High-Throughput Cellular-Based Toxicity Assays for Manufactured Nanoparticles and Nanostructure-Toxicity Relationship Models

(*Task Number: 425.035)*

Subtask 1: "High Throughput Screening"

Subtask 2: "Computational Models"

PIs:

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Objectives

Subtask 1:

- **Validation of high-throughput cellular-based toxicity assays for MNP assessment.**
- **Test QNTR (Quantitative Nanostructure Toxicity Relationship) model using the predictive models developed in subtask 2.**

Subtask 2:

- **Develop QNTR models that correlate the compositional/physical/chemical/geometrical and biological descriptors of MNPs with known toxicological endpoints.**
- **Improve the prediction performance of QNTR models with the availability of new experimental data from subtask 1.**

ESH Metrics and Impact

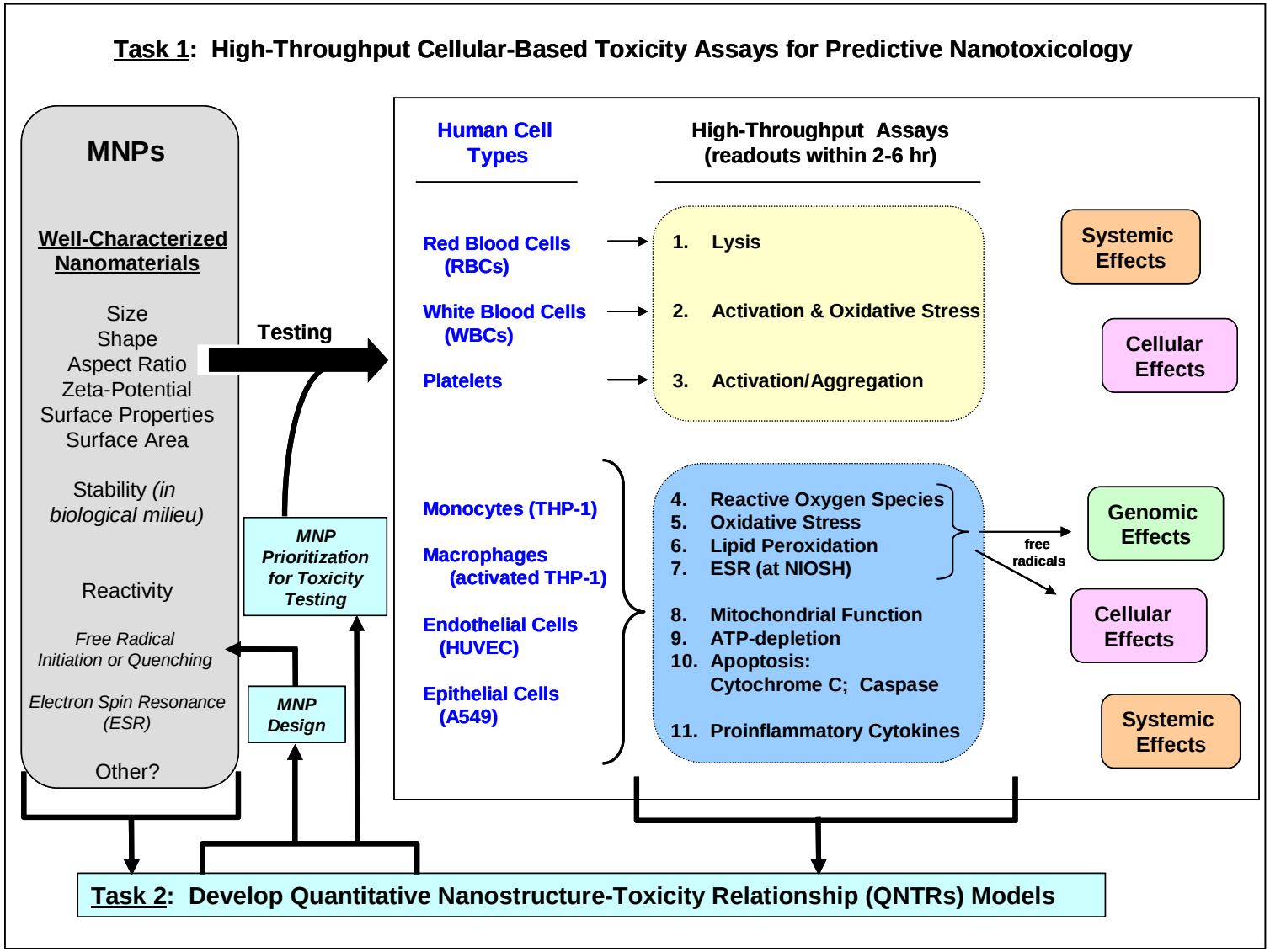
1. *Obtain predictive knowledge of the physical and chemical properties of manufactured nanoparticles.*

2. *Develop relevant in-vitro assays utilizing human cells to predict the toxicity of manufactured nanoparticles.*

3. *Develop predictive computational models that correlate physical-chemical descriptors of MNPs with their toxic effects.*

Impact: Utilize the knowledge gained through above three metrics for improved MNP experimental design and prioritized toxicity testing toward the manufacturing of safe nanomaterials.

