Review Article

"OMICS" STUDIES ON CARBON NANOPARTICLES EFFECTS

ESTUDOS "ÔMICOS" SOBRE OS EFEITOS DAS NANOPARTICULAS DE CARBONO

Liudy Garcia HERNÁNDEZ[1*]; Foued Salmen ESPINDOLA[2]; Luis Felipe Desdin GARCÍA[1]

1. Departamento de Física, Centro de Aplicaciones Tecnológicas y Desarrollo Nuclear, CEADEN, La Habana, Cuba. liudy@ceaden.edu.cu; 2. Instituto de Genética e Bioquímica, INGEB, Universidade Federal de Uberlândia, Uberlândia, MG, Brasil. foued@ufu.br

ABSTRACT: Nanoparticles are being explored for biomedical applications in diagnostics and therapeutics. However, biocompatibility, toxicity and cellular entry ability are critical factors that will determine the utility of nanoparticles in clinical applications. "Omics" technologies can provide powerful tools that are sensitive and complementary for the study of molecular toxicology and for biomedical applications of nanomaterials. Specifically, proteomics could elucidate mechanisms of action and contribute to studies on how nanomaterials affect protein expression in tissues and organs, metabolic pathways, signal transduction mechanisms and target molecules. The objective of this review is to present a small approach about "omics" technologies applied to the study of the effect of carbon nanomaterials in the cell.

KEYWORDS: Nanoparticles. Nanotubes. Nano-onions. Proteomic and Gene expression.

INTRODUCTION

The term "omics" (Figure 1) refers to genomics, proteomics, transcriptomics and other tools used to study the "omics", meaning "totalities". The genome for example, is the total set of genes common to all cells in a body.

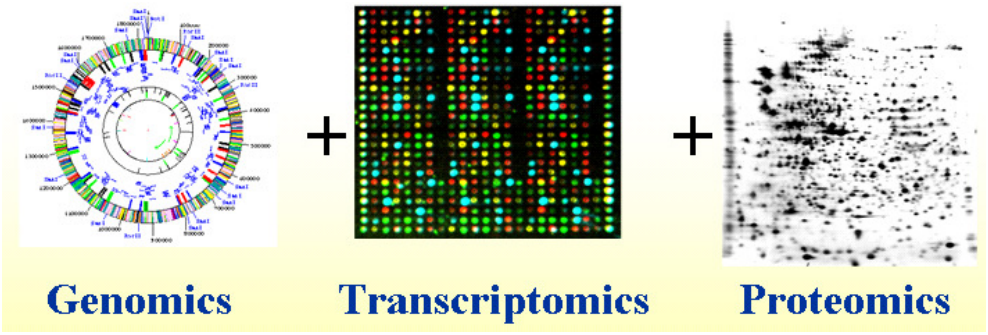

Figure 1. Representative illustrations of some "Omics" technologies.

Genomics is the study of the structure, function and interaction of genes. Developments in this area have mainly stemmed from progress in molecular biology and specifically in automated DNA sequencing and bioinformatics, which is based on the large-scale storage, structure, organization and extraction of information from biological studies. Some genomic techniques are based on blotting. In this technique, DNA is transferred to a solid support such as nitrocellulose followed by hybridization with appropriate nucleic acid probes and can be preceded by polymerase chain reactions (PCR). Southern blotting (SOUTHERN, 1975) and Northern blotting (FERNYHOUGH, 2001) are used for DNA and RNA respectively. Another technology is real-time PCR (RT-PCR) (GIULIETTI et al., 2001). This PCR reaction is a powerful and sensitive tool for detecting and quantifying mRNA in small samples and is also used in genomic assays, genetic diagnosis and quantification of gene expression.

A transcriptome is a set of genes transcribed in a cell at a specific stage that can be used to determine the profile of genetic expression. One

Received: 09/12/14
Accepted: 15/04/15

transcriptomic technique is used to determine expressed sequence tags (ESTs). This technique was used to bulk sequence cDNA (complementary DNA) populations in order to generate thousands of ESTs. These short sequences (300-500 bp) are usually sufficient to identify genes by comparison with sequences in public databases (e.g. GenBank, EMBL). cDNA microarray analysis is the most common transcriptomic technique and can be used to simultaneously analyze thousands of genes.

Hybridization experiments are conducted with DNA chip samples. These samples are labeled either radioactively or with suitable fluorophores and quantified by a Phosphorimager or confocal microscopy. In this way, the expression patterns of thousands of genes can be identified simultaneously during development or in response to environmental stimuli. These data can then be analyzed by bioinformatics techniques to associate groups of coordinately expressed genes and provide important information about their function.

The proteome has been defined as the set of proteins expressed by the genome of an organism, or in the case of multicellular organisms, as the protein complement expressed by specific cells or tissue, more specifically, the set of proteins specifically expressed at a given time under defined conditions (WILKINS et al., 1996). Unlike astatic genome, a proteome is complex and dynamic depending on cell cycle, external conditions and system history. Generally, the proteome is modified according to the specific biological and pathophysiological state of the organism.

