NNI Public Webinar

Characterization and Quantification of Engineered Nanomaterials: Drivers of NanoEHS Research

April 9, 2019

Speaker
Dr. Robert MacCuspie
Director of Science, *Natural Immunogenics Corp.*

Moderator
Dr. Debra Kaiser
Senior Advisor, *Office of Data and Informatics, Material Measurement Laboratory, National Institute of Standards and Technology-NIST*

Welcome

Lisa Friedersdorf
Director,
National Nanotechnology
Coordination Office

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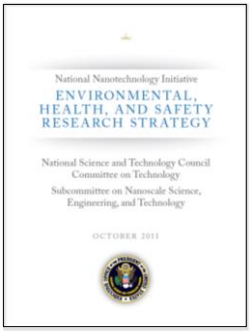
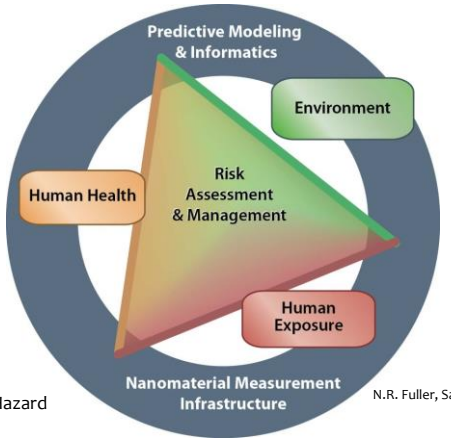
>> Lisa Friedersdorf: Good afternoon. Welcome to this webinar and thank you for joining us. My name is Lisa Friedersdorf and I am the Director of the National Nanotechnology Coordination Office. Today's webinar kicks off the NNI's 2019 nanoEHS webinar series. To mark the 15 years since the authorization of the NNI was signed, the 2019 nanoEHS webinar series will highlight the significant progress that has been made in the understanding of potential environmental, health, and safety impacts of nanomaterials. It's my pleasure to welcome our moderator, Debbie Kaiser, Senior Advisor, Office of Data and Informatics in the Materials Measurement Laboratory at the National Institute of Standards and Technology. Our speaker today is Robert MacCuspie, Director of Science, Natural Immunogenics Corporation.

Before I turn it over to the moderator, I would really like to encourage you to find out more information about this webinar series on nano.gov or our podcast series, *Stories from the NNI,* which can be found on your favorite podcast platform. You're also welcome to follow us on Twitter, at @nninanonews or on LinkedIn. Debbie, Rob, thank you so much for your time this afternoon. With that I will pass it over to Dr. Kaiser. Thank you.

NNI 2011 Environmental, Health, and Safety (EHS) Research Strategy

"A future in which nanotechnology provides maximum benefit to the environment and to human social and economic well-being"





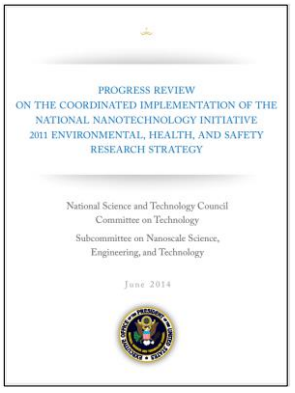
Risk = Exposure x Hazard

N.R. Fuller, Sayo-Art

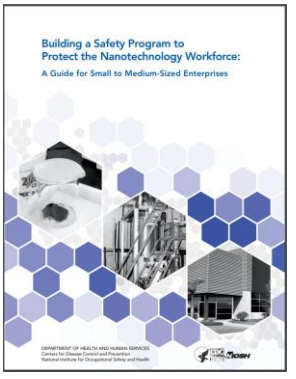
>> Debbie Kaiser: Thank you, Lisa. It's my pleasure to be the moderator for this webinar. I would like to spend a few minutes giving you some background information for the speaker. As many of you may know, in 2011 the NNI released an environmental, health, and safety nanoEHS research strategy, which was essentially a framework for Federal Government investment in nanoEHS. The figure that you see here depicts six different core research areas, within each of which there are research needs. The risk is approximately the product of exposure and hazard either to human health or to the environment. Underpinning the exposure, environment, and ultimately, risk assessment, is a nanomaterial measurement infrastructure as well as predictive modeling and informatics.

NNI Federal Agency Progress on the 2011 Nano-EHS Research Strategy

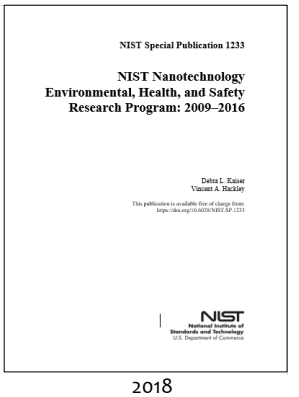
A few examples...



2014



2016

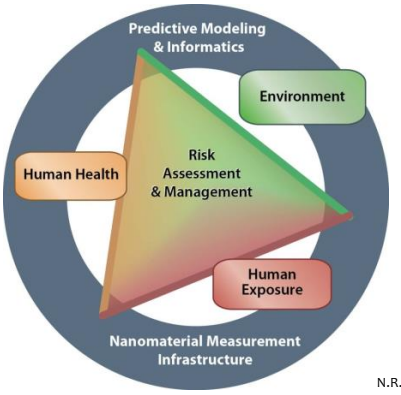


2018

>> Debbie Kaiser: The Federal agencies have conducted and funded a large body of research aimed at the responsible development of nanotechnology. This slide shows some reports. There are many reports indicating the Federal agency progress against the elements of the nanoEHS strategy.

On the left. In 2014 the NNI published a progress review that focused on the coordination of work between the different agencies. In the center is an example from NIOSH, the National Institute for Occupational Safety and Health. NIOSH has published many bulletins, and this one focuses on the nanotechnology work force. Finally, to the right, there is a NIST (National Institute of Standards and Technology) publication which summarizes seven years of the NIST nanoEHS research program. It's a pleasure to say that our speaker Dr. MacCuspie's research is included in the NIST report, as he worked there for a brief time.

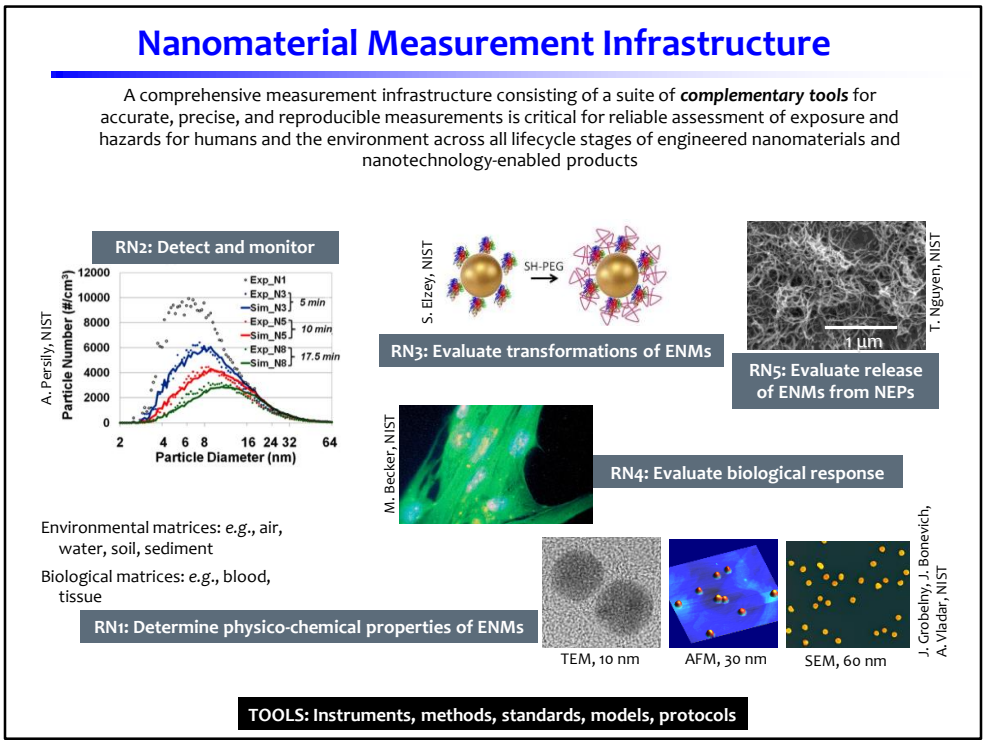
NNI 2011 Nano-EHS Research Strategy



N.R. Fuller, Sayo-Art

Science-based risk assessment and management requires accurate, precise, and reproducible measurements

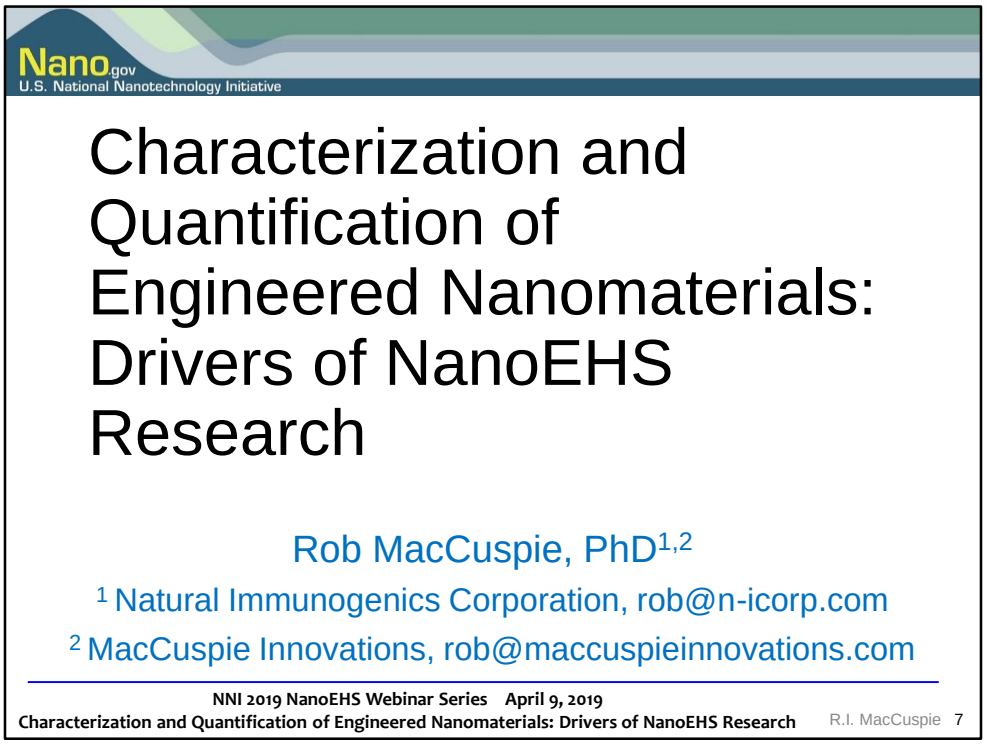
>> Debbie Kaiser: So both the NIST report and Dr. MacCuspie's talk will focus on the nanomaterial measurement infrastructure, which is at the bottom of the figure that you can see here. It is well known that accurate and reproducible measurements are needed for science-based risk assessment and management.



>> Debbie Kaiser: So what is the nanomaterial measurement infrastructure? It consists of a suite of complementary tools (which are shown at the bottom of the slide)--these being instruments, methods, standards, models, and protocols--that enable accurate and reproducible measurements, and these, tying to the previous slide, are critical for the reliable assessment of exposure and hazards, both for humans and the environment. We're talking about the entire life cycles of engineered nanomaterials and nanotechnology-enabled products.

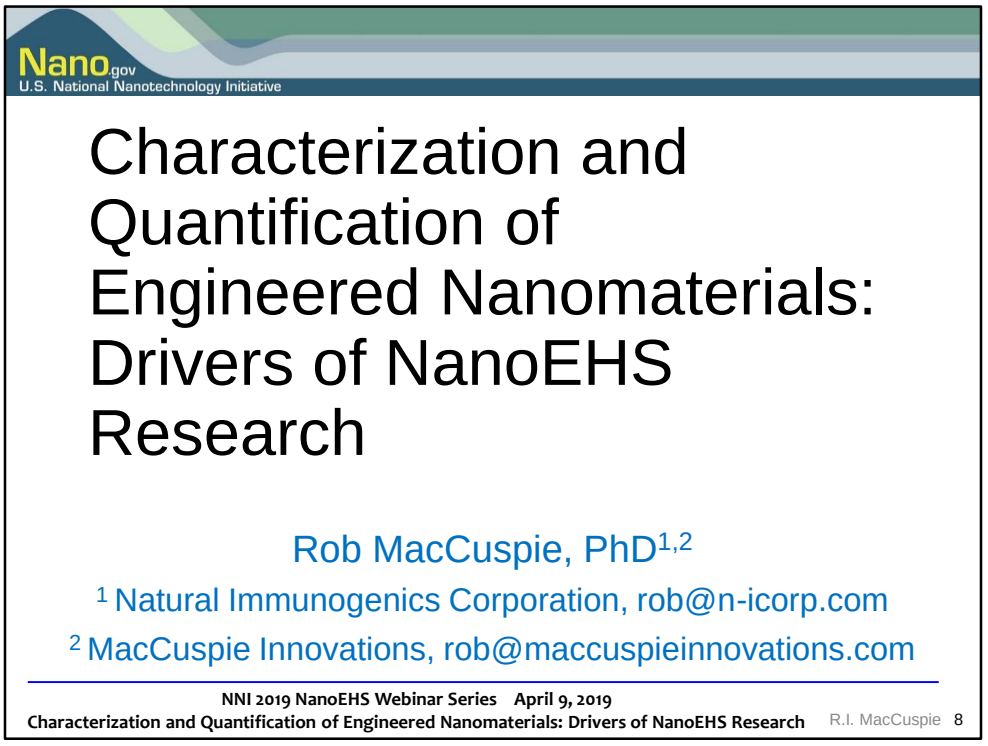
In the gray boxes you see the five different research needs that are under this core research area of the nanomaterial measurement infrastructure. These concern the physico-chemical properties of nanomaterials, detection and monitoring, evaluating transformations and biological responses of nanomaterials, and finally, evaluating the release of nanomaterials from products.

