Summary of Phase II Proposal (Goals and Milestones)

“Big Picture” Goals

Grand challenge: **Minimize the environmental impact of engineered nanomaterials while maintaining their desired function**.

Key requirement: Provide molecular-scale mechanisms for understanding, predicting, and controlling how nanomaterials and their transformation products interact with environmental and biological interfaces.

New approach based on:

1. Developing a molecular-level understanding of how engineered nanoparticles interact with biological systems of environmental relevance;
2. Using the resulting mechanistic insights to predict the impact of nanomaterials based on their intrinsic chemical and physical properties;
3. Using these predictions to design more sustainable nanoparticles which retain functionality without causing negative environmental impact.

Guiding Principles

We aim to develop a set of design rules that will allow us to predict the biological responses induced by NPs in different organisms and to use these design rules to facilitate nanotechnology development in a safe and sustainable manner. Our studies are guided by two principles:

- **Guiding Principle 1 i**s that association and subsequent biological response are controlled by chemical and physical interactions between the NPs and the various surfaces and interfaces present in cells and their compartments (e.g., mitochondria and the cell nucleus). **Thus, we aim to understand the nano-bio interface from a perspective in which we place the surface chemistry of the NP and the surface chemistry of the cell on equal footing**.
- **Guiding Principle 2** is that the number of combinations of technologically relevant NPs with environmentally relevant organisms is immense; this makes it intractable to characterize the interactions of all possible combinations even in a 10-year time span and drives us toward a new paradigm based on molecular-level understanding. This chemical and structural complexity challenge is exacerbated by the fact that NPs are often transformed in the environment through displacement and chemical modification of surface ligands, oxidation/reduction of NP cores, and adsorption of natural organic matter and biopolymers secreted from cells. **Thus, we will take a molecular-level approach based on identifying which specific chemical and physical properties of various classes of NPs and organisms ultimately control their interactions**.

Our four signature goals:

1. **Develop and implement a comprehensive experimental and computational framework** that will provide molecular-level insights into the behavior of NPs in the environment, including the transformation of the core and ligands and the interaction of NPs with molecules and organisms present in the environment;
2. **Develop and implement model laboratory systems**, such as suspended and free standing lipid bilayers and/or other model cell surfaces, that will provide a molecular-level understanding of how NPs interact with different biological surfaces in the environment;
3. **Identify the specific molecular processes that NPs induce in model cell types and organisms** that are representative of the biological diversity in the environment, by using state-of-the-art microscopy and proteomics and genomics tools and linking experimental studies with computational studies;
4. Use the above understanding to **enable the predictive design and synthesis of NPs** that combine technological utility with reduced environmental impact.

Milestones

Year 1

1. Understand, control, and predict how the spatial distribution of charge in nanoparticle polymer coatings determines particle properties and governs biological outcomes. ***e.g.: polyamines (Role of Charge)***.
2. Elucidate the chemical origin for a membrane-mediated nanoparticle transformation process and subsequent biological outcome. ***e.g.: lipid corona (Transformations)***.
3. Design and synthesize a nanoparticle coating that reduces the biological impact. ***e.g.: NMC or QDs (NP Re-design)***.
4. Establish ability to retrieve nanoparticles from complex biological and environmental matrices and analyze acquired surface coatings. ***e.g.: lipids, natural organic matter (NP Coronas)***.

Year 3

1. Use "omics" approaches to compare the molecular changes in model organisms exposed to nanoparticles that elicit different biological responses. ***e.g.: Daphnia vs. Shewanella to cationic polymer NP coatings***.
2. Demonstrate ability to extend single-molecule detection approaches to non-fluorescent materials. ***e.g.: Nanodoublers, Doped oxides***.
3. Develop and release a validated, open-source computational membrane model that includes heterogeneity and dynamics in electrolytes, in the presence of different nanoparticles. ***e.g.: NP-electrolyte-phospholipid triple junction***.
4. Identify the structure and composition of fragments released from nanoparticle-polymer composites, and their biological impact. ***e.g.: CdSe QDs / PMMA***.

Go to **TOP**

RFA Summaries

RFA1. The Enabling Toolkit

Establishes the critical synthesis, analytical characterization, and computational methods that serve as the foundation of activities extending throughout the Center. **RFA1** will establish a **"make, measure, model"** paradigm to:

1. **Develop and implement methods to synthesize and characterize** well-defined, functionalized, and increasingly complex NPs in quantities that enable Center-wide investigations of identical materials.
2. **Establish analytical techniques and instrumentation to investigate NP interactions** with model cell surfaces and intact living cells and organisms with unprecedented spatial and temporal resolution, and chemical sensitivity and specificity, under environmentally relevant conditions.
3. **Develop multi-scale computational methods for molecular-level understanding** of NP transformations, and dynamic and chemical processes that occur at the nano-bio interface in environments relevant to *in vivo* exposures. The integration of experimental and computational approaches will lead to design rules for sustainable nanomaterials.

