UNC ESHELMAN SCHOOL OF PHARMACY

Task Number: 425.035

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

Subtask 1: "High Throughput Screening"
Subtask 2: "Computational Models"

PIs

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Research Objectives

Subtask 1

- Validation of high-throughput cellular-based assays for toxicity assessment of Manufactured NanoParticles (MNPs).
- Test the predictive QNTR (Quantitative Nanostructure-Toxicity Relationships) 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

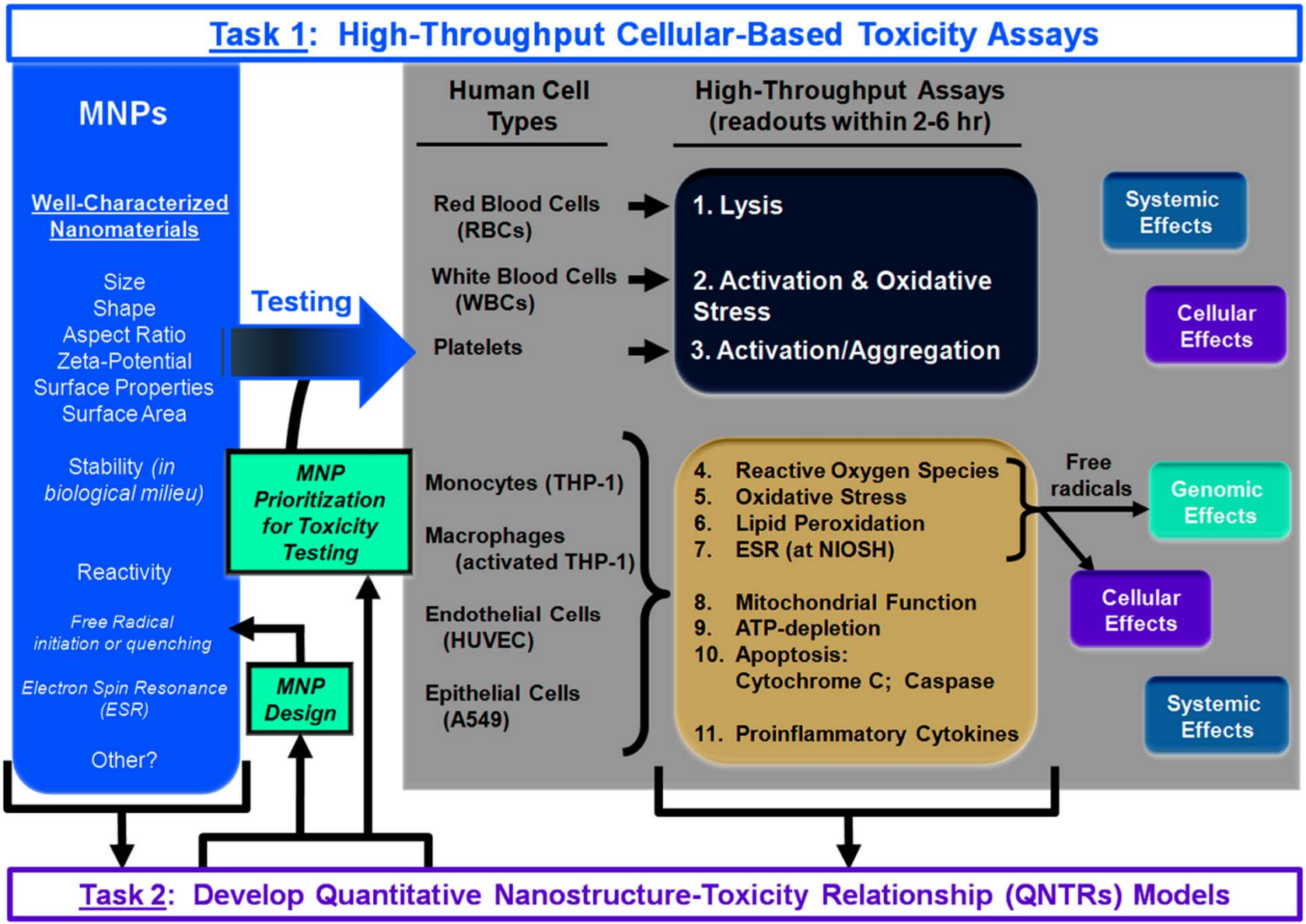
1. ***Obtain predictive knowledge of the physical and chemical properties of MNPs.***

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

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

Research 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

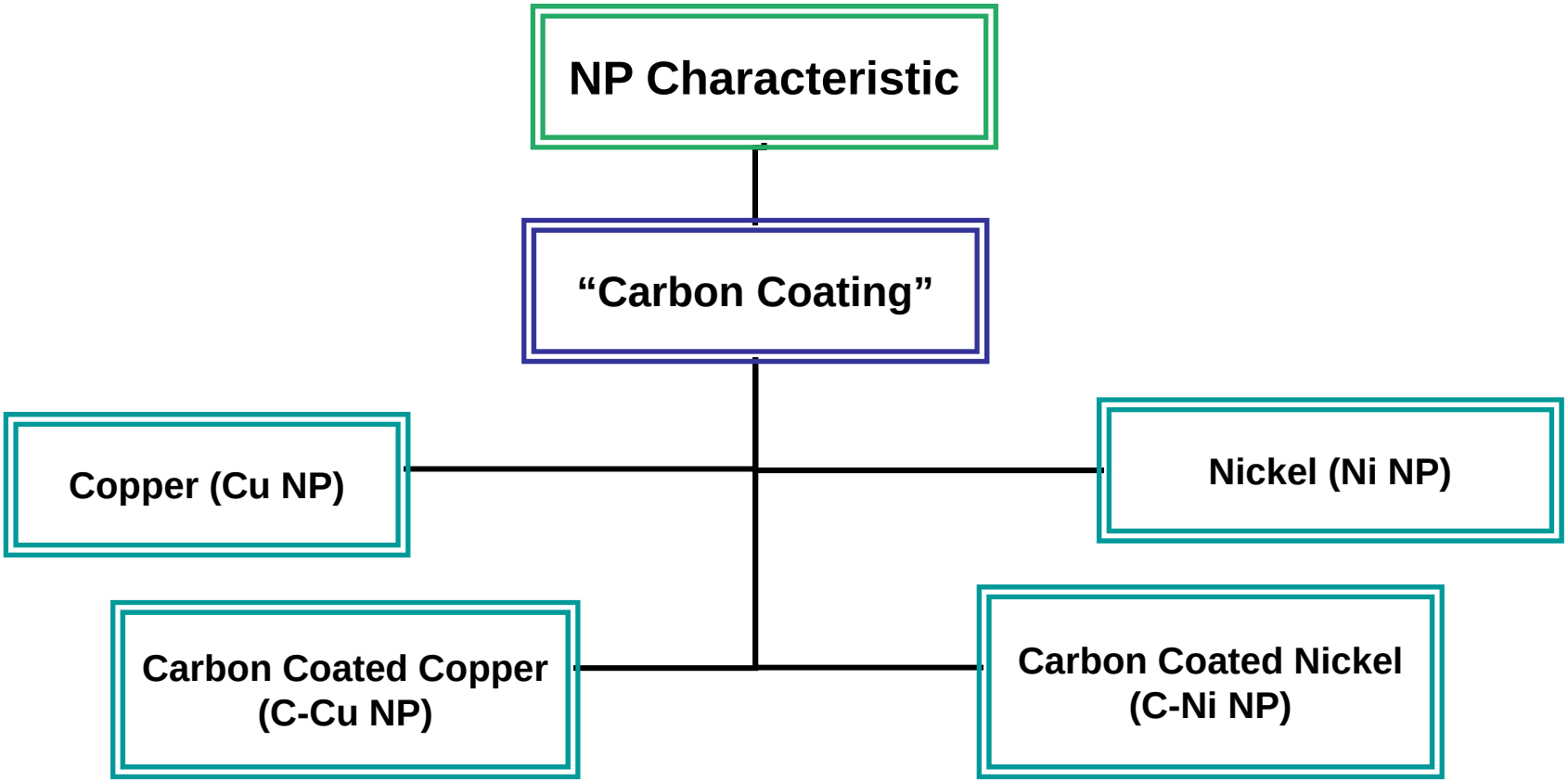


Subtask 1: Potential Cellular-based Assays

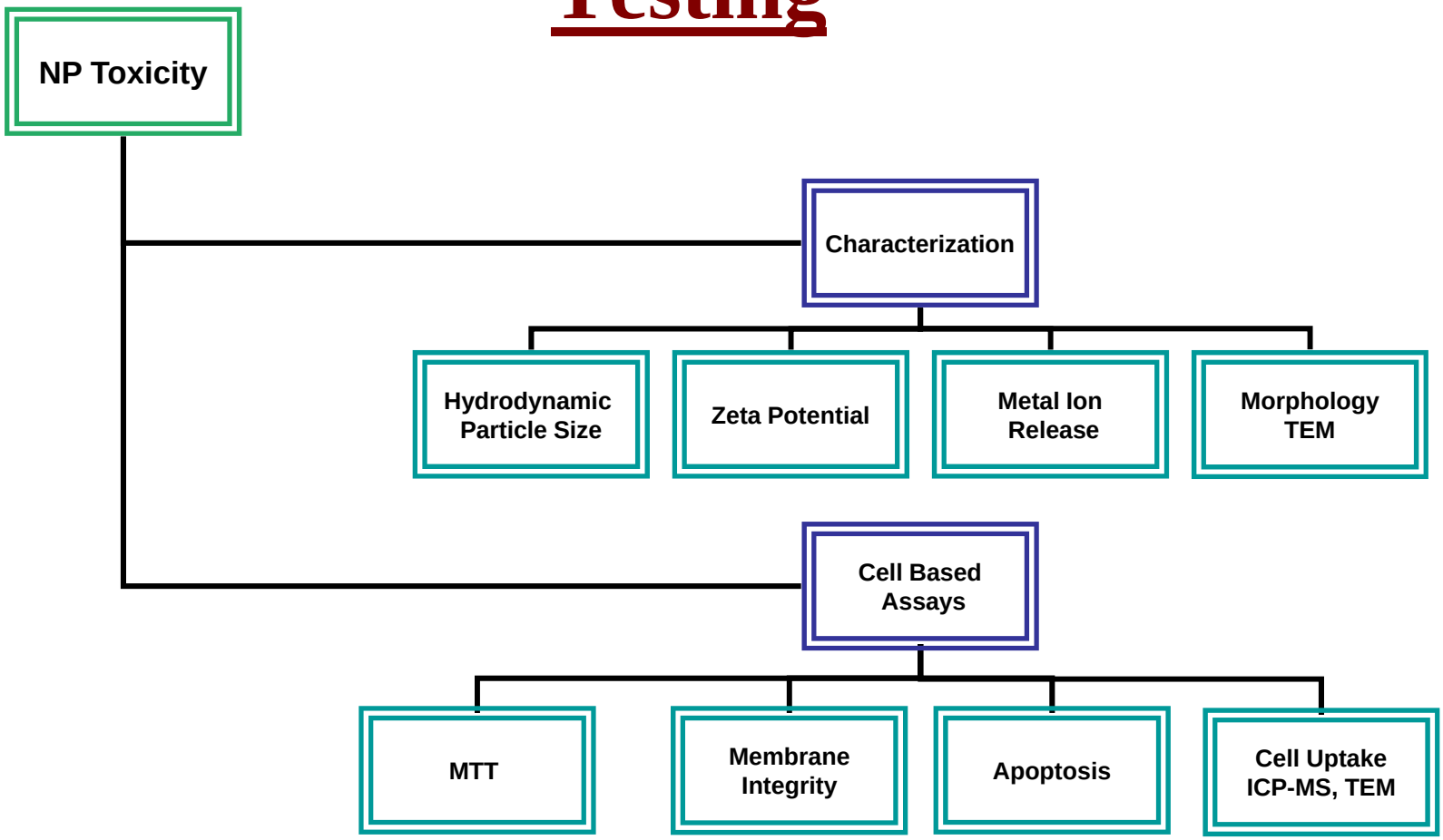
Human Cells	Assay	Description
Red Blood Cells (RBCs)	**Lysis**	Measure oxyhemoglobin at 540 nm
White Blood Cells (WBCs)	**Activation**	Measure reduction of ferricytochrome c caused by produced superoxide anions
	Oxidative Stress	Measure intracellular GSSG/GSH ratio; where GSSG is oxidized glutathione 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 Approach