Our webinar speaker today will address several of these research needs and also will be covering a number of tools that are shown here on this slide.



>> Debbie Kaiser: I'm going to post now the speaker's title slide and introduce him. Robert MacCuspie has been working in the area of nanoEHS for over twenty years and also in the area of nanometrology. Early in his career he worked at NIST, and led a team that ultimately developed the first--and only--silver nanoparticle reference material. This work was done at NIST, and I'm pleased to say that I was the supervisor for that work, although I had nothing do with it.

Rob then moved on to academia where he served as the first faculty member and director of the nanotechnology and multifunctional materials programs at Florida Polytechnic University. In the private sector he founded his own consulting company, MacCuspie Innovations, which has advised numerous small businesses. Rob currently holds the position of Director of Science for Natural Immunogenics Corporation (NIC). He has written over 40 peer-reviewed publications and two book chapters, and he holds one U.S. patent. With that I will let Rob take over with his webinar.



>> Robert MacCuspie: Thank you very much, Dr. Kaiser, for that very kind introduction. And thank you as well, Lisa, for the invitation to present here today. I really appreciate it. It's been really neat to see how characterization and quantification of engineered nanomaterials have led to really significant advances in nanoEHS research over the years.

Disclaimer

The findings and conclusions in this presentation are those of the authors and do not necessarily represent the views of the National Institute of Standards and Technology or Natural Immunogenics Corporation.

Certain trade names and company products may be mentioned in order to specify adequately the experimental procedures and equipment used. In no case does such identification imply recommendation or endorsement by the National Institute of Standards and Technology or Natural Immunogenics Corporation, nor does it imply that the products are necessarily the best available for the purpose.

>> Robert MacCuspie: That reminds that it would not be a presentation unless we had a disclaimer that the findings and conclusions in this presentation are those of the author and don't necessarily represent the views of NIST or Natural Immunogenics. Any trade names of products mentioned, or periodically specified experimental procedures, do not imply recommendation or endorsement by NIST or NIC or imply that they are necessarily the best available for the purpose.

Goals

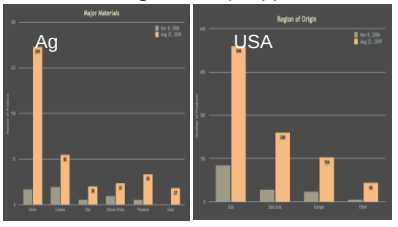
- Highlight a small portion of NanoEHS research on measurements and metrology that NNI has enabled
- Illustrate examples of how research advances in measuring nanomaterials increased NanoEHS understanding and knowledge
- Discuss how these research advances support responsible development and commercialization of nano-enabled products, including through the use of tiered risk assessment strategies

>> Robert MacCuspie: For the talk today I would really like to highlight a small portion of the EHS research in measurements and metrology and try to illustrate some examples of how these have enabled research advances for increasing our nanoEHS knowledge as a field. And I will try to discuss how some of these research advances have also led to supporting responsible development and commercialization of nano-enabled products, including through the use of tiered risk assessment strategies.

What was the context circa 2010 around NanoEHS?

- Public, Regulatory, Economic concerns over environmental, health and safety (EHS) risks associated with nanomaterials
  - EPA/Office of Pesticide Products now regulating silver nanoparticles (AgNPs) as a new antimicrobial ingredient
  - Give consumers confidence products are safe for use
- AgNPs used as antimicrobial in numerous consumer products
  - Plush toys, toothpastes, bandages, dietary supplements

http://gblsharing.app.aol.com/pop-up/
Screen clipping taken: 3/30/2010



Project on Emerging Nanotechnologies, Aug 2009

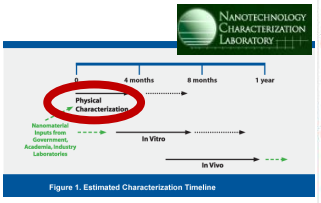
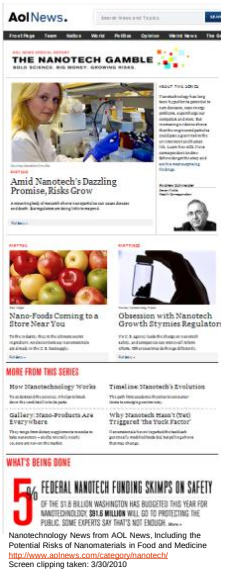


Figure 1. Estimated Characterization Timeline

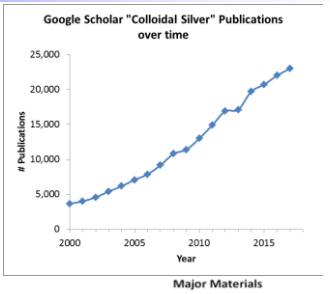


Nanotechnology News from AOL News, Including the Potential Risks of Nanomaterials in Food and Medicine
http://www.aolnews.com/category/nanotech/
Screen clipping taken: 3/30/2010

>> Robert MacCuspie: Many people in the audience may remember roughly 10 years ago the context of the nanoEHS conversation was growing public, regulatory, and economic interests wondering about the environmental, health, and safety risks of nanomaterials. It was in the media, it was well underway with the NNI Initiative, so it was getting a lot of attention. Interestingly, one of the headlines was that about 5% of Federal funding was focusing on EHS for nanotechnology. Some folks felt it needed to be more when it was, in fact, one of the highest percentages we had seen in technology development over time. So it was really neat to see the proactive nature of this.

Why silver nanoparticles? (AgNPs)

- Reported use in consumer products
- Antimicrobial properties
- Research & Consumer awareness
- Workshop findings identified AgNP reference materials were needed
  - NNI 2007, 2010
  - EPA 2009
  - NIST/US Army Corps ERDC 2009
  - NanoRelease 2011
- Requests from FDA, NTP, EPA, CPSC, others for NIST Reference Materials
  - EPA Pesticide Regulations (12/2011, first conditional registration of known nanoAg product)



Method: scholar.google.com search term "(colloidal silver) OR (nano* AND silver) OR (nanoAg) OR (AgNP)" on 6/21/18

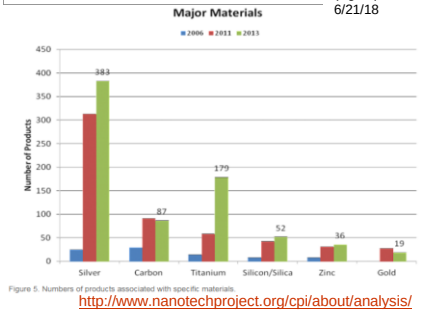


Figure 5. Numbers of products associated with specific materials.

http://www.nanotechproject.org/cpi/about/analysis/

>> Robert MacCuspie: Silver nanoparticles were one type of nanomaterial in particular that was gaining a lot of attention for their use in consumer products. The Nanotechnology Consumer Products Inventory from the Project on Emerging Nanotechnologies at the Woodrow Wilson Center was widely cited as showing the greatest number of consumer products identified as having a nanomaterial, identified silver nanomaterials, primarily for their antimicrobial properties.

As you can see in this chart, the research interests and publications have continued to grow over time, as well, and many workshops were identifying a need for silver nanoparticle reference materials (RMs) to provide a common test benchmark for all of these studies that were going on---a well-characterized, common test material, something people could include to have much more confidence when they entered comparative results.

NIST Nanoscale RMs and SRMs

Appendix E: Nanoscale Reference Materials

RM: Reference Material; SRM: Standard Reference Material; NP: nanoparticle; CNT: carbon nanotube

Material Type	Identifier(s)	Form	Reference Property	Nominal Value	Release Date	Total # Units Sold*
gold NPs	RM 8011	in aqueous suspension	mean diameter	10 nm	12/17/07	555
	RM 8012			30 nm	12/17/07	615
	RM 8013			60 nm	12/17/07	613
TiO_2 NPs	SRM 1898	dry powder	specific surface area	55 m^2/g	6/14/12	118
silver NPs	RM 8017	freeze-dried	mean diameter	75 nm	12/6/14	67
silicon NPs	RM 8027	in toluene suspension	mean diameter	2 nm	2/4/14	9
single-wall CNTs	SRM 2483	dry soot	mass fraction	impurity elements	11/14/11	62
	RM 8281	in aqueous suspension	length	"long", "medium", "short"	7/9/13	15
multiwall CNTs	SRM 2484	dry soot	mass fraction	impurity elements	6/1/17	0

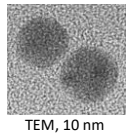
*As of July 2018

https://nvlpubs.nist.gov/nistpubs/SpecialPublications/NIST.SP.1233.pdf

>> Robert MacCuspie: NIST has actually developed many nanoscale reference materials, including gold nanoparticles, titanium dioxide (TiO_2), silver, silicon, and single-walled and multiwalled carbon nanotubes, with a variety of data provided on their certificates of analysis.

NIST Gold Nanoparticle RMs

RMs 8011, 8012, 8013: Gold Nanoparticles, nominal diameters of 10 nm, 30 nm, and 60 nm



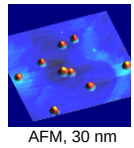
TEM, 10 nm

Feedstock and unit form

- Citrate-stabilized Au nanoparticles in an aqueous suspension, two 5 ml ampoules per unit

Measurement methods employed for reference values

- Atomic force microscopy, scanning electron microscopy, transm microscopy
- Electrospray-differential mobility analysis
- Dynamic light scattering, small angle X-ray scattering



AFM, 30 nm

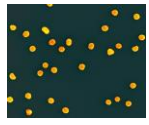
Measurement sample preparation

- Different for each method; dispersion key for microscopy metho

NIST RMs
8011, 8012, 8013

Reference Values

- Particle size by determined by six methods above
- Different techniques measure different aspects of particle size



SEM, 6o nm

Technique	Analyte Form	Particle Size (nm)
Atomic Force Microscopy	dry, deposited on substrate	55.4 ± 0.3
Scanning Electron Microscopy	dry, deposited on substrate	54.9 ± 0.4
Transmission Electron Microscopy	dry, deposited on substrate	56.0 ± 0.5
Differential Mobility Analysis	dry, aerosol	56.3 ± 1.5
Dynamic Light Scattering	liquid suspension	
backscatter, 173° scattering angle		56.6 ± 1.4
90° scattering angle		55.3 ± 8.3
Small-Angle X-ray Scattering	liquid suspension	53.2 ± 5.3

Technique	Analyte Form	Particle Size (nm)
Atomic Force Microscopy	dry, deposited on substrate	8.5 ± 0.3
Scanning Electron Microscopy	dry, deposited on substrate	9.9 ± 0.1
Transmission Electron Microscopy	dry, deposited on substrate	8.9 ± 0.1
Differential Mobility Analysis	dry, aerosol	11.3 ± 0.1
Dynamic Light Scattering	liquid suspension	13.5 ± 0.1
Small-Angle X-ray Scattering	liquid suspension	9.1 ± 1.8

NIST

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>> Robert MacCuspie: The gold nanoparticles were very useful for their very monodispersed size distribution---10, 30, and 60 nm nominal diameters. Six different measurement techniques were applied to help illustrate the differences in the underlying measurements used to report a size value for the nanomaterials.

