Food Matrices: Oral delivery of bioactives with polymeric nanoparticles: bioavailability and tissue distribution of delivered active ingredient, nanoparticle translocation, and overall nanoparticle bioreactivity

PI: C. M. Sabliov[1], Co-PIs: T. Morgan[2], J. Blumberg[3], C. Astete[1], and M. Trif [4]

[1]Louisiana State University Agricultural Center, Baton Rouge, LA, [2]Louisiana State University, Baton Rouge, LA, [3]Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA, [4]Biochemistry Institute, Bucharest, Romania, European Union

Project period, duration and locations: 08/01/2010-07/30/2014, Baton Rouge, LA, Boston, MA, and Bucharest, Romania. Project cost: $599,864

Abstract

The impact of nanotechnology on the food sector is expected to be tremendous--better quality and safer nanofoods with enhanced nutritional and health benefits are envisioned and advocated by researchers and industry experts. It is critical that nanotechnology demonstrate superior potential benefits that outweigh risks to insure its acceptance by the food industry and the public. To date, little work has been done *in-vivo* to evaluate the bioreactivity of polymeric nanoparticles such as poly(lactic-co-glycolic acid (PLGA) and PLGA/Chitosan (Chi), and the effects of increased bioavailability of entrapped bioactives. Since the bio-nano interaction and cytotoxicity of polymeric nanoparticles have not yet been extensively studied in humans and safety of these systems is a major concern, it is important to understand the effect of various parameters on particle absorption, translocation and (potential) bioreactivity, as well as the effect of the particle type and properties on the pharmacokinetics and biodistribution of the entrapped bioactive. The thrust of this research is to assess the bioreactivity (biotoxicity, inflammatory effects and immune-reactivity) of polymeric nanoparticles specifically designed for delivery of biologically active compounds via food, with a focus on quantifying the bioreactivity as a function of nanoparticle properties and dose, type of entrapped active ingredient, and characteristics of the meal with which administered (**Figure 1**). The results are critical in building the interdisciplinary, cross-borders knowledge base required to design nanoparticulate systems for safe and efficient delivery of bioactives via foods. The proposal addresses ***Research Area 2***: Improve the scientific understanding of engineered nanoscale additives and ingredients that may be intentionally introduced into food for delivery of important micronutrients and modification of sensory attributes.

Objectives

The overriding goal of the project is to assess the bioreactivity (biotoxicity, inflammatory effects and immune-reactivity) of polymeric nanoparticles specifically designed for delivery of biologically active compounds via food, with a focus on quantifying the bioreactivity as a function of nanoparticle properties and dose, type of entrapped active ingredient, and characteristics of the meal with which administered.

The following objectives are proposed:

(1) Synthesize and characterize polymeric poly (lactic-co-glycolic acid) (PLGA) and PLGA-Chitosan (PLGA/Chi) nanoparticles of controlled properties (size and zeta potential) with entrapped hydrophilic and hydrophobic bioactives;
(2) Measure *ex-vivo* and *in-vivo* nanoparticle intestinal uptake and translocation as a function of composition (PLGA and PLGA/Chi) and nanoparticle properties (size, zeta potential);
(3) Study the effect of bioactive solubility, type of delivery system, and type of meal with which administered on pharmacokinetic profiles and tissue distribution of flavanol epigallocatechin gallate (EGCG) and xanthophyll lutein (LT);
(4) Determine *in-vivo* acute and chronic biotoxicity, inflammatory effects, and immuno-reactivity associated with nanoparticles; (5) Develop collaboration with EU biochemistry laboratory on polymeric nanoparticle *in-vitro* biotoxicity studies.

Experimental Approach

Polymeric nanoparticles of well-controlled sizes, surface properties, and material compositions with entrapped hydrophobic (LT) and hydrophilic (EGCG) bioactives will be synthesized out of PLGA and PLGA/Chi by (double) emulsion evaporation method. Pharmacokinetics of entrapped EGCG and LT in 3 forms (purified, PLGA, and PLGA/Chi) provided via 2 types (normal and high fat) meals will be quantified by HPLC, *in-vivo*. *Ex-vivo* and *in-vivo* experiments will be performed to quantify NP bioadhesion and translocation by confocal microscopy. *In-vivo* NP bioreactivity will be measured by clinical pathologic assessment of serum chemistry parameters and histopathologic evaluation of target organs of mice following acute and chronic exposure to PLGA and PLGA/Chi nanoparticles of two sizes. The mechanisms involved in GI delivery of hydrophobic and hydrophilic bioactives with polymeric nanodelivery systems of controlled characteristics (size and zeta potential) will be elucidated and insights on nanoparticle bioreactivity associated with nanoparticle uptake will be provided. The results are critical in building the knowledge base required to design nanoparticulate systems for safe and efficient delivery of bioactives through food applications.