Despite the importance of genomic and transcriptomic data, only the proteomic level makes it possible to glimpse the biochemical functionality and diversity of the real products of genetic expression (proteins). This diversity exists because a gene can produce multiple distinct proteins from the alternative splicing of primary transcripts, polymorphisms, post-translational modifications and other protein processing mechanisms. Studies on yeast and mammalian cells show that mRNA (messenger RNA) quantity is not directly correlated with protein levels (GYGI et al., 1999; CHEN et al., 2002; ORNTOFT et al., 2002).

Proteomic analysis is usually carried out by combining protein separation and identification techniques. Electrophoresis and chromatography techniques can be used for proteomic separation (Figure 2), whereas mass spectrometry is preferred for identification.

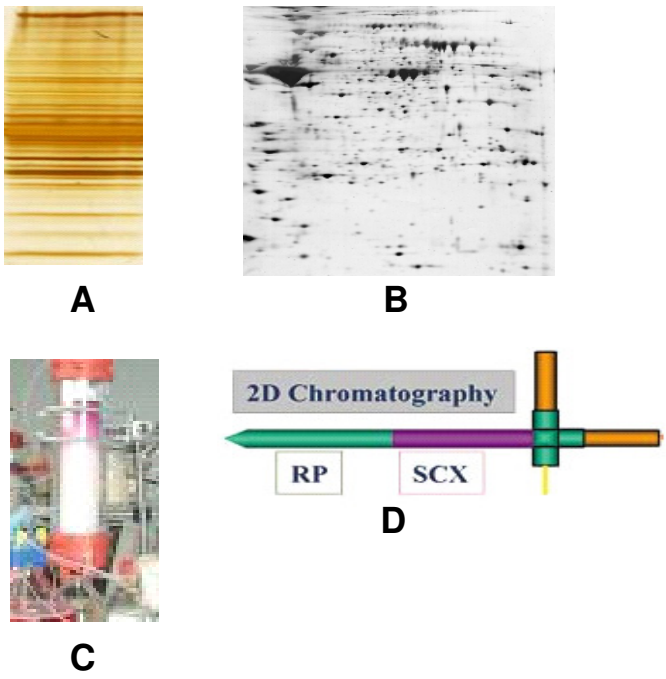

Figure 2. Illustrations of protein separation techniques. A: One-dimensional gel electrophoresis, B: Two-dimensional gel electrophoresis, C: Column of one-dimensional chromatography, D: Scheme of two-dimensional chromatography, RP: Reversed-phase, SCX: Strong cation exchange.

Two-dimensional electrophoresis (2-DE) is a powerful separation technique that allows simultaneous resolution of hundreds or even thousands of proteins under specific conditions. These proteins are separated by two independent characteristics: molecular mass and isoelectric point (O´Farrell et al., 1975). Isoelectric focusing (IEF) is used in the first dimension of 2-DE to separate proteins by charge and SDS-PAGE (sodiumdodecyl sulphate-polyacrylamide gel electrophoresis) is used in the second dimension to orthogonally separate proteins by molecular weight.

The main limitations of 2-DE are the under representation of high molecular weight protein and hydrophobic proteins, the limited ability to detect proteins with few copies per cell (GYGI et al., 2000) and the difficulty to obtain reproducible electrophoretic patterns for proteins with highly alkaline isoelectric points (HOVING et al., 2002). These constraints have been substantially alleviated by pre-fractionating samples (GORG et al., 2002 and LOCKE et al., 2002), decreasing IPG (immobilized pH gradient) concentration (CANDIANO et al., 2002 and BRUSCHI et al., 2003) and decreasing the electroendosmotic effect of alkali gels (HOVING et al., 2002).

To overcome some of the limitations of 2-DE, alternative techniques have been integrated into mass spectrometry as new platforms for proteomic analysis. One promising technique is to use Liquid Chromatography-Mass Spectrometry (LC-MS) for the direct analysis of complex mixtures of peptides obtained by enzymatic digestion of total protein extracts. This allows peptides with similar elution times to be automatically selected, isolated and sequentially fragmented within the spectrometer without overlapping fragmentation patterns. Thus, sequence information for thousands of peptides can be obtained using two-dimensional LC-MS/MS assay (WASHBURN et al., 2001).

DEVELOPMENT

Nano-onions as carbon nanostructures

Concerns about nanotechnologies are related to the fact that at nanoscale, the mechanical, electronic, optical, chemical, biological and other properties of a substance can significantly differ from the properties exhibited at macroscopic and microscopic levels. Furthermore, nanomaterials with identical chemical compositions but different shapes can also have different properties (WIESNER et al., 2006).

Another source of uncertainty is that exposure to dust and toxic substances is measured and expressed as mass per unit volume (typically mg/m^{-3}). However, the effects of nanoparticles (NPs) seem to be more closely related to surface area than to mass (STONE, 2009).

Carbon nanostructures have risen to importance since the discovery of fullerene C60 (KROTO et al., 1985). Investigations in recent years have demonstrated the great capacity for carbon to exist in different allotropic forms with varied nanometric textures, dimensions and fascinating properties.

Carbon nano-onions (CNOs), also known as bucky onions, multi-shell-fullerenes and onion-like fullerenes are the least studied family of carbon allotropes (XU, 2008). They have received more attention recently because new synthesis methods have been developed that can produce sufficient CNOs for study and exploration for potential applications.