General Framework of the Proposed Approach

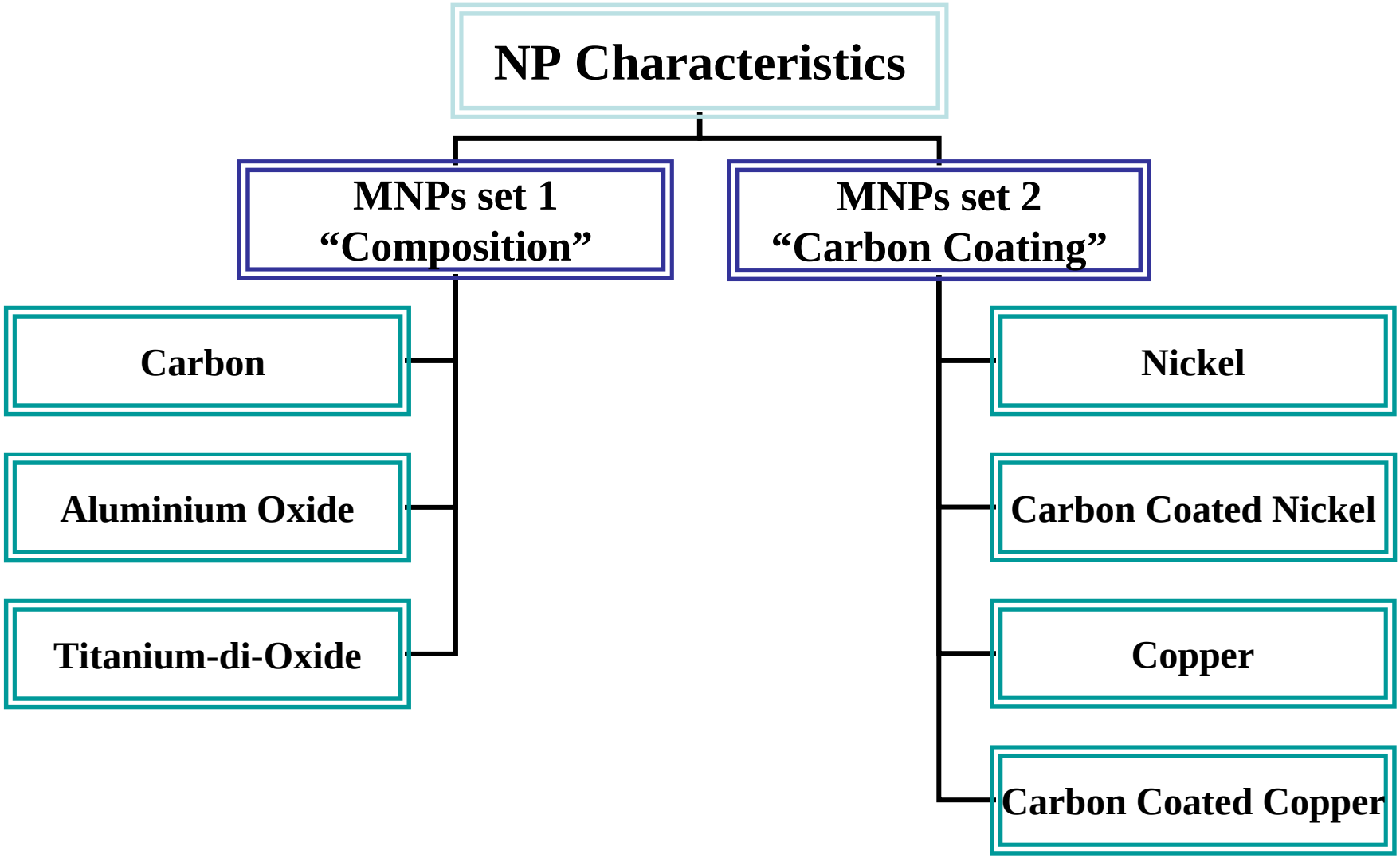


Subtask 1: Potential Cellular-based Assays

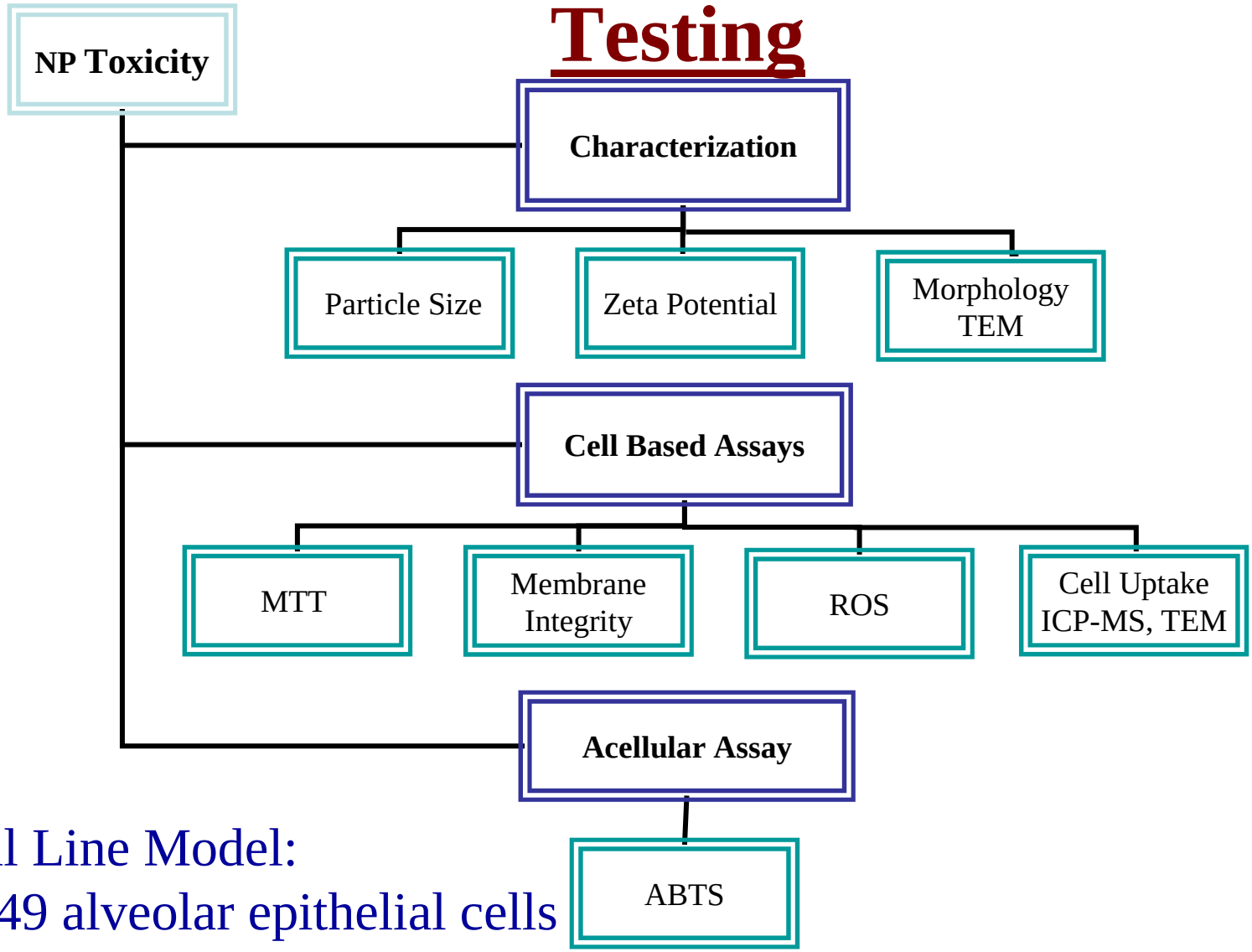
Human Cells	Assay	Description
Red Blood Cells (RBCs)	**Lysis**	Measure oxyhemoglobin at 540 nm
White Blood Cells (WBCs)	**Activation**	Measure reduction of ferricytochrome c caused by produced superoxide anions
	Oxidative Stress	Measure intracellular GSSG/GSH ratio; where GSSG is oxidized glutathione and GSH is reduced glutathione
Platelets	**Activation**	Flow cytometry to measure PAC-1-FITC binding to activated platelets
	Aggregation	Whole Blood Impedance Aggregometry

Human Cells	Assay	Description
Monocytes (THP-1) **Macrophages (activated THP-1)** **Endothelial Cells (HUVEC)** **Epithelial Cells (A549)**	**Reactive Oxygen Species**	1) Measure intracellular fluorescence produced with H_2DCFDA or carboxy-H_2DCFDA loaded cells; 2) Measure (a) cellular ESR
	Oxidative Stress	Measure intracellular GSSG/GSH ratio; where GSSG is oxidized glutathione and GSH is reduced glutathione
	Lipid Peroxidation	Lipid Hydroperoxide (LPO) Assay
	Mitochondrial Function	MTT assay & JC-1 assay
	ATP-depletion	ATPlite 1step® Assay Kit (PerkinElmer)
	Apoptosis:	
	Cytochrome C	Cytochrome C immunoassay
	Caspase-3	Caspase-3 Fluorometric Assay (R&D Systems); Quantify caspase-3 activation by cleavage of DEVD-AFC substrate
	Proinflammatory Cytokines	Cytokine assays by ELISA; NFKB, IL-1β, TNF-α, IFN-γ, IL-8

Subtask 1: Current Method Approach



Subtask 1: Current Scheme for Toxicity Testing



Cell Line Model:
A549 alveolar epithelial cells

Characterization: MNPs Set 1 and 2

NP Type	Manufacturer	Particle Size* Range (nm)	Particle Size in DI water (nm)	Zeta Potential (mV)
Carbon	American Elements	55-100	611.7 ± 510.5	-21.1 ± 4.6
Aluminum oxide	Alfa-Aesar	40-50	488.8 ± 318.7	-17.7 ± 7.4
Titanium-di-oxide	NanoAmor	30-40	511.7 ± 336.7	-25.3 ± 5.2
Nickel	NanoAmor	20	834.6 ± 495.1	2.76 ± 0.7
Carbon coated Nickel	NanoAmor	20	466.6 ± 179.6	-16.4 ± 1.8
Copper	NanoAmor	25	662.2 ± 139.3	-9.0 ± 2.4
Carbon coated Copper	NanoAmor	25	412.1 ± 210.9	-6.21 ± 0.7

