

# Nanomaterials



## Immunological Properties of Engineered Nanomaterials and Some Challenges in Their Preclinical Characterization

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# Outline

## Presentation outline

- Definitions
- Nanoparticles in Daily Life
- Nanoparticles for Cancer Diagnosis and Therapy
- Nanomedicine Overview
- NCL overview
- Immunological Properties of Nanomaterials
- Challenges in Preclinical Characterization

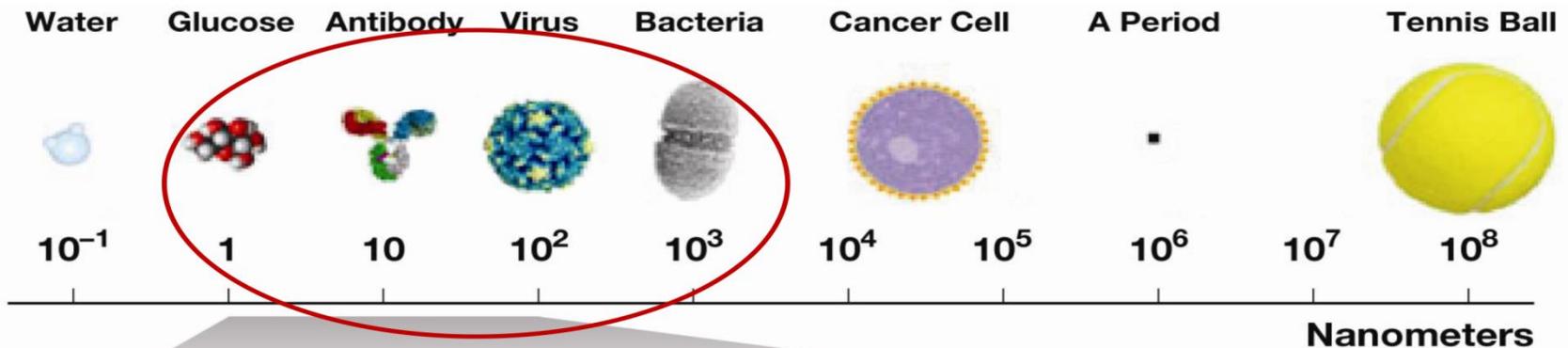
# What is nano?

## What is Nano?

### Nanotechnology:

“Research and technology development at the atomic, molecular or macromolecular scale leading to the controlled creation and use of structures, devices and systems with a length scale of approximately **1 – 100 nanometers** (nm).” (Source: National Nanotech Initiative)

“Whether a material or end product is engineered to exhibit properties or phenomena, including physical or chemical properties or biological effects, that are attributable to its dimension(s), even if these dimensions fall outside the nanoscale range, **up to one micrometer (1,000 nm)**” (US FDA)



# Nanoparticles

## Nanoparticles in Daily Life

### ■ Consumer products

- 800+ “manufacturer identified” products from 400+ companies in 20+ countries
- Clothing, wound dressings, washing machine liners
- Sunglasses (lens coatings)
- Sporting equipment



*Turtle Wax™ makes a nanotech car wax*



*The Adidas Lone Star™ track shoe includes a lightweight spike-plate made of carbon nanotubes*



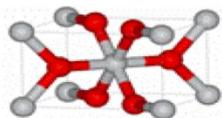
*Nanosilver is in supplements and used to treat clothing*



*Speedo LZR™ Racer Swimsuit is treated in a nanotech cold-plasma process that reduces water absorption.*

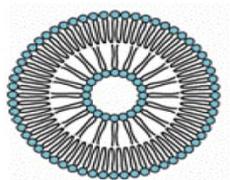
# Nanoparticles in daily life

## Nanoparticles in Daily Life

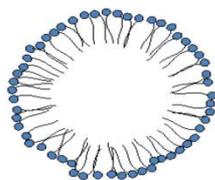


TiO<sub>2</sub>

- Nearly all translucent sunscreens contain nanoscale TiO<sub>2</sub> or ZnO<sub>2</sub>

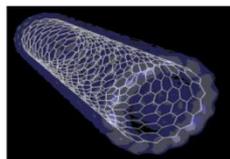


Liposomes



Nanoemulsions

- Liposomes and emulsions are commonly used in cosmetics (L'Oreal holds more than 60 patents related to nanotechnology)

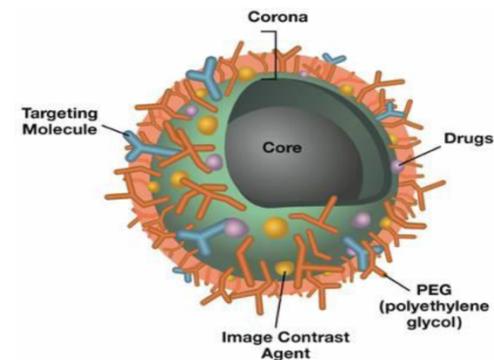


Carbon nanotube

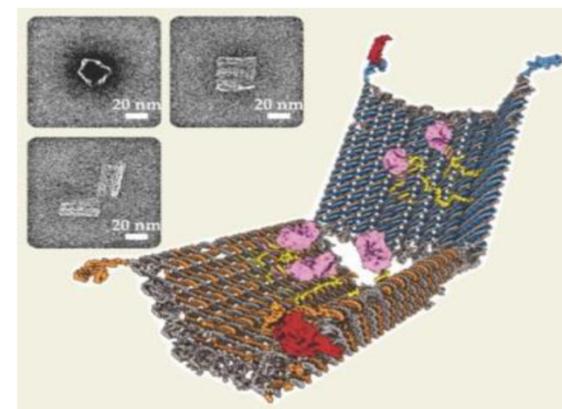
- Carbon nanotubes are used as structural materials

## Cancer Nanotechnology

- Improve solubility; act as a carrier for hydrophobic drugs.
- Multifunctional capability
- Tumor targeting (reduced toxicity)
- Robotic tasks such as sensing, computation, and actuation; triggered responses.



McNeil, (2005), J. Leuk. Biol., 78:585-594



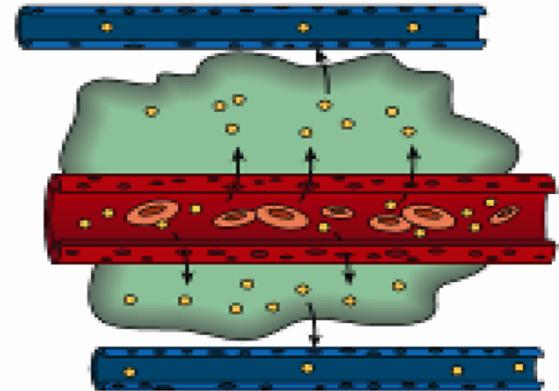
Douglas et al., (2012) Science, 335 831-834.

# Targeting

## Targeting

### EPR Effect = Passive Targeting

- Particulate size causes tumor accumulation
  - Optimization is a mechanical problem
  - “Transport Oncophysics” focuses on understanding the mass transport properties and time dynamics of evolution of biological barriers in cancer.



### Active Targeting

- Surface chemistry allows functionalization w/ targeting molecules
  - Antibodies, e.g. Herceptin
  - Small molecules, e.g. folic acid
  - Cytokines, e.g. TNF- $\alpha$

# Category



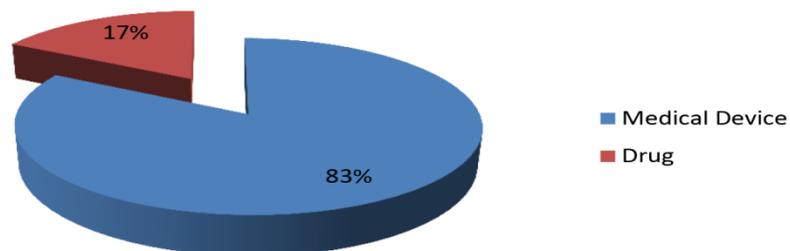
NCI Alliance for  
Nanotechnology  
in Cancer

## Nanomedicine by Category

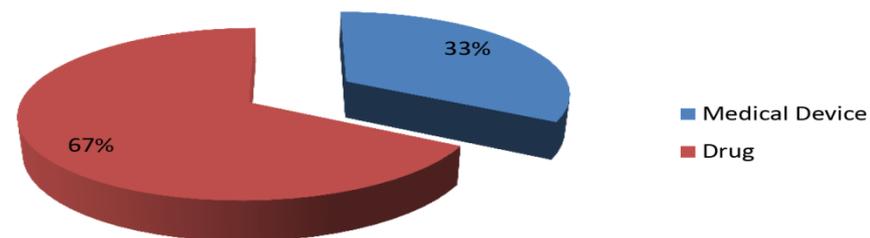


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Nanotechnology  
in Cancer

### Investigational



### Commercial



Proportion of nanoparticle-based drugs and medical devices is different between investigational and approved products

# Nanoparticle type

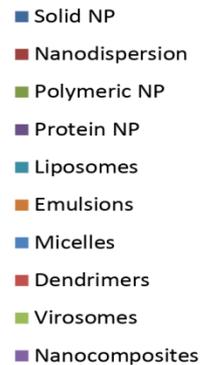
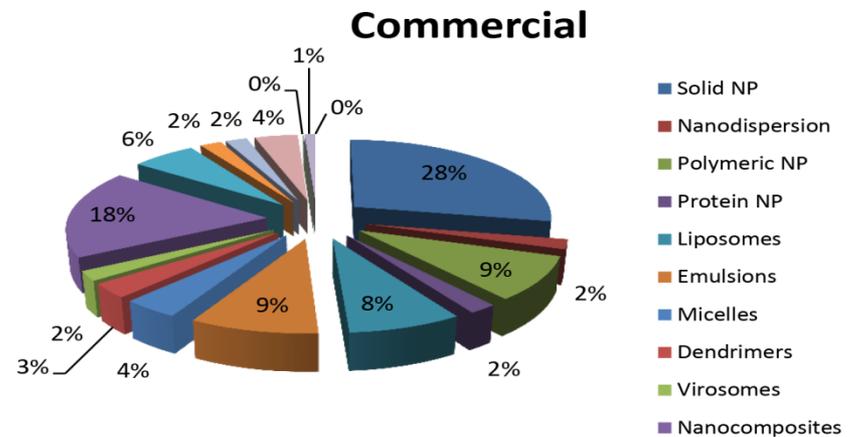
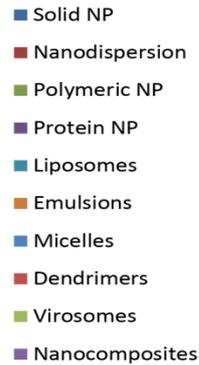
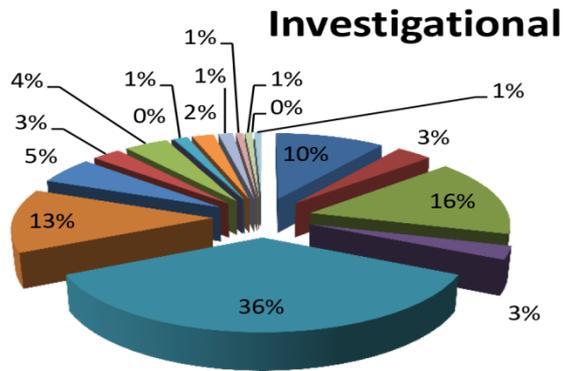


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## Nanomedicine by Nanoparticle Type