RFA2. Nanoparticle Transformations and Interactions with Model Biological Interfaces

Use the techniques of RFA1 to investigate NP interactions with model systems such as bilayers and membranes that simulate biological interactions. **To gain predictive, molecular-level insight we will**:

1. **Determine the mechanisms, dynamics, and energetics of NP interactions with key membrane components**. Molecular-scale understanding of these interactions will provide mechanistic insight into NP and membrane properties that drive cellular uptake and alter membrane function, and will help guide the design of next generation NPs tailored to interact (or avoid interaction) with specific biological targets.
2. **Develop a detailed understanding of the structure and dynamics of NP surfaces**. The chemical species exposed at the NP surface, whether intentional or acquired in the environment, are expected to strongly impact NP interaction with biological systems. The arrangement and dynamics of ligands on NP surfaces and the nature and orientation of molecules acquired from the environment need to be understood to predict and control the interaction of nanomaterials with biological systems.
3. **Determine the kinetics, mechanisms, and products of chemical and physical transformations of nanomaterials in environmental and biological media**. Development of sustainable nano-enabled products requires the ability to predict the rates and products of transformation.

RFA3. Molecular Interactions of NPs with Intact Cells and Organisms

Assess the interactions of individual cells, tissues, and whole organisms with NPs that the Center develops in RFA1.
Goals for RFA3:

1. **Identify critical molecular processes** by which NPs interact with membranes, intracellular molecular complexes, and organelles in intact cells.
2. **Determine molecular mechanisms** by which organism interact with NPs and the ultimate population-level consequences resulting from these molecular interactions.
3. Characterize how environmental matrices and NP transformations in the environment and within organisms impact their molecular effects on cellular and organism functions.
4. **Provide mechanistic insights** to RFA1 and RFA2 that will inform the design of NPs with reduced environmental impact.

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Materials, techniques, organisms in proposal

Specific nanoparticles

- KNbO3 “nanodoublers”
- Fe3O4
- Mesoporous silica
- Mesoporous silica-coated Fe3O4
- CdSe quantum dots
- CdSe/ZnS quantum dots
- Gold
- Nanodiamond
- LiNixMnyCo1-x-yO2

Complex Nanomaterials.

- bimetallic,
- metal oxides,
- III-V semiconductors, and
- non-centrosymmetric materials such as (“nanodoublers”)
- lanthanide-doped oxides
- core/shell structures
- polymer-NP composites
- atomic layer deposition (ALD) to produce very thin shells of oxides

Protective inorganic shells

- ZnS
- Al2O3
- TiO2
- Mesoporous silica

NP Properties

- charge density
- charge delocalization
- NP shape
- ligand stability
- ligand flexibility
- ligand density
- Multivalency

Surface Ligands

Ligand Types

- amine-terminated thiols, gold
- silanes, oxides
- alkenes, nanodiamond
- phosphonates, semiconductor QDs
- quaternary amine (positive point charge),
- dialkyl imidazolium (positive distributed charge),
- primary amine (positive charge with H-bonding potential),
- carboxylate or a sulfate (negative charge with different p*K*a values)

- alkylated
- PEGylated
- chelates
- short multimers
- large polymers

Ligand Properties

- positively and negatively charged ligands
- localized vs. delocalized surface charge
- hydrophobicity/ hydrophilicity
- molecular recognition
- multivalency
- mixing charged and neutral ligands
- branched ligands

Techniques

- QCM-D
- OWLS
- SHG
- SFG
- AFM
- electrochemical impedance spectroscopy
- NMR
- isothermal titration calorimetry
- fluorescence correlation spectroscopy
- Super-resolution microscopy
- Single molecule fluorescence microscopy
- stochastic optical reconstruction microscopy (STORM)
- structured illumination microscopy (SIM)
- STORM-AFM
- SIM- nanoSIMS (secondary ion mass spectrometry)
- confocal Raman microscopy
- hyperspectral imaging

Computational Methods:

- atomistic quantum-mechanical calculations
- equilibrium and non-equilibrium sampling techniques
- coarse-grained (CG) and dynamic models
- coupling of CG dynamics, kinetic Monte Carlo, and local atomistic computations

Model membranes

- lipid monolayers at air-water interfaces
- free-standing lipid bilayers suspended across apertures
- tethered and supported lipid bilayers
- suspended and supported vesicles
- vesicles in solution

- Macromolecular assemblies of intact cell surfaces

Unique cell membrane chemistries:

- The presence of lipopolysaccharides in gram-negative bacteria
- Teichoic acids in gram-positive bacteria
- Cholesterol and proteins in eukaryotic organisms
- Immune recognition molecules in vertebrates
- Phospholipids
- Glycosphingolipids
- transmembrane proteins
- cell wall polymers
- Growth factor receptors.
- Integrins/adhesion complexes.
- Extracellular matrix proteins.
- Ion channels.
- Aquaporins.

Model organisms.

- gram-negative bacterium *Shewanella oneidensis*
- gram-positive bacterium *Bacillus subtilis*.
- **fruit fly** *Drosophila melanogaster*
- **harlequin fly** *Chironomus riparius*
- **water flea** *Daphnia magna*
- **rainbow trout** (*Onchorhynchus mykiss*)
- cell lines for gills (RTgill-W1) and macrophages (RTS-11)
- **Zebrafish**

Properties to quantify:

- molecular order
- orientation distributions
- ligand densities
- interfacial electrostatic parameters
- dynamics of surface species on NPs
- reversibility
- binding constants
- corona formation and surface transformations
- surface-mediated transformations
- alterations to membrane structure
- local curvature at the NP-membrane contact point
- surface ligand density
- surface ligand desorption

NP properties that induce biological responses:

- Chemical composition
- Surface charge.

- Electronic structure.
- Galvanic corrosion effects.
- Increased ROS production due to enhanced charge separation.
- Valence and conduction band energies facilitate formation of reactive oxygen species in the presence of visible light
- Macromolecular crowding.