* Provided by Manufacturer

Sample preparation:1 mg/ml suspensions in DI water; bath sonicated for 6 x30 sec

MNPs Set 1
ABTS and ROS Assay

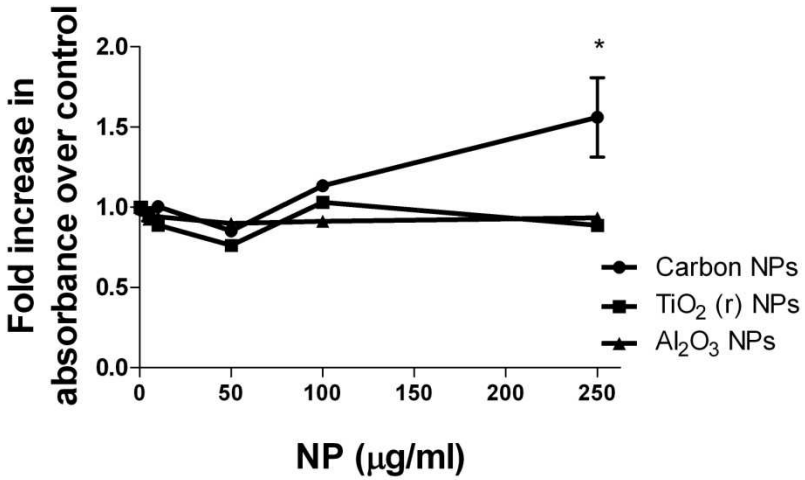


Fig. 1 ABTS assay is an *in-vitro* acellular assay that gives an indication about reactivity of MNPs. ABTS conversion to ABTS radical is observed by measuring increase in absorbance at 734 nm (characteristic of ABTS radical). MNPs in various concentrations were incubated with ABTS, 60 mM solution in a 96 well plate for 24 hr. Data are corrected for absorbance from blank NPs. (* $p < 0.05$ as compared to control)

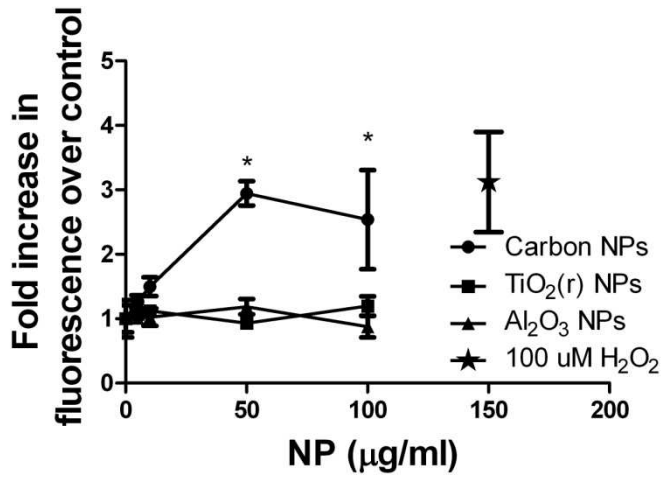


Fig. 2 ROS or Reactive Oxygen Species assay gives an indication of the ability of MNPs to induce oxidative damage in cells. A549, human alveolar epithelial cells (25,000 per well), were incubated with NPs for 4 hr. Observed fluorescence is due to cleavage of carboxy H2DCFDA to fluorescein inside cells. Data corrected for fluorescence from blank NPs. H_2O_2 was used as positive control.
(* $p < 0.05$ as compared to control)

ABTS = 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid diammonium salt)

MNPs Set 1
Mitochondrial Function

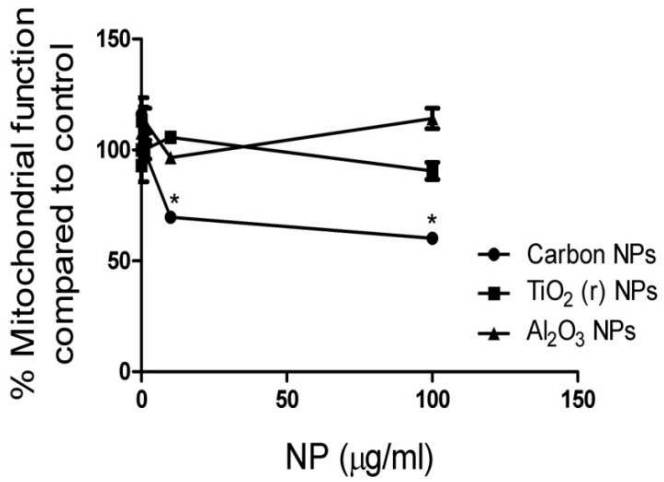


Fig. 3 MTT dye reduction assay measures ability of MNPs to alter with the mitochondrial function. A549, human alveolar epithelial cells (25,000 per well), were incubated with NPs for 24 hr. Observed absorbance is due to reduction of MTT dye inside viable cells. Data corrected for absorbance from blank NPs.
(* $p < 0.05$ as compared to control)
Note : Carbon NPs are 98% pure. Impurities include, among a few others: Si (0.1%), Fe (0.08%), Cr (0.06%), Ni (0.05%). Among carbon, TiO_2, Al_2O_3 NPs; Only carbon NPs have transition metal impurities.

Conclusions: MNPs Set 1

- Particle size measurement by dynamic light scattering shows that NP sizes are different from those provided by the manufacturer.
- ABTS assay was successfully developed as an *in-vitro* acellular assay to assess the free radical forming potential of NPs. The assay is simple, adaptable to 96 well plate and cost effective.
- Carbon NPs appear to be more toxic as compared to other NPs as shown by *in-vitro* MTT and ROS data.
- Carbon nanoparticles probably act as a vehicle to carry iron/nickel in cells. This is a very plausible scenario as hydrophobicity provided by carbon NPs facilitate iron entry in cells.
- Results from ABTS assay correlates well with ROS and MTT cytotoxicity data.

MNPs Set 2
MTT and Membrane Integrity Assay

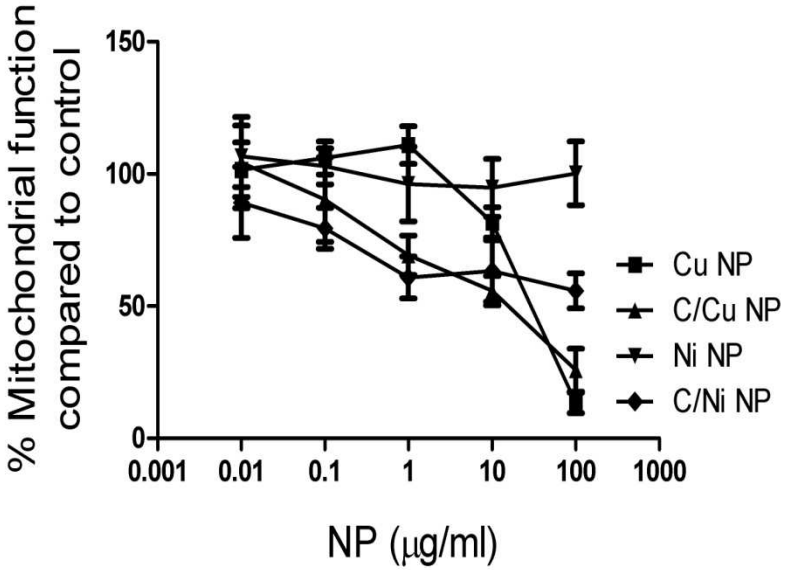


Fig. 4 MTT dye reduction assay measures the ability of MNPs to alter with the mitochondrial function. A549, human alveolar epithelial cells (25,000 per well), were incubated with NPs for 24 hr. Observed absorbance is due to reduction of MTT dye inside viable cells. Data corrected for absorbance from blank NPs. Ni NPs significantly differ from C/Ni NPs at all doses and Cu NPs significantly differ from C/Cu NPs at 0.1, 1 and 10 µg/ml.