Subtask 1: Current Scheme for Toxicity Testing



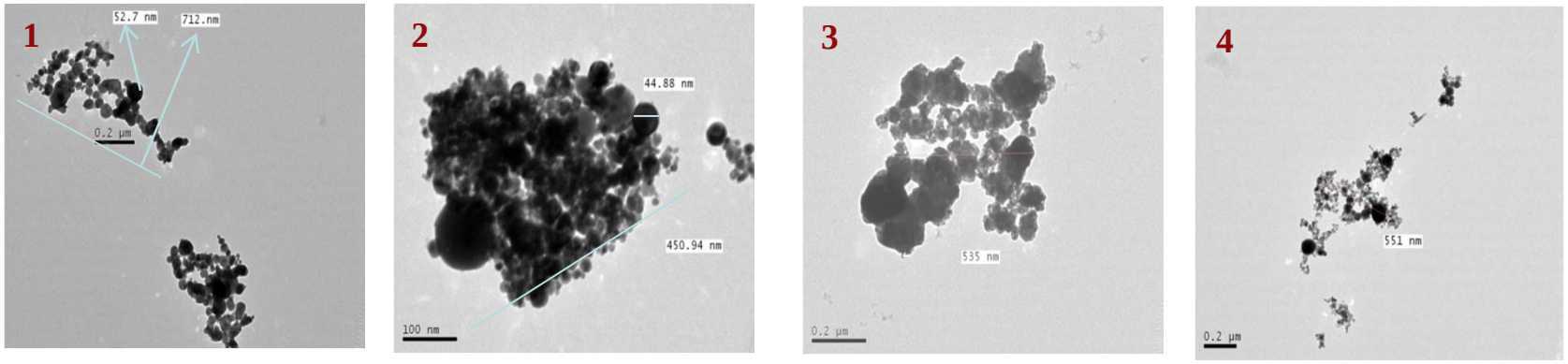
Cell Line Model:
A549 alveolar epithelial cells

Characterization: NPs

NP Type	Manufacturer	Particle Size* (nm)	Particle Size in DMEM with 10% FBS	Particle Size in DMEM (nm)	Particle Size in DI water (nm)	Zeta Potential (mV)
Nickel	NanoAmor	20	559.4 ± 213.7	2778.3 ± 107.2	1210 ± 180	2.8 ± 0.7
Carbon coated Nickel	NanoAmor	20	427.1 ± 149.7	953.7 ± 382.3	728.2 ± 281.2	-16.4 ± 1.8
Copper	NanoAmor	25	272.2 ± 92.6	537 ± 167	1175.3 ± 369.1	-9.0 ± 2.4
Carbon coated Copper	NanoAmor	25	252.8 ± 7.5	446.7 ± 121.4	392.3 ± 89.9	-6.2 ± 0.7

* Provided by Manufacturer

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

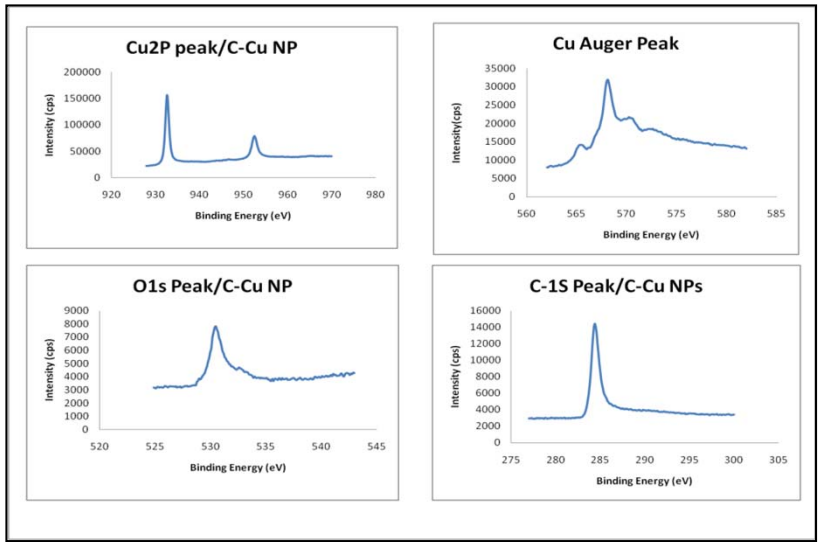


1. Ni 2. C-Ni 3. Cu 4. C-Cu MNPs. Nanoparticles were suspended at concentration of 10 µg/ml in DI water for this analysis. The average particle sizes measured by TEM correlates with the dynamic light scattering data.

XPS Analysis: Cu NP and C-Cu NP

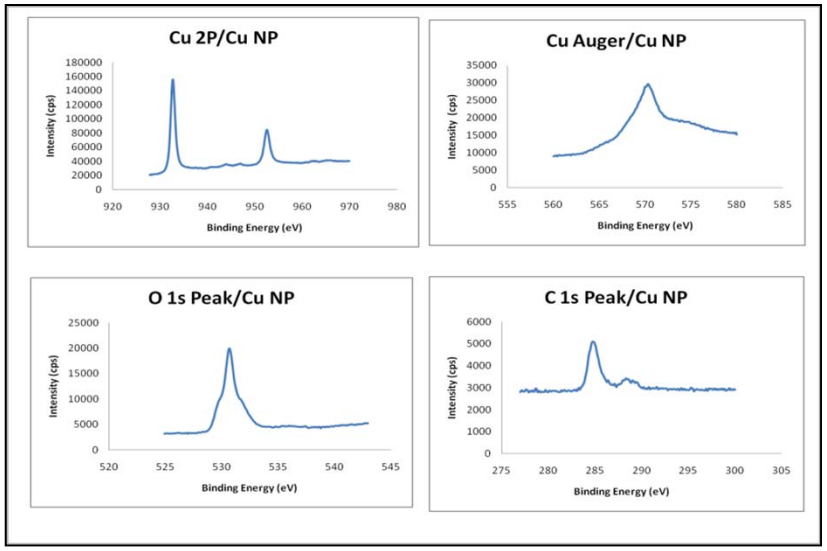
XPS analysis was carried out for analyzing the composition of top 5 nm of the nanoparticles

C-Cu NP



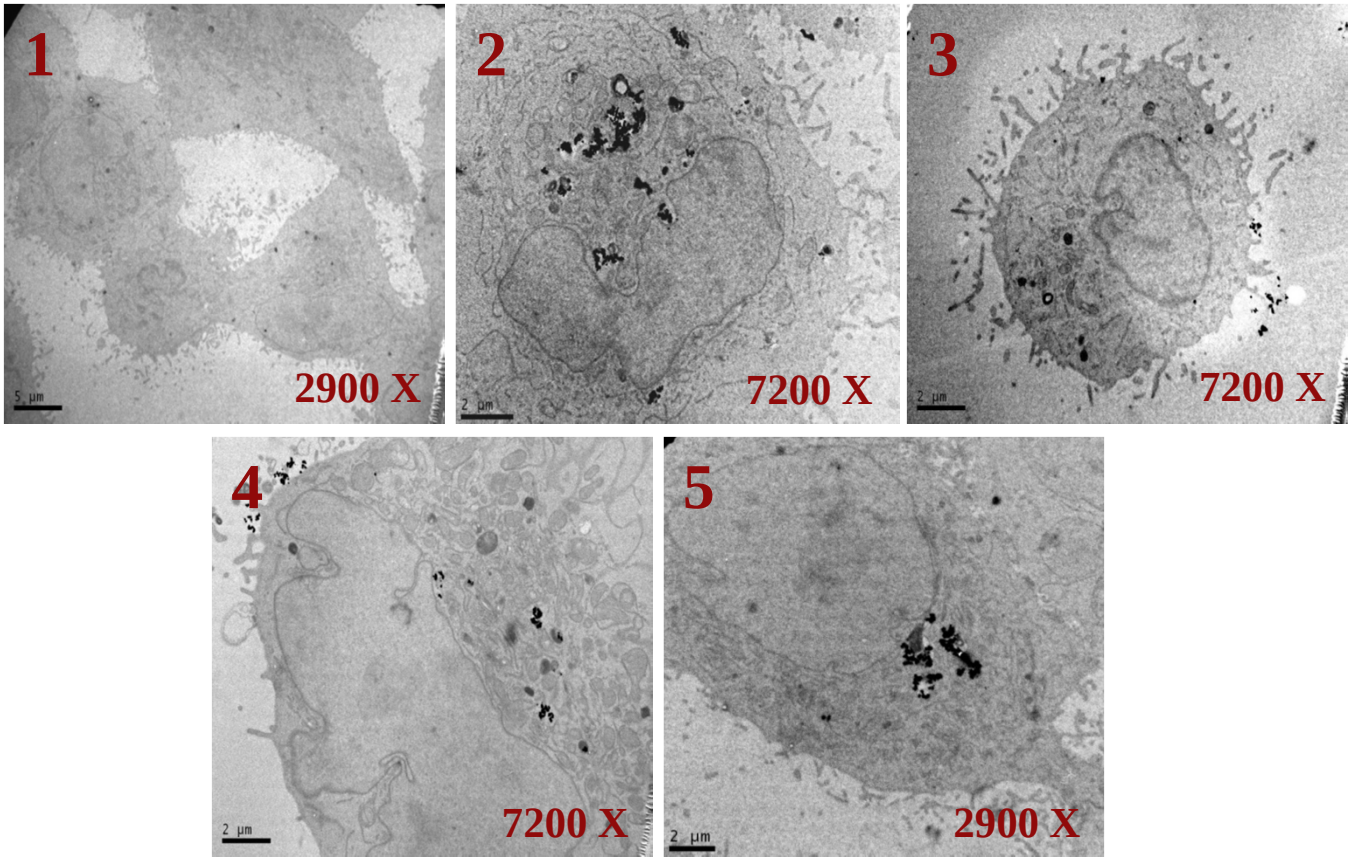
CU 2P peak was observed at 932.7 eV and Cu auger peak was observed at 570.3 eV. The relative percent atomic concentrations of various elements were Cu 2P (36.3), C1S (54.95) and O1S (8.68).