Environmental factors:
- pH
- UV illumination
- natural organic matter differing in sulfur and nitrogen content

Biological mechanisms potentially influenced by NPs:
- Attachment
- Uptake
- Internalization
- Protein binding.
- Endocytosis
- Phagocytosis

Biological responses:
- DNA damage
- Membrane disruption/damage
- Mitochondria damage
- Change membrane-bound protein conformations
- Disrupt ion transport
- Initiate compensation mechanisms in the cell
- Trigger immune molecules
- Oxidative stress

Measuring NP-bio interactions.
- Super-resolution microscopy
- Single-molecule fluorescence microscopy
- Confocal Raman microscopy
- Dark-field scattering microscopy
- RNA-Seq and RT-PCR to monitor changes in gene expression
- Protein sequencing.
- Mass spec to measure metabolites.

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RFA1. The Enabling Toolkit

The goal of RFA1 is to develop methods and advanced instrumentation that enable the synthesis and characterization of well-defined science- and technology-enabling NPs as well as *in situ*, real-time characterization, and quantification of NP interaction with biological and environmental systems at the molecular level. **Our vision** is to create an integrated suite of materials, methods and tools to enable sustainable NP production and use.

RFA1 will establish a "make, measure, model" paradigm to:

1. **Develop and implement methods to synthesize and characterize** well-defined, functionalized, and increasingly complex NPs in quantities that enable Center-wide investigations of identical materials.
2. **Establish analytical techniques and instrumentation to investigate NP interactions** with model cell surfaces and intact living cells and organisms with unprecedented spatial and temporal resolution, and chemical sensitivity and specificity, under environmentally relevant conditions.
3. **Develop multi-scale computational methods for molecular-level understanding** of NP transformations, and dynamic and chemical processes that occur at the nano-bio interface in environments relevant to *in vivo* exposures. The integration of experimental and computational approaches will lead to design rules for sustainable nanomaterials.

We will integrate a suite of in situ and ex situ characterization methods available across CSN institutions and develop new, innovative methods and advanced instrumentation to further enable molecular-level characterization of nano-bio interfaces. **By using identical NPs and standard methods across the Center**, we can:

1. Synchronize experiments,
2. Correlate results from disparate techniques, and
3. Perform cross-checks and counterfactuals that will enhance the validity of our approaches and the reliability of data interpretation.

General aims:

- Precise control over composition, size, and shape.
- Provide well-characterized, standardized NPs that form the basis of synchronized cross-Center studies.
- Create new, innovative NPs that will allow our team to take full advantage of the RFA1 toolkit.
- Develop novel synthetic methods for controlling the types of chemical groups exposed at the NP surfaces.
- Discriminate the types of NP interactions driven by external ligands (e.g., electrostatics) from those driven by the core material (e.g., van der Waals forces).
- Investigate how the detailed nature of the nanocomposite and the environment influences the rate and form of released NPs.

1. Synthesize and Characterize Complex Nanomaterials

The ability to synthesize a wide range of both standard and innovative materials provides us the ability to make NPs that are uniquely tailored to address scientific questions while maintaining relevance to emerging nano-enabled technologies.

We will investigate a balanced portfolio of "***science-enabling***" and "***technology-enabling***" NPs:

1. Gold NPs whose size, shape, and surface ligands can be readily modified,
2. Lanthanide-based NPs that are non-linear optical agents and sub-diffraction-limited imaging agents,
3. Diamond NPs whose ultra-stable surface chemistry provides stability against ligand loss and whose Nv allows fluorescence detection and
4. LiNixMnyCo1-x-yO2 NPs whose controllable redox potential (based on extent of lithiation) allows us to test how NP redox potential impacts cellular redox processes.
5. Redox-active metal oxides common in “e-waste”,
6. Graphene-based materials,
7. NP-polymer composites containing III-V quantum dot-polymer nanocomposites.

Specific enabling NP technologies:

- Synthesize NPs from non-centrosymmetric materials such as KNbO3 (“nanodoublers”) to enable imaging of NPs with non-linear optical methods.
- Synthesis of lanthanide-doped oxides or core/shell structures will allow us to extend single-particle optical imaging techniques to include materials that are not inherently fluorescent.
- Prepare Fe3O4 and mesoporous silica-coated Fe3O4 NPs for use in magnetic extraction experiments to allow molecular analysis of adsorbates on the NP surface after biological exposure.

Ligand chemistries:

- One general ligand assembly strategy uses amine-terminated thiols, silanes, alkenes, or phosphonates to form amide bonds with carboxylic acid-containing compounds on NPs comprised of gold, oxides, nanodiamond, or semiconductor QDs, respectively.
- The CSN team will generate NPs functionalized with positively and negatively charged ligands such as a quaternary amine (positive point charge), a dialkyl imidazolium (positive distributed charge), a primary amine (positive charge with H-bonding potential), carboxylate or a sulfate (negative charge with different p*K*a values).
- The importance of average charge density will be examined by mixing charged and neutral ligands on NPs and by using branched ligands to present patches of particular functional groups on the surface.
- The importance of the hydrophobicity/hydrophilicity of the NP ligands will be compared across a series of alkylated or PEGylated surface ligands.
- Incorporation of synthetically generated multivalent ligands, including chelates or short multimers (e.g., dimers, trimers) or large polymers will facilitate evaluation of multivalent effects.