NIST Silver Nanoparticle Reference Material

RM 8017:
Silver Nanoparticles, nominal diameter 75 nm

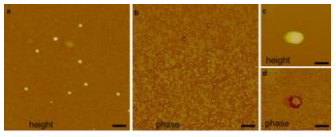
National Institute of Standards & Technology

Report of Investigation

Reference Material 8017

Polyvinylpyrrolidone Coated Silver Nanoparticles

(Nominal Diameter 75 nm)



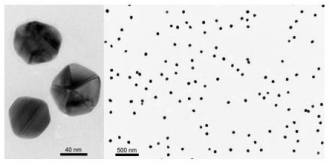


Table 1. Reference Values and 95 % Uncertainty Intervals for Mean Silver Particle Size[a,b]

Methods	Analyte Forms	Particle Size (nm)
Atomic Force Microscopy (AFM)	dry, deposited on substrate	70.1 ± 6.0
Transmission Electron Microscopy (TEM)	dry, deposited on substrate	74.6 ± 3.8
Ultra-Small-Angle X-ray Scattering (USAXS)	liquid suspension	67.9 ± 0.8
Dynamic Light Scattering (DLS)[c]	liquid suspension, diluted	105.6 ± 4.6

>> Robert MacCuspie: The silver nanoparticles were a very interesting project because, as we'll hear about, silver metal particles release silver ions; they undergo a surface oxidation and dissolution process over time. So in order to ensure that the size distribution will be uniform for the customer and it will have a stable five-year shelf-life, the team came up with the idea of freeze drying and packaging under a vacuum to prevent these transformations from happening on the shelf, and then the user would just add water to get a singly dispersed uniform set of silver particles.

Intercomparison of Sizing Results

What size are my nanoparticles?

Are they the same as my colleagues'?

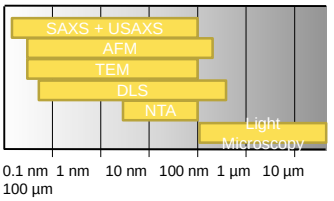
Going beyond "Minimum Characterization"

- What are "stock" conditions?
- Underlying Metrology
- Timing: Stability and Transformations
- Attention to detailed experimental reporting
- Using consensus protocols

>> Robert MacCuspie: This is important because in research we're looking at these sized-based effects, often. So we're asking what size are my nanoparticles and are these the same size as my colleagues'? We want to compare our results to what's been done in the literature and understand what other folks are seeing. And so, oftentimes, this requires going beyond so-called "minimum characterization levels" and looking at what are the working stock conditions and the underlying metrology of the measurements. And the timing of the sample prep to the measurement, as well, becomes important.

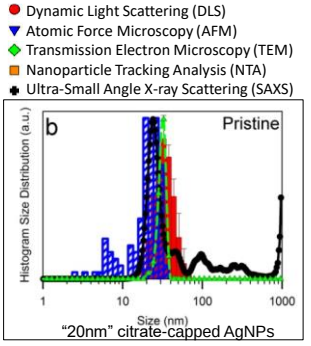
Underlying Metrology

- What is being measured and reported?
  - Different techniques provide different measurands
  - Different techniques weight size distributions differently
- Instruments have different operating ranges
  - No single technique "sees" everything for every case
- "Conflicting" results may be from underlying metrology
- Report size distributions!



	TEM	AFM	SAXS / USAXS	DLS	NTA
Measures size of:	Ag only	Ag & dehydrated Coating	Ag only	Ag & coating & solvent	Ag & coating & solvent
Measurement basis	Number R	Number R	Volume R^3	Volume^2 R^6	Number R
Strength / "see"	Small NPs	Very Small NPs	Balanced	Infreq. Large NPs or Agglom	Mixtures of sizes
Weakness / "miss"	Infrequent structures	Infrequent structures	Conc. < ~0.01 mg/mL	Small NPs in mixtures	NPs < ~30 nm

Adapted from: R.I. MacCuspie, "Characterization of nanomaterials for nanoEHS studies", Ch.4, *Nanotechnology Environmental Health and Safety, 3rd ed.*, M. Hull, D. Bowman, 2018



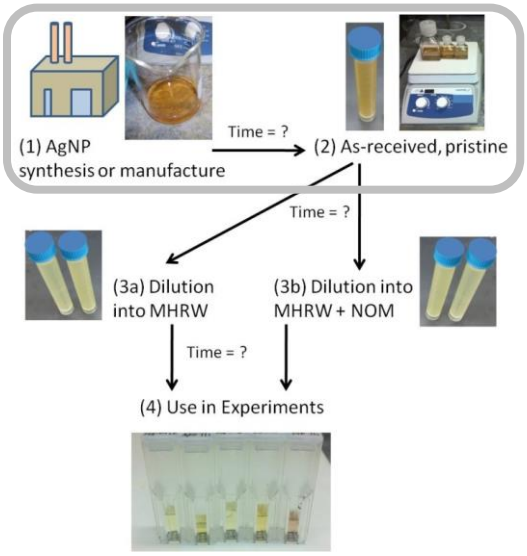
MacCuspie, Rogers, *et al., J. Environ Monit* (2011), DOI:10.1039/C1EM10024F

>> Robert MacCuspie: Looking at the underlying metrology, you can see that in cases like for TEM (transmission electron microscopy) compared to DLS dynamic light scattering, TEM measures the silver metal core, the diameter of that particle, and get a number basis for that measurement, average size. The strength of this is you get to see those small nanoparticles. But if you have infrequently occurring structures, you may not see those, whereas with dynamic light scattering, you're measuring both the silver core and any hydrated coating on the surface of the particle and sometimes a layer of solvent molecules associated with the Brownian motion of those particles.

You get the intensity or volume-squared basis for your measurement due to the Rayleigh scattering of the light with a photo detector. So it's proportional to the radius to the sixth power, which makes the strength of DLS the ability to see those infrequent agglomerates or infrequent large nanoparticles. But it kind of provides a challenge for seeing the small particles in a polydisperse solution. By understanding how to pair the right techniques, you can really get a full view, a much better view, with multiple, orthogonal measurements.

What Are "Stock" Conditions?

- Ambiguous meanings
  1. Vendor's label?
  2. Measure as-received?
  3. Measure diluted into…??
- Timing
- Detailed reporting is critical

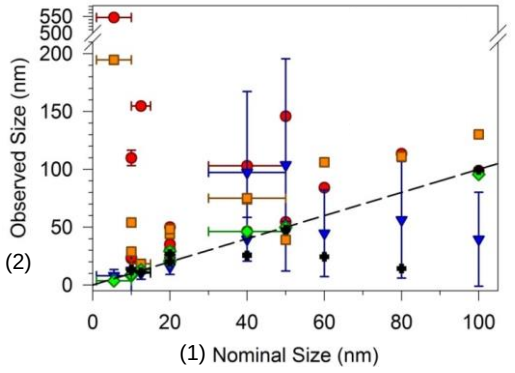


R.I. MacCuspie, K. Rogers, *et al.*, *J. Environ Monit* (2011), DOI:10.1039/C1EM10024F

>> Robert MacCuspie: This relates to how we characterize and communicate what we have. Also in terms of if you were to purchase materials or receive them from a collaborator, there could be a measurement made at the factory by the manufacturer and then a measurement made upon receipt in a lab or dilution into various working buffers and stocks before its use in experiments. There are potential transformations that could influence what the size and size distribution is, so it's important to compare this.

Comparing Results

- Divergence between two "stock" conditions
- Not all AgNPs are created equally
  - *e.g.*, narrow vs. wide size distribution
- Are these "conflicting" results?



Dynamic Light Scattering
Atomic Force Microscopy
Transmission Electron Microscopy
Nanoparticle Tracking Analysis
Ultra-Small Angle X-ray Scattering

R.I. MacCuspie, K. Rogers, *et al.*, *J. Environ Monit* (2011), DOI:10.1039/C1EM10024F

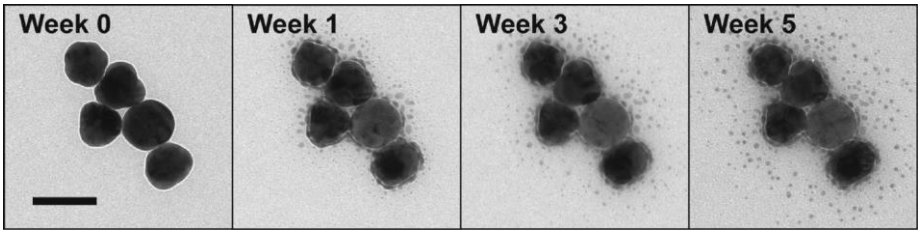
>> Robert MacCuspie: Here is a brief example comparing some products as measured by the manufacturer and then measured as they were received in two different laboratories. This one particular study highlighted a variety of techniques; on the dotted line if the recipient got the exact answer that the manufacturer did and put on the label, you would see all the data would fall on that line.

As you can see, transmission electron microscopy seems to be the gold standard technique for particle sizing that most people tend to be using. We tend to get quite nice agreement and you also get good agreement with several other techniques, in many cases. But as you begin to see some disparity between what would be predicted from the label and the measurements you can look at, what is the role of the technique, or is it actually telling you something more useful about the types of particles that you have?

And so we can begin to reconcile what are sometimes apparently conflicting results when you understand more of these transformations.

Sample Prep – Timing Matters for TEM Measurements

- Storage of microscopy samples leads to artifacts
- Incidental AgNPs could affect size distribution results
- Reporting of timing between sample prep and microscopy measurements is critical!

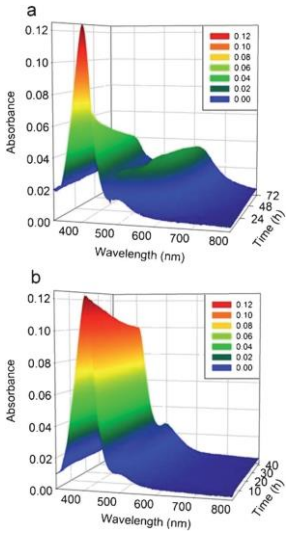


Glover, Miller and Hutchison, *ACS Nano*, 2011, v. 5(11), p. 8950-8957 doi: 10.1021/nn203131

- Consistency of sample prep timing can reduce apparent batch-to-batch variation

>> Robert MacCuspie: The timing prep is critical. In the case of transmission electron microscopy it was reported that if you did not store the TEM grids under a dry, humidity-free environment, that over time the humidity layer that was absorbed in a very thin layer to the surface could actually allow the dissolution of ions and then their renucleation to form satellite nanoparticles surrounding these large original particles. So these incidental particles that began to show up on the grid over time could influence the reported average size or size distribution. So it became revealed that careful sample handling and short turn-around times led to better accuracy and less apparent batch-to-batch variation.

Sample Prep – Timing Matters in Environmental Water



- Dilution buffer composition matters
- Fulvic acids(a) or humic acids(b) mixed with EPA MHRW
- Citrate capped, nominally 20 nm diameter
  - Varied degrees of agglomeration
  - Varied degrees of single AgNP loss
  - Effects after 24 hours

R.I. MacCuspie, K. Rogers, *et al.*, *J. Environ Monit* (2011), DOI:10.1039/C1EM10024F

>> Robert MacCuspie: In terms of the timing as well, looking at the absorbance of these particles, their surface plasmon resonance changes over time, and various working buffers, you could begin to see as a function of the nature of the buffer, whether it had various types of natural organic matter or moderately hard reconstituted water, you could see changes to the degree of singly dispersed particles based on the surface plasmon resonance and changes in the degree of agglomeration based on those red-shift and absorbance peaks.

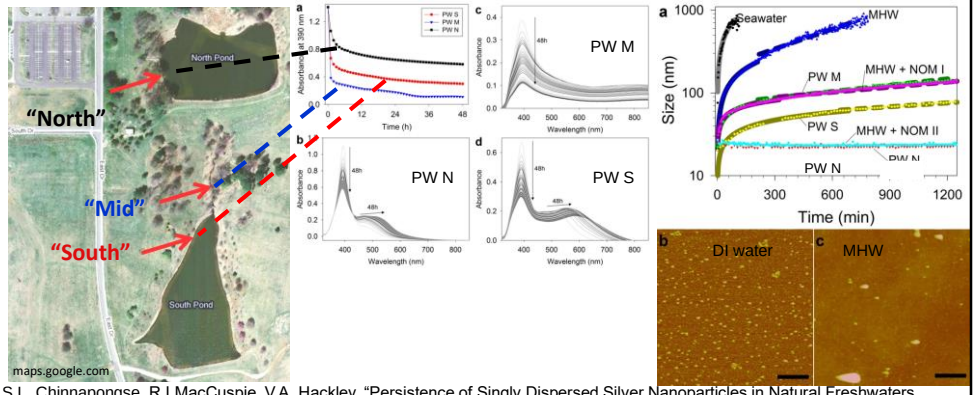
You begin to not only understand what you are starting with and how it might be changing in relevant exposure conditions for EHS studies, but you could also begin to ask questions [*see next slide*]...

What is the fate of AgNPs in Natural Waters?