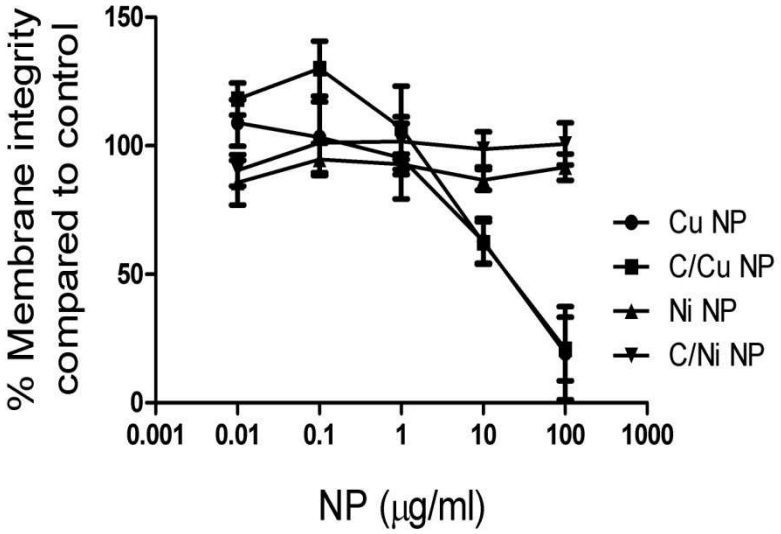


Fig. 5 Membrane Integrity assay measures the ability of MNPs to alter with the plasma membrane integrity. A549, human alveolar epithelial cells (25,000 per well), were incubated with NPs for 24 hr. Neutral red dye absorbs at 540 nm in lysosomes of cells with intact plasma membrane. Data corrected for absorbance from blank NPs. Ni NPs and C/Ni NPs do not appear to alter membrane integrity whereas Cu NPs and C/Cu NPs are equally toxic.

MNPs Set 2

TEM Characterization of MNPs

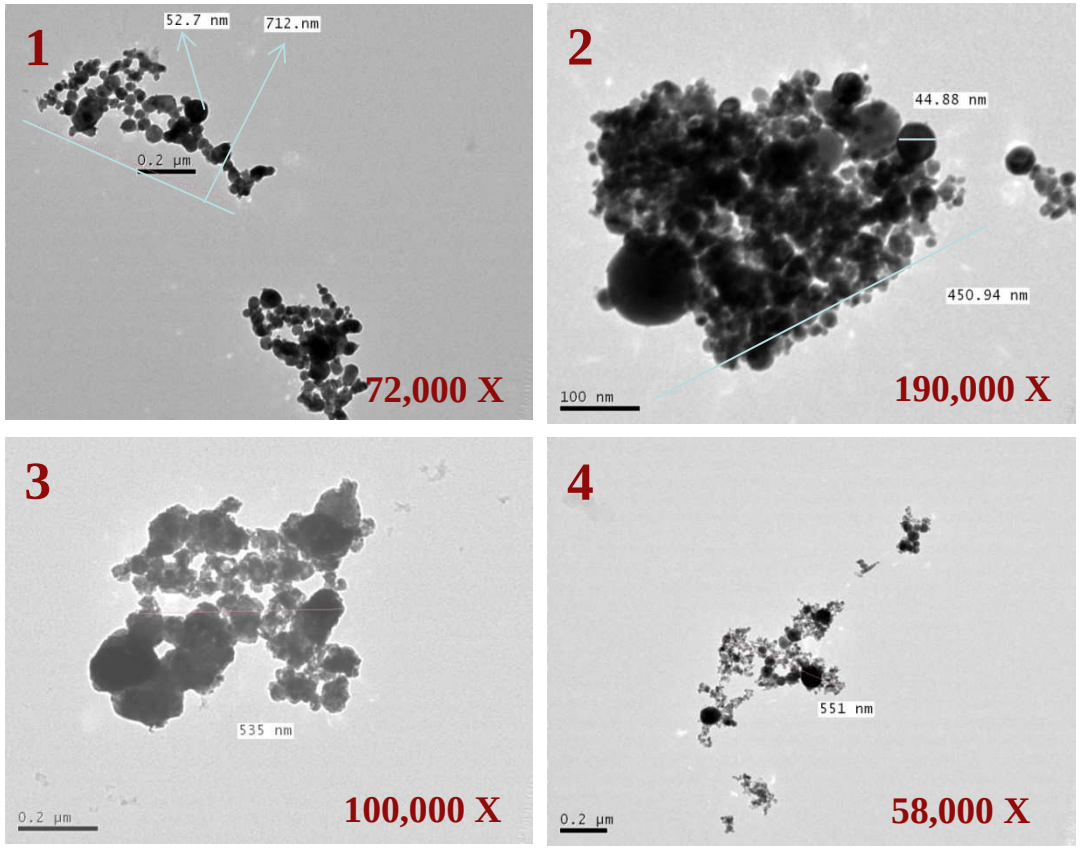


Fig. 6 TEM Images of 1. Ni , 2. C/Ni, 3. Cu and 4. C/Cu MNPs. Nanoparticles were suspended at concentration of 10 µg/ml in DI water for this analysis. The average particle sizes measured by TEM correlates with the dynamic light scattering data.

MNPs Set 2
Cell Uptake Analysis by TEM

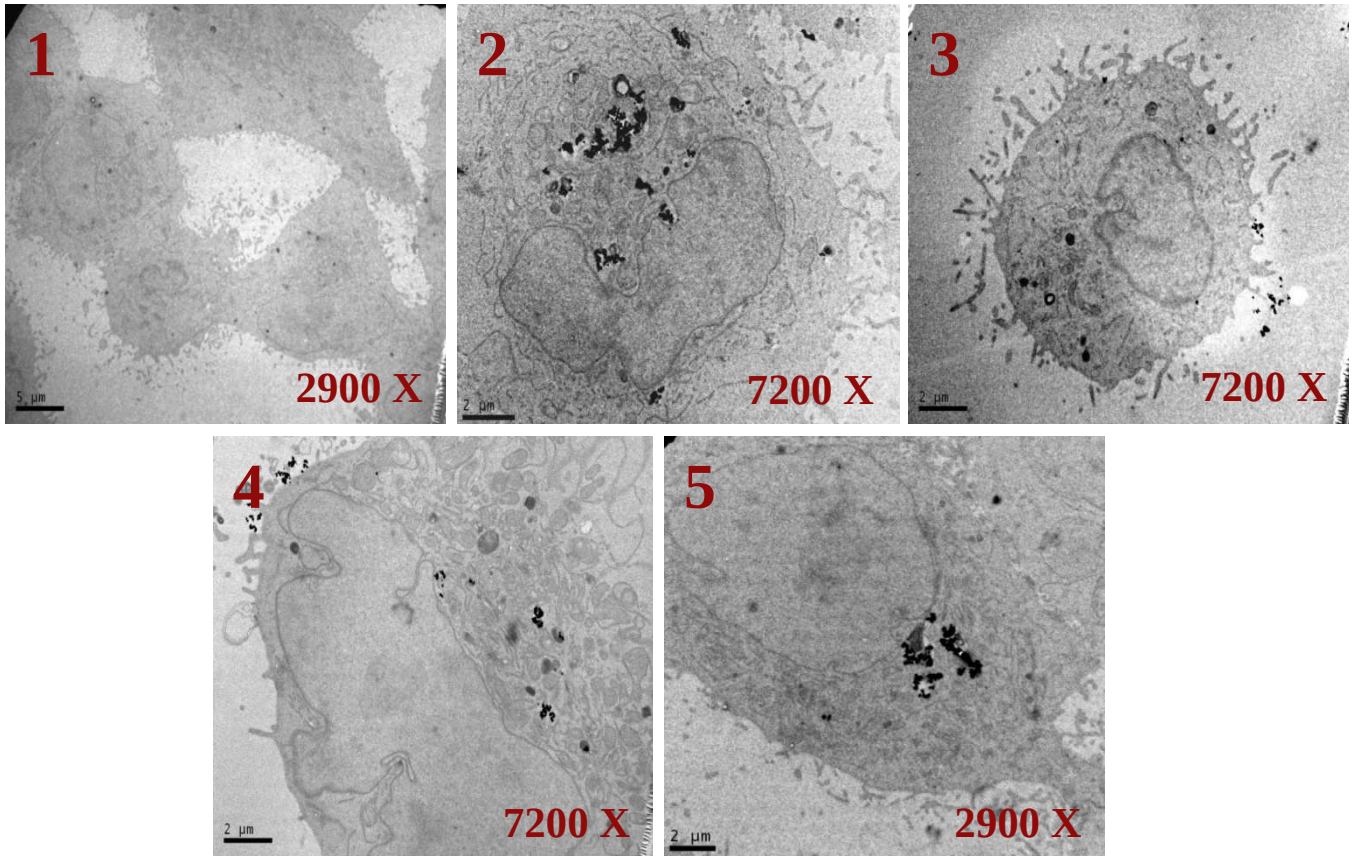


Fig. 7 TEM Images of 1. Control A549 cells , 2. Cu treated , 3. C/Cu treated , 4. Ni treated and 5. C/Ni treated A549 cells. Cells were treated with nanoparticles at concentration of 10µg/ml for 8hr.

