fluorescent graphene with biotissuues at single cell level is extremely challenging. To partly overcome this problem, we propose novel integrated noninvasive, super-resolution, 3-D photothermal (PT) and photoacoustic (PA) imaging of graphene-based nanomaterials in different environments at single nanoparticle levels (*e.g.*, in powders, solutions, cells, plants, fish, and animals) that is impossible with conventional assays.. This research is highly relevant to the FDA mission and will validate the accuracy of novel nanotoxicity testing, and eventually, of nanomaterial's safety.

The unique capability of these methods were successfully demonstrated for label-free imaging and identification of carbon nanotubes (CNTs) for verification purpose and then graphene inside single cells (**Fig. 5B-C**), which were not visible using conventional optical microscopy (**Fig. 5A**). Moreover, by a combination of confocal PT imaging and PT spectroscopy, we provided a rough characterization of a ~2 μm graphene flakes containing from 2-3 to 8 graphene layers (**Fig. 5D**). In further study , we plan to evaluate these emerging imaging techniques with the goal of using them for real-time toxicity assessment at sub-cellular levels. This will allow us to collect data on graphene-based nanomaterial uptake, biodistribution and toxicity for pure and doped graphene-based nanomaterials in various biological environments (cells, *C. Elegans*, plants, fish, and animals). This effort will support the development of universal analytical techniques that can be used for rapid quantification and toxicity study of many other nanomaterials.

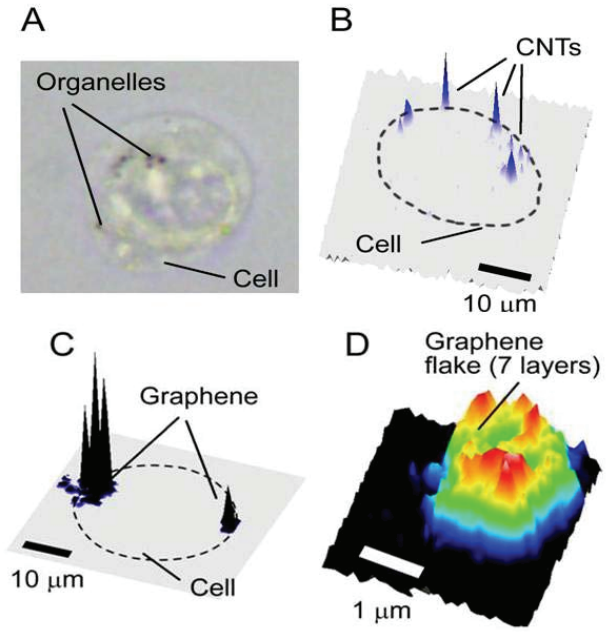

FIG.5 (A) Transmission image of MDA-MB-231 cell with intrinsic scattering background. **(B)** PT image of CNTs in the cell (incubation: 30 min, 37^0). **(C)** PT image of graphene at the cell membrane (30 min, 37^oC). **(D)** 3-D PT image of a single graphene flake.

4. BIOLOGICAL ASSAYS

Biological assessments are being conducted at the NCTR and the Universities. University research is funded by the State of Arkansas. There are a several *in vitro* and *in vivo* biological assays that will be used to evaluate the biological interaction and toxicity of chemical and substances, including nanomaterials. In addition to characterization discussed above

there are three key aspects to properly performing a biological or toxicity test that will impact our understanding of the toxicity of nanomaterials including graphenes.

First is to have the appropriate assay for determining the biological activity and toxicity. Although understanding the behavior and toxicity in any cell system or animal model will contribute to our understanding of the potential hazard of these materials, regulatory agencies have developed a battery of tests that have been validated as acceptable and predictive of the hazardous properties of many chemicals. Although the tests differ between agencies, common tests include genotoxicity in systems such as the *Salmonella typhimurium* histidine-reversion assay (a.k.a. Ames test), mutagenicity in mouse lymphoma cells, and thymidine kinase mutation in a number of cell types. Other tests include assays for cytotoxicity in a number of prokaryote and eukaryote cell lines, hormonal activity (anti- or pro-estrogenic; anti- or pro-androgenic) *in vitro* and *in vivo*, and toxicity assays *in vivo* for genotoxicity, mutagenicity, neurotoxicity, behavior toxicity, immunotoxicity, reproductive/developmental toxicity, inhalation toxicity, and carcinogenicity. The Arkansas Research Consortium in Nanotoxicity (ARCN) has the capability to perform these toxicity assays, and will utilize this expertise to develop data and understanding of the hazard of graphenic nanomaterials.

Second is the evaluation of the behavior of the nanomaterial in the dosing solution and buffers used in biological assays. This is a very key analysis for assays that are being used for regulatory decisions. The dispersibility of the graphene test materials in various solvents is being explored, with the goal to provide laboratories in the ARCN and elsewhere with a specific standard operating procedure (SOP) that will allow reproducible dispersion and aggregation. The second phase of this effort is developing SOPs for the introduction of hydrophobic graphene into tissue culture media and buffers used in the toxicity assays. The goal is development and distribution of methods to confirm the size distribution and stability (e.g. aggregation, oxidation) of the graphenic test materials. This type of analysis is critical in biological studies to be considered by regulatory agencies for hazard and risk assessments.

Third is proper conduct of the biological assay and quantitative determination of the nanomaterial in the test cell. There are several examples in the scientific literature where a nanomaterials interfered with a toxicity assays by either adsorbing an analyte used to quantify toxicity or through spectral properties that interfered with the assay. As a result, each toxicity assay must properly control for interference from the graphenes. One of the keys to hazard and risk assessment is determining the dose-response effect in the system. A consensus is building that nanomaterial toxicity must be expressed as the result of careful quantification of the amount of nanomaterial that interacted with the test cells. As a result, laboratories in the ARCN are focused on evaluating the sensitivity and selectivity of various spectroscopic methods to detect graphenic materials in cells, organisms and environmentally relevant matrices.

5. REFERENCES

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