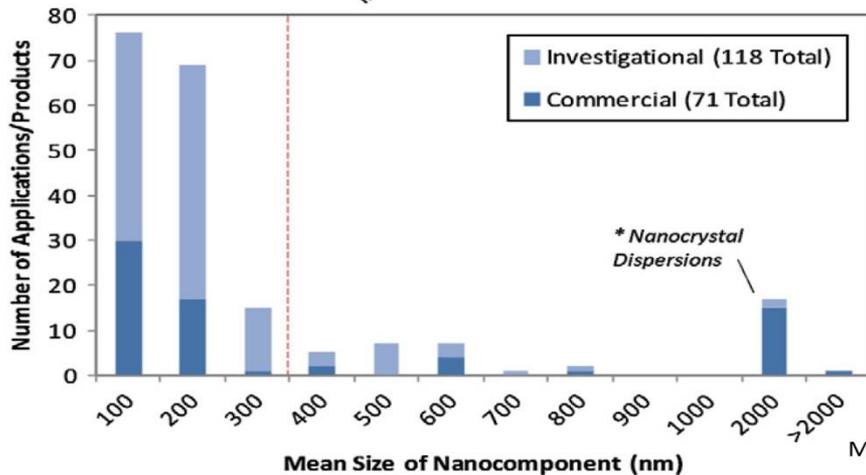
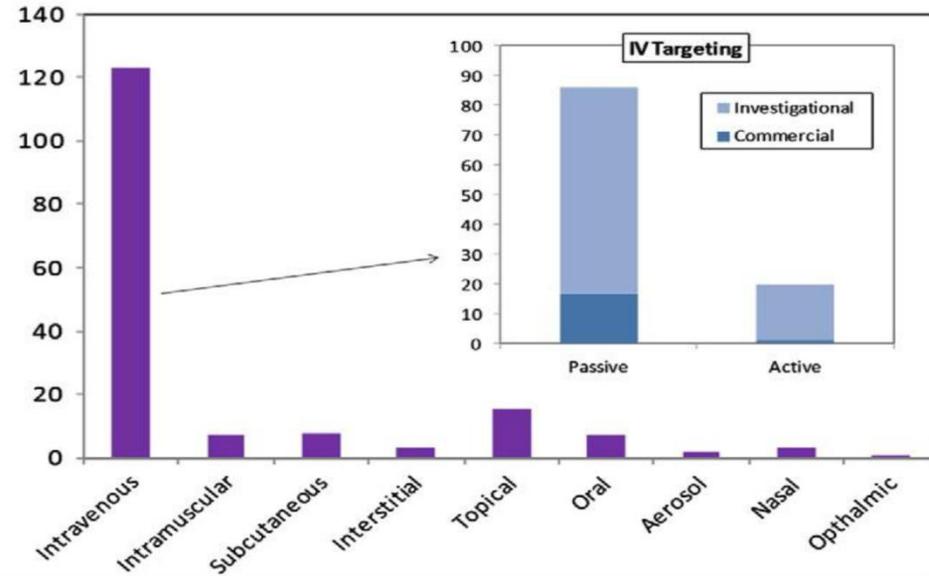
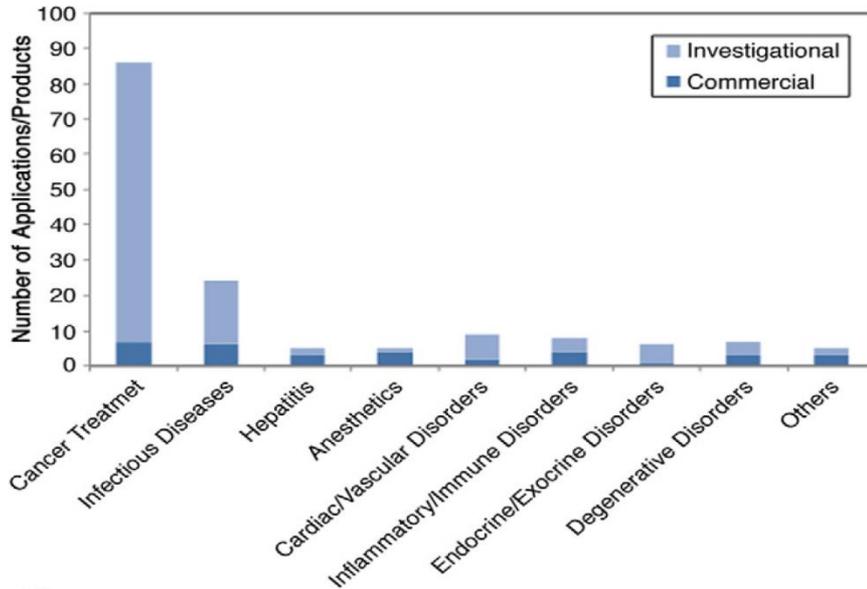
NCI Alliance for  
Nanotechnology  
in Cancer



- Dominant (> 10% of total) Investigational Nanomedicines: Liposomes > Polymeric NP > Emulsions > Solid NP
- Dominant Commercial Nanomedicines: Solid NP > Nanocomposites

# Properties

## Nanomedicine properties



### Common features of Nanomedicines:

- Primary market is cancer therapy
- Intravenous administration
- <350 nm in size
- Neutral, hydrophilic surfaces
- Spherical

# Funding



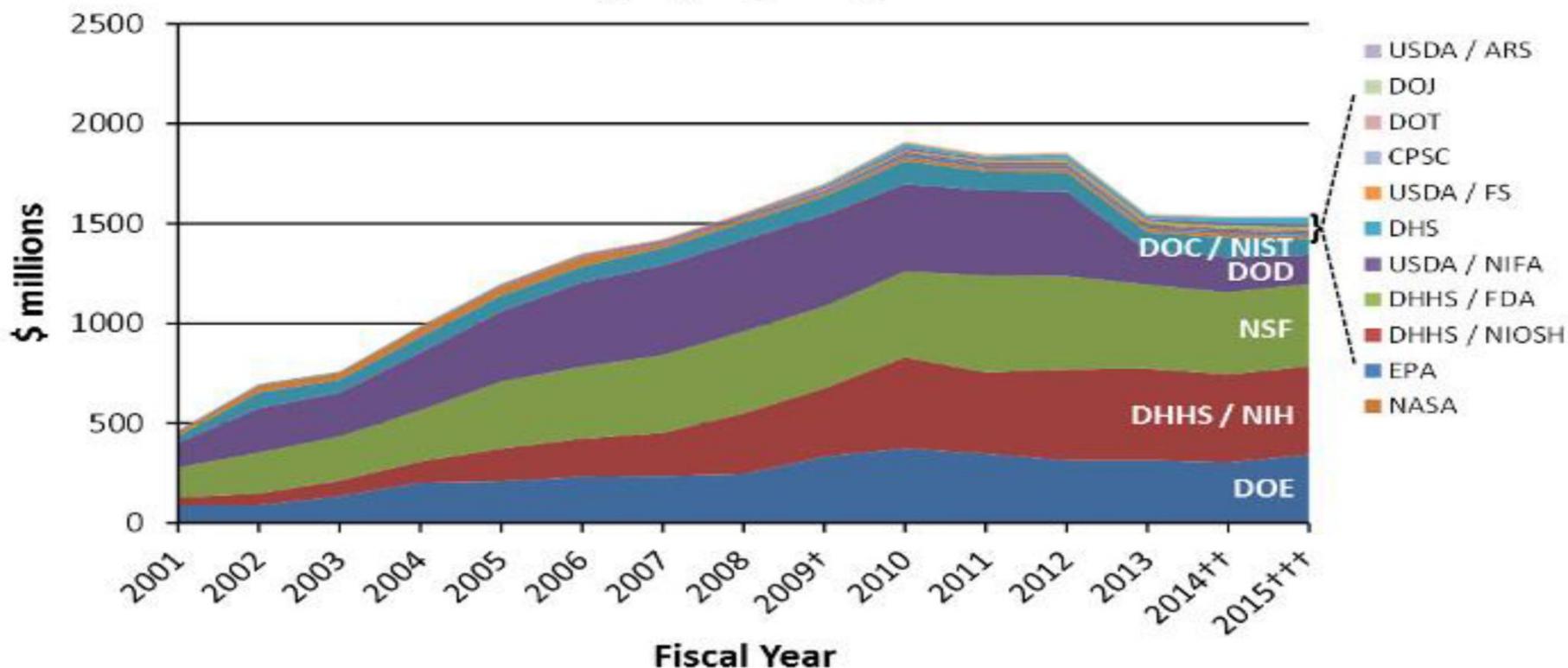
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in Cancer



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in Cancer

## NNI Funding

### NNI Funding by Agency, 2001-2015



† FY '09 does not include American Recovery and Reinvestment Act funds for DOE (\$293 M), NIH (\$73 M), NSF (\$101 M), and NIST (\$43 M)  
 †† FY '14 estimate based on 2014 enacted levels; may change as operating plans are finalized  
 ††† FY '15 request

Source: <http://www.nano.gov>

# Drug development

## Drug Development

Drug Discovery

Clinical Use



*Butler D. Nature. 2008 Jun 12;453(7197):840-2*

“Valley of Death”

- Translation of basic research concepts into clinic is not a fast and easy process
- Crossing “Valley of Death” requires efforts from both academia and industry

## NCI Nanotechnology Characterization Lab

The NCL was established in 2004 as an interagency collaboration among NCI, NIST, and FDA. The lab's mission is to accelerate the translation of promising nanotech cancer drugs and diagnostics.

### R & D Community

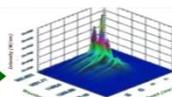


Government  
Big Pharma  
Academic PIs  
Biotech Companies

*Physicochemical  
Characterization*

*In Vitro  
Characterization*

*In Vivo  
Characterization*



### Support



Clinical  
Regulatory  
Commercialization

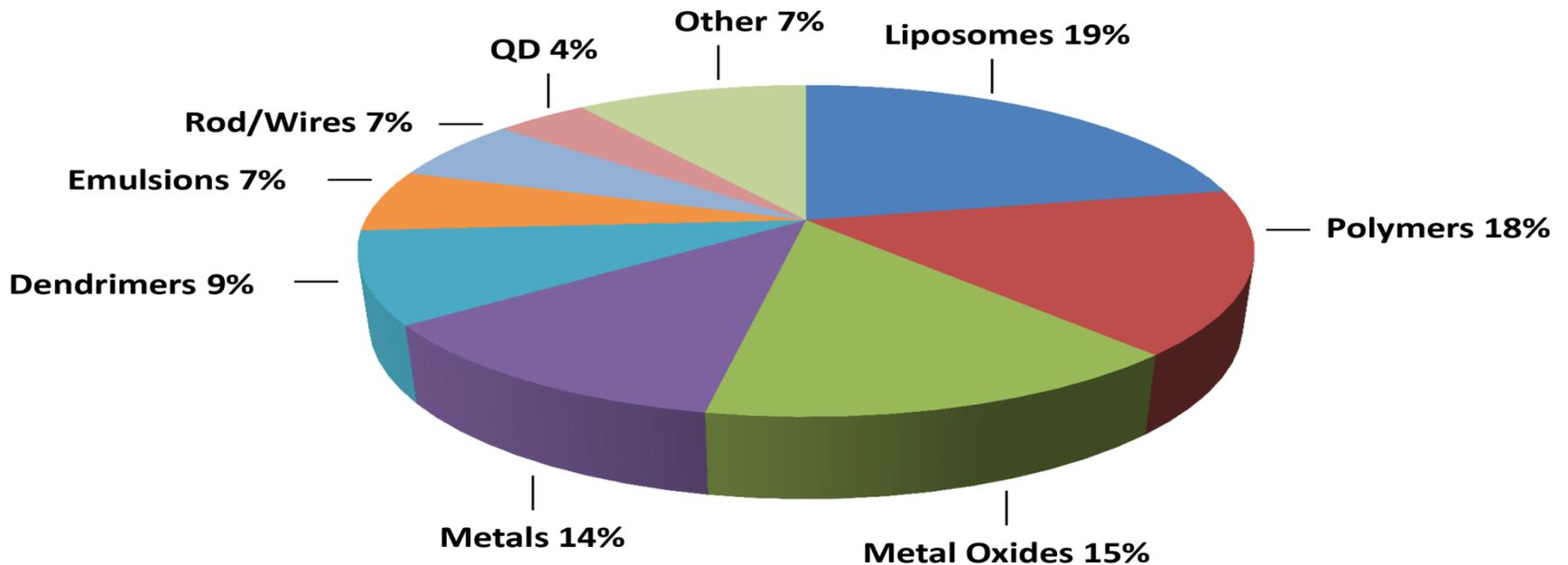
### Objectives:

- Characterize nanoparticles using standardized methods;
- Conduct structure activity relationships (SAR) studies;
- Facilitate regulatory review of nanotech constructs;
- Engage in educational and knowledge sharing efforts.