Department of Biological and Agricultural Engineering
Room 149 E.B. Doran Building, LSU, Baton Rouge, LA 70803
Phone: 225-578-1055, Fax: 225-578-3492

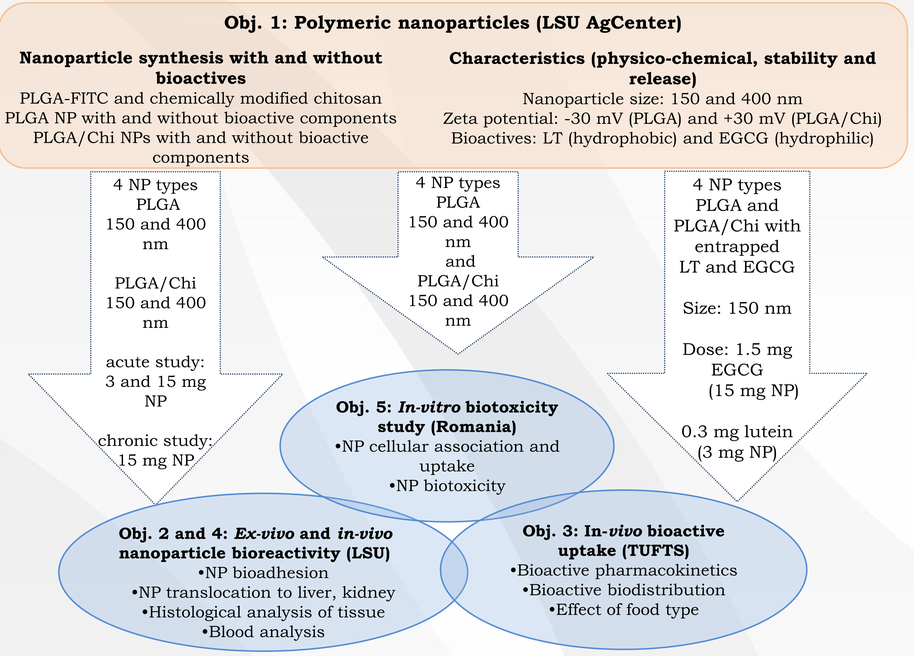


Figure 1. Planned studies, including nanoparticle synthesis and characterization, *in-vitro* biotoxicity studies, *ex-vivo* bioadhesion studies, *in-vivo* nanoparticle translocation and bioreactivity, and *in-vivo* bioactive uptake studies

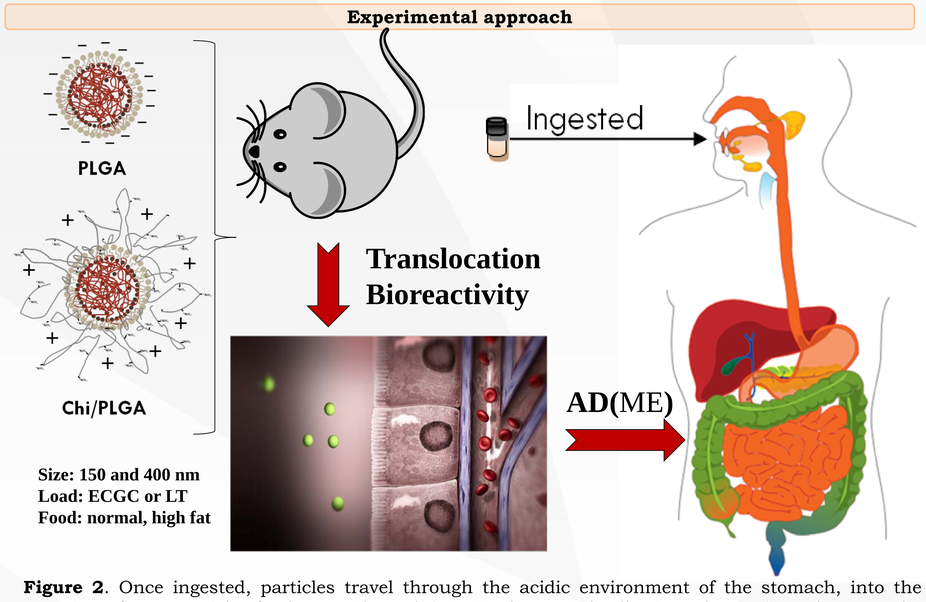


Figure 2. Once ingested, particles travel through the acidic environment of the stomach, into the intestine. If nanoparticles freely pass through intestinal mucosal cells via endocytosis, or between the mucosal epithelial cells, they enter the capillaries or lacteals and are translocated to other organs in the body. Transcytosis of smaller nanoparticles is generally higher than that of bigger nanoparticles of similar surface charge. Positively charged nanoparticles adhere to the intestinal mucosa and are uptaken by adsorptive endocytosis more prevalently than negatively charged PLGA nanoparticles. The enhanced bioavailability of the entrapped bioactive delivered with polymeric nanoparticles associated with translocation of the nanoparticles can trigger toxicity or immune and inflammatory responses following nanoparticle ingestion. With this research, we propose to study the absorption, distribution, and bioreactivity of polymeric nanoparticles made of PLGA and PLGA/Chi *in-vivo*, as well as the *in-vivo* distribution and effects of the enclosed bioactives.