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2. Molecular-level Characterization of NP Interactions in Complex Environments

We will develop new methods to answer questions such as:

1. Which forces control NP interactions?
2. How can we identify where the NPs are in complex media?
3. How can we obtain spectroscopic information about NP-bilayer interactions?

2a. Nanometer-resolution Dynamics of Nanomaterials in Biological Systems. Super-resolution and single molecule fluorescence microscopies can characterize the interactions of NPs with biological systems with 20-120 nm resolution.
2b. Nonlinear Optical Methods to Measure Chemical Specificity in NP-Bio Interactions. A key goal of our work is to expand the ability to obtain chemically specific information at nano-bio interfaces; In particular, we want to understand how NPs influence the ordering of molecules within biological membranes.

2c. Enhancement of Commercial Instruments. Another aspect of our efforts is to work directly with instrument designers to advance the state-of-the-art of measurement tools for the nano-bio community.

Properties to characterize/quantify:

- **Mass changes on model membranes** upon NP exposure (QCM-D, OWLS),
- **Degree of lipid disruption** (SHG, SFG, AFM, electrochemical impedance spectroscopy),
- **Binding constants and kinetics** for biomolecule/NP interactions (NMR, isothermal titration calorimetry, fluorescence correlation spectroscopy),
- **NP position and dynamics** in biological membranes, cells, and organisms (various sub-diffraction optical imaging techniques).
- Identify the **interactions and intracellular fate** of fluorescent NPs in tissues and cultured cells.
- Probe **changes that occur in protein expression patterns** or subcellular structures.
- Diagnose **membrane disruption** and establish NP location in an intact cell or organism, which is key to understanding how to relate molecular studies to organism-level studies.
- Detection of **changes in the integrity of the cell membrane** or in the NP itself, such as degradation of a NP-polymer composite or shedding of fluorescent ligands.

Types of measurements.

- Detection of individual NPs as they interact with specific molecules, identified by their fluorescent tags, at the surface of an intact cell or a model membrane.
- Establish correlative STORM-AFM by integrating single molecule fluorescence and atomic force microscopy in one system.
- Super-resolution and single molecule fluorescence microscopies to characterize the interactions of NPs with biological systems with 20-120 nm resolution.
- NanoSIMS will enable detection of NPs within cells based on their elemental content, while SIM will provide information about the cellular context using fluorescent proteins and tagged subcellular structures, together providing spatial information with nanometer scale resolution.
- Use second-order spectroscopy to provide *in situ* spectroscopic information on SLBs.
- Expand the unique structure- and interface-selective properties of SFG and SHG to probe lipid ordering with sub-minute time resolution.
- Using the world's only sub-wavenumber resolution SFG spectrometer, we will add analysis of accurate vibrational lineshapes to our toolkit.
- SHG will be used to probe NP attachment to model membranes, in live cells, and in multicellular organisms.
- We will use resonance enhancement or double resonance approaches, or enhance off-resonance sensitivity using sub-50 nm diameter KNbO3 "nanodoublers" to image at video rates and with 250 nm spatial resolution.
- The same 1 MHz repetition rate amplifier microscope to be built for this purpose will be used for nonlinear optical voltamicroscopy and is sensitive enough to track very low interfacial potentials (10^{-10} V) in real time and space when using heterodyned imaging.
- Pair experiments with atomistic calculations to describe the molecular dipole moments and hyperpolarizabilities within the electrical double layer at the lipid bilayer-solution interface in the absence and presence of attached NPs.

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3. Computation and Simulation of NP Interactions Across Length and Time Scales.

A potential broader scientific outcome will be the ability to use the new modeling framework for computationally driven rational design of NPs towards sustainability. A validated multiscale framework providing a connection between molecular scale tunability and meso- to macroscale structure and function would be useful beyond the applications of interest to the CSN, potentially including emergent catalytic behavior and transport of various species within organisms. We will initially model functionalized nanogold and nanodiamond using this multiscale framework and expand to include emerging NPs within the CSN.

Computational approaches:

- atomistic quantum-mechanical calculations - atomistic force fields based on accurate quantum chemistry computations, including advanced density functional theory
- develop novel equilibrium and non-equilibrium sampling techniques to efficiently characterize the binding free energy of biomolecules to NPs under complex environmental conditions.
- construct effective coarse-grained (CG) and dynamic models guided by atomistic simulations to model NP assembly and their interaction with complex membranes
- establish an efficient computational framework that features an intimate coupling of CG dynamics, kinetic Monte Carlo, and local atomistic computations

Go to **TOP**

RFA2. Nanoparticle Transformations and Interactions with Model Biological Interfaces

The major goal of RFA2 is to understand, control, and predict how NPs attach to, penetrate, and alter model cell surfaces. We will study how the core composition, size, aspect ratio, and the types of chemical functional groups exposed at NP surfaces impact their interaction with model biological interfaces. To achieve this goal, we will acquire detailed chemical and structural views of these interactions by pairing experiments with novel computational methods that place the surfaces of NPs and membranes on an equal footing.