Ideal nano-onions consist of a series of concentric graphite levels separated by a 0.34 nm space that contains a nucleus-like C60 molecule. Actual nano-onion structures usually differ from this model by assuming quasi-spherical or polyhedral shapes (UGARTE, 1992). The submerged arc discharge method (SAD) (SANO et al., 2001), with distilled water and graphite electrodes, can be used to produce significant numbers of CNOs with diameters ranging from 3 to 50 nm. Figure 3 shows a CNO example obtained by CEADEN (Centro de Aplicaciones Tecnológicas y Desarrollo Nuclear) using the SAD method.

The Lawrence Berkeley National Laboratory has proposed using CNOs obtained by SAD as a new antitumor therapy. The laboratory used micro-array analysis of gene expression to conclude that EGFR (epidermal grow factor receptor) expression reduced fourfold in skin cells treated with CNO (DING et al., 2005). EFGR is overexpressed in over 20% of human breast cancer cases. This study also showed that nano-onions caused less cell stress than nanotubes. Other studies have shown that functionalized single-wall nanotubes are effective at detecting and treating breast cancer (SHAO et al., 2007; XIAO et al., 2009).

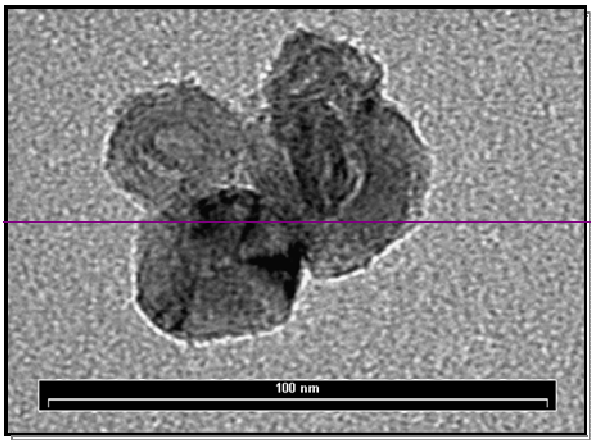

Figure 3. Transmission Electron Microscopy image of nano-onions synthesized in CEADEN using SAD (DARIAS et al., 2011).

Nanomaterial entry into cells depends on size, shape and surface.

Size is one of the most important characteristics of nanomaterials, because with a decrease size the number of particles per unit mass increases. Thus, nanomaterials can cross biological barriers to regulate absorption, distribution, metabolism and excretion. Once inside the body, a NP can enter a cell, interact with biomolecules and destabilize normal cell function. However, this can be an advantage for medicine and for the development of new nanodevices to treat certain diseases (FERRARI, 2005; SAHOO et al., 2007). Size is critical to a nanomaterial's ability to enter a cell. Cell entry can be accomplished through many mechanisms including diffusion through the plasma membrane directly, or through channels. Another mechanism is endocytosis, which is an energy-dependent mechanism that can follow different paths. Nanomaterials of the right size and shape need to interact with membrane receptors that facilitate receptor mediated endocytosis (PATEL et al., 2007). Investigations show that NPs up to 200 nm are usually assimilated by endocytosis. On the other hand, 50 nm particles are more rapidly assimilated than smaller (<14 nm) and larger (up to 500 nm) diameter particles (REJMAN, et al., 2004; CHITHRANI, et al., 2006; CHITHRANI, et al., 2007; GRATTON, et al., 2008), (**Erro! Fonte de referência não encontrada.**).

Nanomaterial shape may also affect the rate of entry into a cell. Spherical nanoparticles enter more quickly than nanotubes, while penetration of the latter is strongly influenced by size (CHITHRANI et al., 2006).

NP size and shape are determined by the surface area associated with a specific mass. Spheres have slightly smaller surface areas than octagonal structures of the same size. Large surface areas increase catalytic activity because surface atoms tend to have unsatisfied high-energy bonds. These bonds rapidly react with other molecules to gain stability. Thus, the greater the surface area of a NP, the higher its reactive potential. In addition, if these nanomaterials can enter cells, there is greater likelihood that the surface area will interact with biomolecules causing cell damage or oxidative stress. Finally, the performance of an NP may have no relationship with their biological impact in a living organism (OSTIGUY et al., 2008).

Could new technologies such as "omics" help identify potential toxicities and supplement current nanomaterial testing?

Without question, "omics" technologies can contribute significantly to the study of nanomaterial toxicology and biomedical applications. Specifically, proteomics could elucidate mechanisms of action and help determine the effects of nanomaterials on protein expression in tissues and organs. Which alterations cause the transduction mechanisms, gene regulation and metabolic pathways? What are the molecular targets of such changes?