Expected results/benefits, outputs, and outcomes

The optimum conditions for synthesis of PLGA and PLGA/Chi of well defined sizes and surface charges will be identified and nanoparticles in sufficient amounts will be made for the *in-vitro*, *ex-vivo* and *in-vivo* studies (Objective 1). The size and charge of the particles will be shown to play a significant role in particle intestinal uptake and tissue distribution, whereas relative tissue distribution of the nanoparticles will be constant across particle properties (Objective 2). The pharmacokinetics of the entrapped drug (Objective 3) will be explained based on the intestinal uptake of the particles (Objective 2) and bioactive release profiles (Objective 1). Both PLGA and PLGA/Chi, regardless of size will be shown to have no detectable to minimal detectable toxic, pro-inflamatory and imuno-reactive properties within target organs (Objective 4).

Particle translocation will be measured, as will degenerative, inflammatory, or immunoreactive properties (bioreactivity) of the nanoparticles. Pharmacokinetics (C_{max}, T_{max}, AUC, relative bioavailability) and biodistribution of the two bioactives will be quantified and explained on the basis of the nanoparticle uptake study. Bioreactivity and bioactive uptake results will be reported relative to rigorously measured AD(ME) characteristics for all PLGA and PLGA/Chi NPs studied. Specifically, of the ADME characteristics, absorption (A) and distribution (D) data will be reported.

Knowledge of nanoparticle translocation and eventual bioreactivity effects (including biotoxicity, immune and inflammatory effects) corresponding to the nanoparticle ADME characteristics, and ultimately to the nanoparticle physical and chemical properties is essential to label particles 'safe'. This is especially true of polymeric nanoparticles, designed and built on the premise that they are biocompatible and biodegradable and that minimum to no harm occurs when they enter the body. The AD (of the ADME) characteristics of the nanoparticles studied will be reported and can be further used to assign a bioreactivity or safety mark to other nanostructures of similar physical-chemical and AD(ME) characteristics, made of biopolymers commonly used in foods. The results of the proposed work will provide a platform for a broad understanding of bioreactivity of polymeric nanoparticles, orally delivered via foods.

Project Timeline

Table 1. Project timeline and major accomplishments

Tasks/Time (months)	1 - 12	12-24	24-36	36-48
Synthesize PLGA-FITC and chemically modified chitosan Synthesize and characterize PLGA and PLGA/Chi with entrapped LT and EGCG of controlled properties (size and zeta potential)	X	X	X	X
Study NP stability and release of entrapped hydrophobic and hydrophilic model bioactives from PLGA and Chi/PLGA NPs under gastric and intestinal simulated environment (**Paper I**)	X	X		
Ex-vivo assessment of PLGA and PLGA chitosan bioadhesion and intestinal uptake as a function of size and charge (**Paper II**)		X		
In-vivo experimental assessment of bioactive uptake and biodistribution as a function of type of nanoparticle system, nanoparticle properties, bioactive characteristics and food type (**Paper III**)			X	
In-vivo assessment of translocation of PLGA and Chi/PLGA NPs as a function of delivery system properties (**Paper IV**)			X	X
In-vivo studies of PLGA and Chi/PLGA NP bioreactivity; the effects of nanoparticle type, size, and charge (**Paper V**)			X	X
Cellular biotoxicity of PLGA and PLGA/Chi (**Paper VI**)			X	X
Project monitoring and evaluation	X	X	X	X

References

[1] Comittee, S. A. T. (2010). "*Nanotechnologies and Food.*" Retrieved from http://www.publications.parliament.uk/pa/ld200910/ldselect/ldsctech/22/2202.htm.
[2] Taskforce, F. (2007). *Nanotechnology: A Report of the U.S. Food and Drug Administration Nanotechnology Task Force.*