MNPs Set 2
Cell Uptake Analysis by ICP-MS

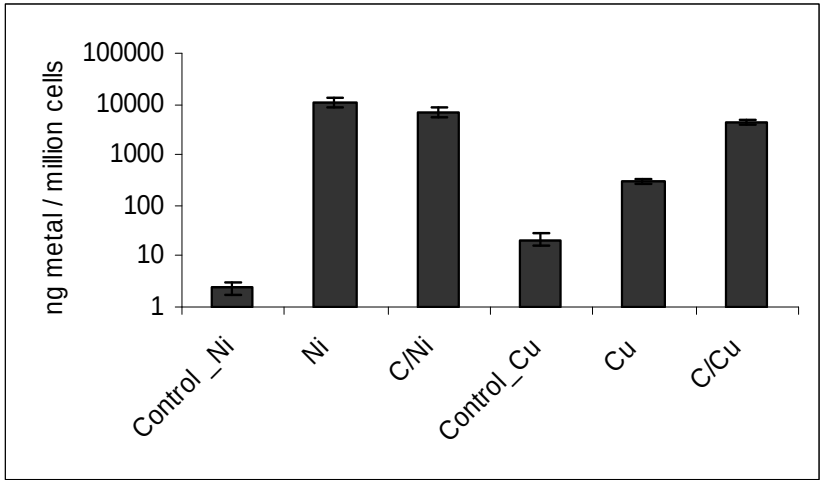


Fig. 8 Preliminary data on Cell uptake analysis of Ni and Cu from Ni, C/Ni and Cu and C/Cu NPs respectively. Control_Ni and Control_Cu signify the amount of respective metal content in untreated control A549 cells. Nanoparticles were tested at concentrations of 10µg/ml for 8 hr in A549 cells. Ni uptake from Ni and C/Ni NPs is comparable but Cu uptake from C/Cu NPs is an order of magnitude higher than uptake from Cu NPs. Correlation of uptake data with observed toxicity in cell based assays awaits data on detailed physico-chemical characterization.

Conclusions: MNPs Set 2

- Average particle size measured by DLS of all MNPs in Set 2 are on an average 20-fold higher than provided by the manufacturers.
- Ni NPs do not alter mitochondrial function and membrane integrity although their uptake by cells is comparable to C/Ni NPs.
- C/Ni NPs alter the mitochondrial function but not membrane integrity.
- Cu NPs alter mitochondrial function at 100 µg/ml but can alter membrane integrity even at 10 µg/ml dose.
- Rounded morphology of Cu NP treated cells and results from membrane integrity and ICP-MS suggest that Cu NPs might act on cell surface at lower dose, possibility of alterations with cell adhesion.
- C/Cu NPs alter mitochondrial function and membrane integrity to the same extent.
- Cu and C/Cu NPs appear to be more toxic than Ni and C/Ni NPs.
- Correlation of uptake data with observed toxicity in cell based assays awaits data on detailed physico-chemical characterization.

Subtask 2: Research Hypothesis

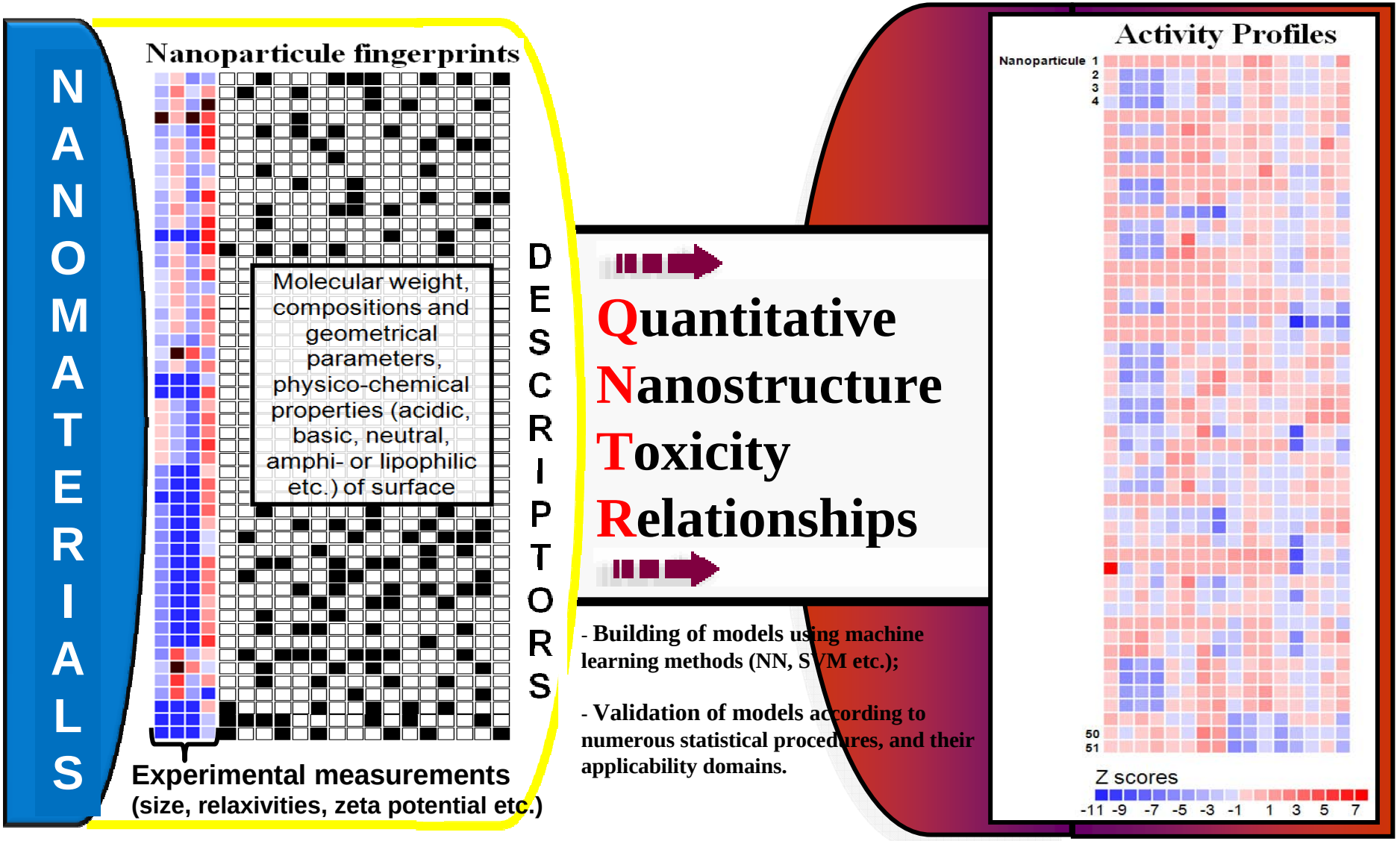
- The effects of MNPs on different types of human cells depend on the compositional/physical/chemical/geometrical properties of the MNPs.