Hypotheses or guiding assumptions:

- Multivalent interactions may allow NPs to interact with membranes even in the presence of repulsive electrostatic interactions.
- The conformational entropy associated with ligands and polymer wrappings on NPs can contribute substantially to NP interaction with biological membranes. The importance of these effects depends critically on solution composition.
- Ligand self-organization on NP surfaces can lead to motifs that yield differences in wetting behavior and impact the orientation of bound proteins influence the interaction of nanoparticles with membranes.
- Even when NPs are coated with ligands or inorganic shells, the underlying NP core still influences the NP-cell surface interaction.
- The interaction of NPs with membranes can induce responses through several pathways, including: (1) Changing the mechanical and thermodynamic properties of the membrane. (2) Selective removal of membrane components. (3) Altering the folding and function of membrane-associated proteins.
- Removal of surface ligands, dissolution of protective inorganic shells, or both may significantly impact NP environmental interactions by controlling the rate of dissolution of the core or by enhancing photochemical production of reactive oxygen species (ROS).
- Electron transfer reactions and photochemical reactions in the environment can result in NP dissolution, change in composition, or loss or transformation of ligands.
- The high surface energy of NPs promotes adsorption of molecules from biological and environmental media in the form of biomolecular and natural organic matter (NOM) coronas, but molecular views of such coronas have remained obscure.
- During their lifecycles, nanocomposites are expected to experience chemical stresses (e.g., pH extrema, harsh oxidants/reductants, UV irradiation) that may release NPs into the environment.
- NP release depends on both exposure conditions and nanocomposite physicochemical characteristics.
- Protective inorganic shells are used in many technological applications of NPs because shells can mitigate surface reactions and promote sustainable design.
- While nm-thick shells can stabilize NPs against degradation in their engineered environment, **little is known** about their longer-term fate in bio/environmental matrices.

Experimental techniques applied to understand nano-bio interface

- 2D NMR to distinguish “alloy,” “patchy,” and “core-shell” ligand arrangements on NP surfaces.
- SFG to determine molecular orientation distributions.
- Fluorescent ligands and FRET to investigate the impacts of NPs on the assembly of ion channels, membrane receptors, and transporters and determine the extent to which NPs alter their conformations. Parallel experiments with cells.

- Apply multidimensional NMR to obtain detailed information about how biomolecules are displayed within coronas, such as determining whether changes in protein folding on the NP surface lead to the display of otherwise hidden antibody binding sites.
- Investigate the state of NP surface ligands, characterizing loss of order using NLO methods
- Characterize ligand exchange dynamics using FRET and NMR.
- "Stitch" cross-linkable ligands onto NP surfaces to investigate how to intentionally reduce ligand loss and mitigate core degradation.

Multi-scale computational methods applied to understand nano-bio interface

- Large-scale (tens of nm, sub-μs) atomistic simulations to characterize the structure and thermodynamics of NP-membrane interfaces.
- Force field parameters will be carefully calibrated based on high-level QM (Mason) and QM/MM (Cui) calculations.
- CG models to analyze the impact of surface ligand properties (e.g., charge, rigidity) on NP assembly and NP-membrane interactions.
- Atomistic simulations of NP interactions with membrane proteins will help establish testable hypotheses regarding how NPs affect electron transfer.
- Quantum mechanical, QM/MM, and reactive force field simulations will provide valuable mechanistic insights into ligand stability at NP surfaces, using embedding approaches as necessary.

Go to **TOP**

1. Synthetic Membrane Models and Organism-derived Membranes.

Experimental platforms will be tailored to the specific questions addressed. We will use free-standing membranes that span apertures, tethered and supported lipid bilayers, monolayers at the air-water interface, and suspended and supported vesicles. We will complement the experimental studies with multi-scale computational methods to provide further atomistic insight into dynamic processes at the interface of nanomaterials and model cell surfaces.

2. Mechanistic Studies of Nanoparticle Interaction with Model Biological Interfaces

1. What is the relative influence of surface ligands vs. NP core on interaction with membranes?
2. When is the concept of "locally high concentrations" important for binding interactions?
3. What are the relative importance of the hydrophobic effect and multivalent and electrostatic interactions in controlling NP-membrane interactions?
4. How do hydrophobic effect and multivalent and electrostatic interactions depend on media composition?
5. How do NPs change mechanical and other properties of membranes upon interaction?

Goals: Influence of nanoparticle/membrane properties

- Determine mechanisms, dynamics, and energetics of NP interaction with key membrane components.
- Determine NP and membrane properties that drive cellular uptake and alter membrane function.
- Investigate the effects of membrane structure and composition on interaction dynamics.
- Search for hotspots associated with changes in membrane electrostatics and structure upon NP binding.
- Probe spatial and temporal evolution of NP position, electrostatics, and ligand coverage.

- Understand how the local concentration of constituents at the NP-membrane-solution triple junction drives NP-membrane interactions.
- Relate quantitative measurement of physicochemical properties such as NP surface charge density or core composition with the degree of interaction with model membranes having different embedded charges.

The interaction of NPs with membranes can induce responses through several pathways:

- Changing the mechanical and thermodynamic properties of the membrane.
- Selective removal of membrane components.
- Altering the folding and function of membrane-associated proteins.
- NH3+ groups on PAH-Au NPs interact with the phosphatidylcholine headgroups of lipids.
- Preferential association of NPs with model peripheral membrane proteins.

NP core composition may influence:

- Strength of van der Waals interactions.
- Trapping of charge in mid-gap states at the NP-ligand interface.
- Presence/absence of image-charge effects.
- Adsorption/desorption/diffusion of ligands.