Cu NP



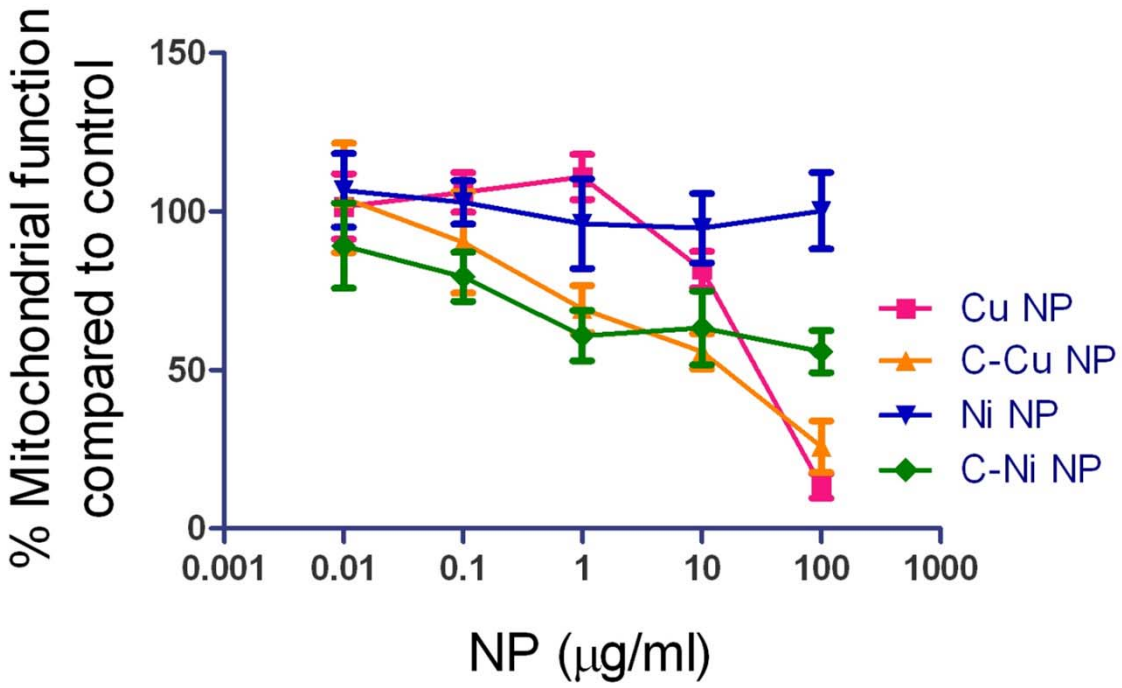
CU 2P peak was observed at 932.7 eV and Cu auger peak was observed at 568.07 eV. The relative percent atomic concentrations of various elements were Cu 2P (48.33), C1S (17.67) and O1S (34).

Cell Uptake Analysis by TEM



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 10 µg/ml for 8 hr.

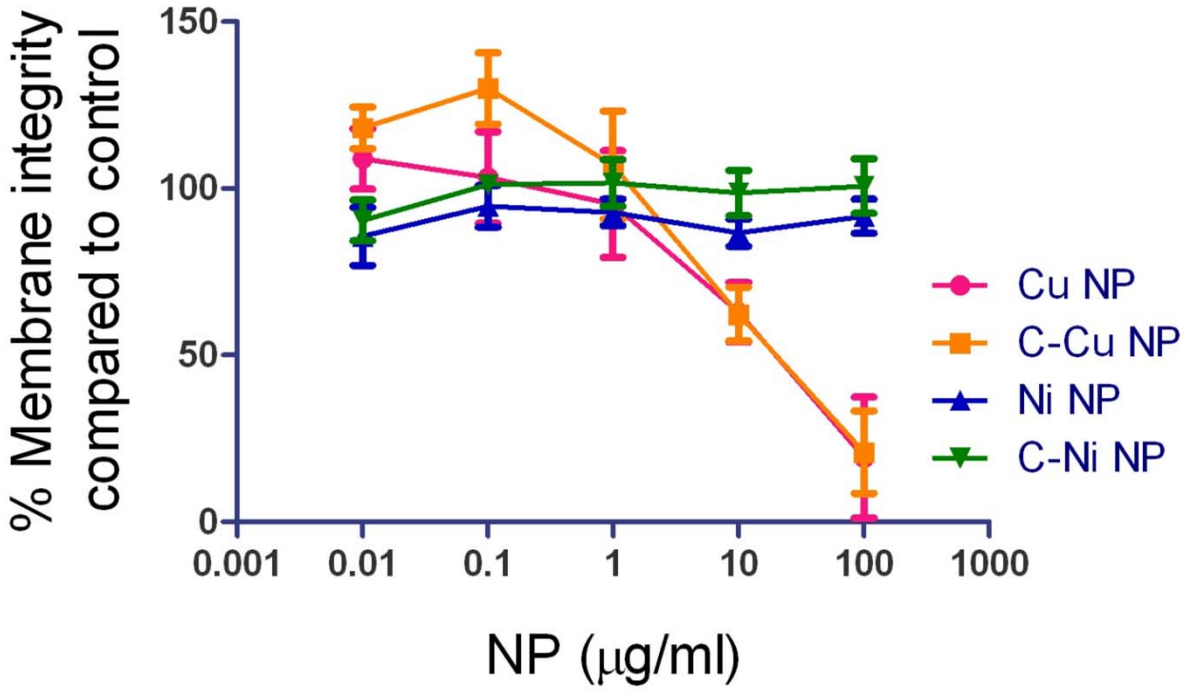
Mitochondrial Function



- A549 cells (25,000 per well), incubation with NPs for 24 hr.
- Data corrected for absorbance from blank NPs.
- Ni NPs significantly differ from C-Ni NPs at all doses.
- Cu NPs significantly differ from C-Cu NPs at 0.1, 1 and 10 µg/ml.

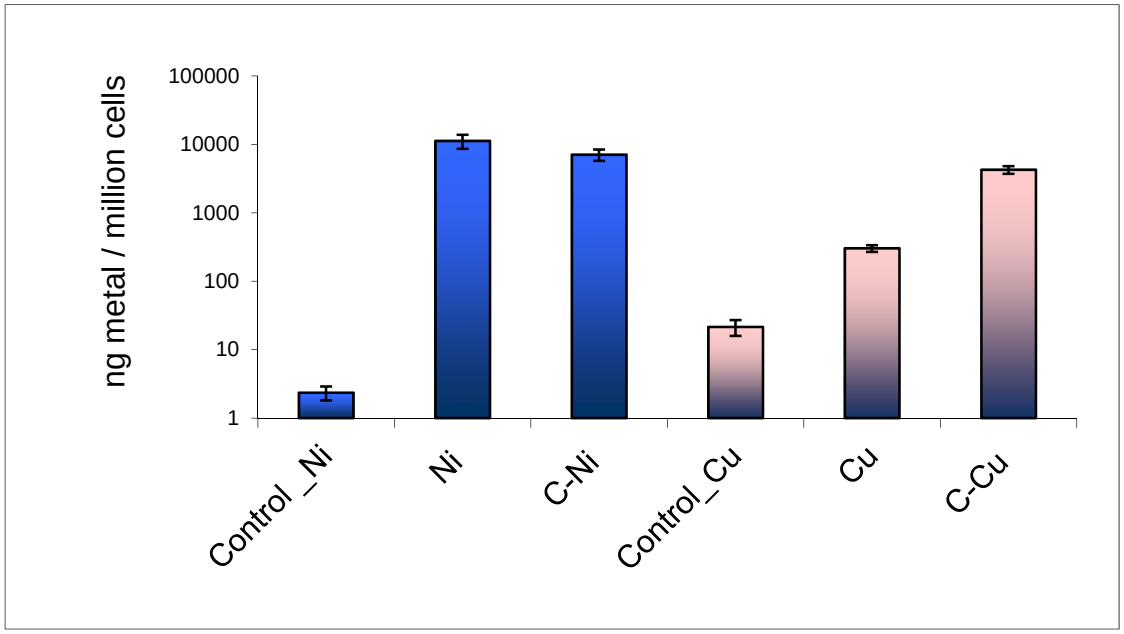
($p < 0.05$ as compared to control)

Neutral Red Membrane Integrity Assay



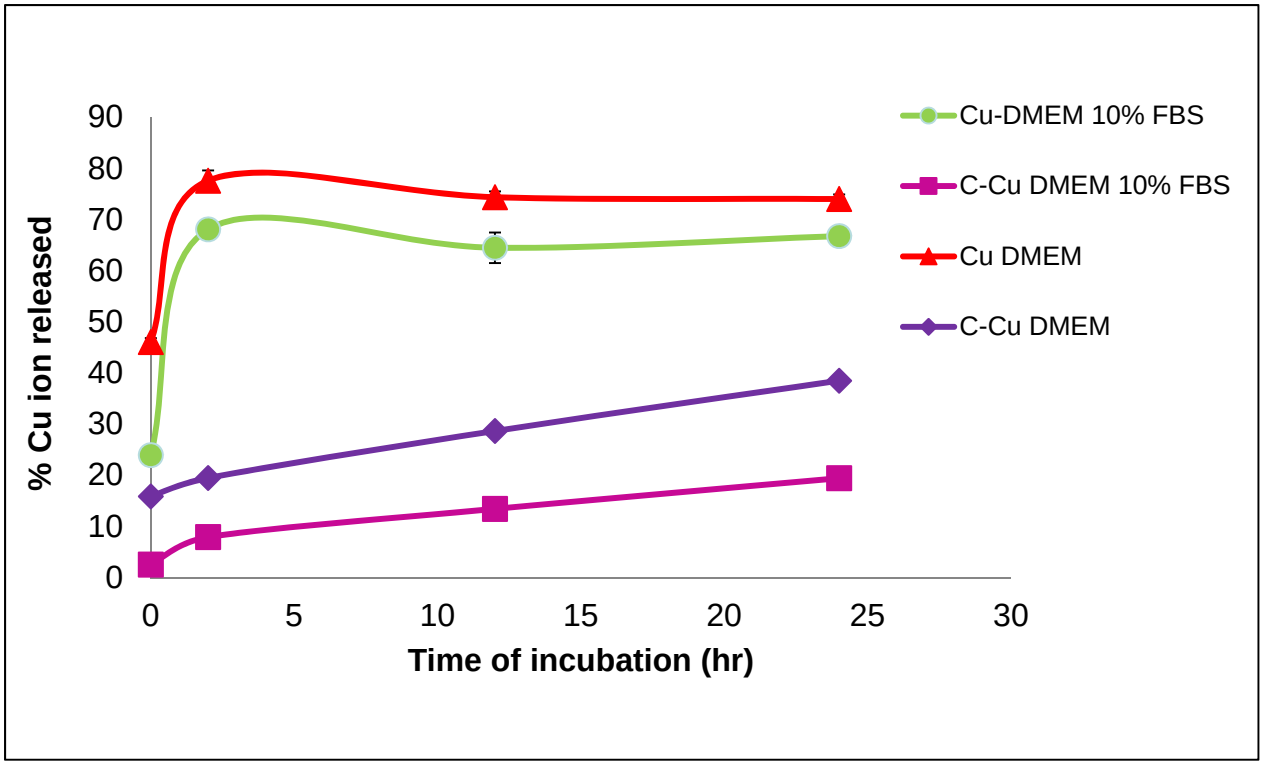
- A549 cells (25,000 per well), incubation with NPs for 24 hr.
- Data corrected for absorbance from blank NPs.
- Ni NPs and C-Ni NPs do not alter membrane integrity.
- Cu NPs and C-Cu NPs were found to be equally toxic in this assay.