**Nanotech expertise & resources in multiple disciplines brought together in one location to serve ALL nanotech oncology researchers.**

# Nanomaterials

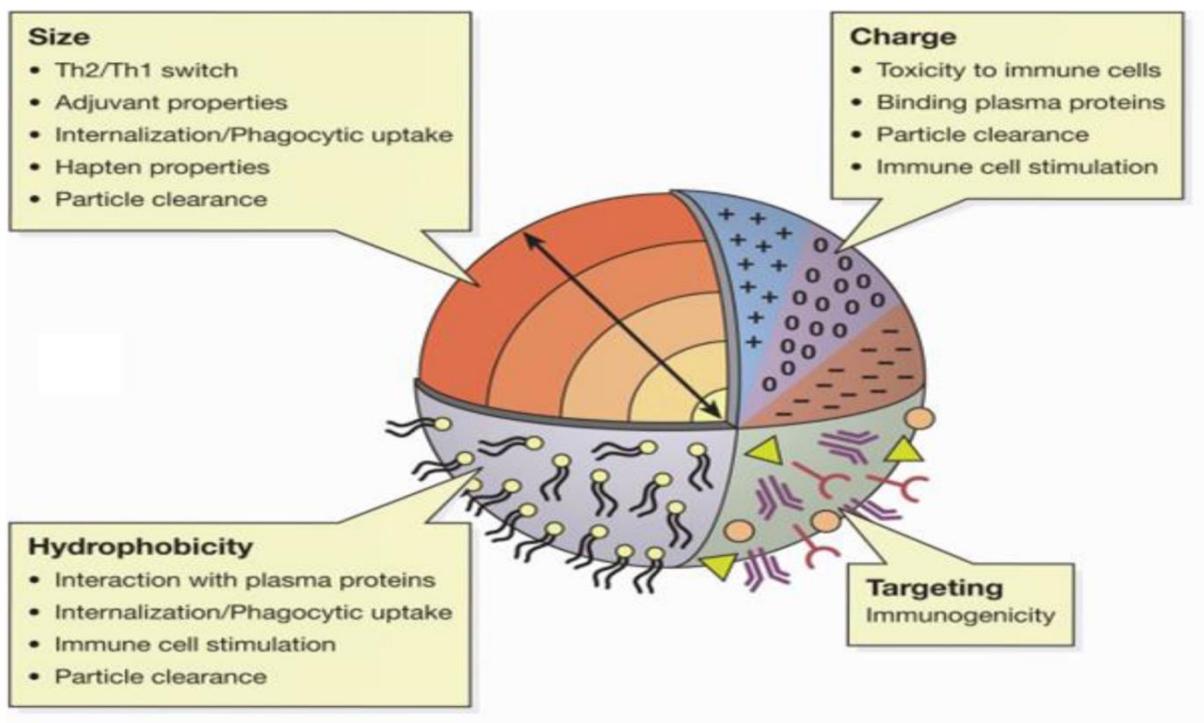
## NCL Characterized Variety of Nanomaterials



*Other nanomaterials include fullerenes, micelles, carbon nanotubes, nanocrystals, and more.*

- More than 300 different nanoparticles have been submitted to the NCL
- Case studies using some of these materials are reviewed in this presentation

## Physicochemical Properties



**Physicochemical properties determine nanoparticle immunotoxicity**

# PARTICLE SIZE

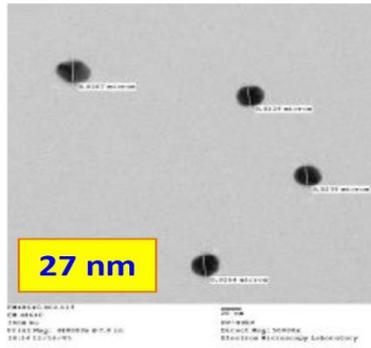
Size is important. Less surface amines leads to less platelet aggregations

# Size

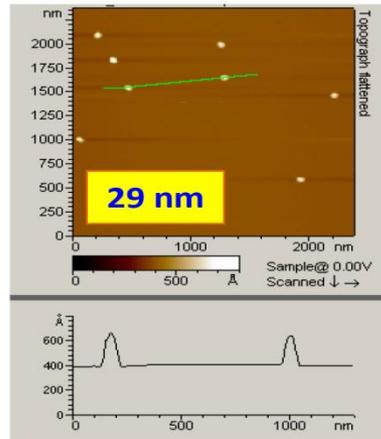
## Size in a Biological Context

### 30 nm Gold colloids in PBS

TEM

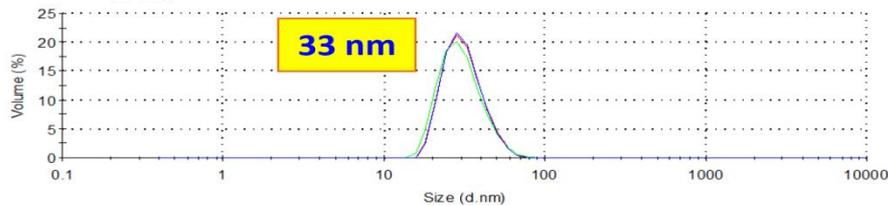


AFM



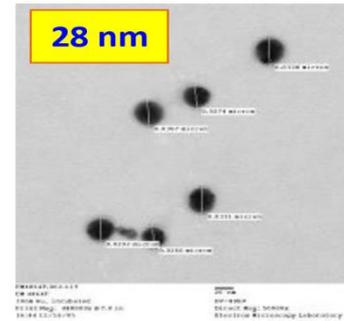
DLS

Size Distribution by Volume

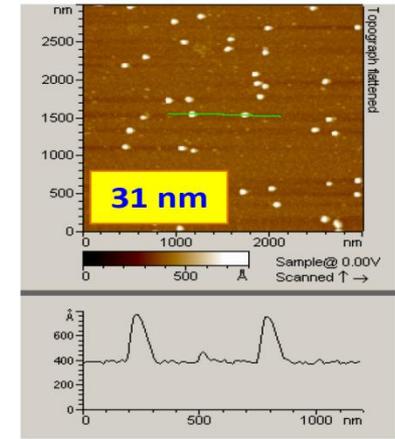


### 30 nm Gold colloids incubated in plasma

TEM

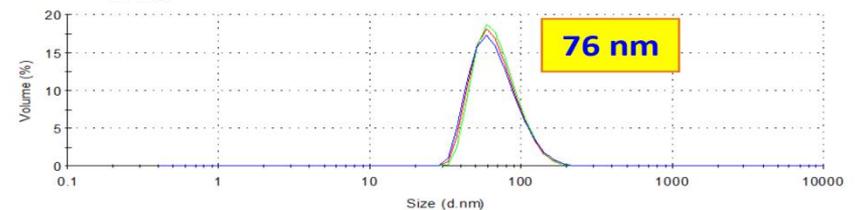


AFM



DLS

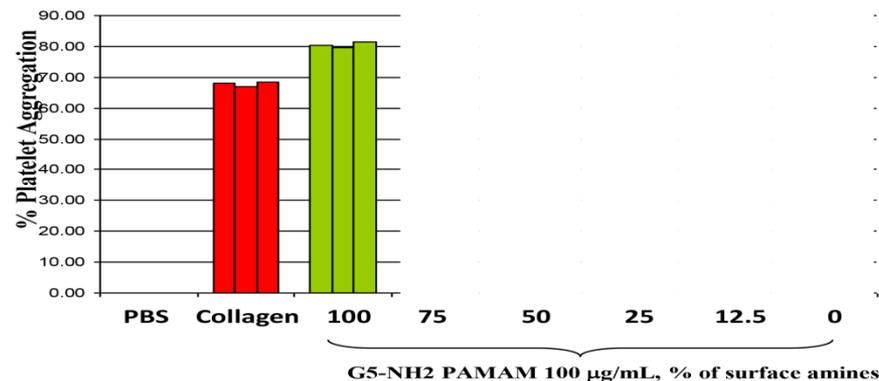
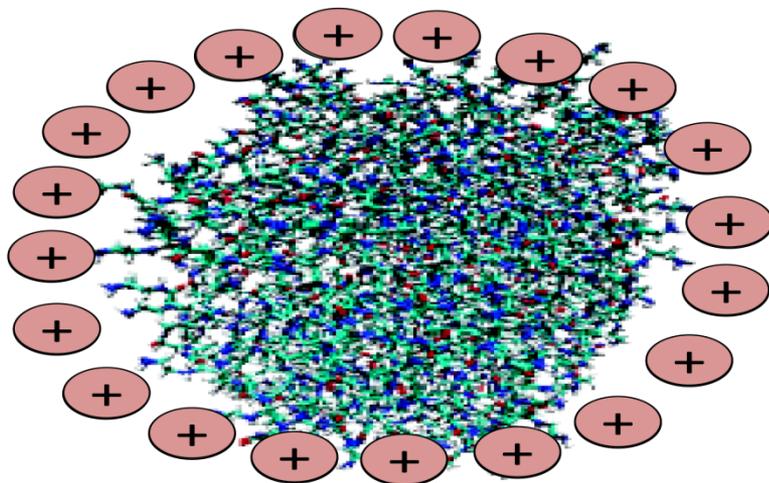
Size Distribution by Volume