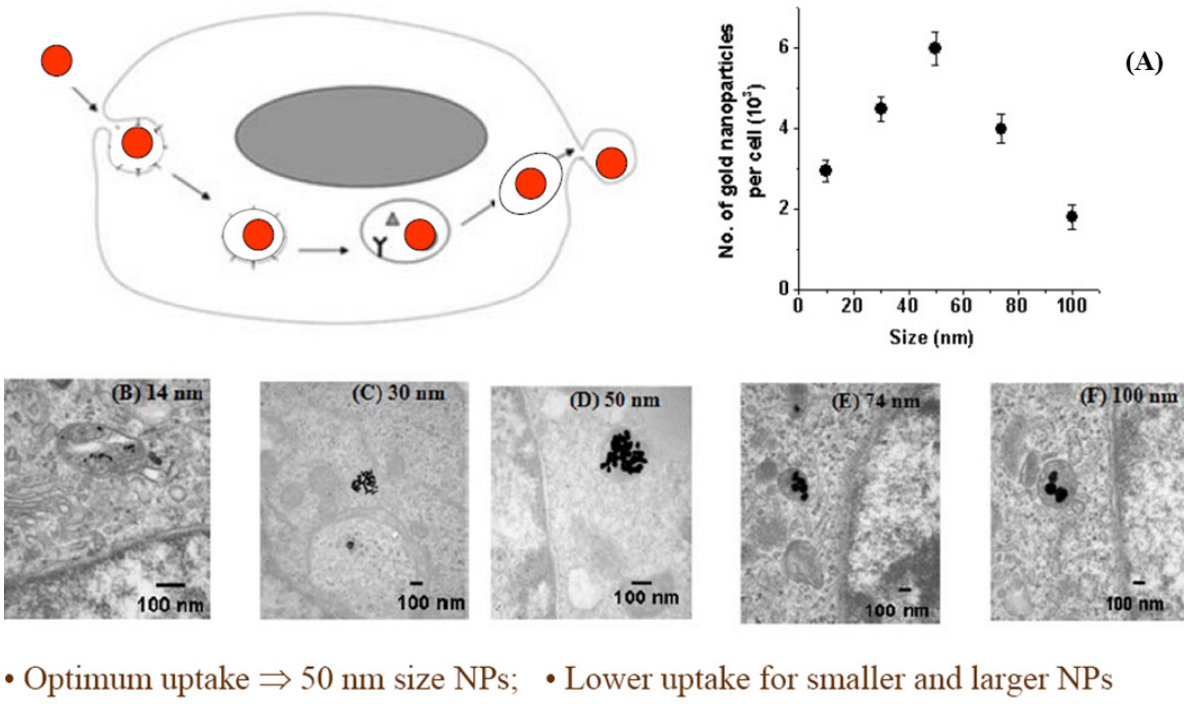

Figure 4. (A) Dependence of cellular uptake of gold nanoparticles as a function of size. (B-F) Transmission electron microscopy images of gold nanoparticles with sizes 14, 30, 50, 74, and 100 nm trapped inside vesicles of a Hela cell, respectively (Chithrani et al., 2006).

"Omic" approaches studies on carbon nanoparticles.

Analyzing gene expression with DNA microarrays shows that multiple cellular pathways were disrupted after exposing skin fibroblast cells to cytotoxic doses of carbon nanomaterials (multiple-wall carbon nano-onions MWCNOs and multiple-wall carbon nanotubes MWCNT) (DING, et al., 2005). These studies showed material-specific toxicogenomic profiles and quantitative and qualitative differences in gene expression profiles for each material and at different doses (6 and 0.6 µg/mL for MWCNO and 0.06 and 0.6 µg/mL for MWCNT). Exposure to both nanomaterials showed different standards of gene expression related to cellular transport, metabolism, cell cycle regulation and stress response. MWCNT presence altered gene expression and led to strong immune and inflammatory response in fibroblast cells of the skin. MWCNOs, on the other hand, altered gene expression related to response from external stimuli. This study demonstrated that MWCNTs seem to cause more cellular stress than MWCNOs. At high doses, MWCNTs caused an innate immune response in cells whereas MWCNOs did not, which also shows that cells react differently to nanomaterial structure.

Other studies using microarrays demonstrated that only 50 genes in human fibroblasts (0.2% of all genes tested) had statistically significant changes in expression (more than 2 fold) when treated with silicon quantum dots coated with polyethylene glycol (PEG-silica-coated Qdots) (ZHANG et al., 2006). Expression changed in genes involved in carbohydrate binding, intracellular vesicle formation and stress response. Surprisingly, PEG-silica-coated Qdots, unlike carbon nanotubes, did not modify gene expression related to strong immune and inflammatory response associated to heavy metal toxicity. Experimental evidence in this study showed that even at high doses, CdSe/ZnS quantum dots induced negligible toxicity in properly protected cells. In summary, this study showed that PEG-silica-coated Qdot shave minimum impact on cells and are a promising alternative to uncoated quantum dots.

Proteomic analysis using 2-DE and mass spectrometry was used to generate a preliminary protein profile of human epidermal keratinocyte cells (HEKS) exposed to multiple-wall carbon nanotubes (MWCNTs) (Witzmann and Monteiro-riviere 2006). The expression of 36 proteins changed after 24 hours of exposure ($P<0.01$) and the expression of 106 proteins changed after 48 hours. In both cases, protein expression decreased significantly (~67%). Peptide mass fingerprinting was used to identify differentially expressed proteins. Many of these proteins showed complex

cellular responses to MWCNT exposure. In addition to proteins associated with metabolism, cellular signaling and stress, a consistent effect was observed in the expression of cytoskeletal elements and components of vesicular traffic. This study showed that MWCNTs are able to alter protein expression in an epithelial target cell, which is the main route for occupational exposure to manufactured nanotubes.