Cell Uptake Analysis by ICP-MS



- Control_Ni and Control_Cu signify the amount of respective metal content in untreated control A549 cells.
- Nanoparticles were tested at concentrations of 10 µg/ml for 8 hr in A549 cells.
- Ni uptake from Ni and C-Ni NPs is comparable.
- Cu uptake from C-Cu NPs is an order of magnitude higher than uptake from Cu NPs.

Cu Ion Release from Cu and C-Cu NPs



Kinetic analysis of Cu ion release quantified from Cu and C-Cu NPs at 50 µg/ml concentration in 2 ml cell culture medium with and without 10% FBS at 37º C.
C-Cu NP releases on average 6-7 fold lower amount of Cu when compared to C-Cu NPs due to carbon coating. Coating of nanoparticle surface due to presence of proteins in FBS may be responsible for lower release of Cu from both Cu and C-Cu NPs

Conclusions-Subtask 1

- NPs were characterized for particle size, charge, morphology, quantitative and qualitative cell uptake.
- Particle size analysis shows that the NP sizes were about 20 fold higher than the average particle size provided by the manufacturer. Surface composition analysis confirmed the presence of carbon coating on the surface of C-Cu and C-Ni NPs.
- Cu and C-Cu NPs were found to be more toxic than Ni and C-Ni NPs.
- For C-Cu NPs, the presence of carbon coating retards the release of Cu ions and facilitates cell uptake.
- Although, both Cu and C-Cu NPs are eventually equally toxic at higher doses in MTT and membrane integrity assay; comparative higher cell uptake, lesser Cu ion release and more gradual alteration of mitochondrial function exhibited by C-Cu NPs suggests the mechanism of toxicity of C-Cu NPs seems to be initiated from within the cells as compared to a more cell surface interaction based mechanism for the Cu NPs.

Subtask 2: Research Hypothesis

1. **The biological effects of MNPs on different types of human cells depend directly on the physical/chemical/geometrical properties of the MNPs.**

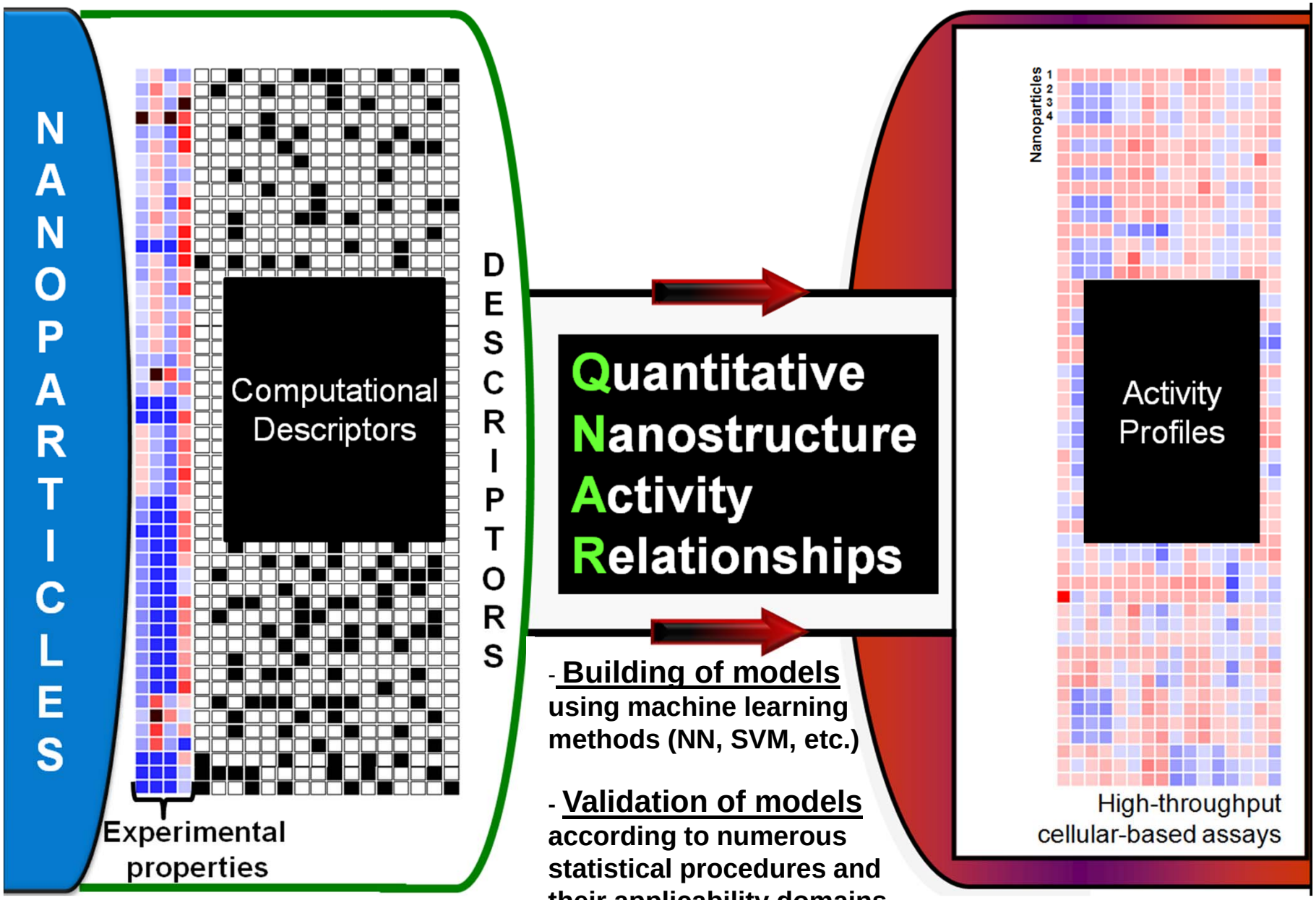
2. **High-throughput cellular-based assays with endpoints within 2-6 hr provide useful and predictive information about long-term biological properties of MNPs.**

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

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

→ **Fundamental, comprehensive and predictive knowledge of the nanotoxicology of MNPs;**

→ **Improvements of experimental design and prioritized toxicity testing to obtain safer and more efficient MNPs.**



- **Building of models** using machine learning methods (NN, SVM, etc.)

- **Validation of models** according to numerous statistical procedures and their applicability domains.

Fourches D, Pu D, Tropsha A. *Comb Chem High Throughput Screen*. **2011** Jan 26. [Epub ahead of print]

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2010 Oct 26;4(10): 5703-12.

ARTICLE

Quantitative Nanostructure—Activity Relationship Modeling

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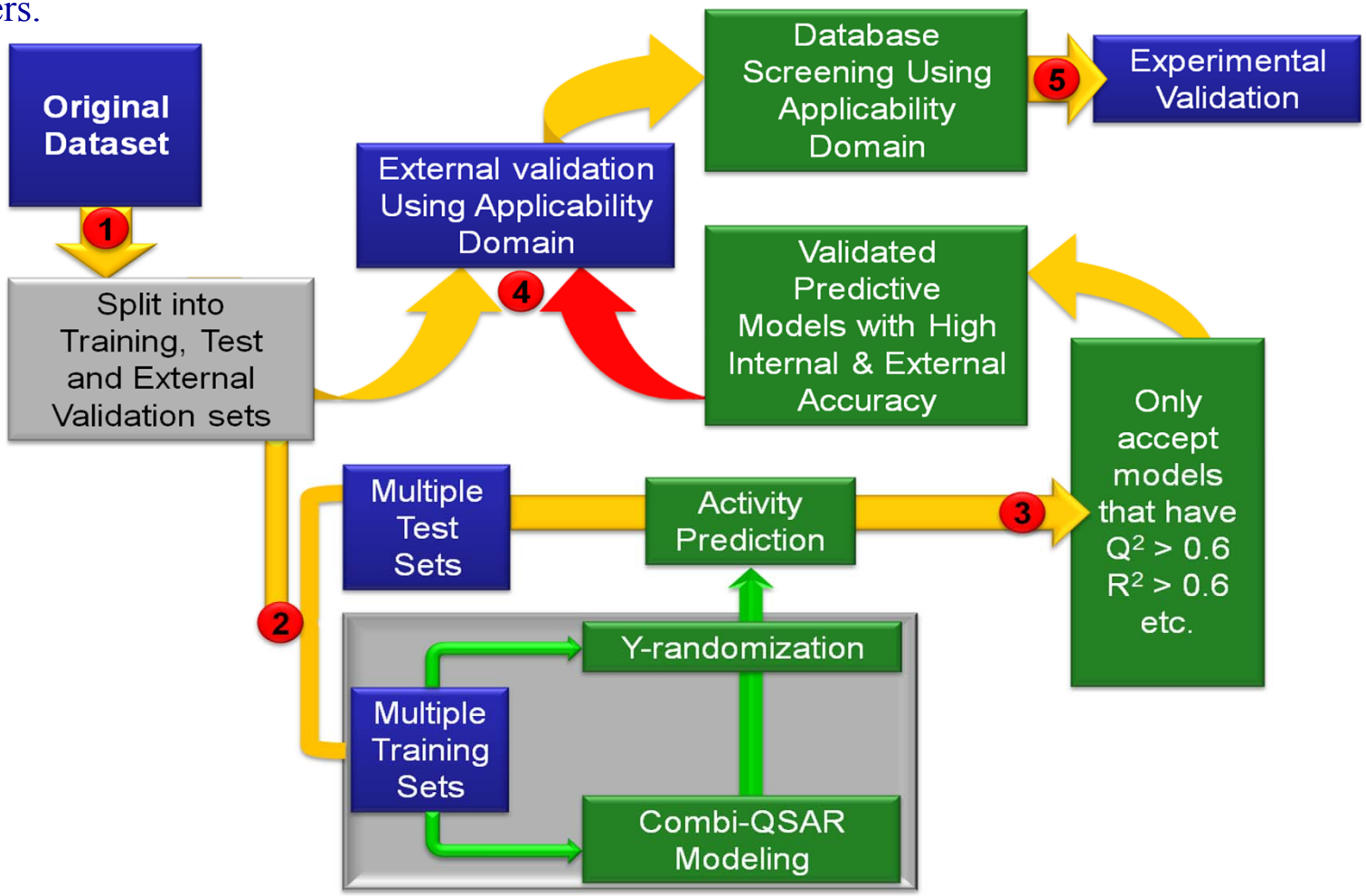
ABSTRACT Evaluation of biological effects, both desired and undesired, caused by manufactured nanoparticles (MNPs) is of critical importance for nanotechnology. Experimental studies, especially toxicological, are time-consuming, costly, and often impractical, calling for the development of efficient computational approaches capable of predicting biological effects of MNPs. To this end, we have investigated the potential of cheminformatics methods such as quantitative structure—activity relationship (QSAR) modeling to establish statistically significant relationships between measured biological activity profiles of MNPs and their physical, chemical, and geometrical properties, either measured experimentally or computed from the structure of MNPs. To reflect the context of the study, we termed our approach quantitative nanostructure—activity relationship (QNAR) modeling. We have employed two representative sets of MNPs studied recently using *in vitro* cell-based assays: (i) 51 various MNPs with diverse metal cores (*Proc. Natl. Acad. Sci.* 2008, *105*, 7387—7392) and (ii) 109 MNPs