Measuring Colloidal Stability in Environmental Waters

Citrate-capped 20 nm AgNPs added to:

- Pond-water from NIST campus (PW), 3 locations geographically clustered
- Seawater (synthetic per ASTM D1141) & EPA Moderately Hard Water (MHW) + Natural Organic Matter (NOM), Suwannee River Standards I and II



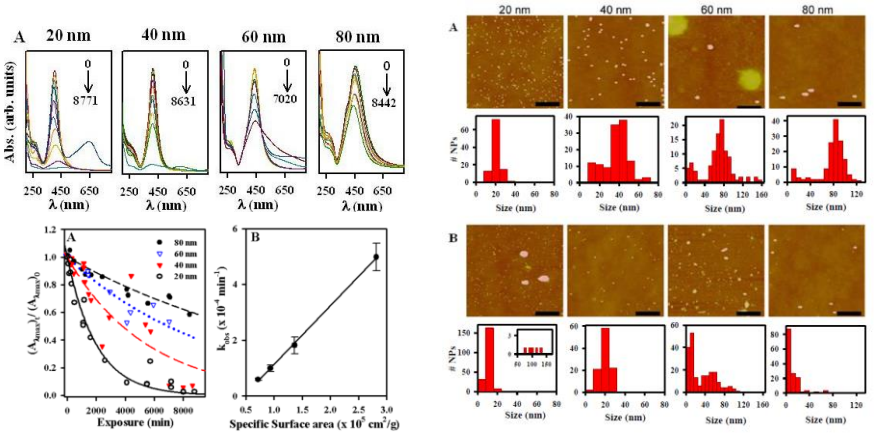
S.L. Chinnapongse, R.I MacCuspie, V.A. Hackley. "Persistence of Singly Dispersed Silver Nanoparticles in Natural Freshwaters, Synthetic Seawater, and Simulated Estuarine Waters." *Science of the Total Environment*, (2011), *409*(12), 2463-2450. doi: 10.1016/j.scitotenv.2011.03.020

>> Robert MacCuspie: You could begin to ask questions like, What would be the fate of silver nanoparticles in a natural surface water? In this particular study from groundwater sources sampled on the NIST campus by a summer undergraduate research fellow found there were some interesting distributions of water chemistries even in a geographically clustered site of water samples and that this did impact the colloidal stability of silver nanoparticles in these natural waters.

By being able to compare that to some well-defined materials, we began to elucidate what these various natural organic matters' roles were in providing the colloidal stability over time. So by being able to make these measurements, we were able to understand whether the silver nanoparticles would remain in the water column, and if so, in what form, or if they were likely to sediment out, and if EHS researchers should be looking more at soil types of scenarios.

Does UV-light Induce AgNP Dissolution?

- UV-light oxidizes AgNPs, increasing dissolution rates
- Dissolution rate is proportional to particle surface area

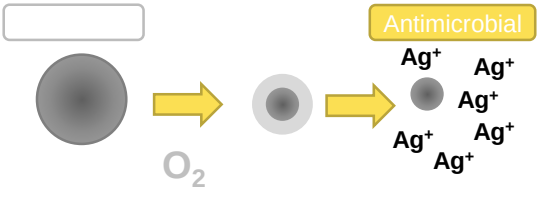


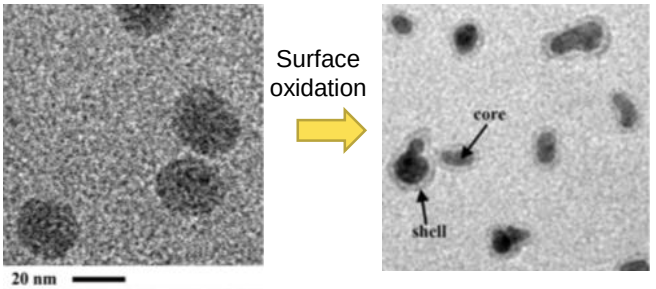
"UV-induced photochemical transformations of citrate capped silver nanoparticle suspensions"
J.M. Gorham, R.I. MacCuspie, K.L. Klein R.D. Holbrook, D.H. Fairbrother., R.D. Holbrook. (2012).

>> Robert MacCuspie: Measurements of the particles also enabled questions like, What role would UV light play for inducing silver nanoparticle dissolution with a photo-oxidation process. It turns out that this was indeed observed. Not surprisingly, the dissolution rate consonant was directly proportional to the specific surface area of the nanoparticles. So for various sizes, as the diameter decreased, they began to dissolve more quickly, of course, and so I was able to find some kinetics about how fast this might happen as a function of UV light.

Surface Oxidation of AgNPs & Dissolution/Ion-Release

- Silver ions (Ag^+) are the antimicrobial form of silver [1]
- Ambient oxygen can react with silver metal surfaces to form a silver oxide shell. [2]
- Antimicrobial silver ions are released
- **Confirmed by liquid cell TEM**



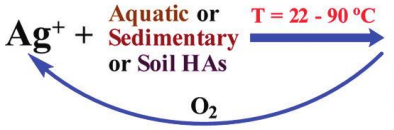


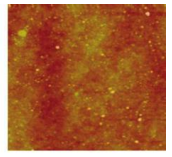
[1] "Negligible Particle-Specific Antibacterial Activity of Silver Nanoparticles", Xiu, et al., 2012, Nano Letters, 12(8), 4271-4275.
[2] "UV-induced photochemical transformations of citrate-capped silver nanoparticle suspensions" Gorham, MacCuspie, et al., 2012, J. Nanoparticle Research, 14(10), 1-16. doi: 10.1007/s11051-012-1139-3

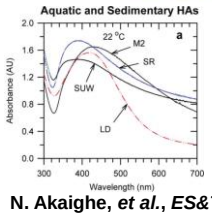
>> Robert MacCuspie: It was also really neat to see some advances in measurements such as liquid cell transmission electron microscopy, to be able to capture one of the first images of these silver oxide shells, this intermediate state in the dissolution of silver nanoparticles. By inducing the dissolution process with UV light and then flowing it through the liquid TEM cell, you can see the lower lighter-grey contrast of the silver oxide shell surrounding the silver metal core with the size distribution decreasing, as was confirmed by the other measurements. So with that release of antimicrobial silver ions, as opposed to the actual particles themselves, that helped to inform other nanoEHS studies that were going on.

Measurements Lead to Discovery of a Silver Cycle

- Silver ions can be reduced by humic acids to form AgNPs
- Multiple measurements confirmed AgNPs' presence
- Perhaps a cycle may exist?
- All AgNPs observed in environment may not be anthropogenic

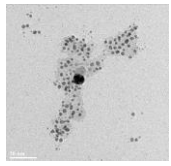






After 8-14 days

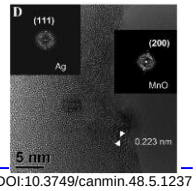
AgNPs



N. Akaighe, *et al.*, *ES&T* (2011), *45*(9), 3895-3901, DOI: 10.1021/es103946g

Summary and Conclusions

In river waters of Texas, 33–89% of the operationally defined dissolved Ag fraction was present in a colloidal form. High affinity of Ag for suspended particulates in river water was



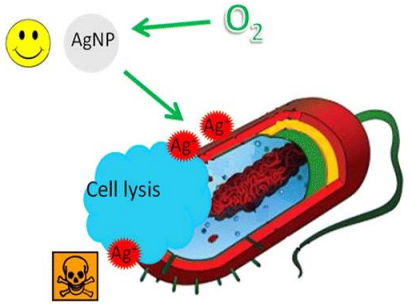
730 ■ ENVIRONMENTAL SCIENCE & TECHNOLOGY / VOL. 31, NO. 3, 1997

DOI:10.3749/canmin.48.5.1237

>> Robert MacCuspie: It was this release of ions that led to questions and kind of a serendipitous discovery that when you place silver ions in the presence of these natural organic matter compounds and leave them on the bench for a couple of weeks at room temperature under ambient conditions, there is enough of a reducing potential to convert the silver ions into new silver nanoparticles, which suggests there might actually be a cycle between the oxidation of the metal particles releasing ions and then those ions nucleating back into new nanoparticles, which could in turn, again, be surface-oxidized and dissolved.

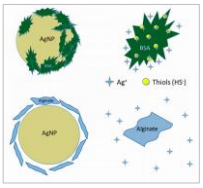
By having a good design of methodologies and the ability to characterize the materials well, this study was able to elucidate this pathway, that there are a variety of transformations. Upon examining the peer-reviewed literature, some environmental waters in the '90s, before the widespread introduction of engineered nanomaterials, in Texas estuaries, were found to have colloidal silver fractions. And also in mine tailings in Mexico they were finding colloidal silver as well as silver ions in mine tailings. So it led to this suggestion that this natural process may be occurring.

Silver Ions are the Active Antimicrobial

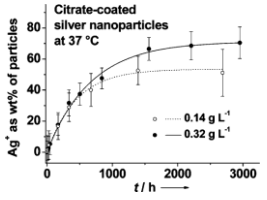


"Negligible Particle-Specific Antibacterial Activity of Silver Nanoparticles." *Nano Letters* 12(8): 4271-4275. Xiu, Z.-m., et al. (2012).

"For nearly a decade, researchers have debated the mechanisms by which AgNPs exert toxicity to bacteria and other organisms. The most elusive question has been whether the AgNPs exert direct "particle-specific" effects beyond the known antimicrobial activity of released silver ions (Ag^+). Here, we infer that Ag^+ is the definitive molecular toxicant. We rule out direct particle-specific biological effects by showing the lack of toxicity of AgNPs when synthesized and tested under strictly anaerobic conditions that preclude Ag(0) oxidation and Ag^+ release."



Influence of Bovine Serum Albumin and Alginate on Silver Nanoparticle Dissolution and Toxicity to *Nitrosomonas europaea*
DOI: 10.1021/es4033106



"Toxicity of Silver Nanoparticles Increases during Storage Because of Slow Dissolution under Release of Silver Ions"
DOI: 10.1021/cm100023p

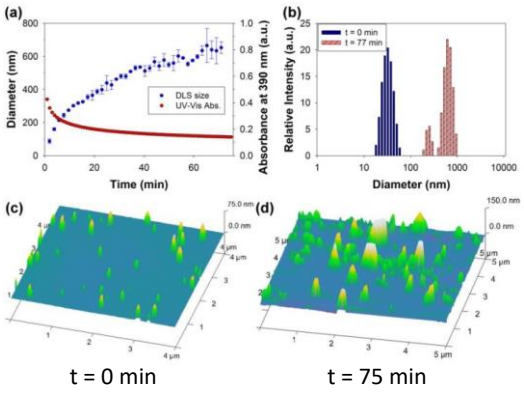
>> Robert MacCuspie: This idea that surface oxidation and dissolution of the nanoparticle released ions also helped researchers understand that the silver ions were the dominant mechanism of antibacterial activity of silver nanoparticles. There was a lot of controversy in the literature and a lot of suggestions that there may be particle-specific effects. And there were a lot of papers that came out and suggested that the dissolution that released the silver ions indeed provided the antimicrobial activity, making the cell wall membrane leaky to the prokaryotic cell and causing a cell lysis event.

It was a very elegant study to remove the oxygen from the environment and observe that it did not cause any harm to the bacteria when they were just metal particles. But then when they did release the ions, they were able to share that it did cause the antibacterial effects. So it was the measurements that enabled that experimental design and confirmation that was indeed what was going on, to get some insights into that debate in the literature.

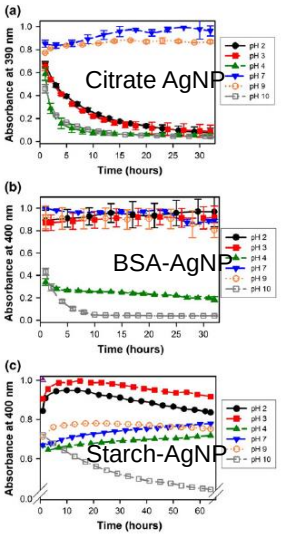
What Happens to AgNPs in Biological Media?

Citrate capped, nominally 20nm diameter AgNPs, PBS 1X

Rapid changes, (< 10 min), Stable fractions?



t = 0 min t = 75 min



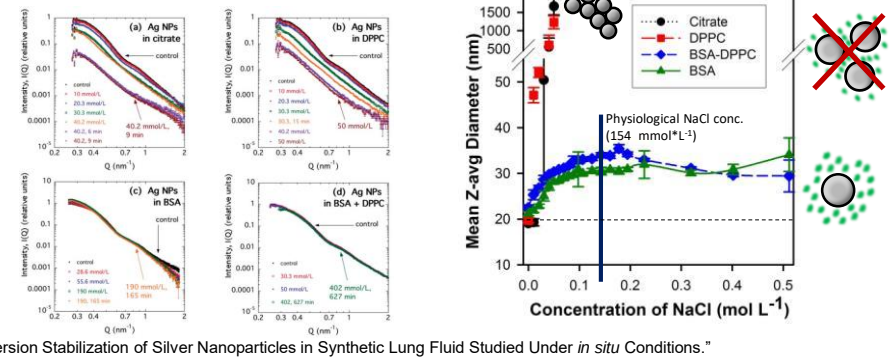
R.I. MacCuspie. "Colloidal Stability of Silver Nanoparticles in Biologically Relevant Conditions." *Journal of Nanoparticle Research*, (2011), DOI: 10.1007/s11051-010-0178-x

>> Robert MacCuspie: That also led to broader questions. Can you measure what happens to silver nanoparticles in biological media? Obviously, the likelihood of ionization depends on the surface coating and the pH and the time that it is in solution. So we were able to understand the kinetics of these transformations and the roles of the surface coatings.