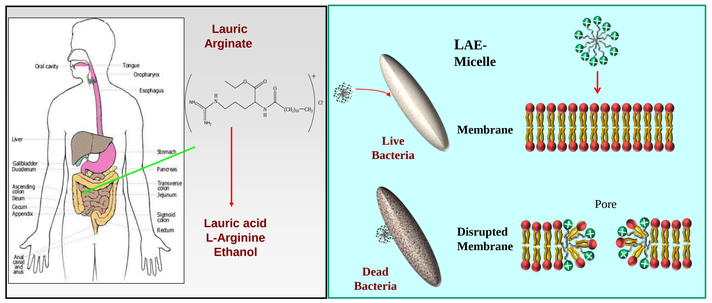
Fabrication, Characterization & Toxicology of Antimicrobial Nanoparticle Delivery Systems

David Julian McClements, Lynne McLandsborough, Hang Xiao

Dept. of Food Science, University of Massachusetts, Amherst, MA 01003

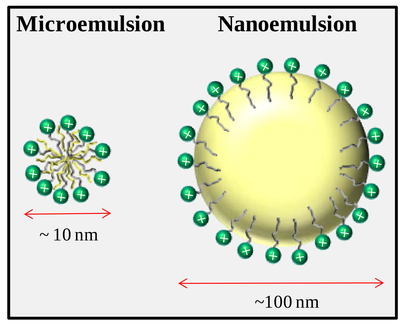
OVERALL OBJECTIVE AND HYPOTHESIS

Lauric arginate (LAE) is a food-grade antimicrobial



- Lauric arginate (LAE) is a food-grade (GRAS) cationic surfactant..
- LAE is a highly potent antimicrobial.
- LAE has a low toxicity because it is normally hydrolyzed rapidly within the human gastrointestinal tract into its natural components.

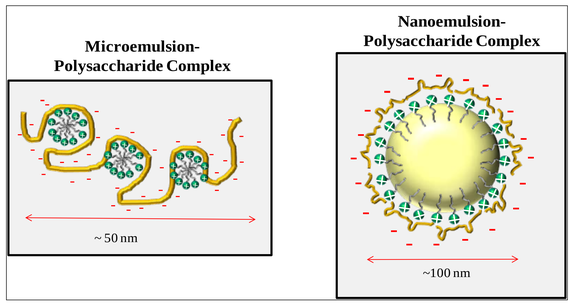
LAE forms positively charged nanoparticles



LAE nanoparticles are positively charged, which causes:

- Bitter/astringent taste : reaction with anionic mucins;
- Precipitation : reactions with anionic food components.

Improving LAE performance through electrostatic complexation



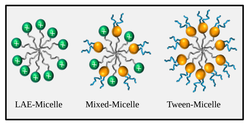
Complexation with polysaccharides influences LAE nanoparticles:

- Reduced interaction of LAE with mouth (beneficial);
- Reduced interaction of LAE with anionic food components (beneficial);
- Reduced antimicrobial efficacy of LAE (detrimental);
- Reduced hydrolysis of LAE in GI (detrimental).

OBJECTIVE 1

-- Design, Fabrication and Characterization of Antimicrobial Delivery Systems

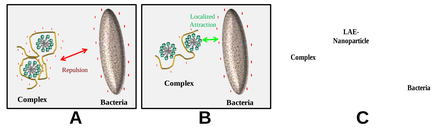
- **Materials:**
  - Surfactants:
    Lauric arginate (cationic), Non-ionic surfactants
  - Carrier oils:
    Corn oil (triglyceride oil), thyme oil (essential oil)
  - Polysaccharides
    Low-methoxy pectin, high methoxy pectin, alginate, carrageenan, gum Arabic, and xanthan.
- **Characteristics of nanoparticle-polysaccharide complexes:**
  - Size
  - Charge
  - Structure
  - Composition



- **Preparation & characterization of LAE nanoparticle-polysaccharide complexes**
  - Impact of LAE **Nanoparticle** properties;
  - Impact of **Polysaccharide** properties;
  - Impact of **Environment** (pH, I, T).

OBJECTIVE 2

-- Antimicrobial Efficacy of Antimicrobial Delivery Systems



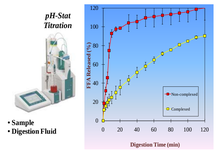
- **Complexes may alter interactions with microorganisms:**
  - **A.** Repulsion;
  - **B.** Localized electrostatic attraction;
  - **C.** Disassociation of LAE nanopariticles.
- **MIC**: determined to compare antimicrobial efficacy.

OBJECTIVE 3

-- Toxicological Assessment of Antimicrobial Delivery Systems

- **In vitro digestion model**

  Simulate human GI tract to elucidate major factors impact LAE hydrolysis:
  - ***Mouth***: + saliva solution (pH 6.8)
  - ***Stomach***: + gastric juice (pH 2.0)
  - ***Small Intestine***: + duodenal juice,
    + bile juice
    + HCO_3 (pH 6.8)



- **In vivo animal model**

  - **Subchronic toxicity studies in mice**
    - monitoring their clinical and behavioral symptoms;
    - Hematology and serum chemistry analysis;
    - Vital organs and tissues: histopathological analysis.
  - **Changes in gut microflora**

    Mouse feces will be used to determine any changes on gut microflora. Denaturing Gradient Gel Electrophoresis (DGGE) is used to detect variations in bacterial populations in mouse feces.

