ANNEXURE I

BUFFERS AND REAGNETS

Phosphate Buffered Saline (PBS)

NaCl - 136mM

KCL - 2.6mM

Na_2HPO_4 - 10mM

KH2PO4 - 1.76mM

Trypsin EDTA

Trypsin - 0.3%

Glucose - 0.03% in PBS EDTA

MTT (Methyl Thiazole Tetrazolium)Reagent

Stock - 5mg/ml (in PBS)

MTT solvent

Triton - 10%

HCl - 0.1 N

Isopropanol

Trypan Blue

Trypan blue- 0.4% in PBS

Cell Fixative

Gluteraldehyde - 4 %

Paraformaldehyde - 2 %

Osmiumtetroxide - 1%

Normal Melting point Agarose (NMA)

Normal Melting point Agarose – 0.75% in distilled water

Low melting point agarose (LMPA)

Low Melting point Agarose – 0.75% in PBS

Lysing solution

NaCl -2.5 M

EDTA -100 mM

Trizma base -10 mM

Electrophoresis buffer

NaOH -300 mM

EDTA -1 mM

Neutralisation buffer

Tris -0.4M

Propidium iodide

Propidium iodide -10 μg/ml in distilled water

CURRICULUM VITAE

07/2007 - present PhD Scholar
Amrita Centre for Nanosciences and Molecular Medicine,
Amrita Institute of Medical Sciences and Research Centre,
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10/2006 – 07/2007 Junior Research Fellow
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AWARDS AND HONORS

The article entitled "Development of novel chitin/nanosilver composite scaffolds for wound dressing applications" has been selected as the **'Best paper of 2010'** in the Journal of Material Science: Materials in Medicine.

LIST OF PUBLICATIONS

Publications arising from the thesis

1. **Sasidharan A**, Panchakarla LS, Sadanandan AR, Ashokan A, Chandran P, Girish CM, Menon D, Nair SV, Rao CN, Koyakutty M. Hemocompatibility and macrophage response of pristine and functionalized graphene. Small, 8, 1251–1263, 2012. **[IF- 8.33].**
2. **Sasidharan A**, Chandran P, Menon D, Raman S, Koyakutty M, Nair S. Rapid dissolution of ZnO nanocrystals in acidic cancer microenvironment leading to preferential apoptosis. Nanoscale, 3, 3657-3669, 2011. **[IF- 5.91]**
3. **Sasidharan A**, Panchakarla LS, Chandran P, Menon D, Nair S, Rao CN, Koyakutty M. Differential nano-bio interactions and toxicity effects of pristine versus functionalized graphene. Nanoscale, 3, 2461-2464, 2011. **[IF- 5.91]**
4. Nair S, **Sasidharan A**, Divya Rani VV, Menon D, Nair S, Manzoor K, Raina S. Role of size scale of ZnO nanoparticles and microparticles on toxicity toward bacteria and osteoblast cancer cells. J Mater Sci Mater Med. Suppl 1:S235-41, 2009. **[IF- 2.325]**
5. **Sasidharan A**, Siddharth S, Panchakarla LS, Menon D, Nair S, Rao CN, Koyakutty M. Genotoxicity of pristine and functionalized graphene in human primary cells. Small (To be submitted).
6. **Sasidharan A**, Panchakarla LS, Chandran P, Menon D, Nair S, Rao CN, Koyakutty M. Cellular and Molecular Characterization of Potential Cytotoxic Mechanisms of Pristine and Carboxyl Functionalized Graphene towards Human Primary cells. ACS Nano (To be submitted).
7. **Sasidharan A**, Chaitanya K, Panchakarla LS, Menon D, Nair S, Rao CN, Koyakutty M. In vivo toxicology, organ bio distribution and histological impact of pristine, functionalized and PEGylated graphene in Swiss albino mice. ACS Nano (To be submitted).