What Form do AgNPs Take in Lung Fluid?

- Multiple measurement techniques provide more information in total than individually
  - (DLS + SAXS + AFM + UV-Vis)
- Synthetic Lung Fluid = bovine serum albumin (BSA) + phospholipid (DPPC)
- 20 nm citrate-capped AgNPs
- *Serum Protein is key to colloidal stabilization*

Porter, *et al.*, "A biocompatible medium for nanoparticle dispersion." *Nanotoxicology* **2008,** *2* (3), 144-154.

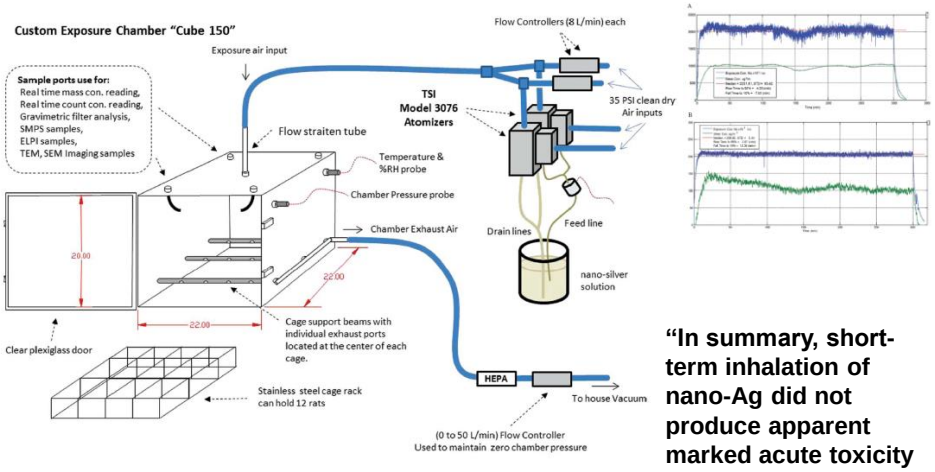


"Dispersion Stabilization of Silver Nanoparticles in Synthetic Lung Fluid Studied Under *in situ* Conditions."
R.I. MacCuspie, V.A. Hackley, A.J. Allen, *Nanotoxicology*, (2011), DOI: 10.3109/17435390.2010.504311.

>> Robert MacCuspie: Also, to look at some specific biological fluids, such as a synthetic lung fluid that was developed by NIOSH and be able to interrogate what was the nature of the dispersion and fate of silver nanoparticles in this lung fluid. By combining the appropriate measurements, we were able to determine that the particles remained singly dispersed and adsorbed a multilayered shell of serum protein on the surface of the particles to provide that colloidal stability. They did not form small clusters or agglomerated particles. They required the proteins to remain colloidally stable, and the contributions from the phospholipids were negligible for the colloidal stability.

But it was this combination of measurements that allowed the confirmation that that is indeed how the particles will behave in that biological compartment.

Measuring Realistic Aerosol Exposure to AgNPs

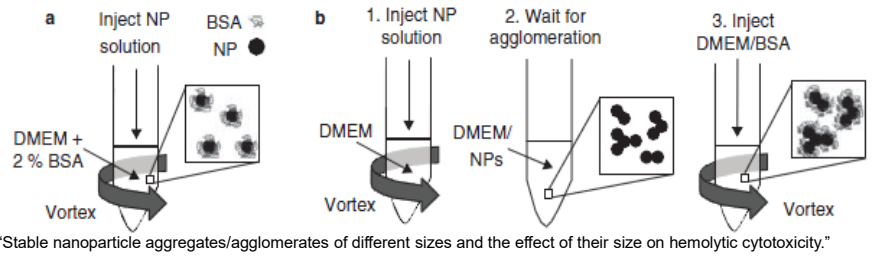


"In summary, short-term inhalation of nano-Ag did not produce apparent marked acute toxicity in this animal model."

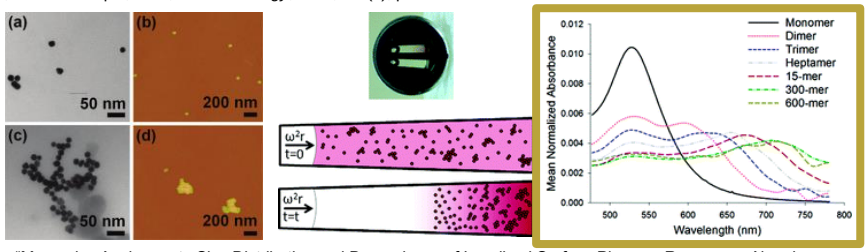
"Pulmonary and Cardiovascular Responses of Rats to Inhalation of Silver Nanoparticles" Roberts, MacCuspie, et al., *J. Toxicology And Environmental Health Part A*, 2013, 76:11, 651-668.

>> Robert MacCuspie: It wasn't enough to just stop there. The questions about how can we measure realistic silver nanoparticle exposures in an aerosol environment for *in vivo* studies also had to be addressed. Some very great work was done in a collaboration between NIST and NIOSH where NIOSH applied this ability to measure the silver nanoparticle concentrations in an aerosol exposure chamber in order to have confidence in one of their conclusions in a publication. There, in summary, the short-term inhalation of silver nanoparticles did not produce apparent marked acute toxicity in this animal model for relevant exposure conditions. So it really helped drive the science, being able to make these measurements and advance these techniques.

Measuring AgNP & AuNP Agglomerates



"Stable nanoparticle aggregates/agglomerates of different sizes and the effect of their size on hemolytic cytotoxicity."
J.M. Zook, R.I. MacCuspie, et al., *Nanotoxicology*, 2011, v. 5(4), p. 517-530.



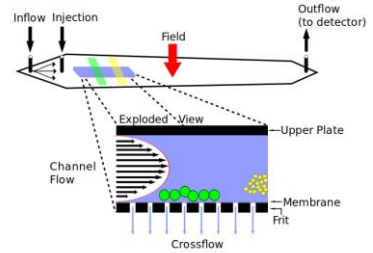
"Measuring Agglomerate Size Distribution and Dependence of Localized Surface Plasmon Resonance Absorbance on Gold Nanoparticle Agglomerate Size Using Analytical Ultracentrifugation"
J.M. Zook, R.I. MacCuspie, et al., *ACS Nano*, 2011, v. 5(10), p.8070-8079.

>> Robert MacCuspie: This was also enabling the ability to measure not just what happens in cell culture media for *in vitro* experiments and understand what the cells might see, but also that you could, by controlling the addition of the cell culture media and the serum protein and the nanoparticles, produce either singly dispersed or stable agglomerates of a controlled size by controlling this process and developing a method to do that.

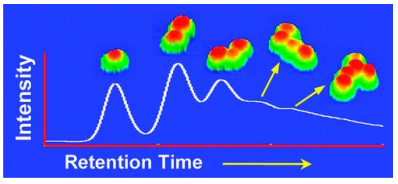
This allowed more control in order to really understand whether it was agglomerates having specific effects or singly dispersed particles in these *in vitro* studies. You could also use measurement techniques like analytical ultracentrifugation to separate these agglomerates and aggregates and measure their optical properties inline and compare those optical signatures of the coupling of the surface plasmon resonances to what one would expect from the new theory calculations, and get some of the first measurements of actual clusters and agglomerates with a controlled dispersion size, dispersion of these agglomerates, to confirm what had been reported from the theoretical literature.

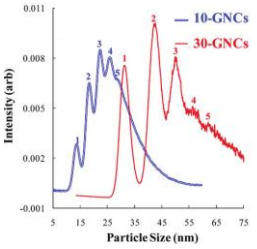
What are the optical properties of clusters of AuNPs?

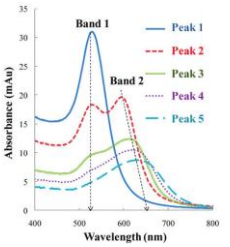
- Asymmetric-Flow Field Flow Fractionation (AF4)
- Separates AuNP clusters by size
- Spectra of purified *n*=1 to *n*=5 AuNP clusters

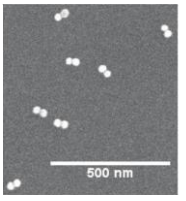


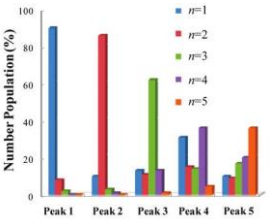
http://en.wikipedia.org/wiki/File:AFFFF_channel.svg









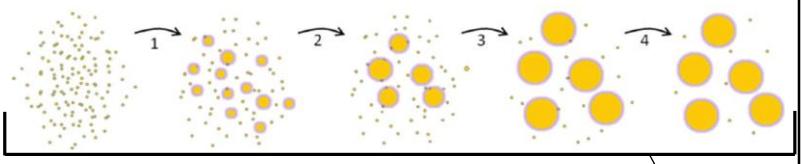


Tsai, Hackley, et al., *JACS*, 2011, v. 133(23), p. 8884 – 8887.

>> Robert MacCuspie: Some other advanced techniques that measurements enabled those confirmations were things like asymmetric flow field-flow fractionation, or AF4. This is a technique that my colleagues at NIST helped really advance for nanomaterials, separating out the various sizes and clusters and aggregates and agglomerates: by providing a perpendicular cross-flow to a semipermeable membrane across the fluid-flow channel and then ramping down that cross-flow, you could elute small particles and increasingly larger particles over time.

By looking at the retention time, you could begin to separate out various sizes or clusters and agglomerate sizes of particles as well, providing an additional confirmation of the coupling of the surface plasmon resonances in the various structures that were formed.

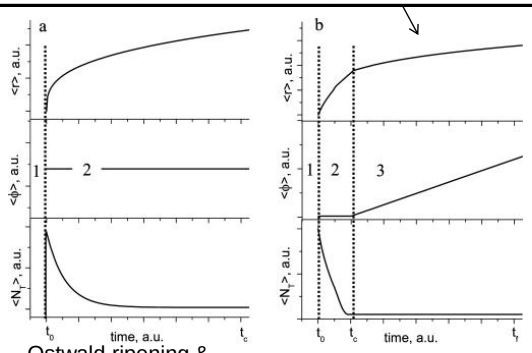
Understanding Nanoparticle Synthesis & Properties



Using 3 measurements, SAXS, TEM and UV-Vis,
Reveals new synthesis routes have different particle growth mechanisms

Ostwald Ripening / Classic Nucleation Theory

New tunable "seeded" growth:
NPs reaches constant while volume of NPs continues growing



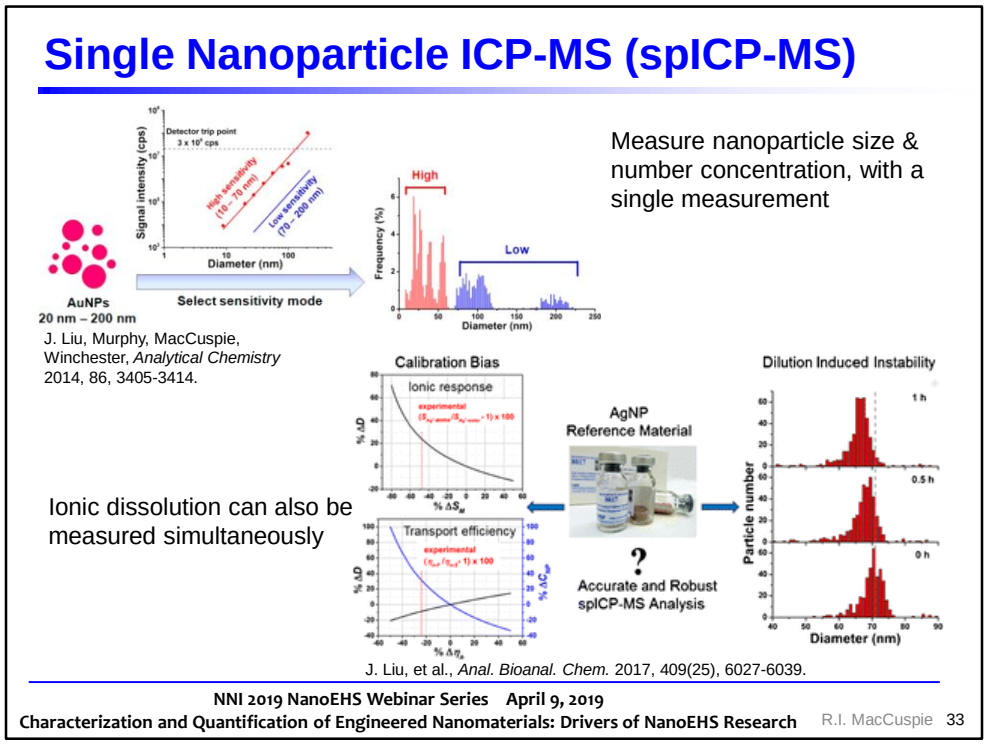
Ostwald ripening & Classic Nucleation Theory

Seeded growth

"In Situ UV/Vis, SAXS, and TEM Study of Single-Phase Gold Nanoparticle Growth"
H. Koerner, R.I. MacCuspie, K. Park, R.A. Vaia, *Chemistry of Materials*, 2012, 24, 981-995.