OBJECTIVE 4

-- Application of Antimicrobial Delivery Systems

Ideal LAE-nanoparticle delivery systems

↓

Incorporate into a model beverage

↓

Test their overall impact on the product (Appearance, stability, antimicrobial effect...)

Developing "Green Nanotechnology" for Eliminating Foodborne Pathogens

RUTGERS UNIVERSITY

Nina C. Shapley,[1] Anubhav Tripathi[2] and Karl R. Matthews[3]

1. Department of Chemical and Biochemical Engineering, Rutgers University; 2. Division of Engineering and Medical Science, Brown University; 3. Department of Food Science, Rutgers University.

Motivation

- Large scale outbreaks of *E. coli* O157:H7 and *Salmonella* associated with fresh produce have nationwide impact.
- Since 1995 FDA has identified eighteen outbreaks caused by *E. coli* O157:H7 associated with lettuce and one outbreak associated with spinach. Other outbreaks: parsley, green onions, basil, cilantro and cabbage.
- Increasingly critical need for rapid and effective methods for destroying multiple types of bacterial pathogens in fresh and fresh-cut produce.
  - Thermal treatments not appropriate for fresh produce.
  - Large scale irradiation limited by cost.
  - Chemical agents (e.g. chlorine wash) produce 1 to 2 log reduction in bacteria population, but not elimination.

Objectives

- To develop a nanoparticle wash treatment with the capability of significantly reducing or eliminating pathogenic bacteria associated with fresh or fresh-cut fruits and vegetables.
- To apply a nanoparticle wash synergistically with a conventional chlorine wash in order to achieve a 3 log reduction or better in the bacteria population.
- To address the priorites of the program, "Nanoscale Science and Nanotechnology to Ensure Safe Food," focusing on novel nanotechnology mitigation measures in minimally processed foods.

Specific Tasks

1) Design, synthesis and characterization of ultra-potent chitosan nanoparticles coated by antimicrobial peptides (e.g. nisin) ("green particles").

2) Evaluation of peptide-enhanced nanoparticles as a lysis agent in realistic food processing environments (neutral to acidic pH, low to moderate temperature, salts or lipids present).

3) Development of a post-harvest nanoparticle-electric field treatment for decreasing the bacterial load of fresh cut leafy greens and tomatoes (improving safety and prolonging shelf life).

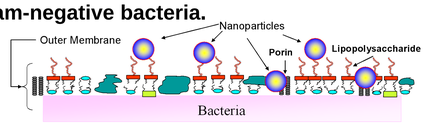
Significance

- Novelty: effective delivery of multiple antimicrobials which are required to positively eliminate both Gram-positive and Gram-negative foodborne pathogens.
- "Green technology": utilizes edible, environmentally benign, and naturally abundant materials.
- Can potentially reduce the concentrations of chemical preservatives and energy intensity required for pathogen lysis in conjunction with conventional antimicrobial treatments.
- New tools: high precision microfluidic platform and nanoencapsulation.

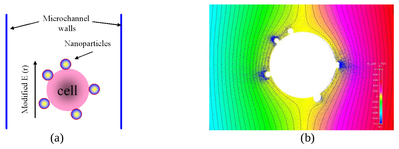
Project Fundamentals

The success of the synergistic technology for pathogen lysis depends on several connected effects.

- Attractive adsorption of nanoparticles on cell surface: Opposite charge attraction between membrane and cationic chitosan, for Gram-negative bacteria.



- Peptide antimicrobial action: Bind to and disrupt bacterial membrane structures. Typically amphipathic structure.
- Lysozyme/nisin controlled release: Nanoparticles deliver high local concentration of peptides (nisin) and proteins (lysozyme) at membrane surface. Proteins protected by encapsulation from denaturing due to hydrophobic components and oil-water interfaces.
- Enhanced electric field-membrane effects: Adsorbed nanoparticles modify electric field gradient near cell membrane, hence affect lysis. $\Delta\phi = 1.5\, a\, E \cos\theta$. Membrane dielectric breakdown voltage ($\Delta\phi$ = 0.2–1.5V).



Electric field lines and potential (color).