- High-throughput cellular-based assays with endpoints within 2-6 hr provide useful and predictive information about long-term biological properties of NPs.

- Toxicological data obtained from *in-vitro* cellular-based toxicity assays will correlate reasonably with *in-vivo* findings.

- Using physical/chemical characterization and toxicological screens for an ensemble of MNPs, it will be possible to develop **predictive Quantitative Nanostructure – Toxicity (QNTR) models.**

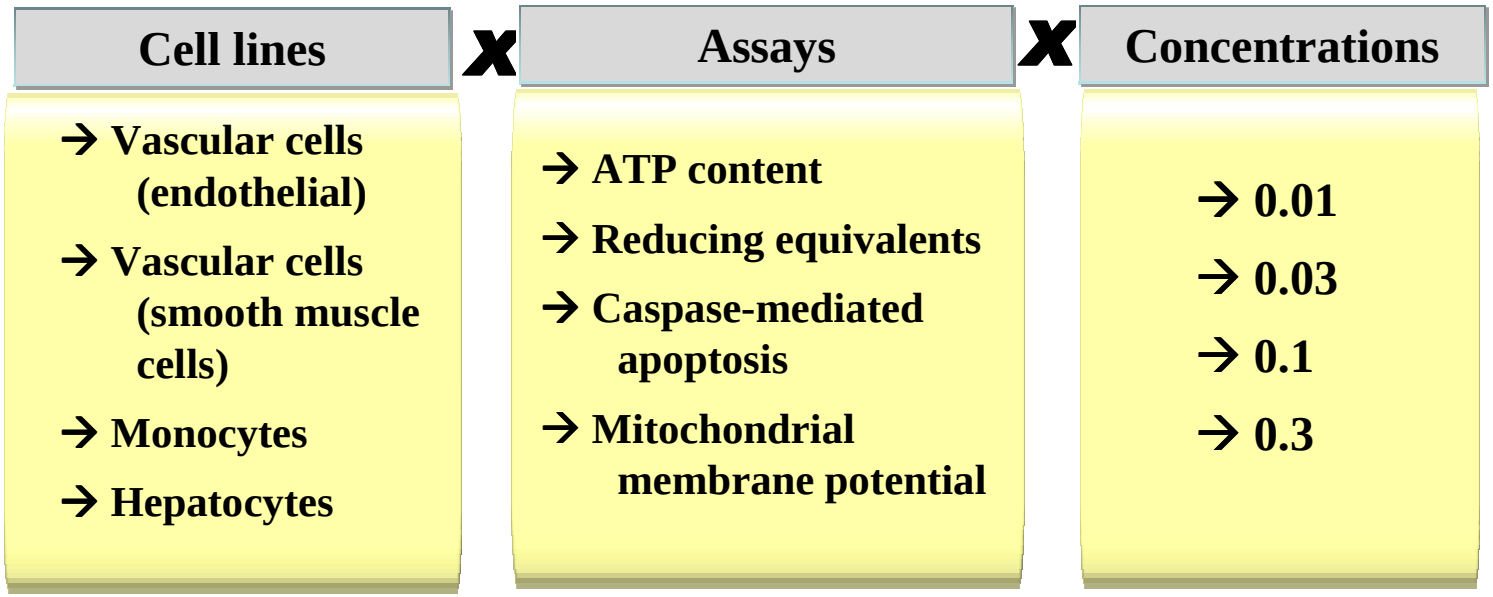
Subtask 2: QNTR Scheme



Case Study 1: QNTR of Whole NPs

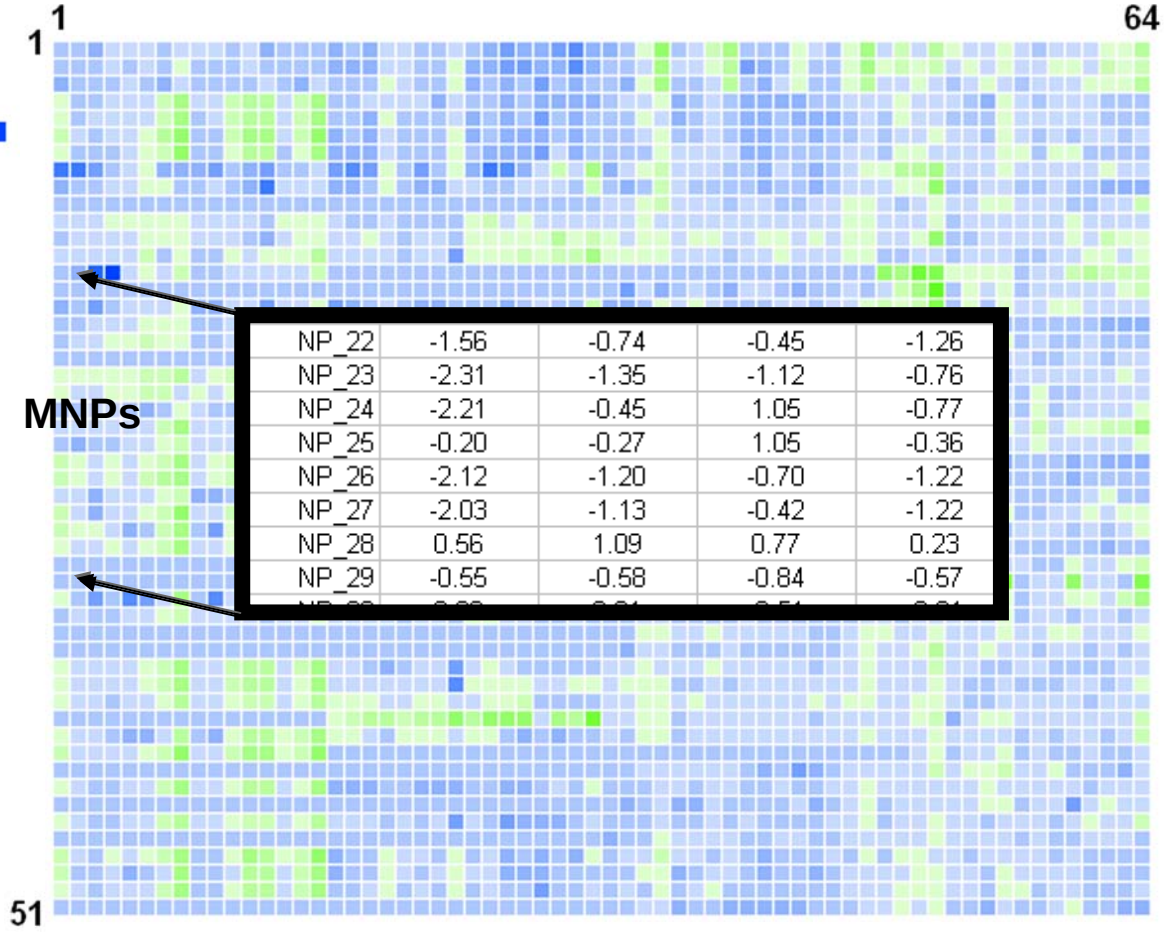
Recently[1], 51 diverse NPs were tested *in-vitro* against 4 cell lines in 4 different assays at 4 different concentrations **(→ 51x64 data matrix).**

MNP	CLIO	PNP	MION	QD	Feridex IV	Ferrum Hausmann
#. particle	23	19	4	3	1	1



[1] ***Shaw et al. Perturbational profiling of nanomaterial biologic activity. PNAS, 2008, 105, 7387-7392***