>> Robert MacCuspie: The incidence in measurements by combining techniques, such as dynamic light scattering with TEM, also led to increased understanding about nanoparticle synthesis and properties. "Seeded" growth synthesis methods began to evolve, compared to the more classic nucleation theory/Oswald ripening synthesis approaches, in order to ensure a greater degree of size control was developed. These measurements enabled both the number of particles per unit volume as well as the mass of those individual particles (their size) to be determined.

And to see that it was indeed a seed growth mechanism that led to a much more uniform size distribution and fewer size impurities. So it is interesting to help guide how those reaction parameters can be tuned by having the right measurements of the mechanism.



>> Robert MacCuspie: Other advanced techniques included single-particle ICP-MS (inductively coupled plasma mass spectrometry). A lot of folks contributed to advances in this space. The idea is that you dilute the particle suspension such that one particle at a time would be introduced into the plasma, you would get a burst of ions into the detection chamber, so you could determine the mass of that single particle by having the appropriate integration time for the measurement. And you could get the mass of individual particles and convert that to their size, as well as getting a number concentration of the particles in a single measurement.

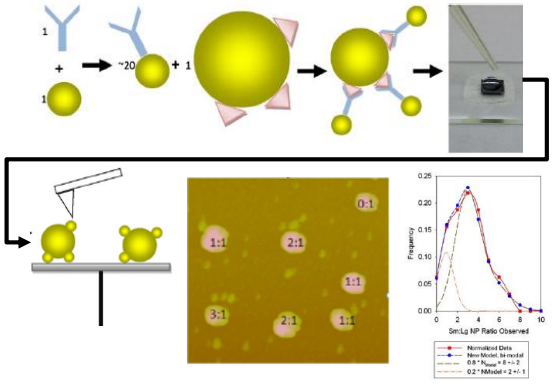
This is quite a powerful technique that was developed. You could even use it in cases with silver nanoparticles where if you have dissolved ions, you would have a baseline above zero measuring your silver ion concentration in every integration time unit. When those bursts come through for the particles you would be able to measure the mass of the particles. It turns out you have to have a little bit higher size limit of detection when you have a dissolved background to get statistically significant measurement results, but what it did enable is the measurement of the amount of dissolved ions as well as the amount of particles and their size distribution of a concentration in a single measurement, which is a very powerful technique for understanding what's happening.

Of course, the sample timing continues to remain important so you don't get dilution-induced instabilities with silver nanomaterials when you are making these single-particle ICP-MS measurements.

Measuring Protein Conformation on NP Surfaces

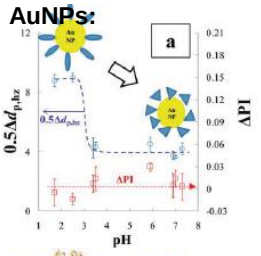
Protein conformation determines biological activity, especially for receptor-binding mechanisms.

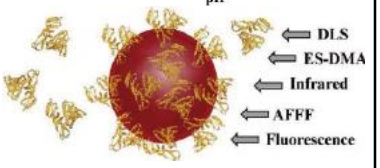
Quantitative Immunostaining by AFM:



R.I. MacCuspie & D.E. Gorka, *Anal. Bioanal. Chem.*, 2013.
C.L.A. Geronimo & R.I MacCuspie, *Microscopy & Microanlaysis*, 2011, 17, 206-214.

pH-dependence of BSA conformation on AuNPs:





"Adsorption and Conformation of Serum Albumin Protein on Gold Nanoparticles Investigated Using Dimensional Measurements and in Situ Spectroscopic Methods", Tsai, MacCuspie, Hackley, et al., *Langmuir*, 2012, 27(6), 2464-2477.

>> Robert MacCuspie: There were also many attempts and successes to identify ways to measure what is on the surface of the nanoparticle. Many researchers identified the need to understand what is on the surface of the nanoparticle because that is what cellular receptors would see when nanoparticles first encounter a cell membrane.

So for things like targeted nanomedicines, you would want to make sure that your targeting molecules are face-up so they can dock to the cell surface receptors. So quantitative immuno-staining was an approach to identify how many of these face-up, if you will, targeting molecules were present on the surface of the larger nanoparticle, using a smaller antibody probe nanoparticle to determine that. Transmission electron microscopy or atomic force microscopy are suitable methods for identifying the number of active targeting molecules on the surface.

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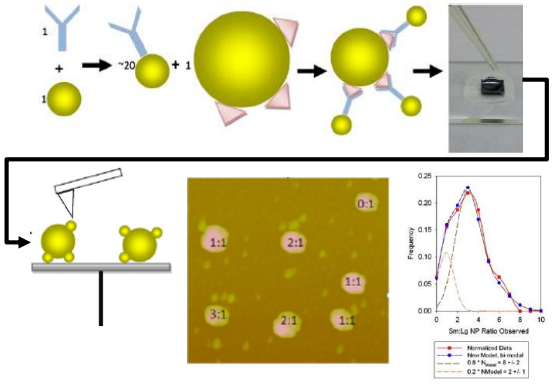

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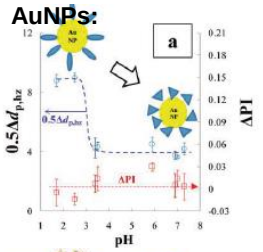

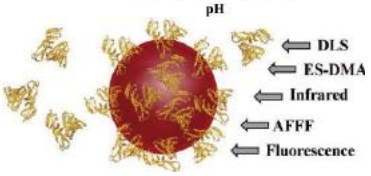

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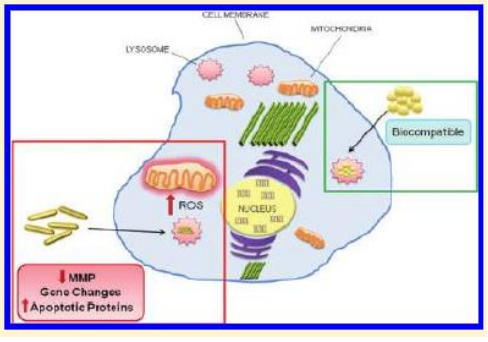
>> Robert MacCuspie: Some other work looked at the pH dependence of serum proteins, their conformational changes on the surface of gold nanoparticles as a function of the pH. And by combining DLS and aerosol measurements, like electrospray differential mobility analysis, with infrared spectroscopy, AF4, and more techniques, there could be confirmation that the serum proteins were remaining on the surface of the particles and they were indeed changing conformation without the amide bond backbone being cleaved.

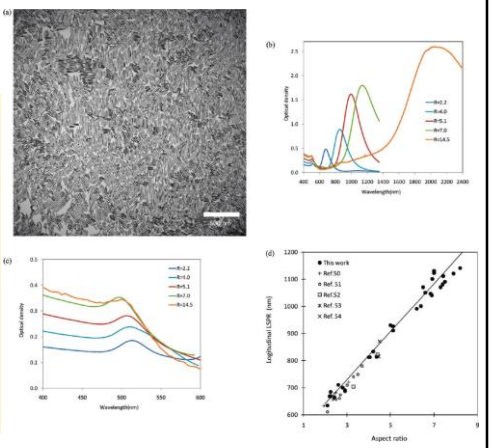
The conformational changes and size changes of the particles as a function of pH could then be understood, so this methodology allowed a much broader understanding of what the behavior might be of proteins on the surface, to really inform what states and behaviors might be in biological contexts.

Does Shape Matter?

Yes. Shape and surface coating both influence biological effects



"Does Shape Matter? Bioeffects of Gold Nanomaterials in a Human Skin Cell Model"
Schaeublin, MacCuspie, et al., *Langmuir* 2012, 28, 3248−3258.

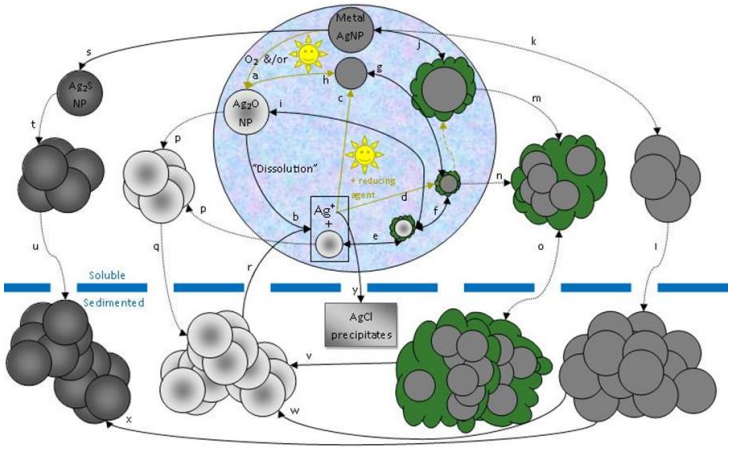


Duan, et al., *J. Phys. Chem. C* 2009, 113, 15524-15532.

>> Robert MacCuspie: Shape matters of course, as well, in addition to the surface coating. And there is a wealth of literature. I just want to acknowledge a lot of this was done in the gold nanoparticle space with gold nanorods. The ability to use advances from Air Force Research Labs to make large quantities of very uniform aspect-ratio gold nanorods allowed studies to be conducted that could help answer some of these questions about whether shape matters in addition to surface coating. It turns out it does have an effect.

Possible Transformations of Silver Nanoparticles

- This understanding was enabled by meeting the NNI measurement research needs

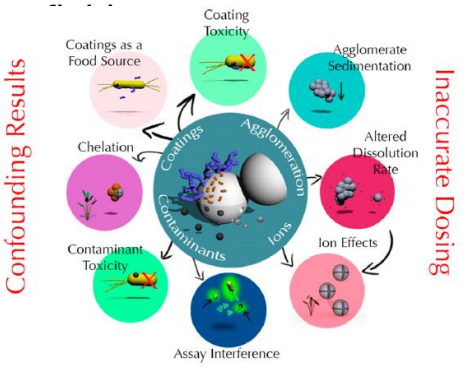


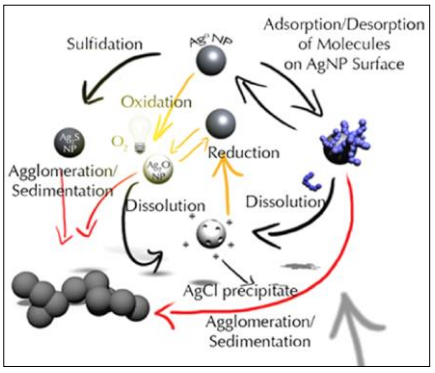
R.I. MacCuspie, "Characterization of nanomaterials for nanoEHS studies", Ch. 4 in *Nanotechnology Environmental Health and Safety, 3rd ed.*, M. Hull & D. Bowman, 2018

>> Robert MacCuspie: As all of these measurements and all of these studies began to come out in the literature, it became apparent that these measurements and these studies were enabling a broader understanding of potential fate and transformations---especially of silver nanoparticles---in relevant conditions, whether they be biological or environmental media. So attempts were made to try to put together a partial list of some of these common transformations: surface oxidation by either oxygen or light; formation of the silver oxide shell and then dissolution of these particles; the role of the surface coatings and other molecules in the environment that can transiently absorb and desorb from the surface of the particle, influencing their aggregation and agglomeration rates and helping guide whether these particles and agglomerates were going to remain stably dispersed as suspensions or sediment down, and understand their stability, as well, to help predict for EHS studies what these changes might be.

Avoidance of Artifacts and Misinterpretations

- Excellent example of how meeting the NNI measurement needs led to deeper understanding and greater technical accuracy for the NanoEHS





"Identification and Avoidance of Potential Artifacts and Misinterpretations in Nanomaterial Ecotoxicity Measurements"
E.J. Petersen, T.B. Henry, J. Zhao, R.I. MacCuspie, T.L. Kirschling, M.A. Dobrovolskaia, V.A. Hackley, B. Xing, J.C. White, *Environmental Science & Technology*, **2014**, *48*, p4226-4246. DOI: 10.1021/es4052999

>> Robert MacCuspie: This led to the ability for review articles to come out to really highlight the best ways to identify and avoid potential artifacts and misinterpretations in nanoEHS studies: the understanding of these potential transformations and how that plays a role, as well, in the biological and environmental senses of whether the coating itself could be a source of toxicity; whether the agglomeration sedimentation rates could influence the results; whether the coatings themselves could be a potential food source for organisms, maybe increasing or decreasing their uptake of the particles; looking at whether ion effects, especially in the case of silver, were separate from the particle effects, and understanding how to differentiate those. And then also being mindful of when you have a surface plasmon resonance if there is optical absorbance, if there were fluorescence assays that might have overlapping optical signatures, and how to validate for those assays to prevent potential interferences in the results.