Book Chapter

Manzoor Koyakutty, **Abhilash Sasidharan**, Shantikumar Nair. Biomedical Applications of Graphene: Opportunities and Challenges. Edited by CNR Rao. Wiley intersciences. **(In Press).**

Other Publications

1. Chandran P, **Sasidharan A**, Ashokan A, Menon D, Nair S, Koyakutty M. Highly biocompatible TiO_2:Gd^{3+} nano-contrast agent with enhanced longitudinal relaxivity for targeted cancer imaging. Nanoscale. 10,4150-61. 2011. **[IF- 5.91]**
2. Madhumathi K, Sudheesh Kumar P.T, **Abhilash S**, Sreeja V, Tamura H, Manzoor K, Nair S.V, Jayakumar R. Development of novel chitin/nanosilver composite scaffolds for wound dressing applications. J Mater Sci Mater Med. 2,807-13. 2010. **[IF- 2.325]**
3. P. T. Sudheesh Kumar, **S.Abhilash**, K. Manzoor, S. V. Nair, H. Tamura & R. Jayakumar. Preparation and Characterization of Novel βChitin/Nano Silver Composite Scaffolds for Wound Dressing Applications. Carbohydrate Polymers, 80, 761-767, 2010. **[IF- 3.463]**
4. Seema Nair, Sam Peter, **Abhilash Sasidharan**, Sujatha Sistla, AKK Unni. Incidence of mycotic infections in diabetic foot tissue. Journal of culture collections. 5, 85-87. 2007.
5. Seema Nair P, Saji.U, **Abhilash. S**, Hypocholesterolemic Activity of Lactobacillus Acidophilus Strains and its Mechanism. Adv Bio Tech, 11,10, 14-18, 2012.

Patent

R.Jayakumar, K. Manzoor, Shantikumar V. Nair. Vinod K. Lakshmanan, P.T. Sudheesh kumar, **S. Abhilash**. The Art, Method, Manner, Process and system of chitosan/hydrogel nano zinc oxide membranes for wound dressing applications, Application No.1025/CHE/2010 A, Filed on 2010-04-12, Publication date 2010-11-05.

CONFERENCE PRESENTATIONS

ORAL PRESENTATION

- **ICMAT 2011**− Suntec, Singapore. **2011**
  Preferential ZnO nanotoxicity against tumor cells.
- **ICMAT 2011**− Suntec, Singapore. **2011**
  Cytotoxicity, Genotoxicity and Inflammatory response of pristine and carboxyl functionalized graphene towards human primary cells.
- **COCHIN NANO 2011**− Kochi, India. **2011**
  In vivo Toxicology, Tissue Biodistribution, and Long Term Fate of Pristine and Functionalized Graphene in Mice.
- **IUMRS-ICAM** − Bangalore, India. **2007**
  Role of ZnO nanoparticles of controlled size and shape on cytotoxicity and antimicrobial activity.
- **NS&NT'07** − Hyderabad, India. **2007**
  Impact of Zinc Oxide and Hydroxyapatite Nanoparticles on cytotoxicity and cellular adhesion on Microbes.

POSTER PRESENTATIONS

- **NANOBIO 2012** − Kochi, India. **2012**
  In vivo toxicology, organ biodistribution and histological impact of pristine and functionalized graphene in Swiss albino mice.

- **ICONSAT 2012** − Hyderabad, India. **2012**
  In vitro and in vivo toxicology, tissue biodistribution, and long-term fate of graphene in human primary cells and mice models.

- **4th Bangalore Nano** − Bangalore, India. **2011**
  Blood compatibility and macrophage response of pristine and functionalized graphene

- **ICONSAT 2010** − Mumbai, India. **2010**
  Nanotoxicology evaluation of Graphene in human primary cells and cancer cells.

- **NANOBIO 2009** − Kochi, India. **2009**
  Rapid dissolution of ZnO nanocrystals in acidic cancer microenvironment leading to preferential apoptosis

- **COCHIN NANO-2009** − Kochi, India. **2009**
  ZnO nanocrystals induce preferential mitochondrial apoptosis in cancer cells.

WORKSHOP ATTENDED

- **"Applications of Flow Cytometry in Nanomaterial Toxicology"** held at IITRC, Lucknow on January 18-22, 2010, India.

- **"Cochin Nano-2011"** held at Cochin on August 14-17, 2011, Cochin, Kerala, India.

- **"Research Methodology"** held at Amrita Institute of Medical Sciences and Research Centre, on 22nd January 2006 Cochin, Kerala, India.

small
NANO MICRO

Hemocompatibility and Macrophage Response of Pristine and Functionalized Graphene

Abhilash Sasidharan, Leela S. Panchakarla, Aparna R. Sadanandan, Anusha Ashokan, Parwathy Chandran, Chundayil Madathil Girish, Deepthy Menon, Shantikumar V. Nair, C. N. R. Rao, and Manzoor Koyakutty**