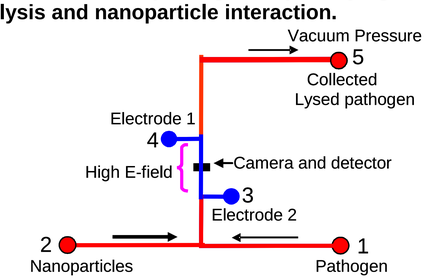
Approach

1) DESIGN, SYNTHESIS AND CHARACTERIZATION OF ULTRA POTENT CHITOSAN NANOPARTICLES COATED BY ANTIMICROBIAL PEPTIDES (e.g. NISIN)
(i) Production of chitosan nanoparticles
(ii) Encapsulation of lysozyme in chitosan nanoparticles
(iii) Surface attachment of peptides

2) EVALUATION OF PEPTIDE-ENHANCED NANOPARTICLES AS A LYSIS AGENT IN REALISTIC FOOD PROCESSING ENVIRONMENTS (NEUTRAL TO ACIDIC pH, LOW TEMPERATURE, SALT OR LIPIDS PRESENT)
(i) Evaluation of decorated nanoparticles as a lysis agent
(ii) A microfluidic setup for quantification of pathogen lysis (For Small Scale Fundamental Experiments Only)
(iii) Determine effect of E fields on *E. coli* in microfluidic system WITHOUT nanoparticles
(v) Understanding the interactions of nanoparticles with bacteria with and without electric fields
(vi) Optimization of parameters
(vii) Realistic food conditions

3) DEVELOPMENT OF A POST-HARVEST NANOPARTICLE-ELECTRIC FIELD TREATMENT FOR DECREASING THE BACTERIAL LOAD OF FRESH CUT LEAFY GREENS AND TOMATOES (IMPROVING SAFETY AND PROLONGING SHELF LIFE)
(i) Exposure of sample to *E. coli* O157:H7 suspension, chlorinated water, nanoparticle suspension. Reduction in *E. coli* measured by enumeration of colonies, with enrichment possible.
(ii) Sensory analysis of odor, color, texture, appearance post-treatment.
(iii) Application of procedure to baby romaine lettuce, spinach, cherry tomato samples.

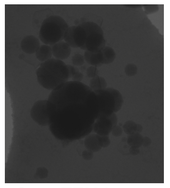
Microfluidic chip design for quantifying bacteria lysis and nanoparticle interaction.



Proof of Concept / Preliminary Data

Nanoparticle Production

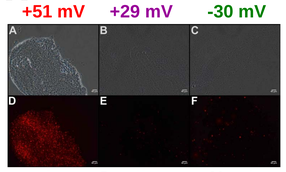
- Uniform chitosan nanoparticles synthesized by ionic gelation method in dilute solutions. Crosslinker: sodium tripolyphosphate (TPP).
- Nanoparticles characterized by zetasizing and TEM.
- Diameter range: 90-500 nm.
- Encapsulated protein quantified by calibrated UV/Vis absorption spectra [ex-280nm/em-340nm].



Cryo-TEM image, field of view = 1.64 x 1.84 µm.

Aggregation

- Rapid aggregation and microbial arrest in *E. coli* solutions of neutral pH when chitosan nanoparticles with positive zeta potential added.
- Established dominant role of electrostatic attraction.
- Nanoparticles more efficient than molecular chitosan; larger clusters form for same mass added.

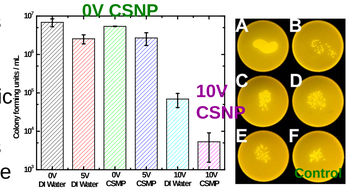


Large, dense clusters | Small, loose clusters | No clusters

Larsen, M.U., Seward, M., Tripathi, A. and Shapley, N.C. (2009) Biocompatible nanoparticles trigger rapid bacteria clustering. Biotechnology Progress 25(4), 1094-1102.

Lysis Experiments

- Chitosan nanoparticles strongly inhibit *E. coli* growth in combination with a low voltage electric field (100 V/cm).
- Chitosan nanoparticles alone ("0V CSNP") cause barely detectable inhibition: mild treatment.

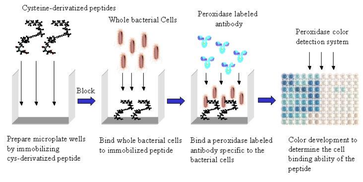


Cell colony counts after electroporation, nanoparticles or both (log scale).

Seward, M., Larsen, M.U., Jones, A., Luryi, A., Shapley, N.C. and Tripathi, A. (2010) Improved antimicrobial activity through synergistic action of microparticulate chitosan and short sustained electric field, in preparation.

Peptide Binding

- Determined native binding affinity and varied binding selectivity of antimicrobial peptides for *E. coli* O157:H7 using whole cell binding assays.



- Demonstrated ability to rapidly select a small set of candidate peptide with preferential binding for the gram-negative *E. coli* O157:H7 vs. gram-positive *S. aureus*
- Collaborator Dr. Charlene Mello, US Army Natick Research Center, specializes in peptide and protein interactions.