Experiments to understand how NP properties influence nano-bio interactions

- Direct comparison of responses elicited by NPs with different charge densities or different core materials with identical surface ligands.
- Comparison of metallic vs. insulating NPs and studies using semiconductor/metal core/shell NPs will provide insight into the influence of image-charge effects.
- Comparison of metallic vs. insulating NPs and studies using semiconductor/metal core/shell NPs will provide insight into the influence of image-charge effects
- Comparison of semiconductor QDs with n- and p-type doping will reveal the influence of charged surface states.
- Extract magnetic-core NPs after interaction with membranes and analyze their surfaces for membrane components.
- Cleavable ligands will be used as a mass spectrometric marker for ligand content.

Experiments to understand how membrane properties influence nano-bio interactions

- The influence of NPs on the rigidity and thermodynamics of lipid monolayers will be assessed via changes in pressure-area relationships and the miscibility of different membrane components.
- Measurements of C–H stretching frequencies and peak ratios will probe ordering of the lipid acyl chains.
- Studies of bilayer membranes spanning apertures will characterize membrane integrity using electrical impedance measurements.
- Vary the membrane composition to determine whether NPs selectively remove components from membranes.
- Determine how membrane properties are altered by NPs with different physicochemical properties (e.g., diameter, charge density, hydrophobic/hydrophilic character). Complementary studies will be performed on vesicles using NMR.
- Membranes incorporating bacterial membrane electron conduits to determine how nanomaterial redox potential determines the extent that NPs perturb transmembrane electron transport.

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3. Nanoparticle Transformations

Many engineered NPs are synthesized with organic ligands that impact their miscibility with polymers or other matrices. Once released in the environment, NPs undergo transformations that may alter NP core and surface composition, and, consequently, their interaction with cell membranes. NP surfaces have complex arrangements of ligands and adsorbed species that control the ability of water and oxidants to access the core.

Goals.

- Determine how the arrangement of ligands on NP surfaces, ligand loss from NPs, and the composition and orientation of molecules in coronas recruited around them influence NP dissolution, redox reactions, and photolysis in environmentally representative media.
- Establish how inorganic components (e.g., salts) as well as proteins and lipids in coronas alter ligand desorption and exchange rates.
- Investigate how ligands and shells impact the dissolution and release of the NP core materials and production of ROS by semiconducting NPs.
- Study rates and mechanisms of redox transformations of nanomaterials, with particular emphasis on those that participate in electron transfer reactions in model membrane or bacterial systems.
- Systematically investigate photolytic transformations of NPs, the production of ROS, and how photolysis impacts NP attachment to membranes. Determine how these interactions evolve as a function of photolysis conditions (e.g., irradiation wavelength and duration), and identify the underlying cause, such as change in NP surface chemistry or ROS production.
- Investigate how ligands and shells designed in RFA1 impact the dissolution and release of the NP core materials and production of ROS by semiconducting NPs.
- Determine the amounts and speciation of NPs released from polymer nanocomposites subjected to a wide range of stresses and evaluate their interactions with membrane surfaces and organisms to determine how their behavior differs from the original NPs.
- Study the interactions of environmentally transformed NPs and NPs released from nanocomposite materials with model membrane systems.

Exchange and Acquisition of Surface Species

Desorption and exchange rates of engineered and environmentally acquired ligands can vary by many orders of magnitude:

- The covalent all-carbon scaffold of diamond yields ligands with no detectable change over months or more.
- Carboxylic acids commonly used to functionalize metal oxides typically desorb within hours in aqueous environments in the absence of excess ligand in solution.
- Thiols, commonly used with gold and other metals and some III-V semiconductors, present an important intermediate.

Transformation of Nanoparticle Cores and Ligands

- Removal of surface ligands, dissolution of protective inorganic shells, or both may significantly impact NP environmental interactions by controlling the rate of dissolution of the core or by enhancing photochemical production of reactive oxygen species (ROS).
- Electron transfer reactions and photochemical reactions in the environment can result in NP dissolution, change in composition, or loss or transformation of ligands.

NP Release from Composites

- During their lifecycles, nanocomposites are expected to experience chemical stresses (e.g., pH extrema, harsh oxidants/reductants, UV irradiation) that may release NPs into the environment. Therefore, their quantity and

speciation (individual NPs, NPs attached to polymeric fragments) need to be determined to effectively redesign them for sustainability.

Go to **TOP**

RFA3. Molecular Interactions of NPs with Intact Cells and Organisms

The vision of RFA3 is to achieve molecular-level understanding of the interaction between NPs and organisms having diverse cellular compositions, architectures and surface chemistries. RFA3 focuses on intact cells and organisms that increase the complexity from the simplified model systems in RFA2 and centers the experiments on realistic systems. These experiments will lead to the discovery of new sets of biological responses to NPs and will enable us to develop an understanding of molecular scale processes controlling these responses.

Limitations or problems that need to be addressed:

1. Existing data are largely limited to simple, homogeneous materials such as Ag and TiO2, rather than the more complex compositions and structures used in emerging technological applications.
2. A molecular-level, mechanistic picture that connects the chemical properties of the NPs with the chemical properties and responses of biological systems.
3. Existing data have limited ability to inform predictive models for interactions with the broad diversity of organisms in the environment.
4. Secreted molecular species may bind NPs, altering attachment to and internalization by cells.
5. Macromolecules secreted by cells differ among organisms.

Guiding hypotheses or assumptions.