Another study used the proteomic approach and human monoblastic leukemia cells (U937) exposed to black carbon nanoparticles to show changes in protein expression and to explore a potential method of safety assessment (HANIU et al., 2009). Cells exposed to black carbon particles (~85 nm) did not show inhibited cell growth for 96 hours. However, 2-DE analysis with U937 cell lysates showed that exposure to the same black carbon particles significantly modified protein expression in 14 protein spots by at least a factor of two. Only one protein was over-expressed, while expression of the other proteins decreased. Ten of the fourteen spots were identified by PMF:transportin, thioredoxin containing domain 5, annexin A2, otubain 1,14-3-3 proteins, etc. The functions of these proteins were associated with metabolism, stress response, signal transduction and cell differentiation. According to this study, black carbon unquestionably caused biological responses that were undetectable by conventional methods. Regarding safety risks, the proteomic approach can detect biological response with greater sensitivity than conventional methods of *in vitro* evaluation.

A proteomic analysis by the same author evaluated the biological response of U937 cells exposed to different types of multiple-wall nanotubes such as raw and purified MWCNTs. These MWCNTs were heat treated at 1800 ºC (HTT1800) and 2800 ºC (HTT2800) (HANIU et al., 2010). Cellular proliferation was strongly inhibited by the unpurified MWCNTs but not by HTT2800. Nevertheless, both unpurified MWCNT and HTT1800, which included some impurities, were cytotoxic. This study showed that 37 proteins had significantly different expression ($p < 0,05$) in 2-DE gels after exposure to HTT1800 with low iron levels, and 20 proteins after exposure to HTT2800 (Figure 4). PMF identified 45 proteins including the heat-shock-1 beta protein, neutral alpha-glucosidase AB and the DNA repair protein MSH2. Modified expression was seen in proteins that have important roles in metabolism, biosynthesis, stress response and cell differentiation. While HTT2800 did not inhibit cell proliferation or produce *in vitro* cytotoxicity, the expression of some proteins involved in stress response was modified. On the other hand, DJ-1, a marker of Parkinson's disease also associated with cancer, was identified after exposure to both MWCNTs. These results demonstrated that MWCNT cytotoxicity is dependent on impurities such as iron and that MWCNTs cause some direct and/or indirect biological responses, *in vitro*.

Other studies demonstrated that exposing HaCaT cells to SiO_2 NPs caused cytotoxicity and modified protein expression (YANG et al., 2010). Proteomic analysis showed that 16 proteins were differentially expressed due to SiO_2 exposure and that the expression levels of these differentially expressed proteins were associated with particle size. These 16 proteins were identified by MALDI-TOF-TOF (matrix assisted laser desorption/ionization mass spectrometry) and were classified into five categories according to function: proteins related to oxidative stress, proteins associated with the cytoskeleton, chaperone proteins, proteins involved in energy metabolism and proteins related to apoptosis and tumors.

More recent results demonstrated that MWCNT induces cytotoxicity in A549 cells only at relatively high concentrations and longer exposure time (JU et al., 2014). Within a relatively low dosage range (30 µg/mL) and short time period (24 h), MWCNT treatment does not induce significant cytotoxicity, cell cycle changes, apoptosis, or DNA damage. However, at these low doses and times, MWCNT treatment causes significant changes in protein expression. A total of 106 proteins show altered expression at various time points and dosages, and of these, 52 proteins were further identified by MS. Identified proteins are involved in several cellular processes including proliferation, stress, and cellular skeleton organization. In particular, MWCNT treatment causes increases in actin expression. This increase has the potential to contribute to increased migration capacity and may be mediated by reactive oxygen species (ROS).

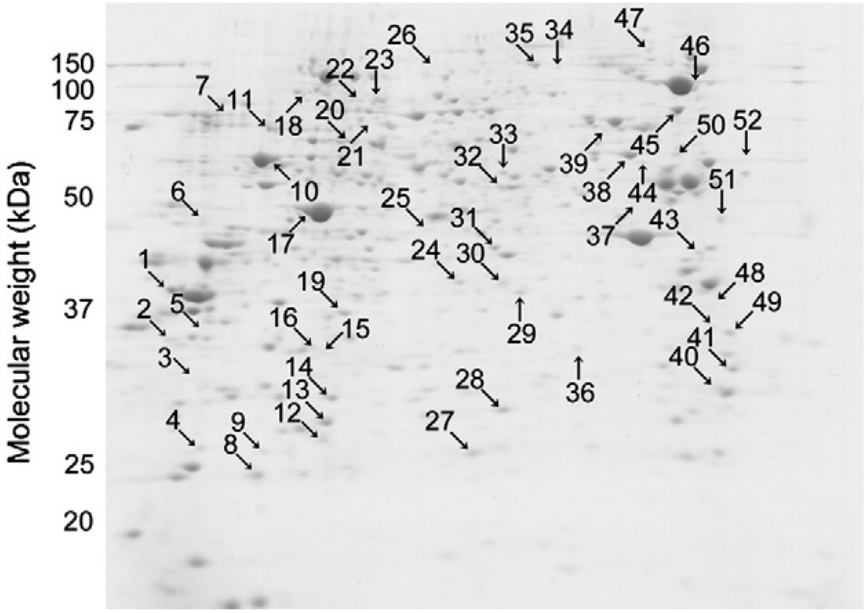

Figure 4. Representative 2-DE gels (pI 9-4.5) of total protein extracts from cell treated with HTT1800, HTT2800 or CB compared to control cells ($n = 4$). Numbered spots were changed more than two-fold with statistically significant differences ($p < 0.05$). (HANIU et al., 2011).