These broader understandings of the community coming together and saying these are the many important considerations we have to pay attention to, help to explain some of the apparent conflicting results of some of the early literature and help us understand how starting with the same materials and starting with what would presumably be similar organisms or scenarios led to different results as a function of some of these other important details. This actually led to being able to mine the literature and get much more out of it as our understanding progressed.

Commercializing Nanotechnologies Responsibly

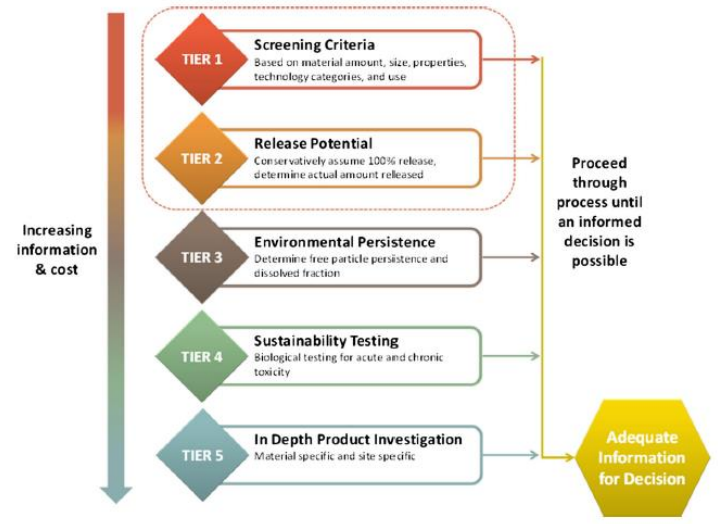
Key business steps to commercialization

- De-risk science around safety
  - NNI NanoEHS research helps support this
- >10X performance or price improvement
- Identify target market need
  - Will somebody pay for the product?
- Obtain funding
  - VC or Angel Investors, SBIR grants, Incubators
- Scale-up: Benchtop to pilot to production scale manufacturing
- Quality Control – validated measurements and methods
- Develop sales, marketing, and education to increase customer base

>> Robert MacCuspie: All of this has led to, in my personal opinion, throughout my career, the opportunity to commercialize nanotechnologies in a responsible fashion. As the science has grown around the safety, supported in large part by the nanoEHS measurements supported by the National Nanotechnology Initiative, this de-risks some of the uncertainty by providing science around the safety. But you not only have to know the product is safe: other literature was showing and demonstrating greater than ten times improvement in performance or price would be required to successfully commercialize nanotechnologies. It would need to be a really significant game changer in order to successfully make it to market.

Folks obviously had to identify the target market in need, identify that somebody as a customer would be willing to pay for these products, and how many would. To do all of the research on a specific formulation and safety and then scale up from bench-top to pilot to production scale, one had to obtain funding as well from a variety of sources—be it investors or grants or incubators. And attention always has to be paid to quality control for validating the measurements and methods to ensure that the manufacturing scale-up is working as intended.

Tiered Approach to NanoEHS Risk Assessment



"Tiered guidance for risk-informed environmental health and safety testing of nanotechnologies" Z.A. Collier, A. Kennedy, R.I. MacCuspie, et al., J. Nanoparticle Research, **2015**, *17*:155. DOI 10.1007/s11051-015-2943-3

>> Robert MacCuspie: One of the ways that derisking the science has helped is by using a tiered approach to nanoEHS risk assessments. This particular tiered approach was developed by the U.S. Army Corps of Engineers ERDC (Engineer Research and Development Center). By looking at whether there is adequate information in the peer-reviewed literature, starting with screening criteria such as measuring the size and amount and properties of a nanomaterial and its intended use, and then looking at the potential for release, conservatively assuming 100% release or being able to measure that amount specifically, one could then look to see if there's adequate information to make a decision on risk assessment. So the measurements are enabling more rapid commercialization in these cases by using a tiered approach.

If there's uncertainty or inadequate information, you would continue to look at environmental persistence, determining if additional persistence or dissolved fraction information needs to be collected; look for additional tiers of biological testing for acute or chronic toxicity, and in some cases, an in-depth product investigation, material-specific or site-specific, or formulation-specific investigations as required, depending on the application. But all of these are increasing time and information and costs to achieve that. So as we've being able to integrate and learn from the peer-reviewed literature this has helped expedite this process by using a tiered approach.

Comparison to Relevant Exposure Scenarios

Study	No Observed Adverse Events?	___X EPA & WHO daily safety gudiance (5µg/kg)	= mg/ kg b.w.	= tsp/day, @10ppm	=gal/day, @10ppm
1 tsp of 10ppm Silver Product	☑	**0.14**	0.0007	1	0.001
Morishita, et al., 2016, "low" dose [1]	☑	**300**	1.5	2,100	2.7
Xue, et al., 2012, "low" dose [2]	☑	**1,500**	7.5	10,500	14
Loeschner, et al., 2011, "low" dose [3]	☑	**1,860**	9.3	13,020	17
Wilding, et al., 2016 [4]	☑	**2,000**	10	14,000	18
Morishita, et al., 2016, "high" dose [1]	☑	**2,000**	10	14,000	18
Loeschner, et al., 2011, "high" dose [3]	☑	**2,520**	12.6	17,640	23
Kim, et al., 2008, "low" dose [5]	☑	**6,000**	30	42,000	55
Xue, et al., 2012, "mid" dose [2]	☑	**6,000**	30	42,000	55
Xue, et al., 2012, "high" dose [2]	lung & liv inflam	**24,000**	120	168,000	219
Kim, et al., 2008, "high" dose [5]	elev liv enz	**60,000**	300	420,000	547

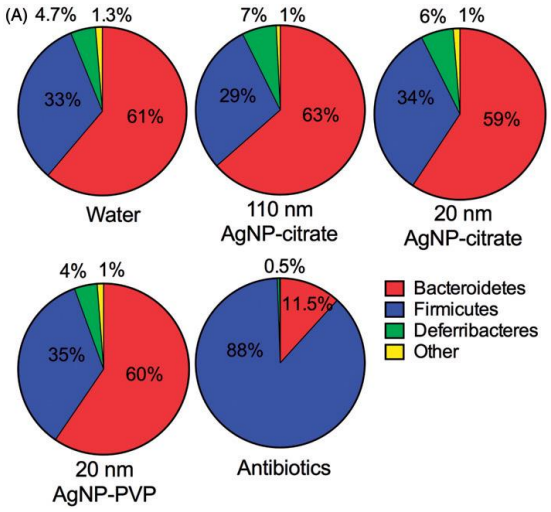
[1] Morishita, Y., et al., *Distribution of Silver Nanoparticles to Breast Milk and Their Biological Effects on Breast-Fed Offspring Mice.* ACS Nano, 2016.
[2] Xue, Y., et al., *Acute toxic effects and gender-related biokinetics of silver nanoparticles following an intravenous injection in mice.* J. Appl. Toxicol., 2012. **32**(11): p. 890-899.
[3] Loeschner, K., et al., *Distribution of silver in rats following 28 days of repeated oral exposure to silver nanoparticles or silver acetate.* Particle and Fibre Toxicology, 2011. **8**(1): p. 1-14.
[4] Wilding, L.A., et al., *Repeated dose (28-day) administration of silver nanoparticles of varied size and coating does not significantly alter the indigenous murine gut microbiome.* Nanotoxicology, 2016. **10**(5): p. 513-20.
[5] Kim, Y.S., et al., *Twenty-eight-day oral toxicity, genotoxicity, and gender-related tissue distribution of silver nanoparticles in Sprague-Dawley rats.* Inhal Toxicol, 2008. **20**(6): p. 575-83.

>> Robert MacCuspie: One example of what this might look like is if one were to compare a 10-milligrams-per-liter or 10-part-per-million silver product, for example, that might be below the EPA and World Health Organization daily safety guidance for how much silver can be safely consumed without any concerns for the risks of argyria or acute or chronic toxicity effects; these safety guidelines have been around for many years.

One can see that the product might be below that safety limit, and then the peer-reviewed literature when you convert it to that mass dose, 5 micrograms per kilogram, you can begin to see that many papers reported no adverse events until tens of thousands of times over those safety limits. And so you can get a sense from the peer-reviewed literature of the relative degree of margin of safety, if you will, by looking at the comparison to the established safety guidelines and what the peer-reviewed literature is showing. So it can give companies a degree of confidence in what the risk level is at those established safety guidelines.

Does Silver Harm the Gut Microbiome Diversity?

- No!
- Gut microbiome diversity is not impacted by silver even at 2,000X the WHO and EPA daily safe exposure limit guidance
- Antibiotic cefoperazone disrupts balance significantly



Wilding, et al., (2016). "Repeated dose (28-day) administration of silver nanoparticles of varied size and coating does not significantly alter the indigenous murine gut microbiome." Nanotoxicology 10(5): 513-520.

>> Robert MacCuspie: This can also lead to studies that help understand common concerns by the public. One study affiliated with the NNI and conducted by the University of Michigan posed the question of whether silver nanoparticles would harm of diversity of species in the gut microbiome. As you can see from their conclusions, by being able to measure well-defined sizes of particles and surface coatings of particles, they could have confidence that a variety of these properties, as you vary them, did not change the diversity of species in the gut microbiome in this particular study at 2,000 times the EPA and World Health Organization daily safety limits for humans.

Prescription antibiotics dramatically altered that diversity of species. Everyone probably is familiar with antibiotic-associated side-effects. Many doctors recommend taking probiotics or eating yogurt, natural probiotics when taking antibiotics to try to help keep the gut flora in a healthy balance. A lot of folks will ask this question, and so by being able to have measurements to do these hypothesis-testing studies helps in understanding the safety profile of engineered nanomaterials.

Quality Control – Part of Responsible Commercialization

- What are the testing costs?
  - Core infrastructure, instrumentation, and other fixed costs
  - Consumables, reagents, waste disposal
  - Labor costs, including skill required
- What are the costs of a product not meeting specifications?
  - Increased customer care costs from complaints?
  - Product recall?
  - Damaged brand reputation?
  - Market share loss?
  - Regulatory, legal, civil penalties?
- What are the costs of carrying quarantined inventory, pending QC release?
- What is the risk of false-positive and false-negative results? How would that impact the number of unneccessary reworks or amount of scrapped material?
- Do regulations require me to perform certain types or frequencies of testing?

R.I. MacCuspie, "Characterization of nanomaterials for nanoEHS studies", Ch. 4 in *Nanotechnology Environmental Health and Safety, 3rd ed.*, M. Hull & D. Bowman, 2018

>> Robert MacCuspie: Looking to the literature and understanding all of these risk assessments as part of responsible commercialization and also quality control is another key part of responsible commercialization. One has to look at the business considerations for quality control (QC), such as what are the testing costs and the core infrastructure and instrumentation and fixed costs that are required, as well as the consumables, reagents, and waste disposal considerations for the testing. And also the labor costs in terms of the degree of skill required to operate the instrument successfully and perform the experiments. And so this leads to the need to make some decisions about what those might be and how frequently and what types to test.

Companies always have in mind what are the costs of a product not meeting specification or an out-of-specification incident beyond just scrap material, to looking at customer care complaints, product recalls, and damaged brand reputation. If the products were not tested for quality control, this could happen, market share losses, and in extreme cases, even potential regulatory or legal or civil penalties for the company. So avoiding those is of key importance for the responsible commercialization of the products. [*Continued*...]

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>> Robert MacCuspie: Companies also consider the cost of carrying the quarantined inventory that is pending the QC release while this testing is done. Turnaround times become quite important: if it's 10 days to send out for testing to get a result back, having the warehouse space and the systems to prevent it from shipping are very important. During the operations process, having a system provide a more rapid turn-around time can help provide cost savings for the company. But also being mindful of the risks of false positives and false negative results and how that would impact the number of unnecessary reworks or additional testing or potentially unnecessarily scrapped material. And of course, regulatory compliance is also a common driver of quality control as well.