- Our choice of which NPs to study will be guided by a balance between using NPs whose cores and ligands are specifically designed to test specific hypotheses and more discovery-based studies aimed at identifying unique phenomena that may arise from more complex structures.
- Understanding the molecular interactions at cell- and organism-NP interfaces to provide critical information needed to make predictions about the impact of not only the current suite of available nanomaterials, but also more complex nanomaterials that could be incorporated into future commercial products.
- Comparison of the molecular interactions across NPs and biological systems with increasing complexity, using the advanced analysis and computational methods developed in RFA1 and the model systems of RFA2, will facilitate a broad understanding of the molecular mechanisms at work across organisms and environments.
- The initial NP interaction at the cell membrane is a key step in determining whether and how the NPs enter the cell and the consequent molecular interactions, ultimately determining fundamental rules that govern NP impacts on that organism.
- NPs with positively charged ligands have a larger impact on growth, reproduction, and gene expression changes.
- Examining the diversity of membrane chemistries across organisms is therefore necessary to build models of chemical interaction of NPs and cells within organisms.
- Differences between organisms change where NPs localize in each cell type, which governs the interactions of the NPs with the intracellular molecules.
- **Internal chemistry of the cell plays an equal role to the surface chemistry of NPs in determining their impact**.
- Direct visualization will allow us to determine the affinity between various tissues/cell types and NPs with particular physicochemical properties (e.g., surface charge, size, shape).
- Macromolecular crowding (where a high localized concentration of a particular functional group can interact with cell surfaces) can affect biochemical pathways.

- Molecular pathways within a cell that are altered by exposures to NPs provide an indication of how nanomaterials interact with specific chemical species in the organism.
- Exposure to specific NP-ligand combinations are expected to lead to unique patterns of up- or down-regulation of key mechanisms involved in reproduction, growth, survival, metabolism, and immune function.
- Transcriptional and proteomic response patterns can provide sensitive, early indications of the eventual ramifications that are not obvious in short-term experiments.
- Molecular pathways that are impacted upon NP-cell interaction can be used to predict the impact of future nanomaterials with similar characteristics.
- Negatively charged NPs have been shown to adsorb proteins in serum by electrostatic interaction and are subsequently taken up by cells.
- The chemical nature of coronas on NPs is determined by a combination of (1) NP surface chemistry, (2) the environment in which the particle is introduced, and (3) transformations due to interactions with cells, tissues, and organs in whole organisms.
- Chemical or photochemical oxidation of InAs quantum dots may accelerate release of In and As oxyanions.
- Adsorption of natural organic matter or secreted biomolecules could form protective layers around NPs that decreases the rate of release of degradation components.

Exploring systems with increasing complexity:

- Explore how altering **NP properties**, including those caused by the environment and modifications by the organism, alter NP interaction with biological systems.
- Determine the **molecular pathways** that are altered in response to NPs of differing chemistries, and how these interactions vary among NPs differing in composition, size, shape, and surface ligands in model species.
- Determine the molecular processes by which NPs interact with biological **systems of increasing complexity**, from cells to unicellular organisms to multicellular organisms.
- Monitor **population-level impacts on model systems** in the form of changes in mortality, immobility, growth, and reproduction to link molecular-level changes to outcomes that are of importance in relationship to the environment.

Model organisms.

- ***Model unicellular prokaryotic organisms*** include the gram-negative bacterium *Shewanella oneidensis* (important for nutrient and metal cycling) and the gram-positive bacterium *Bacillus subtilis* (important for nutrient cycling).
- ***Invertebrate models*** will include a terrestrial model (the **fruit fly** *Drosophila melanogaster*) and two aquatic models (the **harlequin fly** *Chironomus riparius* and the **water flea** *Daphnia magna*).
- ***Vertebrate models*** will focus on **rainbow trout** (*Onchorhynchus mykiss*), using both the whole organism and cell lines for gills (RTgill-W1) and macrophages (RTS-11). **Zebrafish** will be used as a model species that can be genetically manipulated for both cell lines and whole organism studies and has been demonstrated as a model for nanotoxicology studies.

Motivation for choice of organisms:

- The types of molecules and chemical functional groups they expose to the environment.
- Gram-negative and gram-positive bacteria that differ fundamentally in the molecular structure of their cell envelope.
- The differences in the chemistry of the environments in which they are found.
- Prior use in determining interactions of organisms with environmental contaminants.
- Availability of genetically manipulated cell lines or organisms, including those where specific pathways have been either deliberately silenced or altered to mitigate or indicate NP interaction with a specific cellular component.

- Cell lines provide simplified systems to test and make predictions that can inform experiments in whole organism *in vivo* models.
- A gill cell line was chosen because the gill is a likely route of exposure and the cell line has been used in nanotoxicology research.
- Macrophages were chosen because they are immune surveillance cells, are expected to come in direct contact with internalized nanomaterials, and are known to respond differentially when exposed to nanomaterials with differing surface chemistries.

Ways to control nanomaterials properties relevant to RFA3:

- Charge on NPs can be altered changing the terminal functional group exposed on the periphery (e.g., sulfonic acid vs. ethylene glycol vs. tetraalkylammonium groups) or by choosing NPs with intrinsically different zeta potentials (e.g., negatively charged TiO2 vs. positively charged CeO2).
- Charge on NPs can be altered by choosing NPs with intrinsically different zeta potentials (e.g., negatively charged TiO2 vs. positively charged CeO2).
- Electrochemical potential in lithium intercalation materials can be controllably altered by changing the extent of lithiation.
- Intercalating lanthanide ions into these materials to enable fluorescence methods on otherwise non-fluorescent NPs.

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Critical Molecular Processes by which NPs Interact with Intact Cells

Mechanism of NP Binding and Internalization into Cells.