In summary, "omics" technologies promise powerful and complementary tools for the study of nanoparticle toxicity. The sensitivity of many of these techniques makes it possible to elucidate cellular actions and reactions to different nanomaterials and thus facilitate the study of the benefits and harms caused by the interaction of these nanoparticles with the human body.

ACKNOWLEDGEMENTS

We are grateful to the institutions and development agencies that support our work (project NAP-1-11-AENTA CITMA). FSE is grant recipient of Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq and Fundação de Amparo a Pesquisa do Estado de Minas Gerais – FAPEMIG

RESUMO: As nanopartículas têm sido exploradas em aplicações biomédicas para fins diagnósticos e terapêuticos. No entanto, a biocompatibilidade, a toxicidade e a capacidade de entrada na célula são fatores críticos que irão determinar a utilidade de nanopartículas com aplicações clínicas. As chamadas tecnologias "ômicas" podem fornecer poderosas ferramentas sensíveis e complementares para o estudo de toxicologia molecular e para aplicações biomédicas dos nanomateriais. Especificamente a proteômica poderia elucidar mecanismos de ação e contribuir no estudo do efeito de um nanomaterial sobre a expressão de proteínas ao nível de tecidos e órgãos, alterações em vias metabólicas, mecanismos de transdução e de regulação de genes assim como os alvos moleculares da terapêutica. O objetivo desta revisão é apresentar uma breve abordagem sobre as tecnologias "ômicas" aplicadas ao estudo do efeito dos nanomateriais de carbono nas células.

PALAVRAS – CHAVE: Nanopartículas. Nanotubos. Nano-onions. Proteômica e Expressão gênica.

REFERENCES

BRUSCHI, M.; MUSANTE, L.; CANDIANO, G.; GHIGGERI ,G. M.; HERBERT, B.; ANTONUCCI, F.; RIGHETTI, P. G. Soft immobilized pH gradient gels in proteome analysis: a follow-up. **Proteomics,** v. 3, p. 821-825, 2003. http://dx.doi.org/10.1002/pmic.200300361

CANDIANO, G.; MUSANTE, L.; BRUSCHI, M.; GHIGGERI, G. M.; HERBERT, B.; ANTONUCCI, F.; RIGHETTI, P. G. Two-dimensional maps in soft immobilized pH gradient gels: a new approach to the proteome of the Third Millennium. **Electrophoresis,** v. 23, p. 292-297, 2002. http://dx.doi.org/10.1002/1522-2683(200202)23:2<292::AID-ELPS292>3.0.CO;2-5

CHEN, G.; GHARIB, T. G.; HUANG,C. C.; TAYLOR,J.M.; MISEK, D.E.; KARDIA, S.L.; GIORDANO, T.J.; IANNETTONI, M.D.; ORRINGER, M.B.; HANASH, S.M.; Hanash, S.M; Beer, D.G. Discordant protein and mRNA expression in lung adenocarcinomas. **Molecular and Cellular Proteomics,** v.1, p. 304-313, 2002. http://dx.doi.org/10.1074/mcp.M200008-MCP200

CHITHRANI, B. D.; GHAZANI, A. A.; CHAN, W. C. Determining the size and shape dependence of gold nanoparticle uptake into mammalian cells. **Nano Letters,** v. 6, p. 662–668, 2006. http://dx.doi.org/10.1021/nl052396o

CHITHRANI, B. D.; CHAN, W. C. Elucidating the mechanism of cellular uptake and removal of protein-coated gold nanoparticles of different sizes and shapes. **Nano Letters,** v. 7, p. 1542–1550, 2007. http://dx.doi.org/10.1021/nl070363y

DARIAS, J.;CARRILLO, E.; CASTILLO, R.; ARTECHE, J.; HERNANDEZ, L.;RAMOS, M.; DESDÍN, L. Sistema de descarga de arco sumergida para la síntesis de nanoonions de carbono multicapas. **Revista Cubana de Física**, v. 28, p. 80-84, 2011.

DING, L. H.; STILWELL, J.; ZHANG, T. T.; ELBOUDWAREJ, O.; JIANG, H. J.; SELEGUE, J. P.; COOKE, P. A.; GRAY, J. W.; CHEN, F. F. Molecular characterization of the cytotoxic mechanism of multiwall carbon nanotubes and nano-onions on human skin fibroblast. **Nano Letters,** v. 5, p. 2448-2464, 2005. http://dx.doi.org/10.1021/nl051748o

FERNYHOUGH, P. Quantification of mRNA levels using Northern blotting. **Methods in Molecular Biology**, v. 168, p. 53-63, 2001.