Quality Control – Validating Low-Cost High-Throughput Measurements

- Manufacturing requires high-throughput, low-cost measurements
- E.g. – use expensive equipment like ICP-MS to validate low-cost measurements live UV-Vis of SPR

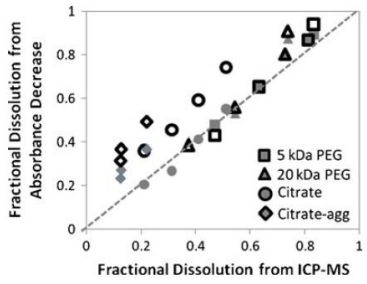

Fig. 1 Comparison of fractional dissolution of AgNPs measured by decrease in absorbance to dissolution measured by ICP-MS. The

Zook, MacCuspie, et al., "Measuring silver nanoparticle dissolution in complex biological and environmental matrices using UV–visible absorbance", Anal Bioanal Chem (2011) 401:1993–2002

- Practical application:
  - Low cost (UV-vis) may be cost-effective option for in-line production measurements
  - High cost (ICP-MS) may still be used on Certificate of Analysis and/or Final QC release

>> Robert MacCuspie: In this conversation, the idea is, can lower-cost, higher-throughput measurements be developed and validated compared to the more expensive or time-consuming techniques? One paper that came out from NIST many years ago looked at this question of validating the UV-Vis (ultraviolet–visible) absorbance measurements of silver nanoparticles, comparing it to ICP-MS metal analysis to look at a lower-cost, higher-throughput method.

By finding out when the method falls on the line of agreement within the uncertainty, there can be a potential to perhaps use a lower-cost measurement inline during production measurements and then still retain a higher-cost method for certificate of analysis or final QC release or for a statistically relevant sampling density in order to decrease the costs for testing, while still having confidence that the quality control measurements would be suitable for the needs of the product. These are the ways that measurements become a key part of the conversation in successfully commercializing nanomaterials in a responsible fashion.

Measurements enable commercialization: Example for Silver Content Quality Control

- Need: Measure product label claim for silver concentration
- Approach: Atomic Absorption Spectroscopy
  - Appropriate Limit of Detection (LOD)
  - Lower cost and higher throughput than ICP-MS
  - Many method standards exist for AAS measuring silver and metals
- Method: (for each QC analysis run)
  - Collect three samples from manufacturing lot
  - Run blank
  - Run calibration curve to NIST-traceable standards
  - Run triplicate analysis on each of the three samples (average of nine data points)
  - Repeat calibration samples at end of measurement (ensures no instrument drift during run)
- Report data on Certificate of Analysis

>> Robert MacCuspie: One example of a method that I helped develop at Natural Immunogenics for their quality control would be measuring a product-label-claim silver concentration using atomic absorption spectroscopy, which has an appropriate limit detection and a lower cost and higher throughput than ICP-MS, especially in terms of the capital equipment expenses. There are also many method standards that exist for measurements of silver and metals in general.

So by doing for the QC analysis runs a triplicate analysis on three samples that were collected from a manufacturing lot, running a blank, and running a NIST-traceable calibration curve at the beginning of each run, then performing the triplicate analysis on each of these three samples to average all nine data points [*Continued...*]

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>> Robert MacCuspie: Measuring a set of calibration samples again at the end of the measurement to make sure there was no instrument drift during the run provides absolute confidence in those measurements. This has actually prevented the number of retesting events and helped in investigations into understanding when additional work may need to be done to ensure that 100% of the product that's being manufactured meet those quality-release criteria every single time with absolute confidence, and then being able to report that data with a very high degree of confidence on their certificate of analysis.

The measurements really provide that, not just the regulatory compliance, but with customers, a great deal of confidence and trust in the companies that are performing these measurements as well, that they understand and care about the safety of their products and the impact for the environment and human health.

Significant Research Progress on Measurement Needs

Nanomaterial Measurement Infrastructure Research Need	Progress
1. Develop measurement tools for determination of physico-chemical properties of ENMs in relevant media and during the life cycles of ENMs and NEPs	☑
2. Develop measurement tools for detection and monitoring of ENMs in realistic exposure media and conditions during the life cycles of ENMs and NEPs	☑
3. Develop measurement tools for evaluation of transformations of ENMs in relevant media and during the life cycles of ENMs and NEPs	☑
4. Develop measurement tools for evaluation of biological responses to ENMs and NEPs in relevant media and during the life cycles of ENMs and NEPs	☑
5. Develop measurement tools for evaluation of release mechanisms of ENMs from NEPs in relevant media and during the life cycles of NEPs	

>> Robert MacCuspie: Now, to kind of bring it back to the wonderful introduction we had by Debbie, our moderator. The various measurement research needs that were laid out in these guidance documents by the NNI really have had significant progress in all areas. There has been tremendous progress---we haven't had time to highlight every single one of these today.

Conclusions

- Measurements have enabled tremendous progress in NanoEHS research over the last 15 years
- NNI provided a framework for impactful research, and delivered science to support policy and regulatory efforts
- Science to support responsible commercialization of nano-enabled products while protecting human health and the environment

>> Robert MacCuspie: They really have made significant progress, which has enabled tremendous advance in understanding how nanomaterials can be safely used in a responsible fashion, protecting environment and human health.

The NNI has provided a wonderful framework for this impactful research and delivering science that not only has supported policy and regulatory decision-making efforts but also the responsible commercialization of products.

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>> Robert MacCuspie: I want to acknowledge that there was a huge number of people that I've been fortunate to collaborate with over the course of my career. I could not include everybody that I've collaborated with who contributed to the data slides in this presentation, but this has been a wonderful opportunity to be part of this great effort over the last 15 years and the many folks who have contributed in very significant ways to it.

Thank YOU for your kind attention!

Questions???

Rob MacCuspie, PhD[1,2]

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>> Robert MacCuspie: Of course, I would like to thank you as well for your kind attention today in attending the webinar. I would be happy to take any questions.

Q/A

What do you feel are future remaining measurement challenges?

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>> Debbie Kaiser: Thank you, Rob. That was an excellent presentation. I'll begin with the first question. What do you feel are future remaining measurement challenges?

>> Robert MacCuspie: Yes, that's a great question. I think that one of the future challenges is, from an industry perspective, can we can get lower costs and higher-throughput measurements that are validated to some of these more expensive techniques? Or can the cost of those instruments and techniques come down so that the initial capital outlays or turnaround times might be more economically competitive to do that testing more frequently? And I think also, looking at how complex nanomaterials and complex local environments dictate the behavior of nanomaterials will continue to be a growing need. One example might be nanomaterials and food matrices and changes that might take place in the gastrointestinal tract. There has been great work that has been started and is continuing to be built upon over the years, and I look forward to seeing more results from that as well.

Q/A

Is there a one-size-fits-all measurement that can predict nanoEHS risk of a nanomaterial?

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>> Debbie Kaiser: Another question- Is there a one-size-fits-all measurement that can predict nanoEHS risk of a nanomaterial?

>> Robert MacCuspie: In my opinion, no, I don't believe one has come out. There is no single one-size-fits-all measurement to predict the risk. In these tiered approaches you are looking for size information, composition information. You have to look at choosing the right techniques for the questions that are being asked. So you might use chemical analysis for the composition or TEM or DLS or AFM for the size, or other techniques as appropriate. So it's the correct combination of these multiple orthogonal measurements to answer the questions that determines the best approach.

Q/A

Are there examples of using read-across approaches in tier 1 assessments?

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>> Debbie Kaiser: I would like to take a moment to encourage our participants in the webinar to submit questions online. One question that has come in, Are there examples of using read-across approaches in tier-one assessments? I think this is referring back to the tiered approach that you worked on in collaboration with ERDC?

>> Robert MacCuspie: Yes. I think read-across is a great approach that you could use for tier one. If you are looking at a general type of material, and there's appropriate literature that's known about that type of material or about any potential size-based effects for that material, then yes, I think that could be a great approach.

Q/A

How well do we know the actual exposure concentrations in nanoparticle form?

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>> Debbie Kaiser: How well do we know the actual exposure concentrations in nanoparticle form, I guess, for some of the common nanomaterials?

>> Robert MacCuspie: Yes, so determining the realistic exposure scenarios, you have to use a little bit of that lifecycle approach type of analysis. But you have to look at, okay, if this is the type of product, what is the intended use, what is the amount of product that would be used, and what is the amount of material/nanomaterial in that product as well? Then one can look at, for example, if it's a dietary supplement, looking at complex food matrices. Or for example, if it's an aerosol exposure, looking at measuring the aerosol concentration. But looking at, let's say, if somebody were using a disinfecting spray, for example, how many sprays they might use in a typical cleaning activity and what the volume of space might be, you can begin to make some reasonable estimates as to what these exposure concentrations might be. [*Continued...*]

Q/A

How well do we know the actual exposure concentrations in nanoparticle form?

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>> Robert MacCuspie: A lot of times, companies will know that very specifically for a product or describe it for their products, so that's really a good way to look at it. But oftentimes it does require some assumptions about the intended use or some knowledge about the intended use in order to accurately predict what those exposure concentrations might be. And then looking at the types of environments where the nanoparticles might be in formulation or released from the products, and understanding what other components might be present or help understand what the potential changes are or might be.

Q/A

Are there nanotechnology degrees, master's degrees or with a specialty in nanotechnology that you're aware of?

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>> Debbie Kaiser: Great. Thank you. There is a question concerning education, and perhaps with your background you can comment. Are there nanotechnology degrees, master's degrees, or degrees with a specialty in nanotechnology that you're aware of?

>> Robert MacCuspie: Yes. I know that there are several graduate degree programs; there are master's degree programs that are being developed as well as Ph.D. programs that exist in nanotechnology. I know in the State University System of Florida, the University of Central Florida recently got a master's in nanotechnology program. It's a concentration at the undergraduate level in many institutions around the country as well. And I believe that there are resources available on nano.gov that I've seen in the past that have identified potential programs that offer these.

Q/A

What are the developments in nanofiber characterization?

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>> Debbie Kaiser: Excellent. There's a question that came in, to switch gears: What are the developments in nanofiber characterization?

>> Robert MacCuspie: Yes. That's a great question. So there are a lot of techniques that are, I think. One of the work-horse techniques for nanofibers has been scanning electron microscopy. It can be very time consuming. Nanofibers present their own characterization challenges, unlike an aqueous suspension where dynamic light scattering gives you an equivalent circle diameter, but it's not the measurement of interest of nanofiber, for example. And so there are a lot of techniques: I know folks have looked at neutron scattering and x-ray scattering techniques as well, to look at the orientation of nanofibers in polymer composites, for example, to try to understand the changes there.

>> Robert MacCuspie: And folks have also employed surface chemical analysis techniques, when appropriate for looking at things like how carbon nanotubes behave in polymers as a function of weathering. NIST has done a lot of great work in that space, as well as others, looking at, for example, in some cases, you may get a mat of carbon nanotubes forming at the surface of the composite as the plastic weathers away. But the carbon nanotubes can be retained in the product and not actually be released. By looking at some of these microscopy and surface chemical analysis techniques.

Q/A

What are the hazards associated with silver nanoparticles?

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>> Debbie Kaiser: We're going to take one final question that just came in. What are the hazards associated with silver nanoparticles?

>> Robert MacCuspie: Yes. So the hazards that have been identified include argyria, which is the condition where you get a cosmetic change where your skin turns a silver or blue color. But it is known that those hazards exist after greater than 10 grams of lifetime exposure to silver, based on the EPA and World Health Organization guidelines. And NIOSH has just come out with a recent Current Intelligence Bulletin draft on the safe use of silver, as well, which has a wealth of information and resources.

>> Robert MacCuspie: But it turns out that in a lot of cases you can find ways to use silver nanoparticles effectively for their antimicrobial properties and be well below these safety limits. So you can actually commercialize silver nanoparticles in a safe and responsible fashion and they actually would end up using far less silver than some of the silver thread materials used in textiles, if you can use a nanosilver coating, for example. So there are ways to successfully mitigate the risks from these particular hazards

Q/A

Activity is one of the hallmarks of nanomaterials. Is that a metric that's being considered for EHS evaluation?

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>> Debbie Kaiser: Great. I see we have 2 minutes left. So I guess I'll ask for a quick response to a question that came in. Activity is one of the hallmarks of nanomaterials. Is that a metric that's being considered for EHS evaluation?

>> Robert MacCuspie: I think the phrase or the word “activity” to me can be very broadly interpreted. So I think when you look at activity, you have to kind of define it with a little bit more focused scope. You know, if you're talking about a biological activity on a certain organism or certain organism’s environment, then you could look at those particular activities as a function of a material. But to me that tends to become something you have to dive down a little bit deeper and look at a little more specifically at that broader metric for the risk, but it’s absolutely something that's important.

Closing Remarks

Moderator: Debbie Kaiser

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>> Debbie Kaiser: Great. Well, I would like to take this opportunity to thank the participants who listened in and particularly those who have submitted questions. I apologize that we are not able to get to all of the questions, but I would like to thank Dr. MacCuspie for an excellent, interesting, and thought-provoking webinar today. Lisa, did you have any closing remarks? I guess I put her on the spot there. So we'll close with this first NanoEHS webinar and we'll look forward to having you participate and having all the participants listen in on the next one.