**Size in a biological context is different.  
Multiple methods needed to identify.**

# Surface Charge

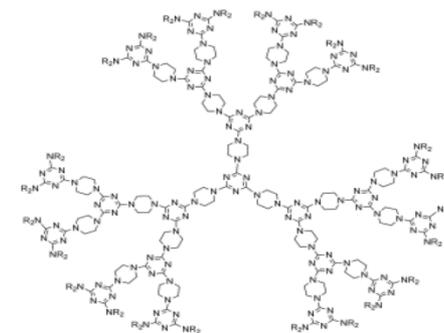
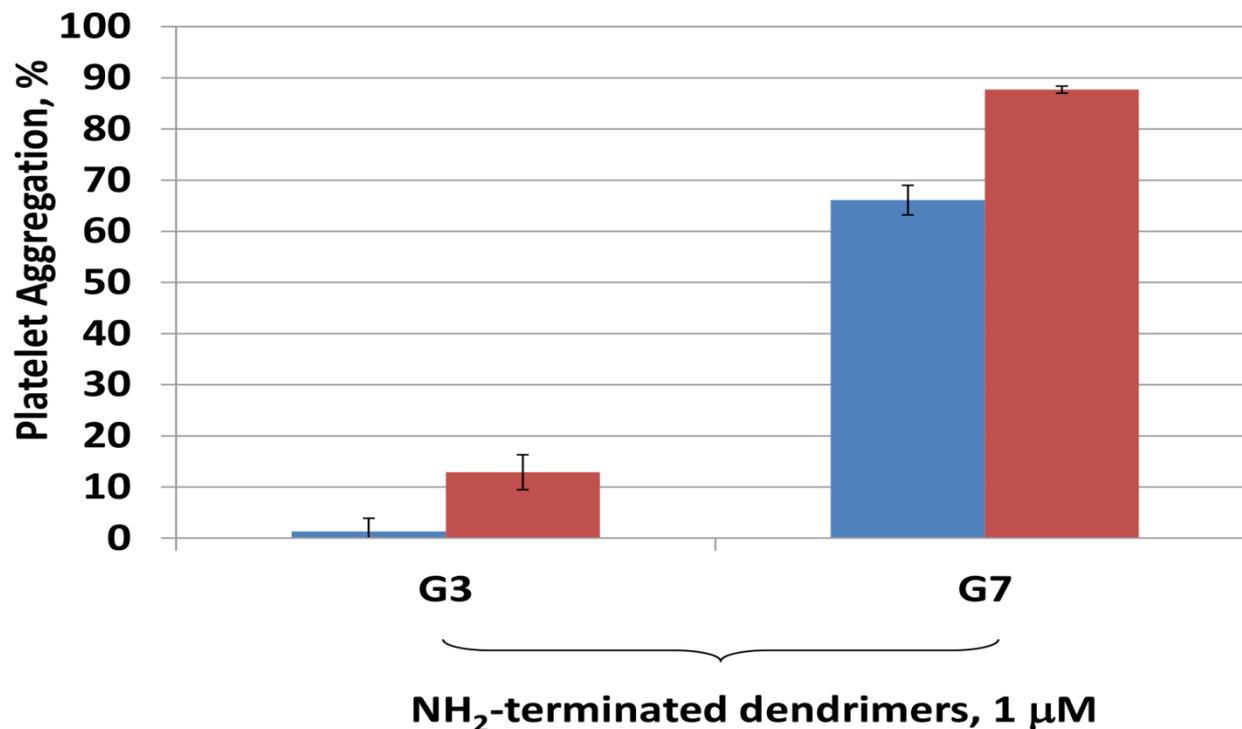
## Surface Charge



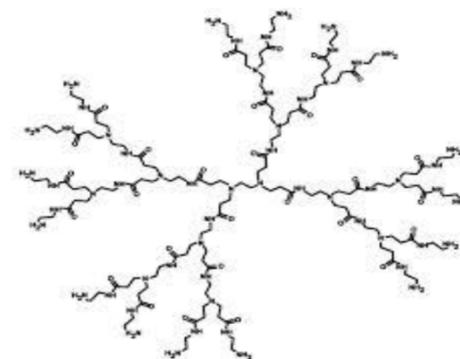
Zeta Potential is important  
Less surface amines = less platelet aggregation

# Composition

## Composition



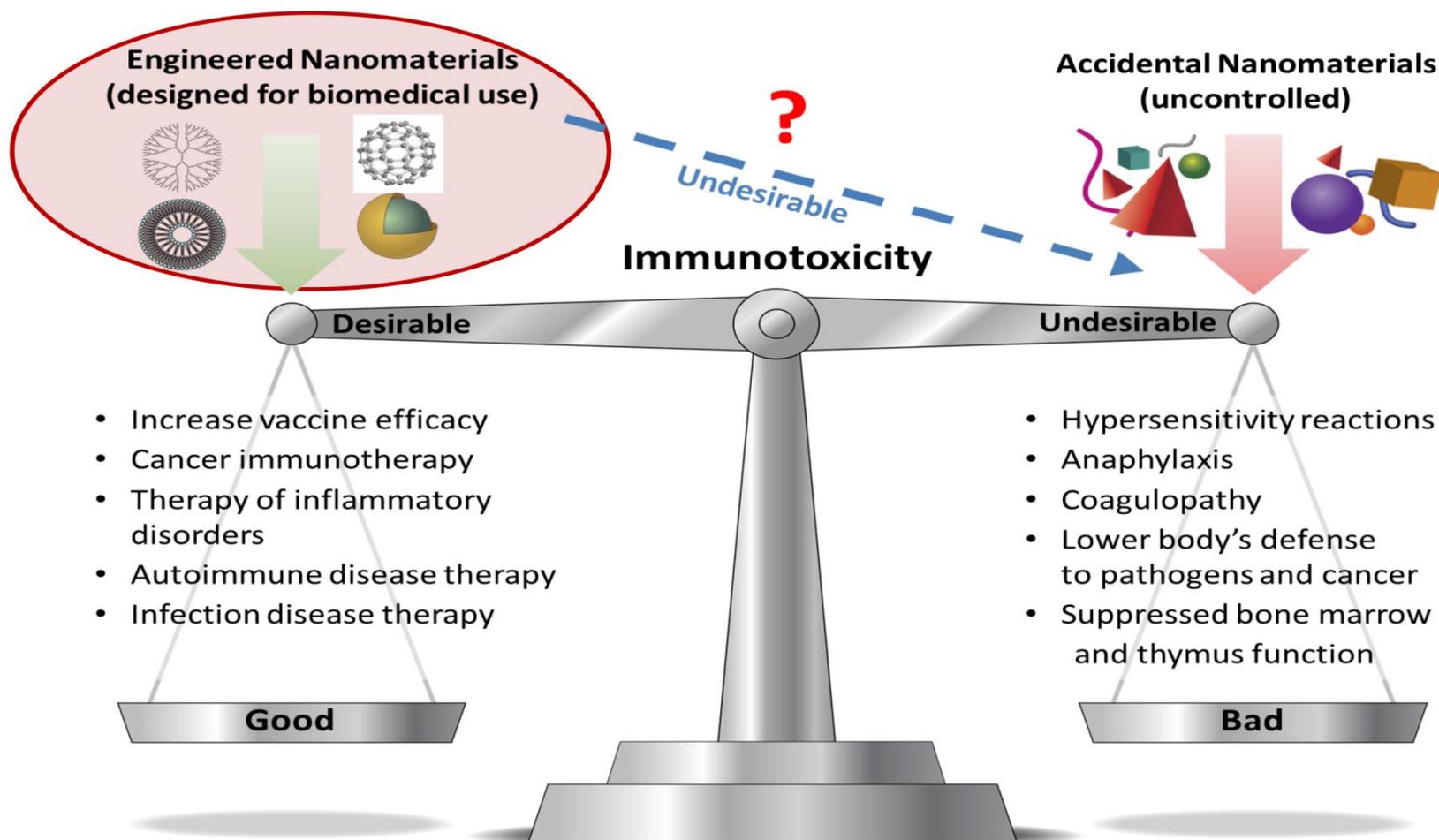
■ Triazine  
■ PAMAM



Triazine dendrimers are less potent in inducing platelet aggregation than their PAMAM counterparts

# Immunotoxicity

## Nanoparticle Immunotoxicity



# Immunotoxicity



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## Nanoparticle Immunotoxicity



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- Nanoparticles can be immunotoxic, however so far no new (i.e. specific to nano) immunotoxicity was reported
- Nanotechnology-based pharmaceuticals are not more intrinsically immunotoxic than other drugs in current clinical use
- Incorporation of conventional pharmaceuticals into nanotechnology-derived platforms helps decrease their immunotoxicity

# Reformulation



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## Reformulation Decreases Immunotoxicity



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**Traditional**

**Nano**

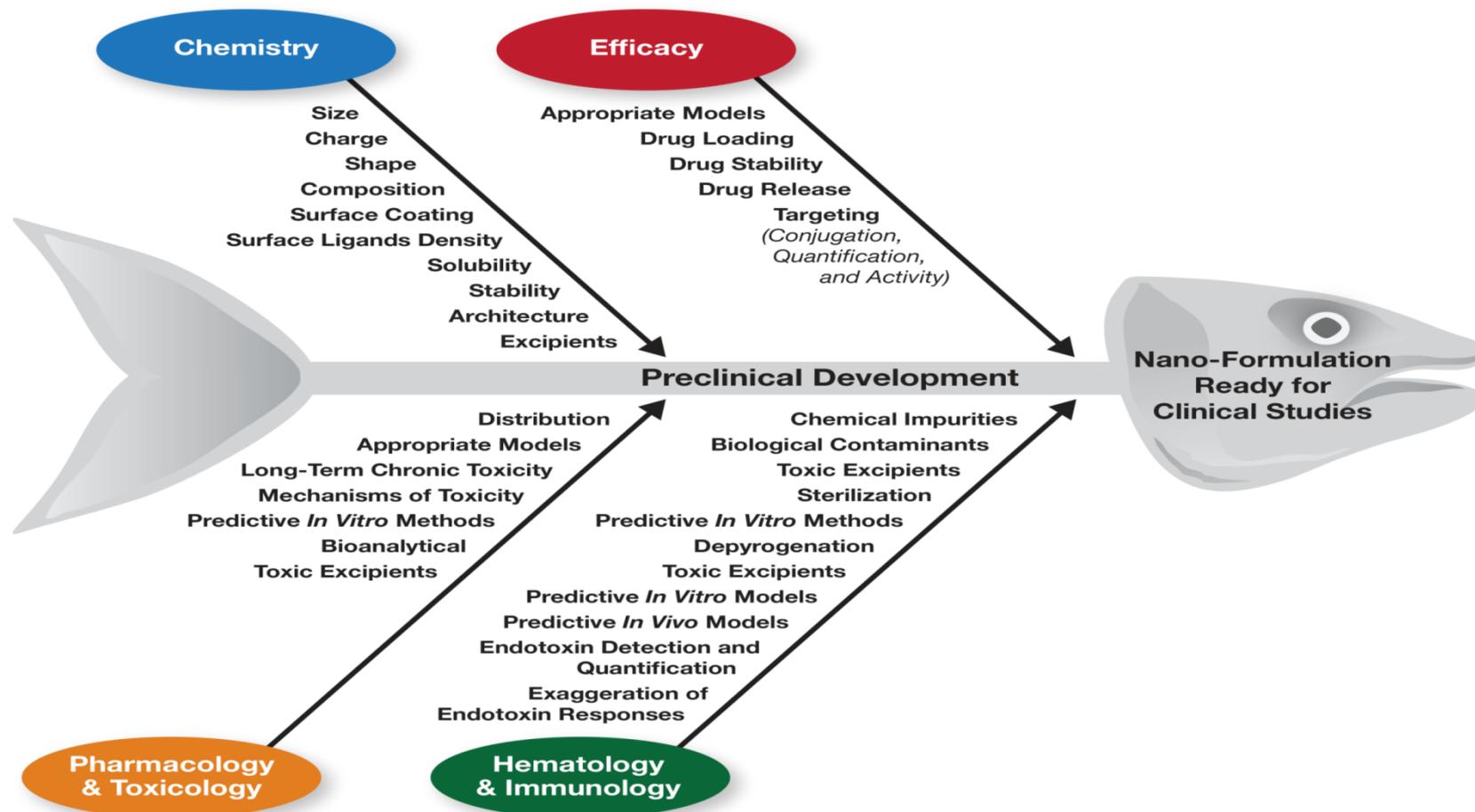
**Small Molecules**

**Therapeutic Proteins**

**Incorporation of conventional pharmaceuticals into nanotechnology-derived platforms helps decrease their immunotoxicity.**