FERRARI, M. Cancer nanotechnology: opportunities and challenges. **Nature Reviews Cancer**, v. 5, p. 161–71, 2005. http://dx.doi.org/10.1038/nrc1566

GIULIETTI, A.; OVERBERGH, L.; VALCKX, D.; DECALLONNE, B.; BOUILLON, R.; MATHIEU, C. An overview of real time quantitative PCR; applications to quatify cytokine gene expression. **Methods,** v. 25, p. 386-401, 2001. http://dx.doi.org/10.1006/meth.2001.1261

GORG, A.; BOGUTH, G.; KOPF, A.; REIL, G.; PARLAR, H.; WEISS, W. Sample prefractionation with Sephadex isoelectric focusing prior to narrow pH range two-dimensional gels. **Proteomics,** v. 2, p. 1652-1657, 2002. http://dx.doi.org/10.1002/1615-9861(200212)2:12<1652::AID-PROT1652>3.0.CO;2-3

GRATTON, S. E.; ROPP, P. A.; POHLHAUS, P. D.; LUFT, J. C.; MADDEN, V. J.; NAPIER, M. E.; DESIMONE, J. M. The effect of particle design on cellular internalization pathways. **Proceedings of the National Academy of Sciences USA,** v. 105, p. 11613–11618, 2008. http://dx.doi.org/10.1073/pnas.0801763105

GYGI, S. P.; ROCHON, Y.; FRANZA, B.R.; AEBERSOLD, R. Correlation between protein and mRNA abundance in yeast. **Molecular Cellular Biology**, v.19, p.1720-1730, 1999. http://dx.doi.org/10.1073/pnas.160270797
GYGI, S. P.; CORTHALS, G. L.; ZHANG, Y.; ROCHON, Y.; AEBERSOLD, R. Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. **Proceedings of the National Academy of Sciences USA,** v. 97, p. 9390-9395, 2000.

HANIU, H.; MATSUDA,Y.; TAKEUCHI, K. Potential of a Novel Safety Evaluation of Nanomaterials Using a Proteomic Approach. **Journal of Health Science,** v. 55, p. 428-434, 2009. http://dx.doi.org/10.1248/jhs.55.428

HANIU, H.; MATSUDA, Y.; TAKEUCHI, K.; KIM, Y. A.; HAYASHI, T.; ENDO, M. Proteomics-based safety evaluation of multi-walled carbon nanotubes. **Toxicology and Applied Pharmacology**, v. 1, p. 256-262, 2010. http://dx.doi.org/10.1016/j.taap.2009.10.015

HANIU, H.; MATSUDA, Y.; USUI, Y.; AOKI, K.; SHIMIZU, M.; OGIHARA, N.; HARA, K.; OKAMOTO, M.;TAKANASHI, S.; ISHIGAKI, N.; NAKAMURA, K.; KATO, H.; SAITO, N. Toxicoproteomic evaluation of carbon nanomaterials in vitro. **J. Proteomics,** v. 74, p. 2703-2712, 2011. doi: 10.1016/j.jprot.2011.03.004.

HOVING, S.; GERRITS, B.; VOSHOL, H.; MULLER, D.; ROBERTS, R. C.; VAN OOSTRUM, J. Preparative two-dimensional gel electrophoresis at alkaline pH using narrow range immobilized pH gradients. **Proteomics,** v. 2, p.127-134, 2002. http://dx.doi.org/10.1002/1615-9861(200202)2:2<127::AID-PROT127>3.0.CO;2-Y

JU, L.; ZHANG, G.; ZHANG, X.; JIA, Z.; GAO, X. JIANG, Y.; YAN, CH.;DUERKSEN-HUGHES,P. J.; CHEN, F. F.; LI,H.; ZHU, X.;YANG, J. Proteomic Analysis of Cellular Response Induced by Multi-Walled Carbon Nanotubes Exposure in A549 Cells. **PLoS ONE.,** v. 9, p. 1, 2014. e84974. doi:10.1371/journal.pone.0084974

KROTO, H.W.; HEATH, J. R.; O'BRIEN, S. C.; CURL, R. F. AND SMALLEY, R. E. C 60: buckminsterfullerene. **Nature,** v. 318, p. 162, 1985. http://dx.doi.org/10.1038/318162a0

LOCKE, V. L.; GIBSON, T.S.; THOMAS, T.M.; CORTHALS, G. L.; RYLATT, D. B. Gradiflow as a prefractionation tool for two-dimensional electrophoresis. **Proteomics,** v. 2, p. 1254-1560, 2002. http://dx.doi.org/10.1002/1615-9861(200209)2:9<1254::AID-PROT1254>3.0.CO;2-A

O´FARRELL, P. H. High resolution two-dimensional electrophoresis of proteins. **Journal of Biological Chemistry,** v. 250, p. 4007-4021, 1975.

ORNTOFT, T.F.; THYKJAER, T.; WALDMAN, F.M.; WOLF, H.; CELIS, J.E. Genome-wide study of gene copy numbers, transcripts, and protein levels in pairs of non-invasive and invasive human transitional cell carcinomas. **Molecular and Cellular Proteomics,** v.1, p. 37-45, 2002. http://dx.doi.org/10.1074/mcp.M100019-MCP200

OSTIGUY, C.; SOUCY, B.; LAPOINTE, G.; WOODS, C.; MÉNARD, L.; TROTTIER, M. **Health Effects of Nanoparticles – IRSST.** Second Edition 2008.