More than 1000 manufacturer-identified, nanotechnology-based consumer products are currently available on the market (The Woodrow Wilson International Center for Scholars, 2010). A growing fraction of them represent green products intended to achieve efficient and less polluting energy sources.[1] However, some manufactured nanoparticles (MNPs) intended for industrial applications may cause toxic effects in humans,[2−4] and public concern about the safety of MNPs is increasing.[5] Induced biological of

Subtask 2: Validated QNAR Workflow

We followed a standardized, predictive QSAR workflow previously established by our group*. Models' prediction power is assessed by external cross-validation techniques as well as rigorous statistical parameters.

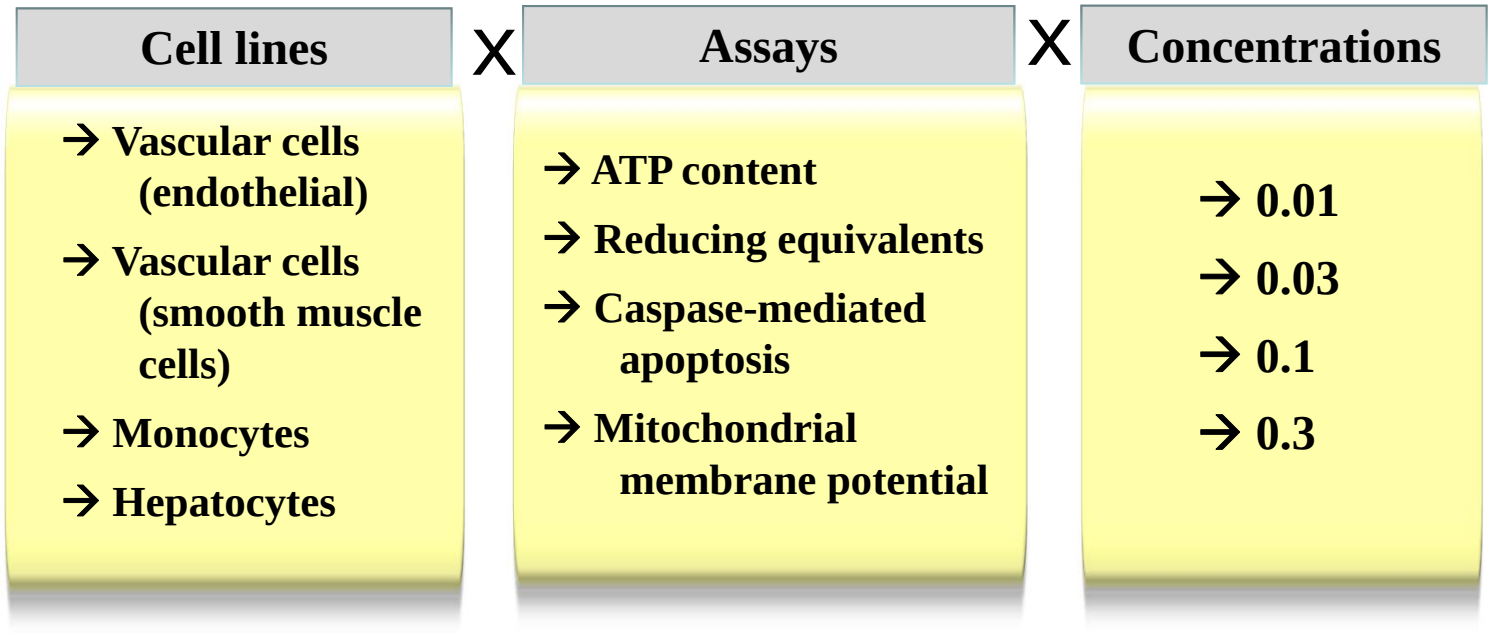


** Tropsha A. Best Practices for QSAR Model Development, Validation, and Exploitation Mol. Inf., **2010**, 29, 476 – 488,*

Case Study 1: QNAR of Whole NPs

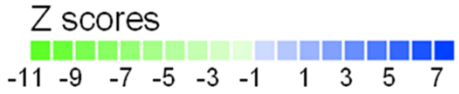
In a recent study[1], 51 diverse NPs were tested *in-vitro* against 4 cell lines in 4 different assays at 4 different concentrations **(→ 51x64 data matrix).**

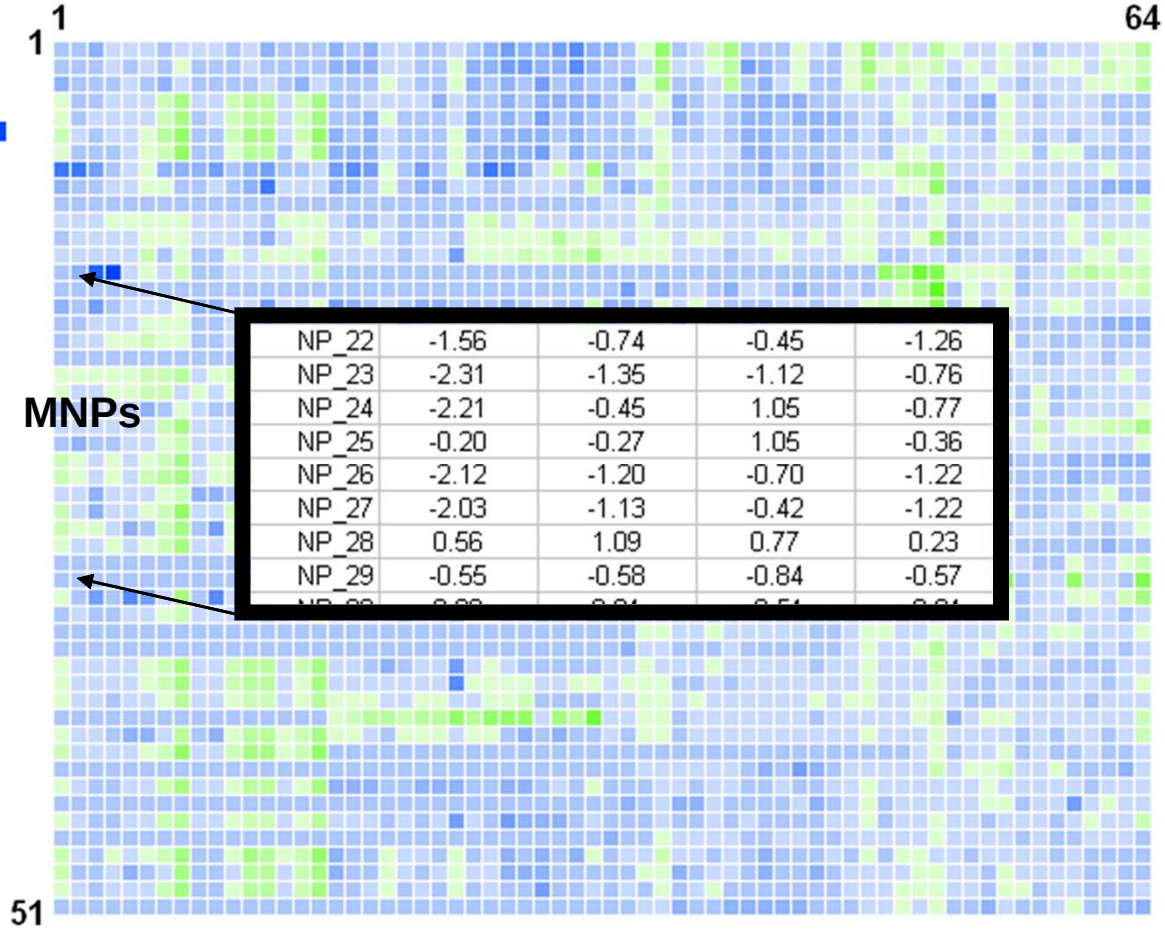
MNP	CLIO	PNP	MION	QD	Feridex IV	Ferrum Hausmann
#. particle	23	19	4	3	1	1



[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: QNAR Matrix and Modeling Results