Graphene and its derivatives are being proposed for several important biomedical applications including drug delivery, gene delivery, contrast imaging, and anticancer therapy. Most of these applications demand intravenous injection of graphene and hence evaluation of its hemocompatibility is an essential prerequisite. Herein, both pristine and functionalized graphene are extensively characterized for their interactions with murine macrophage RAW 264.7 cells and human primary blood components. Detailed analyses of the potential uptake by macrophages, effects on its metabolic activity, membrane integrity, induction of reactive oxygen stress, hemolysis, platelet activation, platelet aggregation, coagulation cascade, cytokine induction, immune cell activation, and immune cell suppression are performed using optimized protocols for nanotoxicity evaluation. Electron microscopy, confocal Raman spectral mapping, and confocal fluorescence imaging studies show active interaction of both the graphene systems with macrophage cells, and the reactive oxygen species mediated toxicity effects of hydrophobic pristine samples are significantly reduced by surface functionalization. In the case of hemocompatibility, both types of graphene show excellent compatibility with red blood cells, platelets, and plasma coagulation pathways, and minimal alteration in the cytokine expression by human peripheral blood mononuclear cells. Further, both samples do not cause any premature immune cell activation or suppression up to a relatively high concentration of 75 g mL^{-1} after 72 h of incubation under in vitro conditions. This study clearly suggests that the observed toxicity effects of pristine graphene towards macrophage cells can be easily averted by surface functionalization and both the systems show excellent hemocompatibility.

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1. Introduction

Graphene is a single-atom-thick two-dimensional (2D) allotrope of carbon with fascinating physicochemical properties that have invoked enormous interest in the scientific community since its discovery in 2004.[1] Graphene exhibits remarkable mechanical strength, high thermal conductivity, and amazing electronic properties leading to a wide range of industrial applications.[2] In addition, graphene and graphene oxide (GO) were also proposed for certain interesting biomedical applications such as drug delivery, gene transfection, biosensing, diagnostics, tissue engineering, and antibacterial properties.[3–8] More recently, Yang et al.[9] have reported successful testing of graphene for in vivo photothermal therapy of cancer using mice models. Graphene is a nonbiodegradable

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COMMUNICATION

Differential nano-bio interactions and toxicity effects of pristine *versus* functionalized graphene†

Abhilash Sasidharan,[a] L. S. Panchakarla,[b] Parwathy Chandran,[a] Deepthy Menon,[a] Shantikumar Nair,*[a] C. N. R. Rao[b] and Manzoor Koyakutty*[a]

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We report the effect of carboxyl functionalization of graphene in pacifying its strong hydrophobic interaction with cells and associated toxic effects. Pristine graphene was found to accumulate on the cell membrane causing high oxidative stress leading to apoptosis, whereas carboxyl functionalized hydrophilic graphene was internalized by the cells without causing any toxicity.

Graphene, the thinnest two-dimensional (2D) allotrope of carbon, is an exciting nanomaterial that has attracted tremendous attention due to its unique physico-chemical properties and diverse potential applications.[1] Graphene is expected to revolutionize the technological advances in electronics, ultrafast computing, solar-energy harvesting, energy-storage and light-weight displays.[2,3] Recently graphene has also been proposed for bio-medical applications such as drug delivery and anti-cancer therapy.[4–8] However, the actual application of any new nanomaterial in biology and medicine is decided critically by its biocompatibility.[9] Amongst the varieties of carbon nanostructures investigated, the *in vitro* and *in vivo* toxicity profiles and biological applications of carbon nanotubes (CNTs) have been explored in great detail.[10–12] It was found that, in addition to the nano-scale features such as size-scale, shape (fibrilous nature), or structure (single- or multi-walled), a critical aspect that significantly influenced the nano-bio interactions and toxicity of CNTs was its dispersion characteristics in physiological medium, which is determined by its surface-chemistry.[13] It is well known that pristine CNTs are highly hydrophobic, whereas surface functionalization (carboxylated, aminated or PEGylated) renders hydrophilicity and dispersibility in aqueous phase, enabling varied interactions with biological systems.[14,15] For example, pristine CNTs were reported to impart serious adverse toxicity effects including reactive oxygen stress, inflammation, immune response and cytotoxicity due to their strong hydrophobic interactions, whereas, functionalized CNTs (*f*-CNTs) showed much less toxicity even to the stem cell population.[16] It was also revealed that despite significant differences in surface chemistry, once water dispersed, *f*-CNTs could enter any type of cells (prokaryotic/eukaryotic, normal/cancer), even under endocytosis inhibited conditions and thereafter permeate through different intracellular barriers to accumulate in the perinuclear region.[17] In effect, these studies imply that surface functionalization of carbon nanostructures has a significant impact on their nano-bio interactions and possible toxicity effects. However, in the case of graphene, the effect of surface functionalization on its interaction with biological systems is not studied so far. Herein, we report very unique interactions of highly hydrophobic, pristine graphene (*p*-G) and carboxyl functionalized, hydrophilic graphene (*f*-G) with monkey renal cells and their differential toxicity effects, which hold great relevance considering the biomedical applications of graphene.