Case Study 1:Initial Activity Matrix

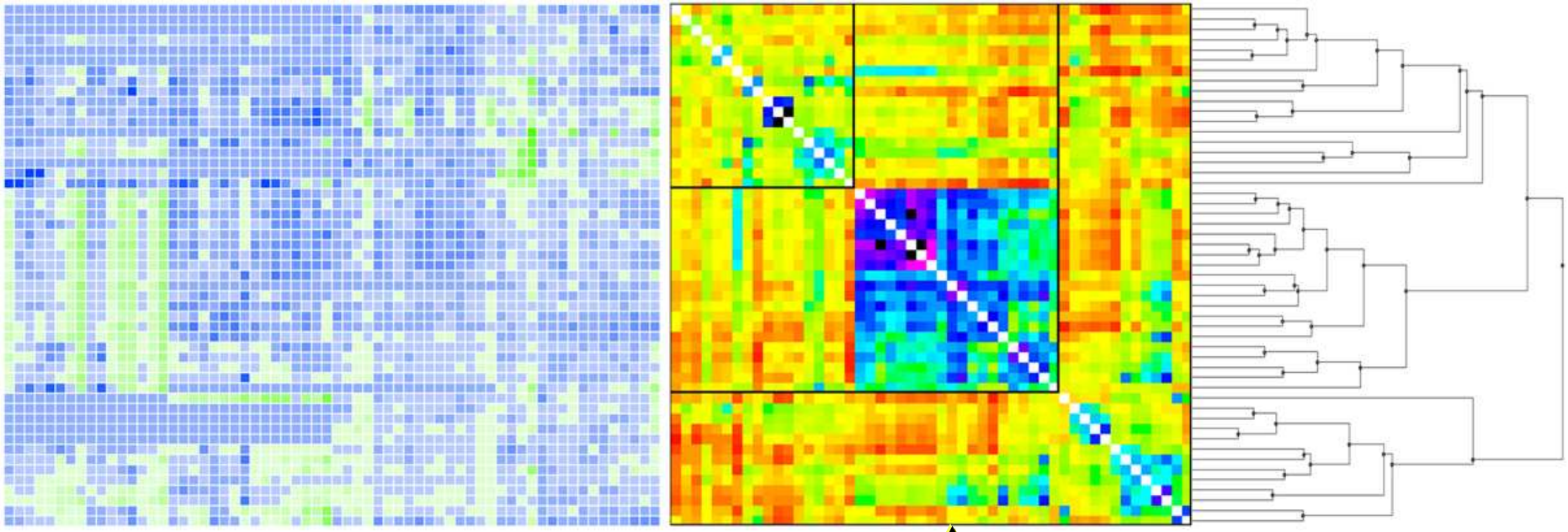


Z scores: assay values were expressed in units of standard deviations of the distribution obtained when cells are treated with PBS (Phosphate Buffered Saline) alone.

$$Z_{NP} = (\mu_{NP} - \mu_{PBS})/ \sigma_{PBS}$$

μ_{NP} : mean of control tests with PBS
σ_{NP} : standard deviation of control tests with tests

Case Study 1: Hierarchical Clustering of The Activity Matrix



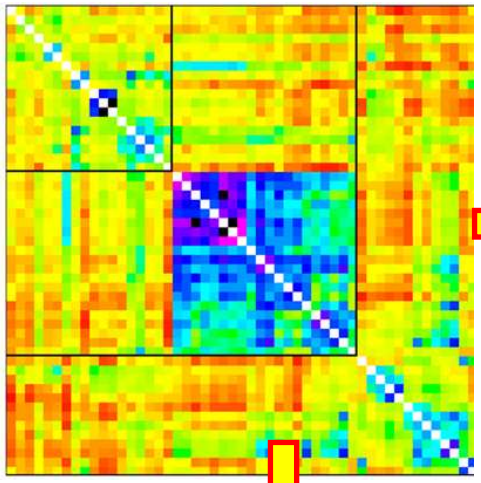
Clustered Activity Matrix

Clustered Distance Matrix

Dendogram

After the normalization of data, ISIDA/Cluster program* was used to cluster the activity matrix (51 * 64), using Johnson's hierarchical method, Euclidean metrics and complete linkage.

Case Study 1: Analysis of Clusters



NP type	CLUSTER 1	CLUSTER 2	CLUSTER 3	Total
CLIO	7	13	3	23
PNP	7	2	10	19
MION	0	4	0	4
Qt-dot	3	0	0	3
Feridex	0	1	0	1
Ferrum Haussmann	1	0	0	1
Total	18	20	13	51

NP Core	CLUST 1	CLUST 2	CLUST 3	Total
Fe_2O_3	5	0	9	14
Fe_3O_4	9	20	4	33
Cd-Se	3	0	0	3
Fe(III)	1	0	0	1
Total	18	20	13	51

A given metal core (i.e, Fe_3O_4) or NP category (i.e, Qt-dot), will induce similar biological effects in most cases, independent of the surface modifications.

Case Study 1: QNTR Matrix and Modeling Results

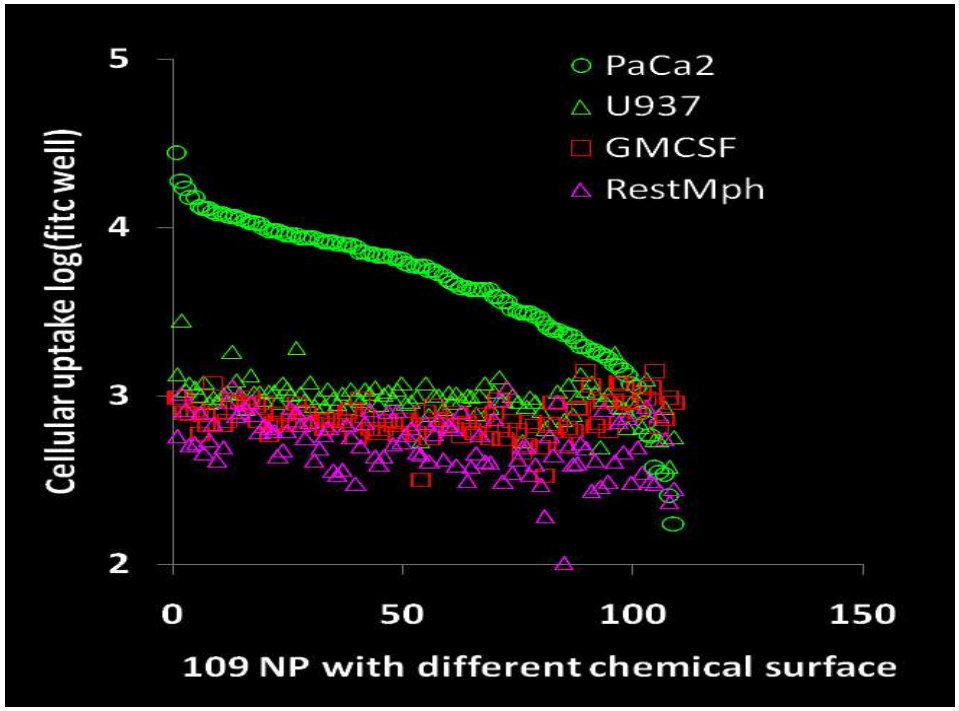
QNTR Matrix

	Effect	Size	Zeta pot.	Relaxitivities
NP-01	High	0.4865	0.5278	0.2941	0.3986
NP-02	Low	0.4054	0.7222	0.4837	0.6476
NP-03	High	0.4324	0.5833	0.3529	1.0000
NP-04	Low	1.0000	0.5833	1.0000	0.7991
NP-05	High	0.3649	0.4722	0.2353	0.9403
NP-06	High	0.3919	0.6111	0.3333	0.9079
NP-07	High	0.5135	0.5833	0.4052	0.6270

Modeling Results

Fold	MODELING SETS				EXTERNAL SETS				
	n	# models	% accuracy internal 5-fold CV	% accuracy	*n*	% accuracy	% CCR[a]	% Sensitivity (SE)	% Specificity (SP)
1	35	11	51.4 – 60.0	71.4 – 82.9	9	**78**	83	67	100
2	35	13	51.4 – 60.0	71.4 – 77.1	9	**78**	75	50	100
3	35	16	57.1 – 62.9	74.3 – 82.9	9	**78**	78	80	75
4	35	11	60.0 – 62.9	77.1 – 88.6	9	**56**	55	50	60
5	36	4	66.7	83.3 – 86.1	8	**75**	67	33	100
[a]CCR – Correct Classification Rate; CCC = ½ (SE +SP)					**44**	**73**	**73**	**60**	**86**