- Measure the location and degree of NP binding and internalization identify the molecules and interaction types responsible for these processes.
- *In situ* visualization of NPs with cells membranes.
- Determine molecules bound to particles following NP extraction from cellular environments.
- Predictions regarding binding and internalization will be tested by using cells that have been genetically altered to present specific cellular components, such as membrane proteins that are suspected to be involved in the interaction.
- Investigate internalization of diamond and/or other fluorescent NPs into cells using super-resolution and single-molecule fluorescence, confocal Raman, and dark-field scattering microscopy.
- Visualization will be done when the biological interfaces are presented with NPs functionalized with single, multiple or multivalent/multifunctional ligands to provide direct insight into the effects of macromolecular crowding.
- Identify and quantify biomolecules that are bound to NPs in different systems by using magnetic Fe3O4 NPs with various surface coatings, allowing them to interact with different cell types and organisms, and then magnetically retrieving the NPs for analysis by mass spectrometry or other methods.
- Visualizations and molecular characterization, coupled with multi-scale computations, will feed back to RFA2 to design realistic membrane models with critical molecular species and to RFA1 to adapt measurement strategies to chemically characterize these interactions and redesign the NPs of interest.
- Once we have identified biological molecules believed to be important for the attachment, internalization, or subsequent intracellular fate of NPs, we will test this hypothesis by using molecular biology approaches to inhibit or silence the expression of these molecules.

- Together with fluorescence tagging of specific proteins and subcellular structures to identify their location in the cell, these approaches will allow us to determine which molecules are responsible for the internalization and intracellular fate of distinct NPs and will inform model membrane design in RFA2.

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Molecular Processes Perturbed and their Consequences for Populations

Molecular pathways within a cell that are altered by exposures to NPs provide an indication of how nanomaterials interact with specific chemical species in the organism. The interactions include:

1. NP binding to membrane proteins and undergoing endocytosis or changing membrane-bound protein conformations so they cause changes in cellular processes,
2. NPs inducing membrane disruption,
3. NPs disrupting ion transport and initiating compensation mechanisms in the cell,
4. NPs triggering immune molecules (e.g., chemokines, cytokines) to influence immune regulation,
5. Internalized NPs damaging DNA, binding proteins, or interacting with cellular functions.

Impact of Nanoparticles in Cells

- Quantitative gene expression for immune or oxidative stress responses, other toxicity assessment assays, will be used to categorize the NPs as toxic or biocompatible, both in the dark and in the presence of light.
- Responses will be compared across cultured cells and the corresponding whole organisms to determine the extent to which the responses of cultured cells predict those of whole organisms.
- Results from acute exposures of cells will be compared to acute and chronic exposures in model organisms to determine how well cell culture responses predict those of whole organisms.
- Monitor gene transcription (mRNA) using quantitative polymerase chain reaction (PCR) and high-throughput next-generation sequencing at several exposure concentrations and time points.
- Monitor protein expression through protein sequencing at several exposure concentrations and time points.
- Monitor small molecule secretion as molecular responses usingmass spectrometric analysis of natural product/secondary metabolites at several exposure concentrations and time points.

Translating molecular-level impacts to population-level consequences

Changes in molecular pathways can be merely adaptive or a greater indication of changes in physiology that has negative consequences. To link molecular-level changes to outcomes that are of importance in relationship to the environment, model organisms will also be monitored for **population-level impacts** in the form of changes in mortality, immobility, growth, and reproduction. We anticipate that these experiments will lead to the discovery of new sets of biological responses to NPs and will enable us to develop an understanding of molecular scale processes controlling these responses.

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Impact of Chemical Transformations from Cells to Organisms

Transformation of the Nanomaterial in the Organism

Impact of Environmentally Transformed NPs

NPs may be altered within an organism by binding of membrane or secreted extracellular components, by enzymatic cleavage, and other pathways.

- Chemically triggered release of NP-bound cleavable ligands will be used to isolate and analyze molecular species adsorbed from the organism/environment following NP introduction into model organisms and cells.
- Analysis of these adsorbed adventitious species and NP properties will be correlated with cell or organism function and/or toxicity.
- These cleavable linkers will enable gentle, selective removal of the corona that has formed on the NP following exposure to a biological sample, enabling us to examine the impact of the adsorbed components on the interaction with the cells and organism.
- Coat magnetic Fe3O4 (ferrite) NPs with shells of other oxides using wet-chemical or atomic layer deposition approaches to retrieve the NPs and to characterize the surface-bound molecules using mass spectrometry-based methods.
- Perform computational modeling to predict the affinity of known media components (e.g., proteins, bioactive lipids, peptides) with the ligand functional groups as well as the stability and energetics of the interacting species.
- Even in cases where protective layers eventually degrade, measurements of release rate will also serve to connect chronic and acute toxicity assays.
- Measure the impact of NPs on individual cells and whole organisms in differing pH and UV illumination conditions, as well as after interaction with natural organic matter differing in sulfur and nitrogen content, to correlate molecular transformation and organism response.
- NPs released from polymer nanocomposites and the interaction of these nanocomposites and their breakdown products will be tested in RFA3 biological systems.

Nanoparticle Redesign to Control Interaction

Guided by information acquired in RFA2 and RFA3, modifications in ligands and coatings will be used to test the hypothesis that NP chemical and physical properties can be altered in specific, predictable ways to minimize biological and environmental impacts while also retaining their practical utility and technological relevance.

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