The two main questions probed in this study include: (i) how do pristine and functionalized graphene interact with eukaryotic cells? (ii) Whether the interactions with *p*-G and *f*-G alter the cellular functions or viability and if so, are they markedly different? Based on the report that functionalization of CNT leads to enhanced accumulation in kidney with subsequent renal clearance,[18] we selected monkey kidney cells, Vero, for probing the above questions. In addition, this study also throws light into the renal toxicity of pristine *versus* functionalized graphene.

Considering that nanotoxicity of CNT was greatly influenced by the residual impurities in the starting materials,[19,20] we have carefully selected highly pure graphite oxide (Alfa Aesar—99.99%) which was subjected to thermal exfoliation, wherein graphite oxide is converted into bilayer graphene and functional groups are converted to O_2, CO, CO_2, *etc.*[21] This as prepared sample is termed as pristine graphene (*p*-G). Carboxyl functionalization of this *p*-G was done by mild acid treatment, resulting in the addition of COOH groups at the surface, which is referred to as functionalized graphene (*f*-G). The samples were characterized using Transmission Electron Microscopy (Fig. 1a) and Atomic Force Microscopy (Fig. 1b), revealing a bi-layered structure with an average thickness of ~0.8 nm. The Raman spectra (inset of Fig. 1b) revealed a characteristic G-band of pure graphene at 1570 cm^{-1} and a band at 2640 cm^{-1}, confirming the phase purity of samples. The most remarkable effect of carboxyl functionalization was seen in the wettability/contact angle measurements where ~0.5 μm thick circular disks of *p*-G and *f*-G were treated with

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† Electronic supplementary information (ESI) available: Experimental details, video files of contact angle measurement, cellular uptake and flow cytogram of ROS. See DOI: 10.1039/c1nr10172b

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PAPER

Rapid dissolution of ZnO nanocrystals in acidic cancer microenvironment leading to preferential apoptosis†

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The microenvironment of cancer plays a very critical role in the survival, proliferation and drug resistance of solid tumors. Here, we report an interesting, acidic cancer microenvironment-mediated dissolution-induced preferential toxicity of ZnO nanocrystals (NCs) against cancer cells while leaving primary cells unaffected. Irrespective of the size-scale (5 and 200 nm) and surface chemistry differences (silica, starch or polyethylene glycol coating), ZnO NCs exhibited multiple stress mechanisms against cancer cell lines (IC_{50} ~150 μM) while normal human primary cells (human dermal fibroblast, lymphocytes, human umbilical vein endothelial cells) remain less affected. Flow cytometry and confocal microscopy studies revealed that ZnO NCs undergo rapid preferential dissolution in acidic (pH ~5–6) cancer microenvironment causing elevated ROS stress, mitochondrial superoxide formation, depolarization of mitochondrial membrane, and cell cycle arrest at S/G2 phase leading to apoptosis. In effect, by elucidating the unique toxicity mechanism of ZnO NCs, we show that ZnO NCs can destabilize cancer cells by utilizing its own hostile acidic microenvironment, which is otherwise critical for its survival.

Introduction

Despite decades of research and development, cancer remains to be a leading cause of human death today.[1] While conventional chemotherapeutic agents interfere with DNA synthesis in cancer cells, recent molecules target aberrant cellular functions involving epigenetics, genetics or protein kinase signalling.[2] However, it is now well known that, in addition to altered intracellular functions, cancer cells are able to create a hostile, acidic and hypoxic microenvironment which plays a critical role in providing extraordinary capacity for cancer to survive, proliferate, invade, metastasize and acquire drug resistance.[3,4] Elevated lactic acid production by glycolytic metabolism and enhanced activity of membrane bound proton pumps that efflux H^+ ions from the cytosol of cancer cells contribute to the unique features of tumor microenvironment.[5] It was shown that the efficacy of many chemotherapeutic drugs which are either neutral or alkaline are reduced due to this acidic extracellular pH (pH_e) and furthermore shelters cancer from being attacked by cytotoxic T cells, which normally mediate immune response against tumor antigens.[6] Among cancer researchers, there is strong conviction that new strategies that selectively target the hostile acidic microenvironment may yield significant improvement in the anticancer therapy.[7,8] A recently concluded meeting of 'International Society of Proton Dynamics in Cancer (ISPDC)' also called for interdisciplinary efforts to target the tumor microenvironment and proton dynamics in cancer.[9]