Case Study 2: QNTR Study of NPs Uptake in PaCa2 Cells



PaCa2: Pancreatic cancer cells

U937: Macrophage cell line

GMCSF: Activated primary human macrophages

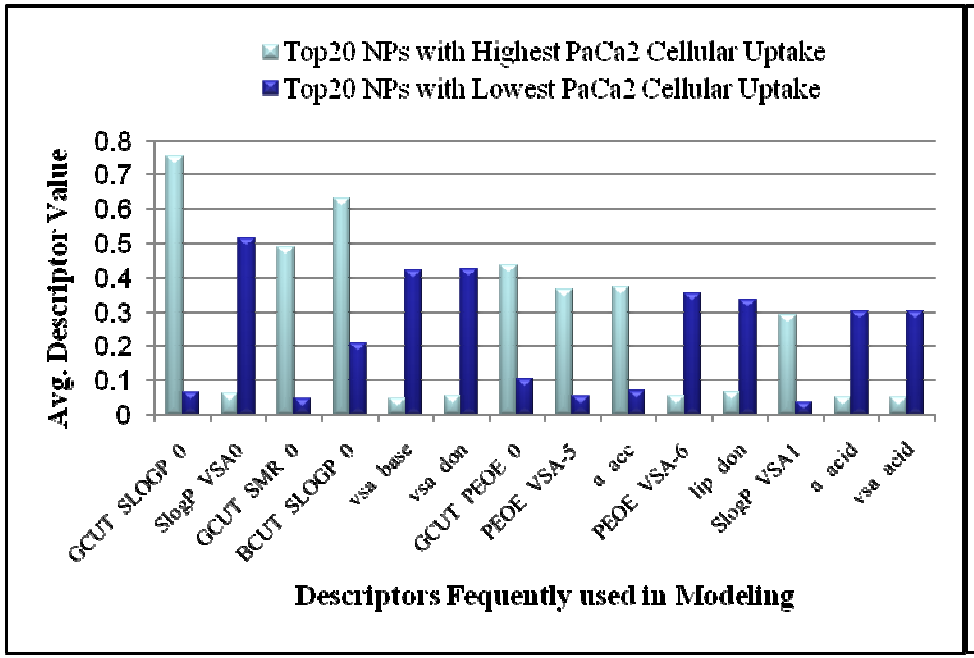
RestMph: Resting primary human macrophages

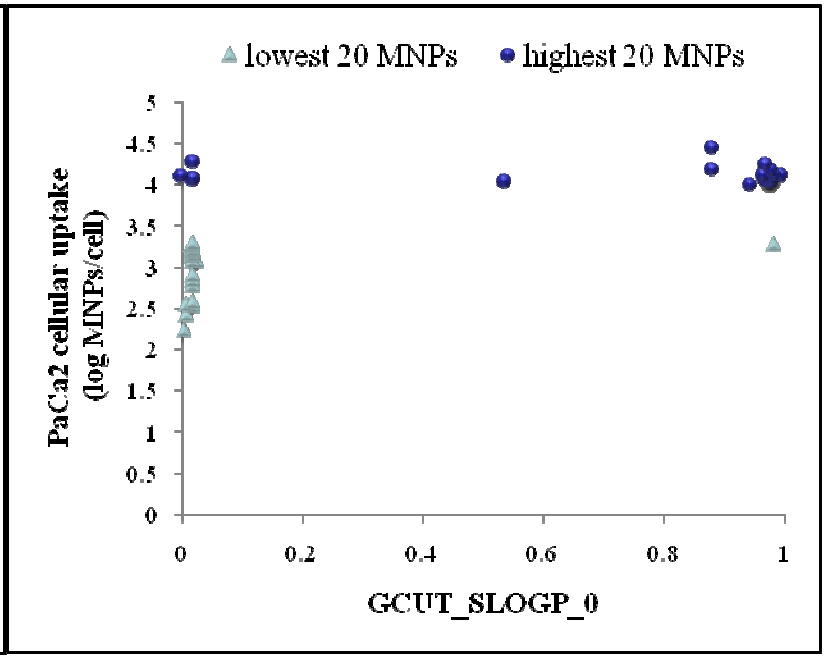
Recently, Weissleder et al.* investigated whether the multivalent attachment of small organic molecules on a same NP can modify its binding affinity to certain cells. 109 NPs possessing the same core (CLIO) were attached with different organic compounds on their surfaces

* *Weissleder et al. Nat. Biotechnol., 2005, 23 (11), 1418-1423*

Case Study 2: Modeling Results And Descriptor Analysis

Fold	# comp. model	# comp. external	w/o AD		w/ AD		
			R_o^2	MAE	R_o^2	MAE	% cov
1	87	22	0.65	0.18	0.67	0.18	86
2	87	22	0.67	0.14	0.73	0.13	91
3	87	22	0.72	0.22	0.75	0.21	82
4	87	22	0.75	0.19	0.90	0.14	64
5	88	21	0.80	0.16	0.78	0.17	76
Average	87	22	**0.72**	**0.18**	**0.77**	**0.17**	**80**





Subtask 2: Conclusions

- Preliminary results demonstrate that QNTR models can successfully predict the biological effects of NPs from their descriptors either experimentally measured (e.g., case 1 study), or calculated (case 2 study).

- To increase the accuracy and impact of models on the experiments, we need additional systematic experimental data (structural and biological).

- QNTR approach may allow rational design or prioritization of novel NPs with desired target (physical and biological) properties.

Industrial Interactions and Technology Transfer

- **Potentially seamless interaction between the ESH Research Center and SRC member companies.**
- **Send nanomaterials to UNC for characterization and analysis.**
- **Analyze experimental data and build predictive QNTR models.**
- **Prioritize MNP design and toxicity testing.**
- **Provide continuous feedback of information for ESH and SRC member companies.**

Future Plans

Next Year Plans

- **Subtask 1:**
  - **Complete the standard outlined assays for MNPs Set 1 and 2.**
  - **Carry out detailed physico-chemical characterization and additional biological assays to identify the cause of differential toxicity profile manifested by MNPs in Set 2.**
- **Subtask 2:**
  - **Establish a database of experimental nanotoxicity data**
  - **Develop extended QNTR models of all available nanotoxicity data**

Long-Term Plans

- **Obtain predictive knowledge of physical and chemical properties of MNPs that affect human cells and utilize this knowledge for improved MNP experimental design and prioritized toxicity testing.**

Publications, Presentations, and Recognitions/Awards

- **Shalini Minocha and Russell J. Mumper, "In-vitro Assays to Assess the Toxic Potential of Manufactured Nanoparticles", 2009 AAPS Annual Meeting and Exposition, Los Angeles CA, November 8-12, 2009.**
- **Shalini Minocha and Russell J. Mumper, "Characterization and in-vitro Evaluation of Potential Toxicity of Commercially available nanoparticles", Chapel Hill Drug Conference, May 13-14, 2009, Chapel Hill, NC, USA.**
- **Denis Fourches, Lin Ye, Russell J. Mumper and Alexander Tropsha. Assessing the Biological Effects of Nanoparticles Using Quantitative Nanostructure – Activity Relationships. Spring 2009 ACS Meeting and Exposition, Salt Lake City, UT, March 22-26, 2009.**
- **Denis Fourches, Dongqiuye Pu, Russell J. Mumper and Alexander Tropsha. Quantitative Nanostructure-Toxicity Relationship (QNTR) Modeling. Nanotoxicology, manuscript in preparation.**
- **Shalini Minocha, Dongqiuye Pu, Alexander Tropsha and Russell J. Mumper, "Systematic Evaluation of Toxicity of Metal Based Nanoparticles", Nanotoxicology, manuscript in preparation.**