# Challenges

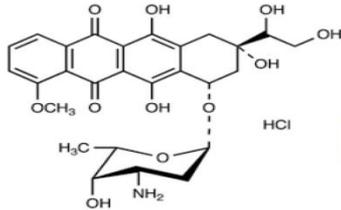
## Challenges in Preclinical Characterization



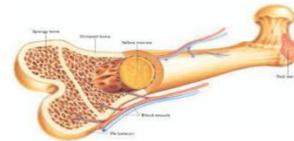
Thorough characterization of numerous parameters is essential prior to moving nanoformulations from pre-clinical to clinical phase

# Biodistribution

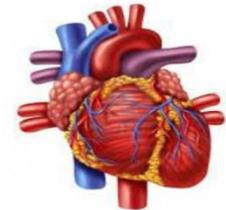
## Change in biodistribution



Biodistribution to

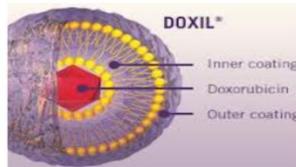


Bone Marrow  
(Myelosuppression)

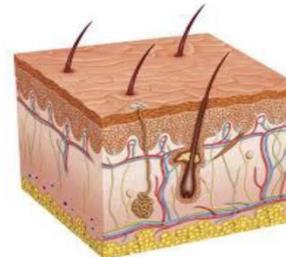


Heart  
(Cardiotoxicity)

Doxorubicin HCl



Biodistribution to



Skin



Palmar Plantar  
Erythrodysesthesia

PEGylated Liposomal Doxorubicin



Biodistribution to



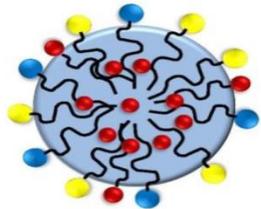
Kidney (Nephrotoxicity)

Doxorubicin-Cyanoacrylate Nanoparticles

**Altered distribution → altered toxicity profile**

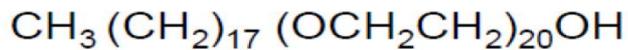
# Excipients

## Excipients



Drug-loaded Nanocapsules

Contains several surfactants, including



Brij 78

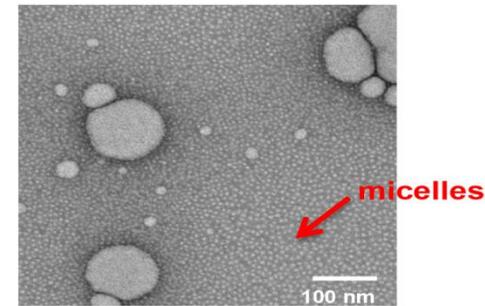
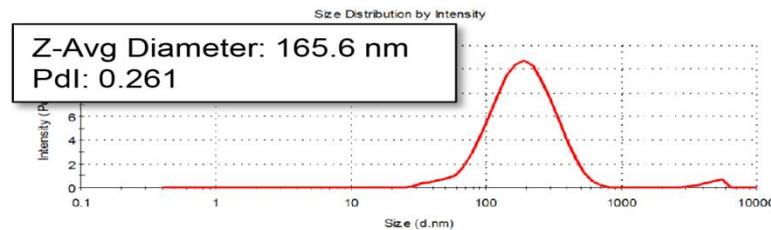
Critical Micelle Concentration  
0.0071 mM



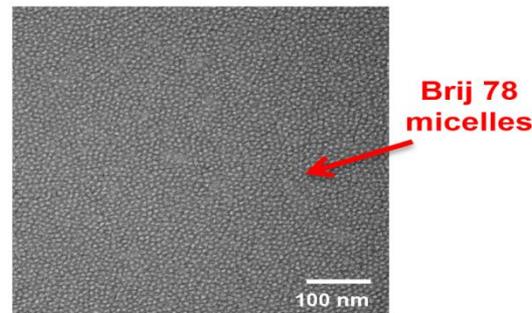
NC PC NP

Hemolytic!

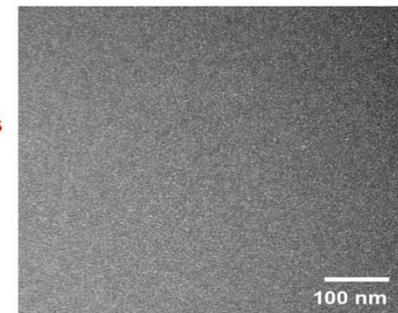
Micelles are not detected by DLS...



...but are clearly visible in TEM when negative stained.



0.02 mM Brij 78  
(Above CMC)



0.002 mM Brij 78  
(Below CMC)

Toxicity is caused by excipient

# Importance



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## Importance of PCC



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- Size and Zeta potential are important determinants of nanoparticle toxicity
- However, not always they allow to discriminate between nanomaterials displaying different biological effects

# Doxils

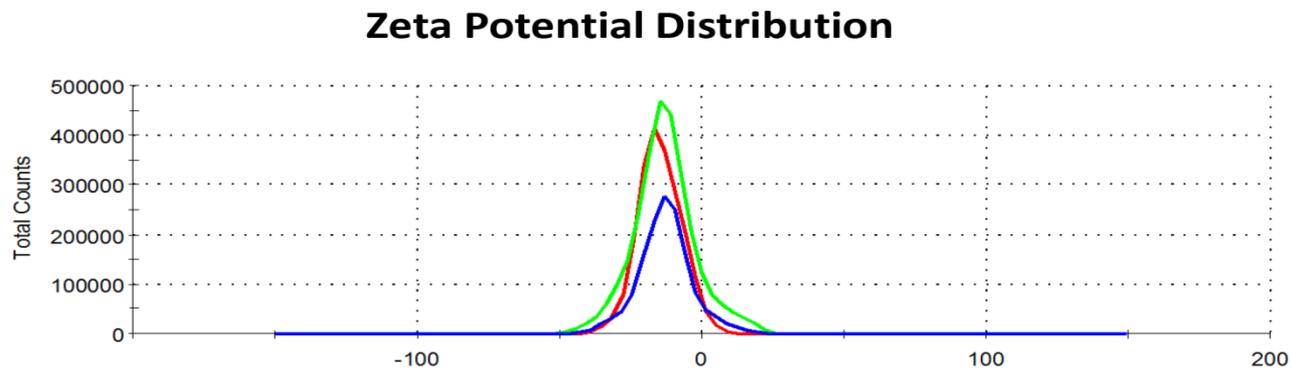
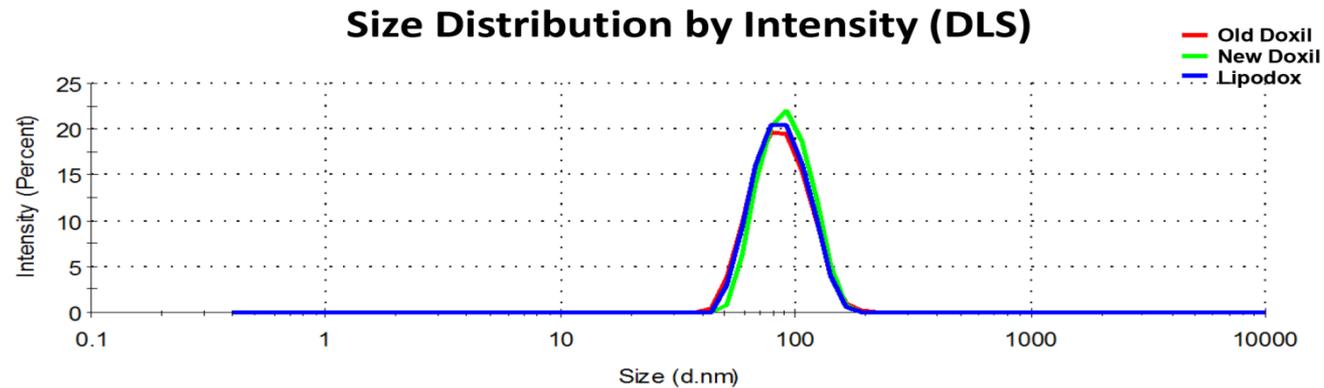
## Case study: Doxils



- Multiple versions of Doxil are available worldwide (Caelyx in EU, SinaDoxosome in Iran)
- Few products are used in the US: Doxil (Janssen Products), Lipodox (SunPharma) and DOXOrubicin hydrochloride liposome injection (SunPharma)
- NCL conducted PCC and complement studies using parent and generic Doxils

# Doxils size

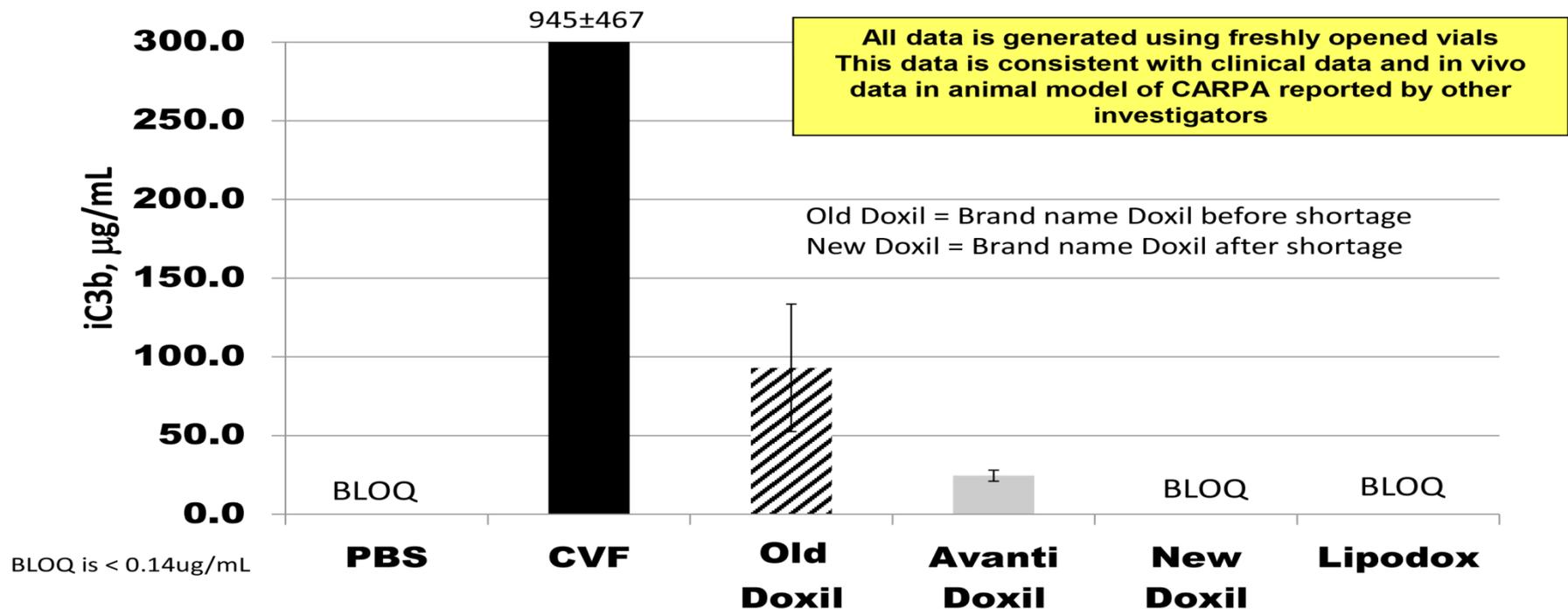
## Case study: Doxils size and zeta potential