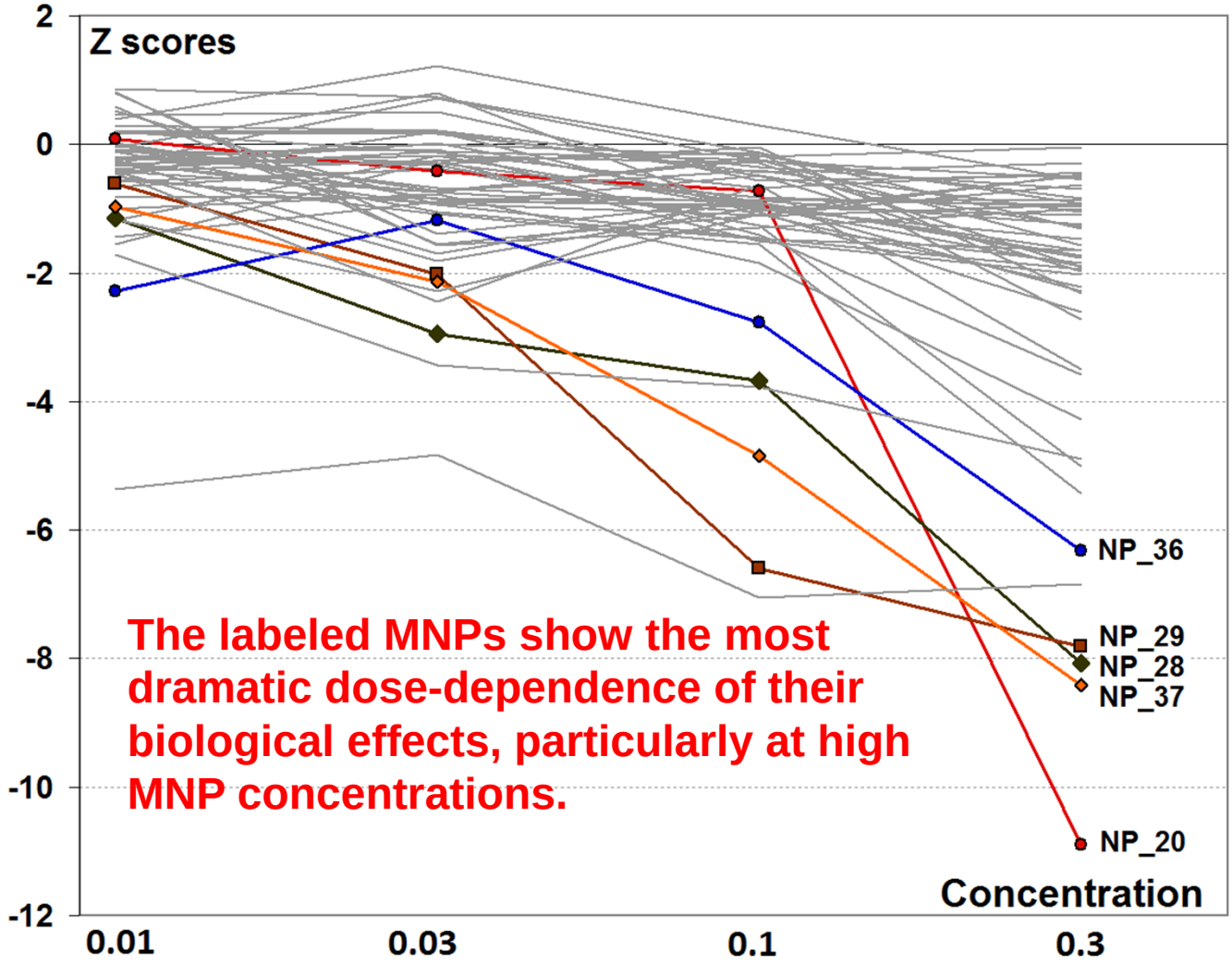
QNAR Matrix

	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**

Analysis of Z score variations for all 51 MNPs tested against AO aorta endothelial cells in the ATP content assay at four different doses

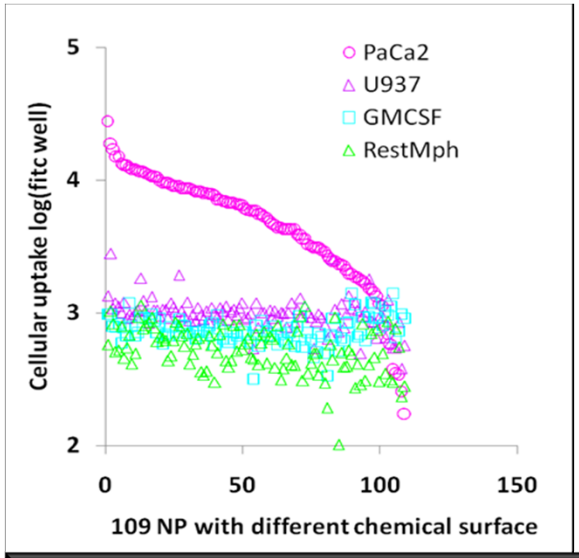


Prediction Outliers For QNAR Models

0.01, 0.03, 0.1, and 0.3 Fe mg/mL for iron-based nanoparticles (NP_1 - 48) respectively; for the three quantum dot-based nanoparticles (NP_49 - 51), concentrations were equal to 1, 3, 30, and 100 nM.

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

In 2005, 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-dextran) were attached with different organic compounds on their surfaces.



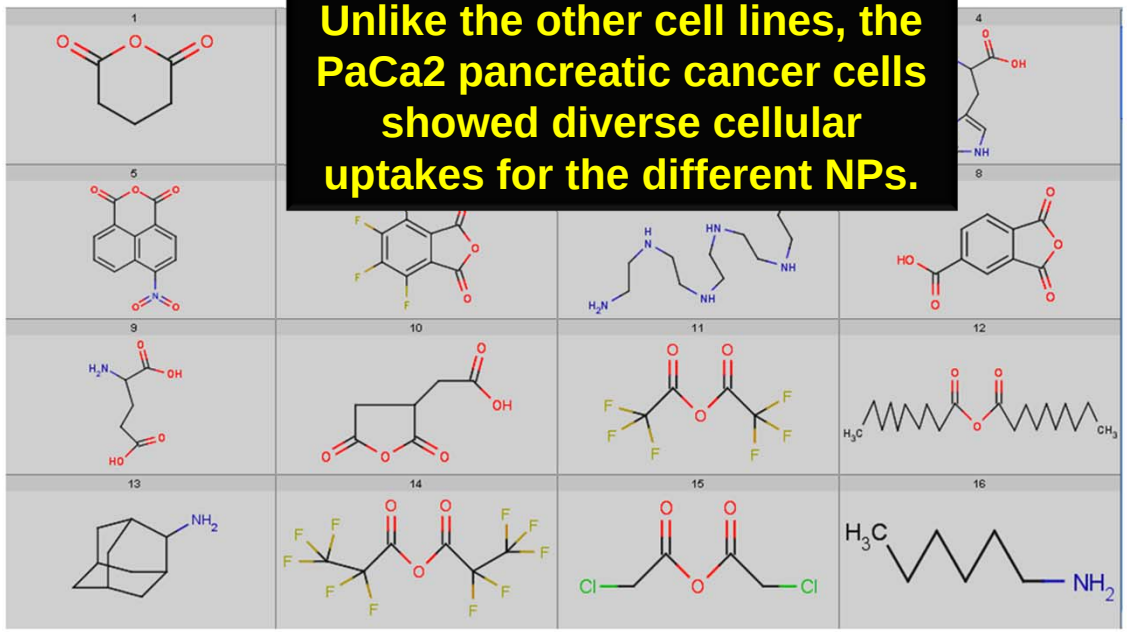
PaCa2: Pancreatic cancer cells

U937: Macrophage cell line

GMCSF: Activated primary human macrophages

RestMph: Resting primary human macrophages

Unlike the other cell lines, the PaCa2 pancreatic cancer cells showed diverse cellular uptakes for the different NPs.

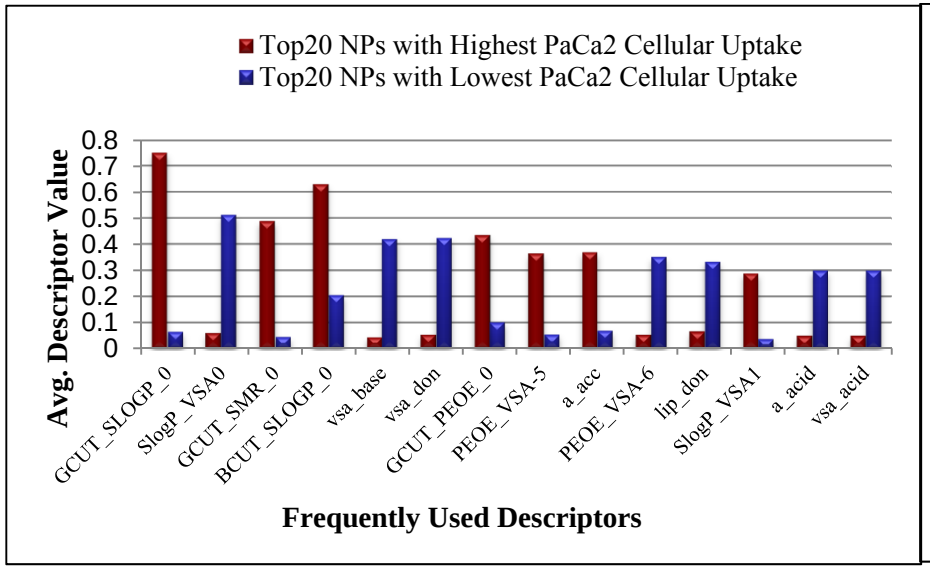


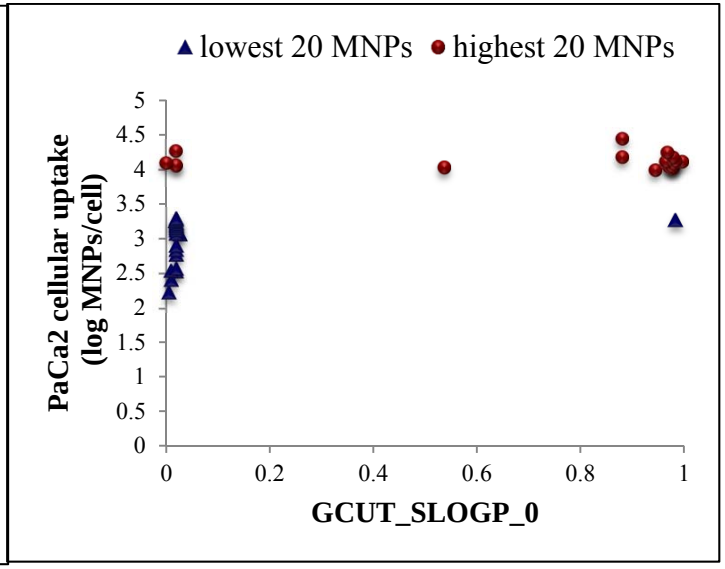
* *Weissleder et al. Nat. Biotechnol., 2005, 23 (11), 1418-1423*

Case Study 2: Modeling Results and Descriptor Analysis

109 surface modifiers and their corresponding PaCa2 uptake.
MML-kNN program using 2D Dragon molecular descriptors
5 fold external cross-validation procedure

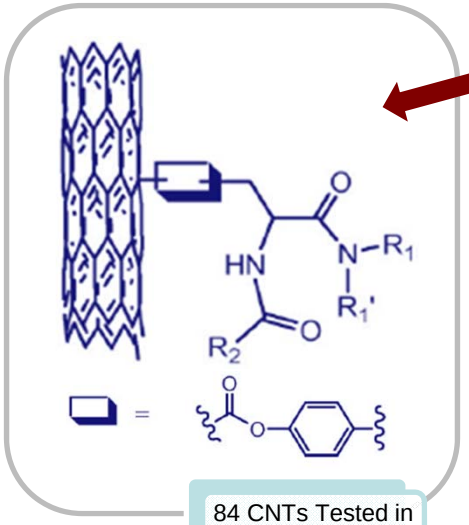
Fold	# comp. model	# comp. external	w/o AD		w/ AD		
			R_o^2	MAE	R_o^2	MAE	% cov
1	87	22	0.65	0.18	0.67	0.18	86
2	87	22	0.67	0.14	0.73	0.13	91
3	87	22	0.72	0.22	0.75	0.21	82
4	87	22	0.75	0.19	0.90	0.14	64
5	88	21	0.80	0.16	0.78	0.17	76
TOTAL			**0.72**	**0.18**	**0.77**	**0.17**	**80**