Emerging field of cancer-nanotechnology offers many novel tools for the fight against cancer. Scheinberg *et al.* recently presented an excellent review on 50 years of nanomaterial development for cancer applications.[10] Unique physico-chemical properties of nanomaterials provide great opportunities to actively interfere or manipulate biological systems. In cancer research, the most discussed applications of nanotechnology are related to targeted drug delivery and contrast imaging.[11] Although, intensive research is being carried out on these areas, the direct utilization of nanomaterials as 'active therapeutic agent' is less explored. Instead, there has been much emphasis on the adverse effect of nanomaterials on the biological systems, termed as nanotoxicology. It was reported that many nanosystems such as quantum dots (QDs; CdSe, CdTe), carbon nanomaterials, metals (Ag, Au, Pt) and metal oxides (ZnO, TiO_2) exhibit characteristic toxic effects towards biological systems.[12,13] One interesting observation, considering the possible therapeutic use of nanomaterials, was that of ZnO nanocrystals (NCs) showing differential toxicity towards bacteria, normal cells and cancer cells.[14–16]

Zinc oxide (ZnO) is an important II–VI group semiconductor material with direct band-gap of 3.37 eV and large exciton binding energy of ~60 meV, making it a promising candidate for

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Role of size scale of ZnO nanoparticles and microparticles on toxicity toward bacteria and osteoblast cancer cells

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Abstract The specific role of size scale, surface capping, and aspect ratio of zinc oxide (ZnO) particles on toxicity toward prokaryotic and eukaryotic cells was investigated. ZnO nano and microparticles of controlled size and morphology were synthesized by wet chemical methods. Cytotoxicity toward mammalian cells was studied using a human osteoblast cancer cell line and antibacterial activity using Gram-negative bacteria (*Escherichia coli)* as well as using Gram-positive bacteria (*Staphylococcus aureus)*. Scanning electron microscopy (SEM) was conducted to characterize any visual features of the biocidal action of ZnO. We observed that antibacterial activity increased with reduction in particle size. Toxicity toward the human cancer cell line was considerably higher than previously observed by other researchers on the corresponding primary cells, suggesting selective toxicity of the ZnO to cancer cells. Surface capping was also found to profoundly influence the toxicity of ZnO nanoparticles toward the cancer cell line, with the toxicity of starch-capped ZnO being the lowest. Our results are found to be consistent with a membrane-related mechanism for nanoparticle toxicity toward microbes.

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1 Introduction

Zinc oxide (ZnO) is currently being investigated as an antibacterial agent in both microscale and nanoscale formulations. Results have indicated that ZnO nanoparticles show antibacterial activity [1–11] apparently greater than for microparticles [1]. While the exact mechanisms of the antibacterial action have not yet been clearly elucidated, suggested mechanisms include, the role of reactive oxygen species (ROS) generated on the surface of the particles [2–4], zinc ion release [5], membrane dysfunction [5, 6], and nanoparticle internalization [7]. The role of ROS needs further study because the influence of light on antibacterial effect related to ROS production is not conclusive. Although one study reported substantial inhibition of bacterial growth under dark conditions, another showed significant antibacterial effect under dark conditions, both studies being on *Escherichia coli*. Furthermore, there is no effect of illumination on the antibacterial effect in certain Gram-positive bacteria [8]. Nevertheless, the excellent study by Sawai et al. [4] clearly showing that ROS concentrations increased with the ZnO content of slurries makes this mechanism worthy of further detailed evaluations. With regard to the role of cell membrane versus cell internalization, one transmission electron microscopy study showed that many particles of 10–14 nm ZnO were internalized [7] after overnight exposure, but membrane damage was also observed. The effect of particle size on internalization of ZnO is not known. Another aspect of relevance to completed studies is the role of the medium in which the exposures are carried out. ZnO can be processed through diethylene glycol (DEG) or aqueous routes. In the former case, DEG can cause damage to bacterial membranes [7], which complicates the interpretation of the role of ZnO.