Old Doxil, New Doxil and Lipodox have comparable hydrodynamic size and slightly negative zeta potential

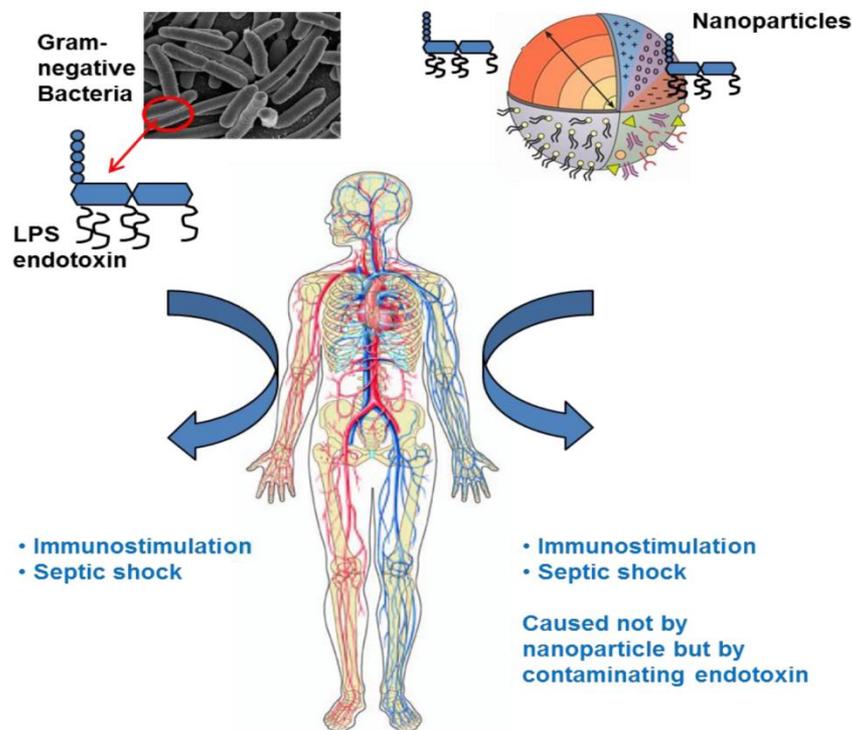
# Complement

## Case Study: Doxil and Complement Activation 2008-2013



- Positive and negative controls were consistent between experiments performed in 2008-2013
- Complement activation by early lots of Doxil was observed *in vitro* and was consistent between December 2008 and March 2011
- Complement activation by Avanti Doxil was lower than historical levels of early lots of Doxil
- Neither new lots of Doxil nor Lipodox activated complement *in vitro*

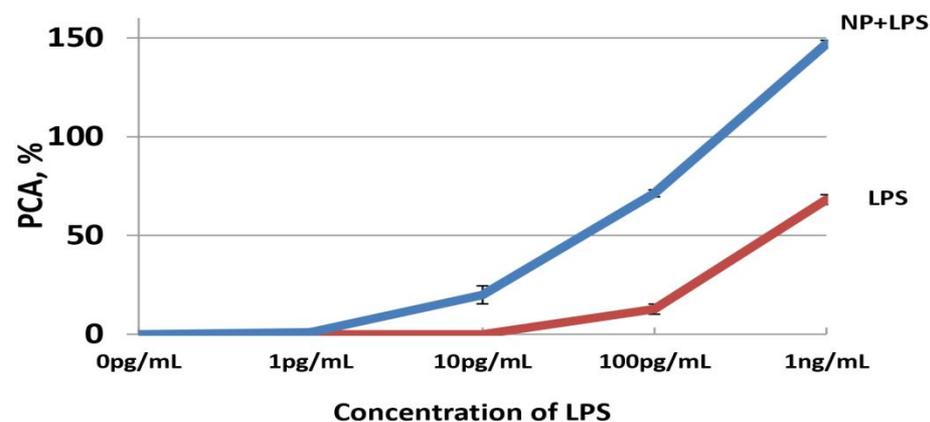
## Importance of endotoxin screening



- Endotoxin contamination is a common issue for engineered nanomaterials
- >30% preclinical nanoformulations fail due to endotoxin

### Endotoxin in Nanoparticles is bad because:

1. Erroneous data => wrong conclusions
2. Confound efficacy
3. Undesirable Toxicity
4. Exaggeration of endotoxin-mediated inflammation
5. Potential problems with immunogenicity of protein based APIs or targeting ligands



*Dobrovolskaia MA et al, Nanomedicine 2012*

Example: Cationic PAMAM dendrimers exaggerate endotoxin-mediated leukocyte procoagulant activity

# Endotoxin

## Endotoxin detection

Type of Interference:

**Inhibition**

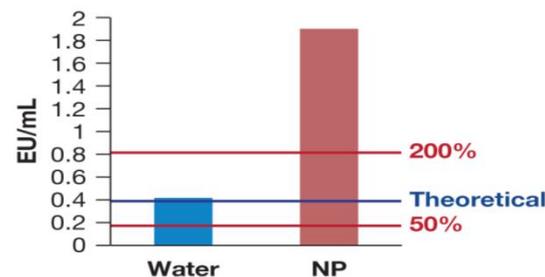
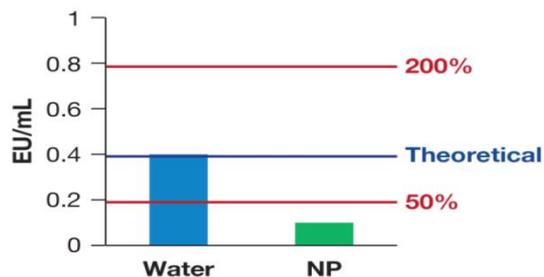
**Enhancement**

Evidenced by:

**Spike Recovery <50%**

**Spike Recovery >200%**

Typical IEC Result:



Commonly Observed  
in the Following  
Nanoparticles:



Cationic Nanoparticles,  
Unfunctionalized CNTs,  
Protein Rich Formulations,<sup>1</sup>  
Anionic Metal Colloids,  
Surfactant Containing  
Formulations<sup>1</sup>



Polymeric Nanoparticles,  
Protein Rich Formulations<sup>1</sup>  
Surfactant Containing Formulations<sup>1</sup>

Reason (Solution):

- Binding of LAL Proteins (Dilution)
- Binding of Endotoxin (Dilution)
- Serine Protease Inhibition (See Legend\*)
- Chelation of Mg<sup>2+</sup> (Add MgSO<sub>4</sub>)
- Competition with Mg<sup>2+</sup> (See Legend\*\*)
- Denaturing LAL Proteins (See Legend\*\*\*)
- Blocking Endotoxin-LAL Interaction (See Legend\*\*\*)

- Binding of LAL Proteins (Dilution)
- Glucans (See Legend<sup>††</sup>)
- Presence of Serine Protease or Serine Protease-Like Activity (See Legend<sup>†††</sup>)

**Nanomedicine Grand Challenge: Nanoparticles interfere with one or more LAL formats**

# LAL results

## Verification of LAL Results with RPT

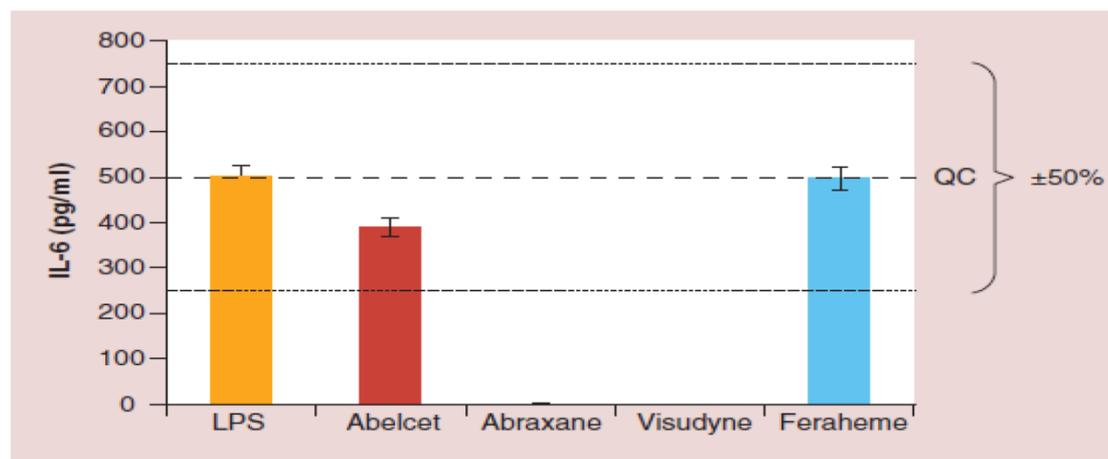
Sample	RPT	End-point Chromogenic LAL	Turbidity kinetic LAL
Nanoformulation	pyrogenic	0.53 EU/mL	21.4 EU/mL
Nanoformulation after purification	Non-pyrogenic	< 0.1 EU/mL	0.0381 EU/mL

- Amount of endotoxin in nanoparticle formulation is verified with RPT.
- Purified nanoformulation is not pyrogenic and does not show discrepancy between different LAL assays.

# Verification of LAL results

## Verification of LAL Results with MAT

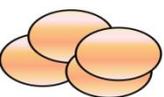
Formulation	Turbidity LAL, EU/mL	Chromogenic LAL, EU/mL	Gel-Clot LAL, EU/mL
Abelcet	0.07	< 0.1	< 0.03
Abraxane	< 0.05	< 0.1	< 0.03
Visudyne	0.118	4.317	< 0.03
Feraheme	< 0.001	< 0.1	< 0.03



**Verification of LAL results with MAT is not applicable to nanoparticles carrying cytotoxic agents**

# Sterilization stability

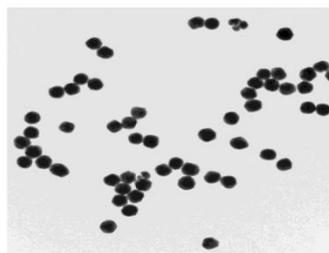
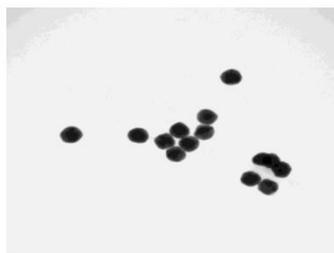
## Sterilization stability



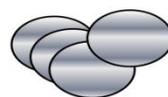
**Citrate-stabilized Gold NP**

Before  $\gamma$ -irradiation

After  $\gamma$ -irradiation



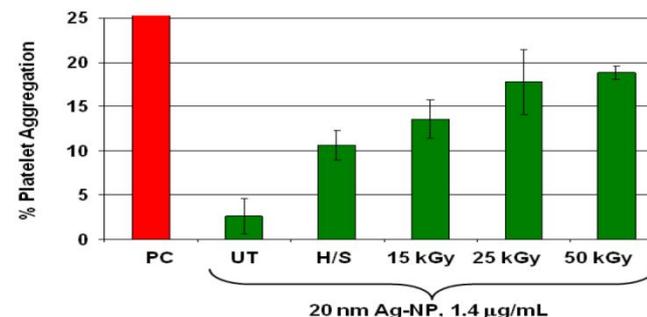
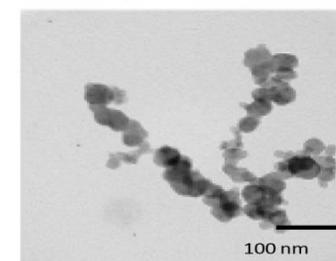
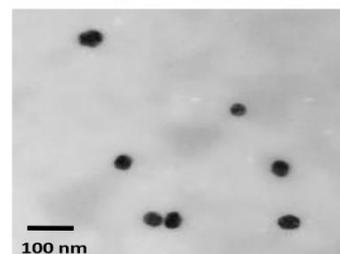
Colloidal gold nanoparticles  
NIST SRM # 8011, 8012 and 8013  
are sterilized by  $\gamma$ -irradiation