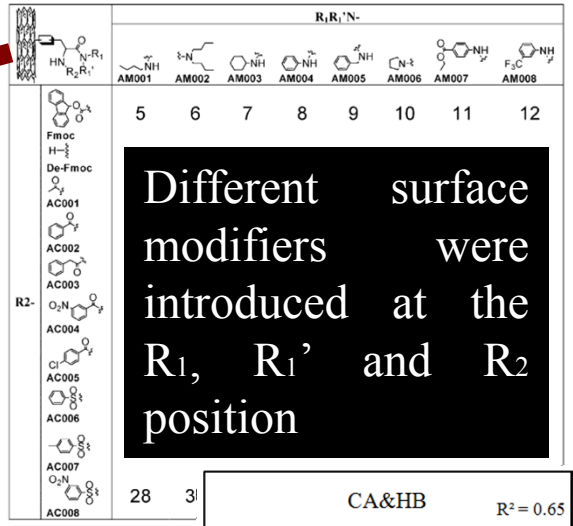




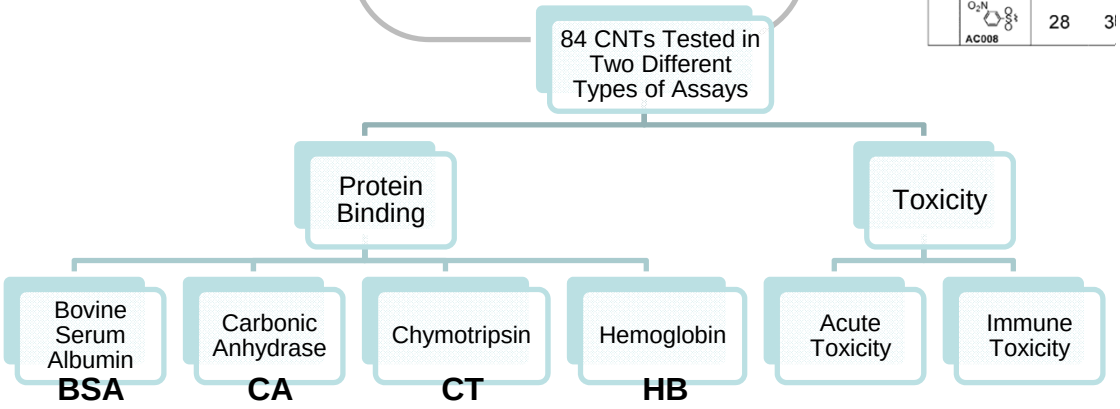
Case Study 3: Modeling of NPs for Protein Binding

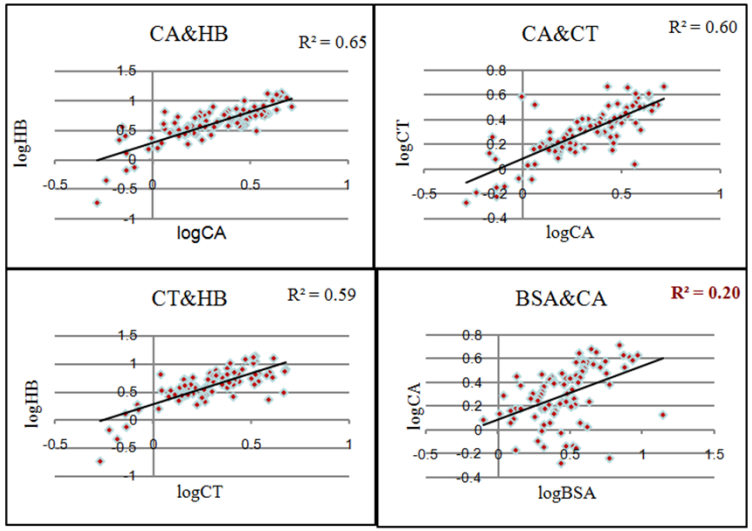
In 2008, Zhou et al* published *in vitro* protein binding, acute toxicity and immune toxicity assays for 84 Carbon NanoTubes (CNTs) decorated with different surface modifiers.





Different surface modifiers were introduced at the R_1, R_1' and R_2 position

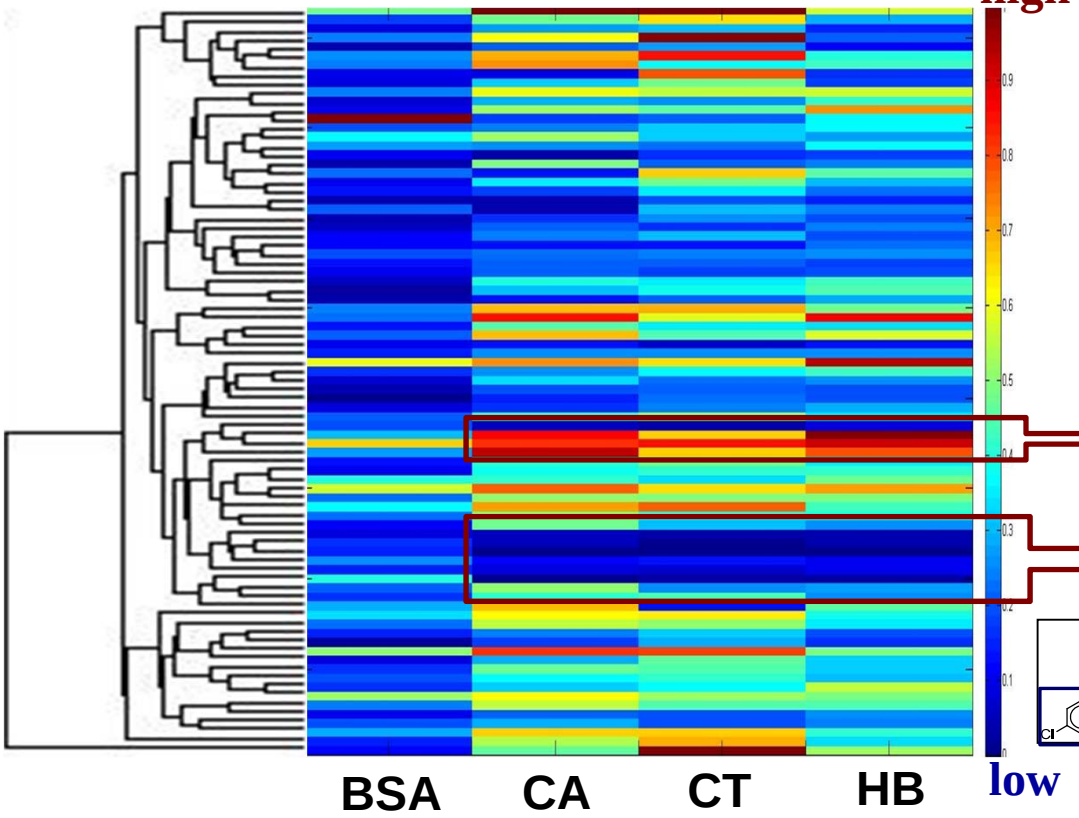




*Zhou et al. Nano Lett., Vol. 8, No. 3, 2008

Case Study 3: Binding profiles sorted according to non-supervised hierarchical clustering of 84 NPs using chemical descriptors

Clustering Uncovers Common Fragments with Distinct Protein Binding Profile.



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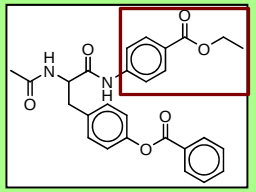
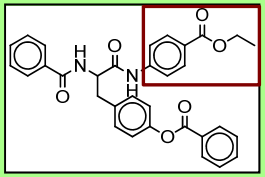


AMOO7AC002



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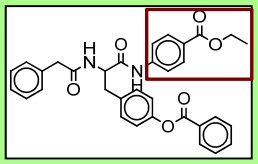

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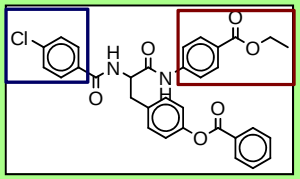
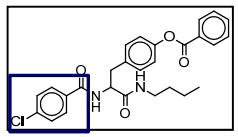


NP ID	BSA Binding	CA Binding	CT Binding	HB Binding
AMOO7AC001	4.0302	4.9276	3.5868	3.3936
AMOO7AC002	4.4012	4.6517	3.5818	4.2787
AMOO7AC003	8.5565	4.4131	4.4598	3.9182
AMOO7AC005	3.0478	0.7487	0.7748	0.3953

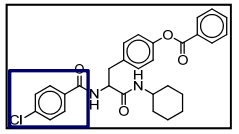
Cluster1: high binding affinities

Cluster2: low binding affinities

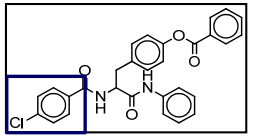
AMOO1AC005



AMOO3AC005



AMOO4AC005



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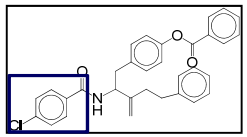

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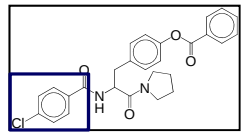
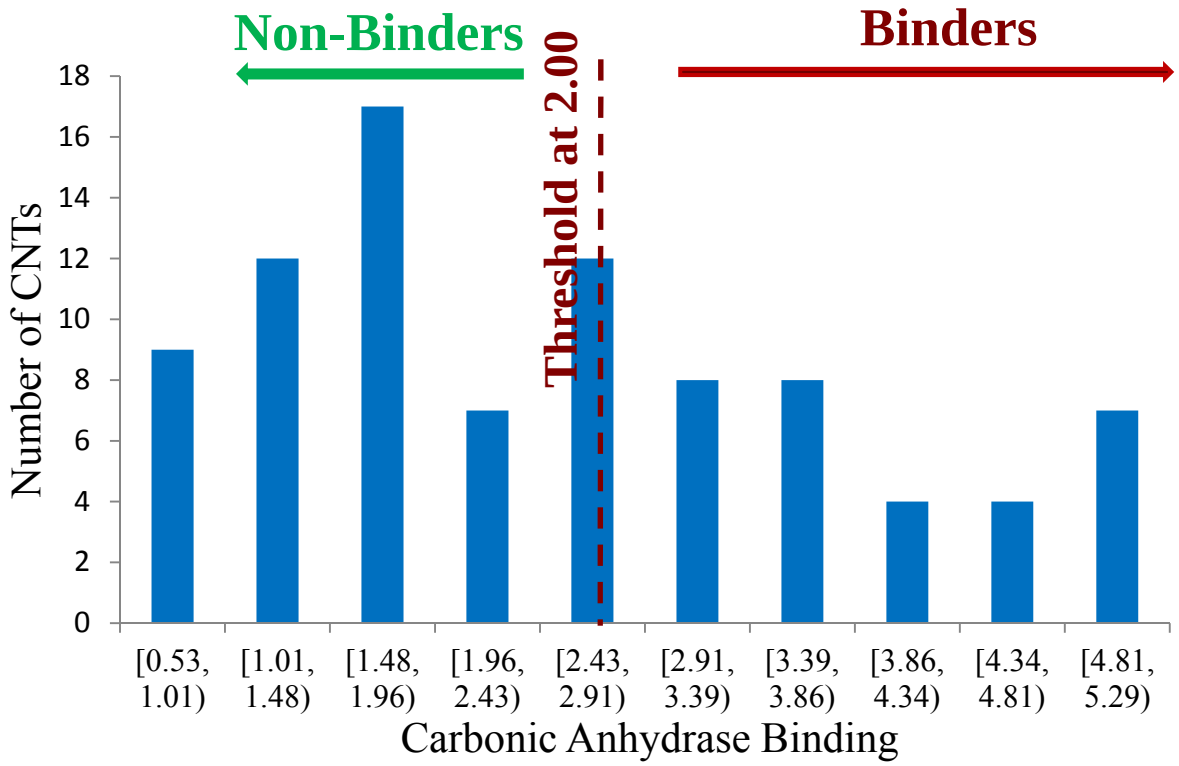


Case Study 3: QNAR Modeling of Carbonic Anhydrase Binding



Each CNT is represented by a single copy of its surface molecule.