PATEL, L.N.; ZARO, J. L.; SHEN, W. C. Cell penetrating peptides: intracellular pathways and pharmaceutical perspectives. **Pharmaceutical Research,** v. 24, p. 1977–1992, 2007. http://dx.doi.org/10.1007/s11095-007-9303-7

WIESNER, M.R.; LOWRY, G.V.; ALVAREZ, P.J.; DIONYSIOU, D.; BISWAS, P. Assessing the risk of manufactured nanomaterials. **Environmental Science and Technology**, V.40, p.4336- 4345, 2006. http://dx.doi.org/10.1021/es062726m

REJMAN, J.; OBERLE, V.; ZUHORN, I.S.; HOEKSTRA, D. Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. **Biochemical Journal,** v. 377, p. 159–169, 2004. http://dx.doi.org/10.1042/BJ20031253

SAHOO, S.K.; PARVEEN, S.; PANDA, J. J. The present and future of nanotechnology in human health care. **Nanomedicine,** v. 3, p. 20–31, 2007. http://dx.doi.org/10.1016/j.nano.2006.11.008

SANO, N.; WANG, H.; CHHOWALLA, M.; ALEXANDROU, I.; AMARATUNGA, G. A.J. Synthesis of carbon "onions'" in water. **Nature,** v. 414, p.506, 2001. http://dx.doi.org/10.1038/35107141

SHAO, N.; LU, S.; WICKSTROM, E.; PANCHAPAKESAN, B. Integrated molecular targeting of IGF1R and HER2 surface receptors and destruction of breast cancer cells using single wall carbon nanotubes. **Nanotechnology,** v. 18, p. 315101-315109, 2007. http://dx.doi.org/10.1088/0957-4484/18/31/315101

SOUTHERN, E. M. Detection of specific sequences among DNA fragments separated by gel electrophoresis. **Journal of Molecular Biology,** v. 98, p. 503-17, 1975. http://dx.doi.org/10.1016/S0022-2836(75)80083-0

STONE, V. **Engineered nanoparticles: Review of health and Environmental safety**. http://nmi.jrc.ec.europa. /Project/ENRHES.htm, 2009.

UGARTE, D. Curling and closure of graphitic networks under electron-beam irradiation. **Nature,** v. 359, p. 707, 1992. http://dx.doi.org/10.1038/359707a0

WASHBURN, M. P.; WOLTERS, D.; AND YATES, J. R., III**. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nature Biotechnology,** v. 19, p. 242-247, 2001. http://dx.doi.org/10.1038/85686

WILKINS, M. R.; PASQUALI, C.; APPEL R.D.; OU, K.; GOLAZ, O.; SANCHEZ, J.C.; YAN, J.X.; GOOLEY, A.A.; HUGHES, G.; HUMPHERY-SMITH, I.; WILLIAMS, K.L.; HOCHSTRASSER, D.F. From proteins to proteomes: large scale protein identification by two-dimensional gel eletrophoresis and amino acid analysis. **Biotechnology,** v.14, p. 61-65, 1996. http://dx.doi.org/10.1038/nbt0196-61

WITZMANN, F.A., MONTEIRO-RIVIERE, N.A. Multi-walled carbon nanotube exposure alters protein expression in human keratinocytes. **Nanomedicine,** v. 2, p. 158-168, 2006. http://dx.doi.org/10.1016/j.nano.2006.07.005

XIAO, Y.; GAO, X.; TARATULA, O.; TREADO, S.; URBAS, A.; HOLBROOK, R. D.; CAVICCHI, R. E.; AVEDISIAN, C. T.; MITRA, S.; SAVLA, R.; WAGNER, P. D.; SRIVASTAVA, S.; HE, H. Anti-HER2 IgY antibody-functionalized single-walled carbon nanotubes for detection and selective destruction of breast cancer cells. **BMC Cancer,** v. 2, p. 351, 2009. http://dx.doi.org/10.1186/1471-2407-9-351

XU, B. Prospects and research progress in nano onion-like fullerenes. **New Carbon Materials,** v. 23, p. 289, 2008. http://dx.doi.org/10.1016/S1872-5805(09)60001-9

YANG, X.; LIU, J.; HE, H.; ZHOU, L.; GONG, C.; WANG, X.; YANG, L.; YUAN, J.; HUANG, H.; HE, L.; ZHANG, B.; ZHUANG, Z. SiO2 nanoparticles induce cytotoxicity and protein expression alteration in HaCaT cells. **Particle and Fibre Toxicology,** v. 19, p. 1, 2010. http://dx.doi.org/10.1186/1743-8977-7-1

ZHANG, T. T.; STILWELL, J. L.; GERION, D.; DING, L. H.; ELBOUDWAREJ, O.; COOKE, P. A.; GRAY, J. W.; ALIVISATOS, P. A.; CHEN, F. F . Cellular effect of high doses of silica-coated quantum dot profiled with high throughput gene expression analysis and high content cellomics measurements. **Nano Letters,** v. 6, p. 800-808, 2006. http://dx.doi.org/10.1021/nl0603350