**Citrate-stabilized Silver NP**

Before  $\gamma$ -irradiation

After  $\gamma$ -irradiation

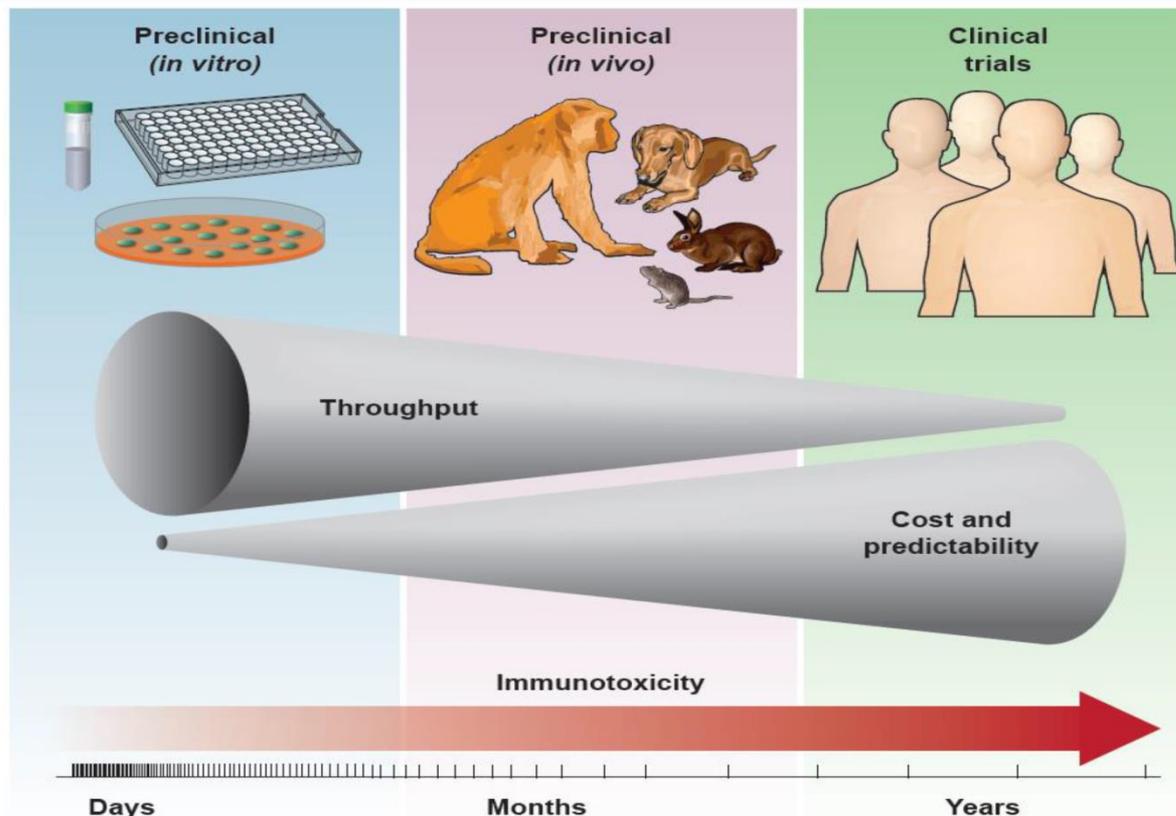


Zheng J, et al J.Nanosci.Nanotech (2011) [www.omiconline.org/2157-7439/2157-7439-S5-001.pdf](http://www.omiconline.org/2157-7439/2157-7439-S5-001.pdf).

$\gamma$ -irradiation disturbs integrity of colloidal  
silver nanoparticles and change their  
biocompatibility

# Correlation

## In Vitro & In Vivo correlation

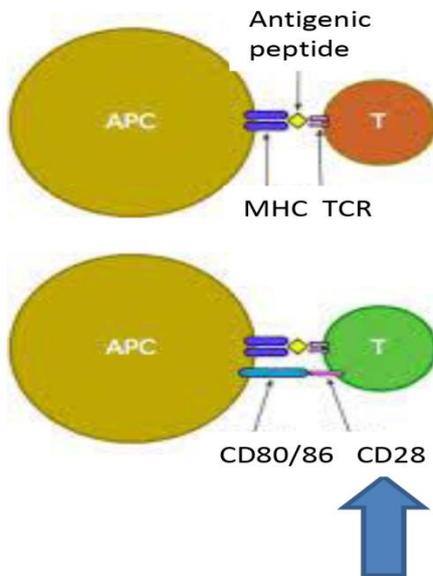


Dobrovol'skaia MA & McNeil SE,  
*J Control Release*. 2013 Jun 3.

- The likelihood of identifying immunotoxicity increases with progression from the preclinical to the clinical phase, however high cost, ethical issues and low throughput of in vivo tests limit their application
  - Growing recognition of the need for rapid screening methods
- A cascade of validated, reliable and predictive in vitro assays would address this need

# Biotherapeutics

## Lessons from biotherapeutics: TGN-1412



TGN1412 = CD28 Super-MAB

Preclinical studies in NHP and rodents did not reveal cytokine storm



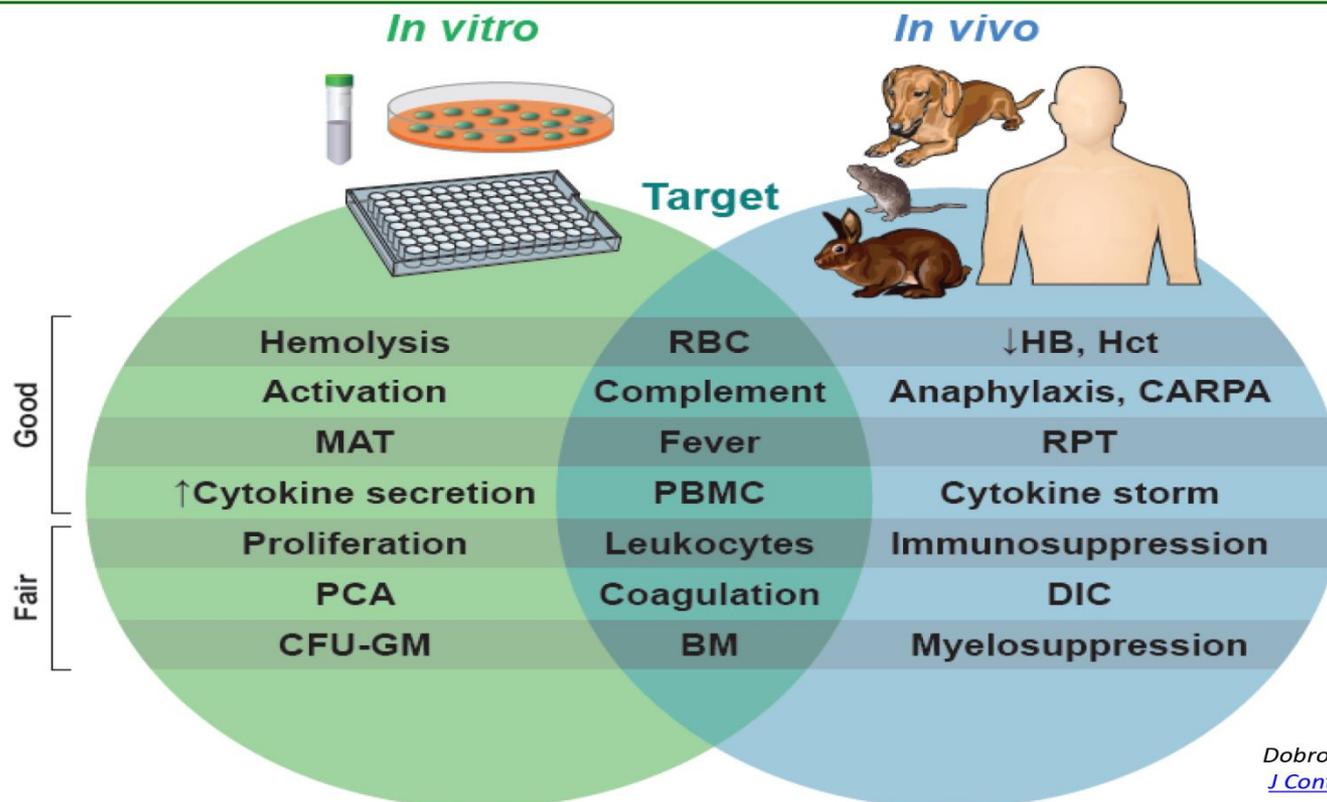
Phase I clinical trial: 6 of 6 volunteers experienced cytokine storm which lead to multiple organ failure



In vitro experiments using human PBMC showed high TNF levels in response to TGN1412

# Correlation

## In Vitro & In Vivo correlation



Dobrovolskaia MA&McNeil SE,  
[J Control Release](#). 2013 Jun 3.

- Common markers for nanoparticle acute toxicities are: hemolysis, complement activation, thrombogenicity, phagocytosis, pyrogenicity and cytokine induction
  - Most of these toxicities can be rapidly assessed in vitro prior to more resource- and time-consuming in vivo studies

# Cytokine storm

## Cytokine storm *In Vitro* & *In Vivo*

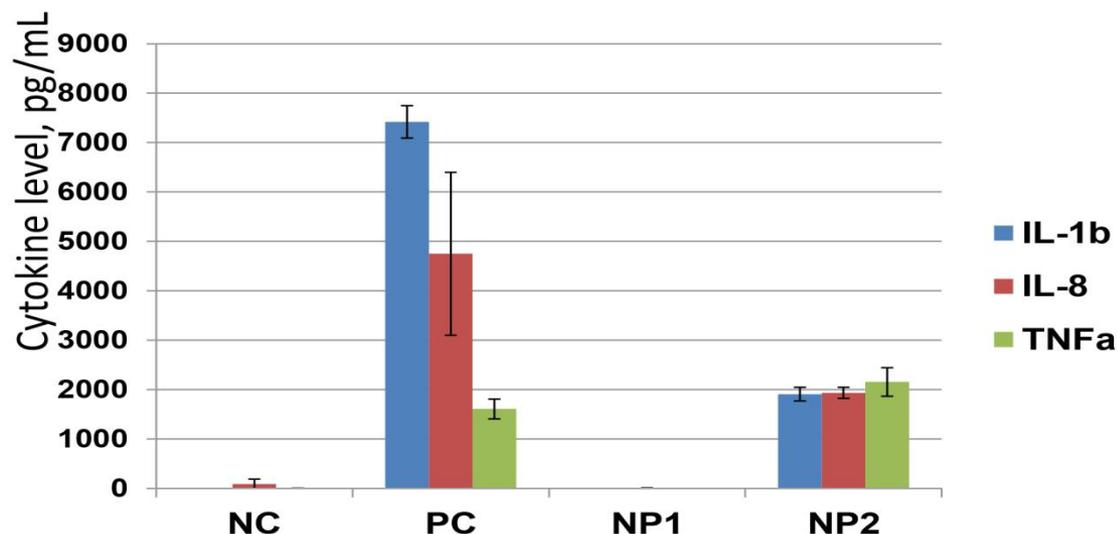
*In vivo*



Metal oxide  
nanoparticles



*In vitro* (human PBMC)