Exploratory research funded by CSREES NRI Nanoscale Science and Engineering for Agriculture and Food Systems Program, (#2007-35603-17738)

Challenges

- Low numbers of *E. coli* O157:H7 associated with plant tissue, possibly below detection levels.
  - Enrich plant tissue samples for non-quantitative information concerning efficacy.
- High cost in comparison to chlorine treatment.
  - Use multiple antimicrobials for synergistic effect reducing the amount of each antimicrobial agent required.
- Utilization of a greater range of conditions (challenge inoculum, pH of chlorine wash, multiple strains of each pathogen) would be ideal.
  - Inclusion of too many variables would be overly ambitious and potentially prevent delivery of a novel technology from this proof of concept study.

Summary

Our study can provide guidelines for combining mild, biocompatible and energy efficient food safety methods synergistically in the design of a "green" nanoparticle treatment step for improved safety of minimally processed fruits and vegetables.

We gratefully acknowledge support from USDA/NIFA AFRI #2010-03453.

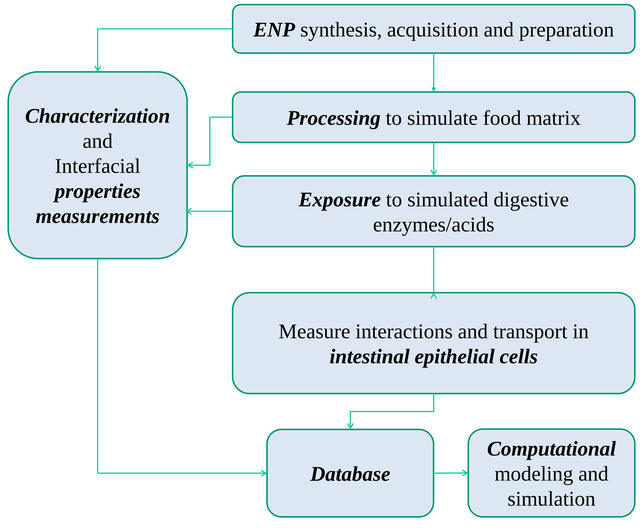
Impact of the Physicochemical Properties of Engineered Nanomaterials on their Cellular Uptake and Potential Toxicity in the Gastrointestinal Tract Environment

Dutta, Prabir K.[1], Waldman, W. James[1], Rathman, James F.[1], Yang, Chihae[2]

[1]The Ohio State University, Columbus, OH 43210 [2]Altamira LLC and FDA CFSAN MD 20740.

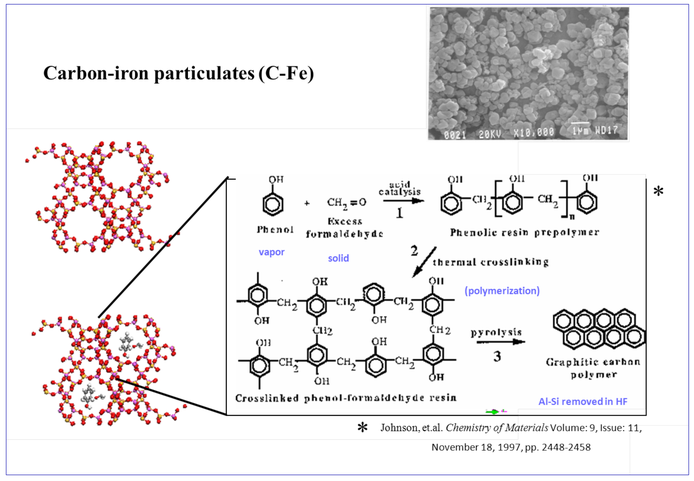
A. Project Overview

Nanomaterials are expected to be increasingly incorporated into food ingredients and food packaging. However, understanding of the potential risks associated with consumption of these materials currently lags far behind developmental aspects of this technology. To address these issues, we propose to test the hypothesis that following ingestion, engineered nanoparticles (ENPs) are modified by the environment of the gastro-intestinal (GI) tract, and that the size, composition, surface charge, crystallinity, and surface chemistry/physics of particles thus modified affect their interactions with intestinal epithelial cells, thereby influencing particle toxicity, bioavailability and tissue distribution.



B. ENP Synthesis (Specific Aim 1)

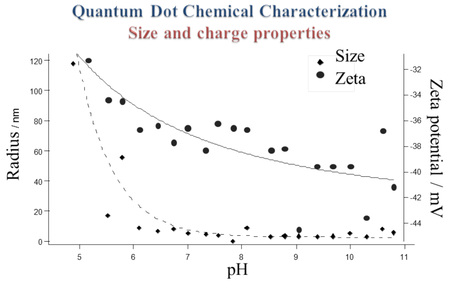
ENPs of ZnO, SiO2, TiO2, silver, and lycopene will be synthesized or acquired, dispersed in matrices which simulate conditions under which they are incorporated into food products, and thoroughly characterized with respect to size, physical and structural form, chemical composition, and surface chemistry/physics. Previous work in our group has focused on quantum dots and carbon particles.