Consensus modeling approach combining different machine learning methods (k Nearest Neighbors, Support Vector Machines and Random Forest) and different types of chemical descriptors (Dragon and MOE).

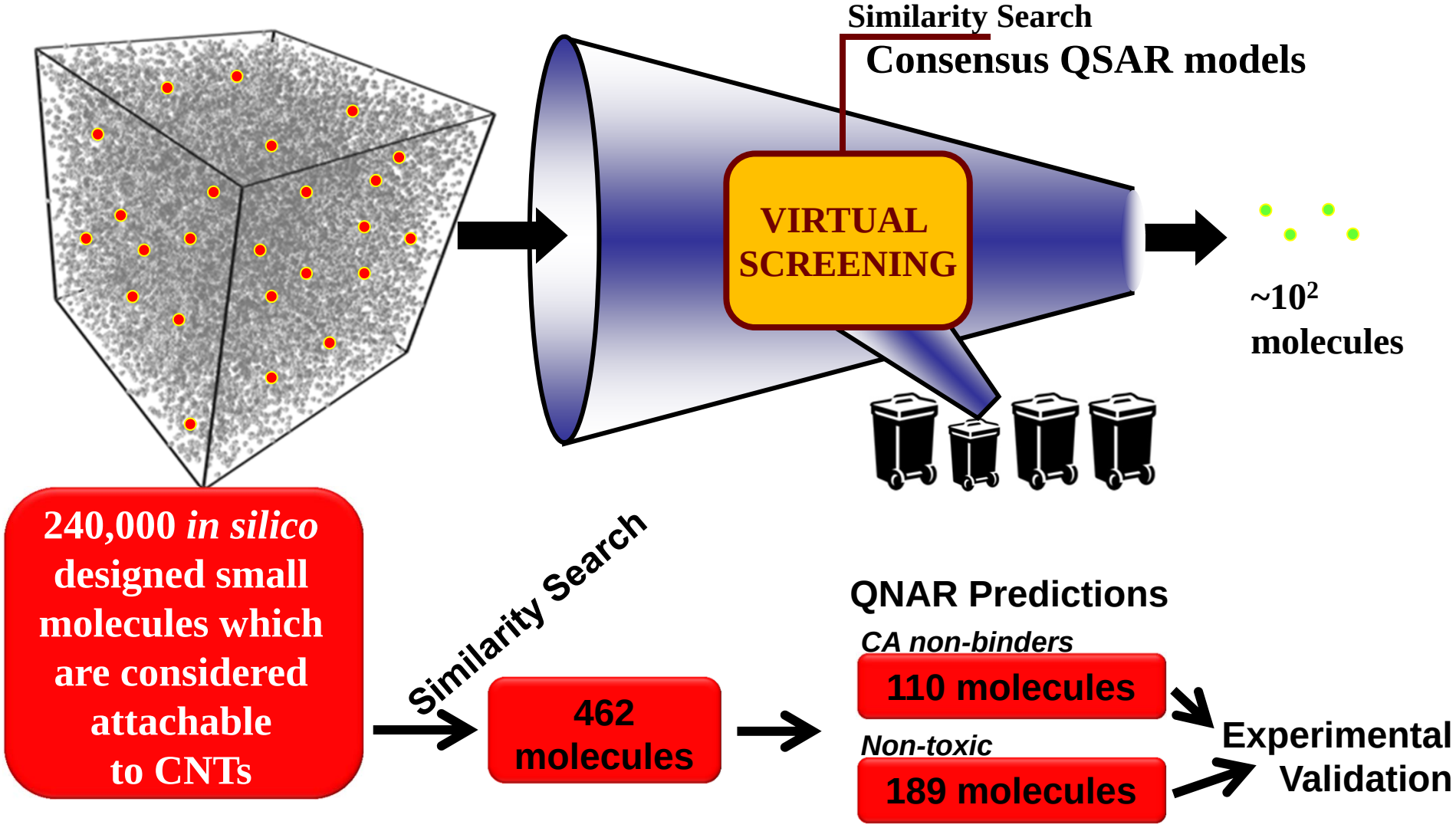
Case study 3: QNAR Modeling of CA Binding

		kNN-Dragon	SVM-Dragon	RF-Dragon	kNN-MOE	SVM-MOE	RF-MOE
F1	Sens.	0.70	0.70	0.70	0.70	0.70	0.70
	Spec.	0.83	0.83	0.83	0.83	0.67	0.83
	Accr.	0.75	0.75	0.75	0.75	0.69	0.75
F2	Sens.	0.80	0.60	0.80	0.80	0.70	0.80
	Spec.	1.00	1.00	1.00	1.00	0.67	1.00
	Accr.	0.88	0.75	0.88	0.88	0.69	0.88
F3	Sens.	0.88	0.75	0.75	0.50	0.63	0.75
	Spec.	0.63	0.44	0.75	0.63	0.50	0.50
	Accr.	0.75	0.63	0.75	0.56	0.56	0.63
F4	Sens.	0.86	0.86	0.86	0.86	0.86	0.43
	Spec.	0.67	0.56	0.67	0.67	0.44	0.67
	Accr.	0.75	0.69	0.75	0.75	0.63	0.56
F5	Sens.	0.63	0.63	0.63	0.63	0.50	0.63
	Spec.	0.64	0.64	0.55	0.45	0.64	0.55
	Accr.	0.63	0.63	0.58	0.53	0.58	0.58
Total	Sens.	**0.77**	0.70	**0.74**	0.70	0.67	0.67
	Spec.	**0.73**	0.68	**0.73**	0.68	0.58	0.68
	Accr.	**0.75**	0.69	**0.73**	0.69	0.63	0.67

Reasonable prediction performances

Case Study 3: Computer-aided design of novel carbon nanotubes with desired biological properties

(in collaboration with Dr. Bing Yan, St. Jude St. Jude Children's Research Hospital)



Subtask 2: Conclusions

- Preliminary results demonstrate that QNAR models can successfully predict the biological effects of MNPs from their descriptors either experimentally measured (e.g., case 1 study), or calculated (case 2, 3 studies).

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

- **QNAR approach may allow both rational design and prioritization of novel MNPs with desired target (physical and biological) properties.**

Industrial Interactions and Technology Transfer

- **Round Robin Effort**

  Carried out characterization and toxicity analysis of CeO_2 nanoparticles at UNC-CH as part of coordinated effort to test the nanoparticles across different universities.
  Active participation in the teleconference with academic and industrial partners.

- **TECHCON 2010**

  Discussion with Brian Raley (Global Foundaries) and SRC members

- The study in subtask 1 provides insights into differential physicochemical properties of the nanoparticles due to surface carbon coating which was also linked to the differences observed in the toxicity mechanisms of these nanoparticles. Furthermore a dose dependent and kinetic analysis of toxicity of Cu and C-Cu NPs is provided to aid informed decisions on the use and monitoring concentrations of these nanoparticles in semiconductor manufacturing operations as the toxicity was not just a function of the released Cu ion fraction.

- **Subtask 2: Chemical identities of CNT modifiers predicted to favor lower protein binding with low toxicity were transferred to Dr. Bing Yan (St Jude Hospital) for experimental validation.**

Next Year Plans

Subtask 1

- **Complete the metal ion release and membrane damage assays for Ni and C-Ni nanoparticles;**
- **Apply the established set of methods to evaluate the toxicity of nanoparticles that are of interest to SRC member companies.**

Subtask 2

- **Establish a database of experimental nanotoxicity data;**
- **Develop extended QNTR models of all available nanotoxicity data (e.g., new datasets are being collected by colleagues at EPA and NTP)**
- **Validate current models by confirmatory experiments.**

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

- "Effect of Carbon Coating on the Physicochemical Characteristics and Toxicity Mechanisms of Carbon Coated and Non Coated Copper and Nickel Nanoparticles" Research manuscript in preparation for submission to ACS Nano.

- Shalini Minocha, Anuraag Sarangi, Alexander Tropsha, Russell J. Mumper. "Physicochemical Characterization and Toxicity Evaluation of Metal-Based Manufactured Nanoparticles." Oral and poster presentations at TECHCON 2010, Austin, TX, September 12-15, **2010**.

- 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**.

- 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**.

- Dongqiuye Pu, Denis Fourches, Alexander Tropsha. Quantitative Nanostructure-Toxicity Relationship (QNTR) Modeling. CMMI Conference, Atlanta, GA, Jan. 4-7.

Publications, Presentations, and Recognitions/Awards (cont.)

- Denis Fourches, Dongqiuye Pu, Russell J. Mumper and Alexander Tropsha. Quantitative Nanostructure-Toxicity Relationship (QNTR) Modeling. *ACS nano*. **2010** Oct 26;4(10): 5703-12. **NOTE: This paper was a subject of spotlight in Nanowerk, http://www.nanowerk.com/spotlight/spotid=18875.php**.

- Denis Fourches, Dongqiuye Pu, Alexander Tropsha. Exploring Quantitative Nanostructure-Activity Relationships (QNAR) Modeling as a Tool for Predicting Biological Effects of Manufactured Nanoparticles. *Combinatorial Chemistry & High Throughput Screening*, **2011**, Jan 26. [Epub ahead of print].

- Denis Fourches and Alexander Tropsha. Quantitative Nanostructure-Activity Relationships (QNAR) Modeling. 241st ACS national meeting, COMP Oral Session, Anaheim **2011**.