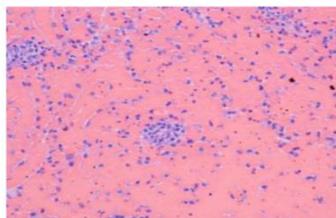
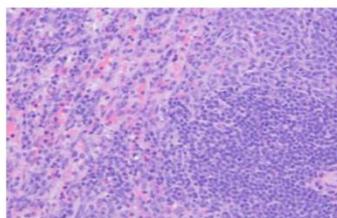


Results are reproduced in vitro using human PBMC

NP = nanoparticle; PBMC = peripheral blood mononuclear cells;  
IL- interleukin; TNF = tumor necrosis factor; LAL = limulus amoebocyte lysate

Control: Normal spleen

NP2: congestion in spleen



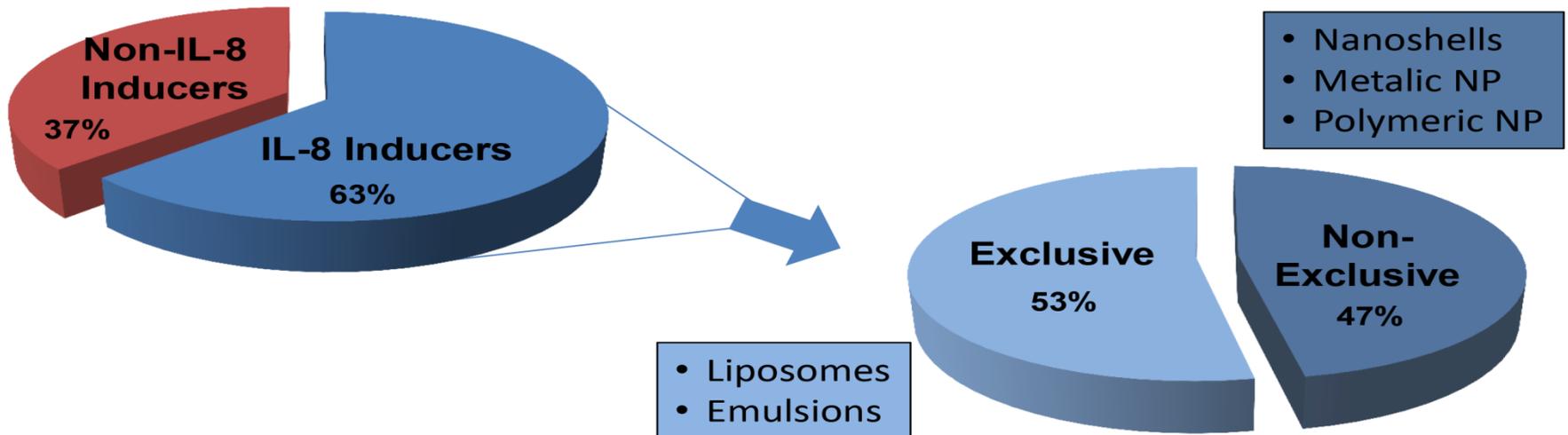
- Endotoxin is undetectable in NP1 and NP2 by gel-clot LAL
  - NP2 but not NP1 is toxic
- Necropsy reveals congestion and multiple organ damage similar to that seen in septic shock
- Analysis of plasma samples reveals elevated cytokines

# Immunotoxicity

## Platform Contribution to Immunotoxicity

10% of NCL tested nanomaterials induced cytokines

↳ > 60% of these particles induced IL-8

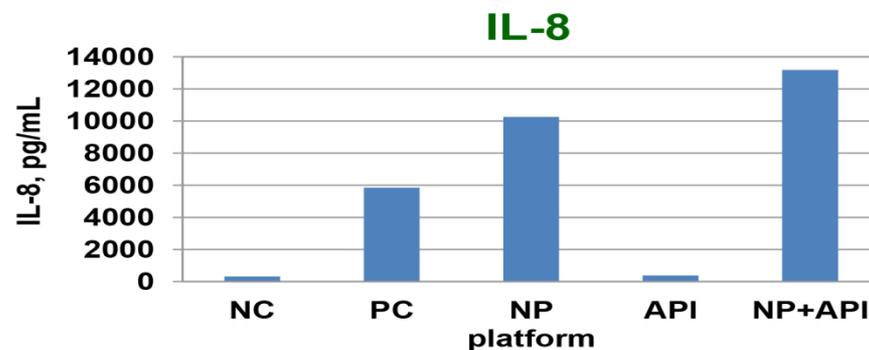
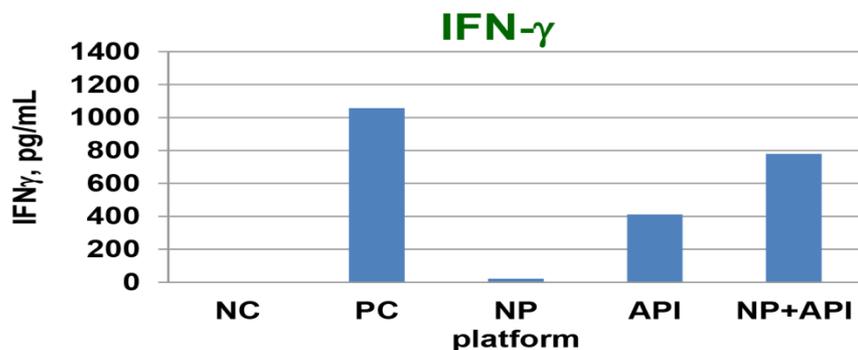
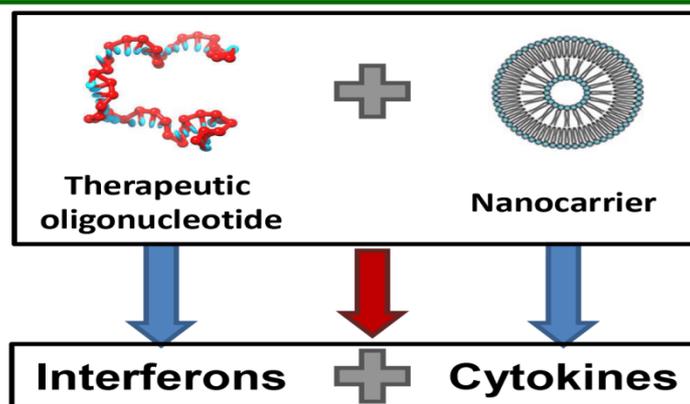


> 50% of IL-8 inducing nanocarriers did so exclusively  
(i.e. w/o inducing other common pro-inflammatory cytokines)

↳ These were typically liposomes and nanoemulsions

# Platform contribution

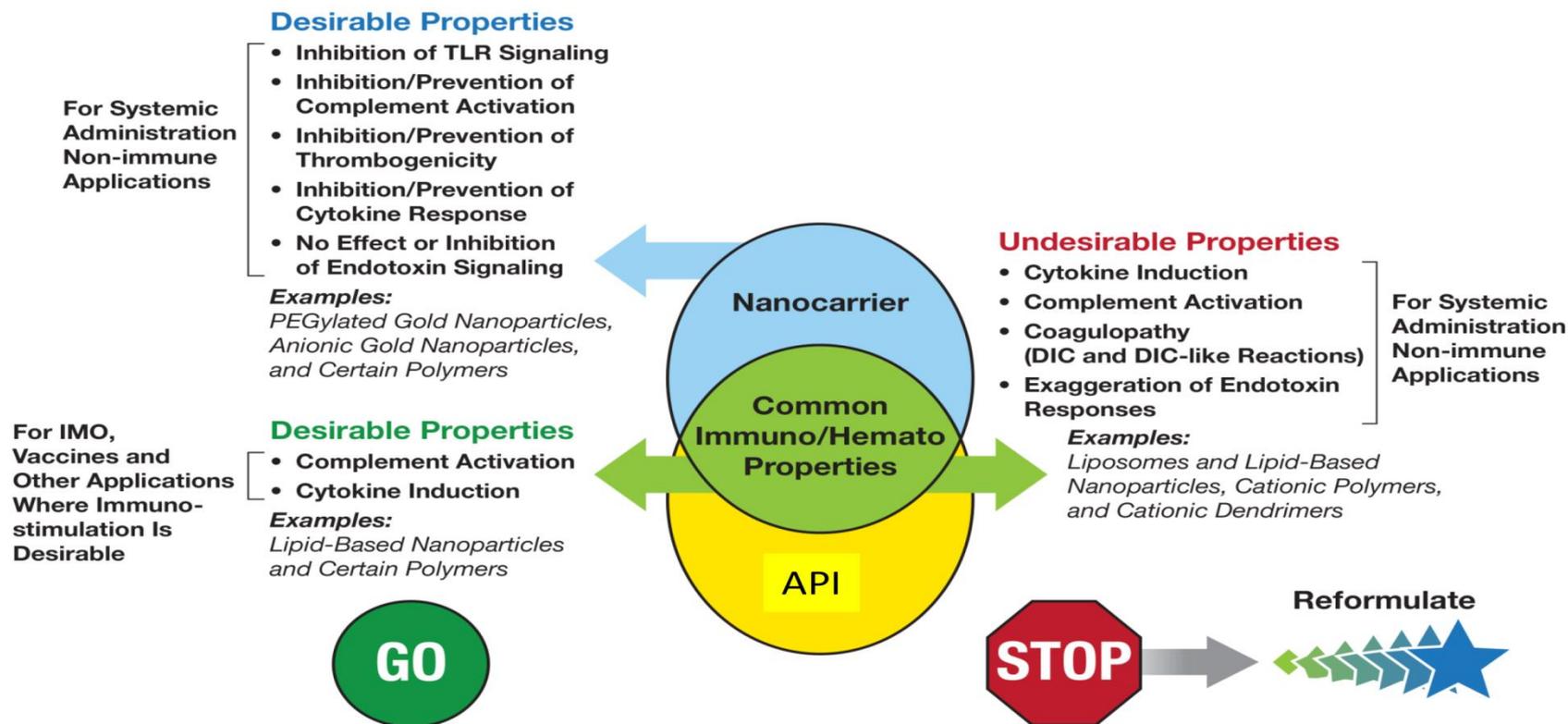
## Platform Contribution to Immunotoxicity



**Some nanocarriers contribute to immunostimulation profile of API**

# Take home message

## Take Home Message



- Immunotoxicity of both API and nanocarrier should be considered.
- Use immunologically reactive carrier when immunomodulation is wanted.
- Avoid such platforms when immunoreactivity is undesirable.

# Summary

## Summary

- Each nanoparticle is unique
- Comprehensive characterization is important (not just DLS and zeta potential)
- Key Factors In Preclinical Development
  - Physicochemical Properties (size, charge, composition)
  - Functional Properties (stability, drug loading, drug release, targeting)
  - Structure-Activity Relationship
  - Endotoxin and sterility
  - Sterilization and depyrogenation
  - Predictive in vitro methods

# Acknowledgments



NCI Alliance for  
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in Cancer

## Acknowledgements



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### Nanotechnology Characterization Lab



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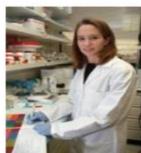
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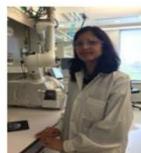
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