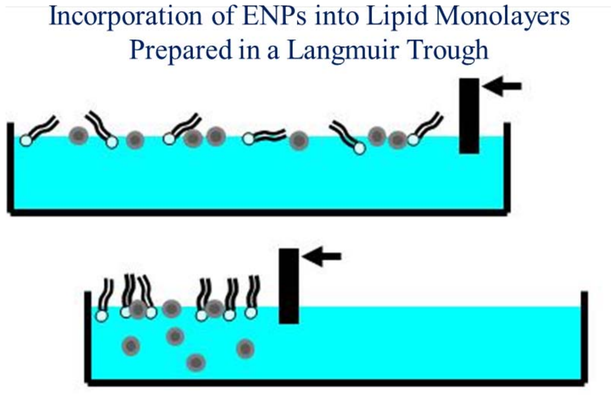


C. Characterization and Interfacial Property Measurements (Specific Aim 2)

Characterization

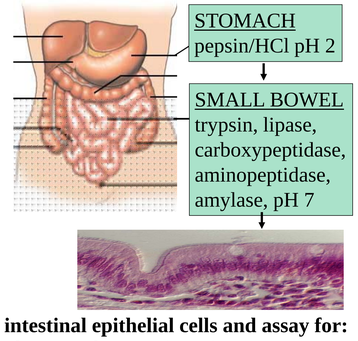
- Size, shape – light scattering, election microscopy
- Surface charge – aggregation-zeta potential
- Bulk structure – spectroscopy, diffraction
- Surface Structure - spectroscopy





D. ENP Digestion and Interactions with Epithelial Cells (Specific Aim 3)

Matrix-modified particles will be subjected to conditions which simulate the environment of the GI tract (digestive enzymes, acidic environment of the stomach, neutral environment of the small bowel) followed by thorough characterization. Interactions of "digested" particles with intestinal epithelial cells will be assessed, specifically particle internalization, epithelial cell toxicity and inflammatory responses, and the impact of particles upon intercellular adhesion and epithelial barrier function.



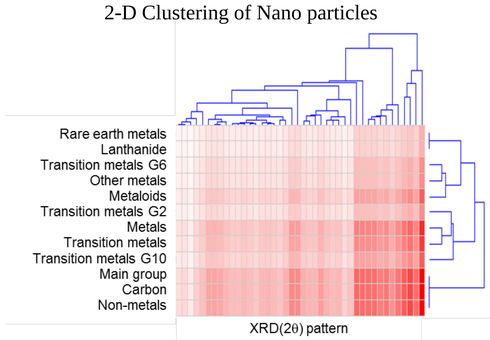
Treat intestinal epithelial cells and assay for:

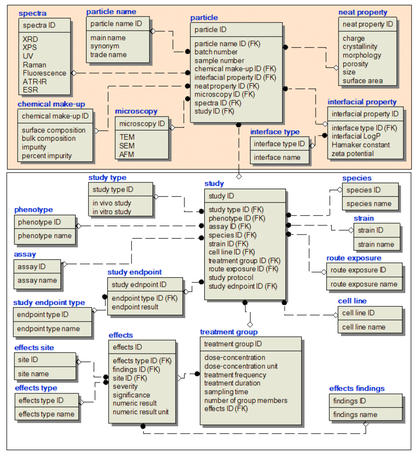
Particle internalization (TEM)
Toxicity (LDH release, Annexin V)
Inflammatory response (ELISA: TNFα, IL6)
Intercellular adhesion/barrier function (E-cadherin staining)

E. Database and Computational Modeling (Specific Aim 4)

Data generated by these studies will be used to develop material descriptors for computational use such as read-across or quantitative structure-activity relationships between ENPs and their biologic activity. This project will develop novel methods to represent ENPs for computational treatment for modeling and read-across. Data generated from this project will also be transformed into a "computable database" model in collaboration with US FDA and EU JRC. Further, computational analysis will assist designing the experiments including the biological assays. In the future, based on the learning from this project, animal studies can be designed to investigate nanoparticle translocation across the gastrointestinal epithelium and into the vascular system, routes of in vivo distribution, sites of accumulation, and routes of elimination.

2-D Clustering of Nano particles



2D nano particle clustering based on physico-chemical properties: XRD pattern (horizontal axis) and chemical groups (vertical axis). Heat-map coloring based on in vitro assay results.

Proposed data model for Engineered Nanoparticle Database