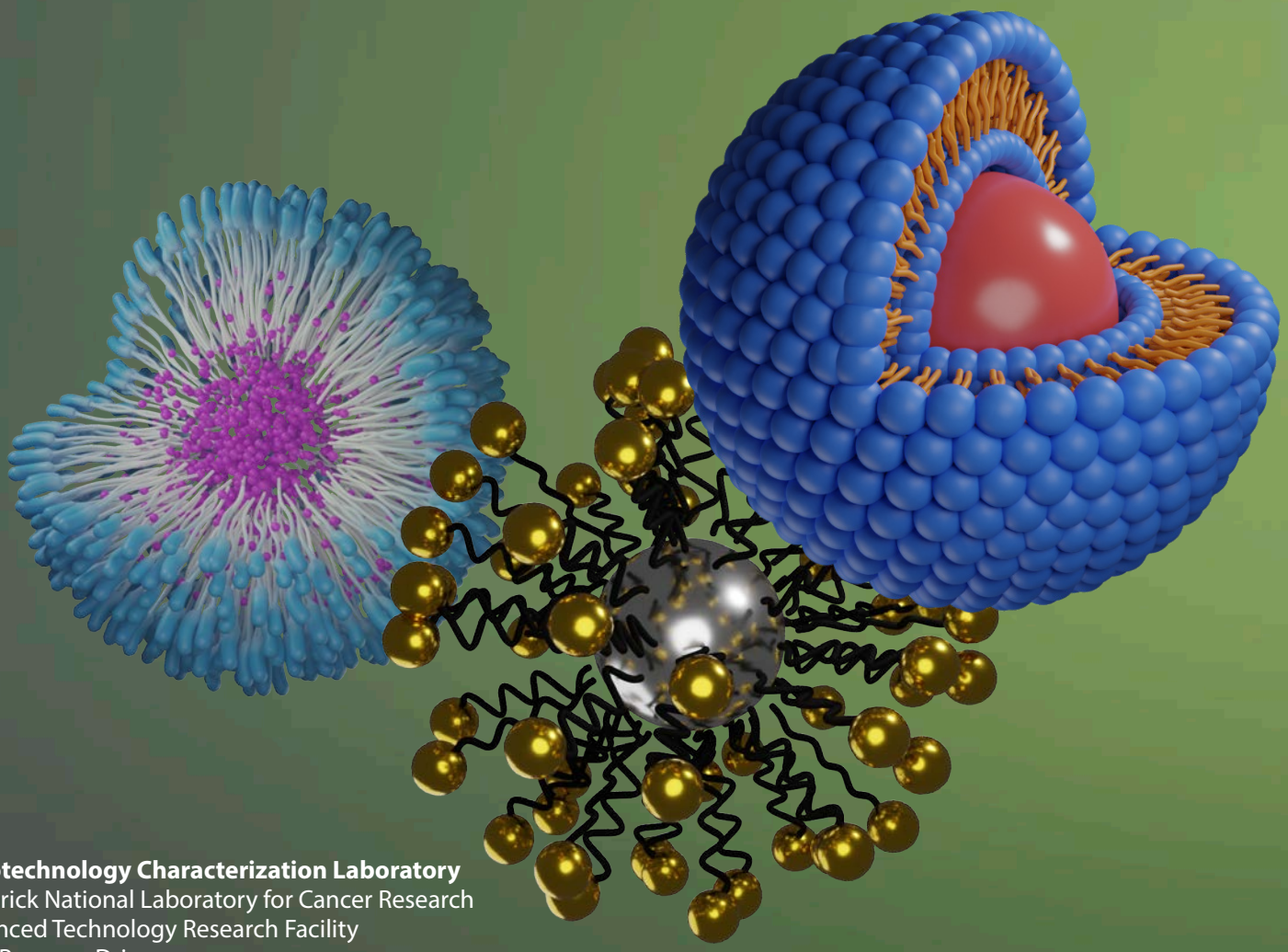
NCL
Nanotechnology
Characterization
Laboratory

Physicochemical Characterization of Nanomedicines



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PHYSICOCHEMICAL CHARACTERIZATION OF NANOMEDICINES AT THE NCL

The Nanotechnology Characterization Laboratory (NCL) has experience with a wide variety of nanoparticle platforms and has characterized nearly every technology being explored in the nanomedicine space.

It is well-known that a nanoparticle's physical attributes can influence pharmacokinetics, toxicity and efficacy. Therefore, a thorough understanding of the physical and chemical traits is imperative in understanding the particle's biological properties.

Using state-of-the-art instrumentation, nanotechnologies coming into the NCL are subjected to a thorough assessment evaluating the particle's characteristics in eight primary categories. In addition to evaluating direct traits of the particle (e.g., size distribution, composition, purity, etc.) aspects such as stability, lot-to-lot reproducibility, as well as assessment of the starting materials are also critical components of the characterization process.

Every nanoparticle is unique. Similarly, the characterization requirements for each particle are unique. Therefore, each nanoparticle brought into the NCL for testing will have a research plan individually tailored to meet the specific needs of that formulation. NCL staff will work alongside investigators to understand the intricacies of the formulation and develop a testing plan that fills any critical gaps in their knowledge and understanding of the formulation.

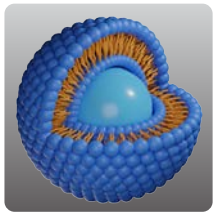
Capabilities and Instrumentation

This brochure highlights the eight most commonly explored attributes of nanomedicine characterization. However, due to the unique nature of nanomedicines, other physical and chemical characterizations may also be required. A more comprehensive listing of the capabilities and instrumentation available at the NCL is outlined on the NCL website.

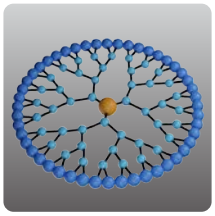
Primary Investigational Areas

- Size/Size Distribution
- Morphology
- Surface Properties
- Composition
- Purity
- Stability
- Batch-to-Batch Variability
- Starting Material Characterization

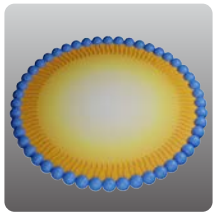
NCL has experience characterizing a wide variety of nanoparticle platforms.



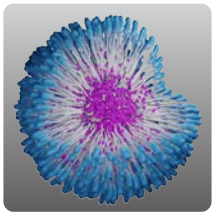
Liposomes



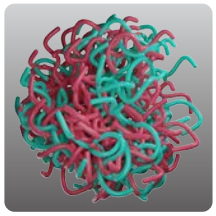
Dendrimers



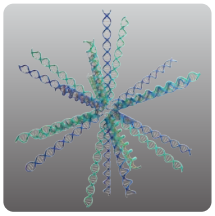
Lipid-based Nanoparticles



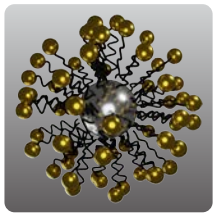
Polymeric Nanoparticles



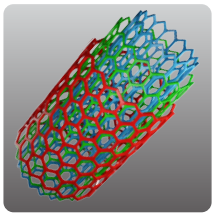
Polyelectrolyte Complexes



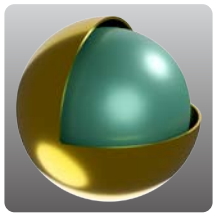
Nucleic Acid Nanoparticles



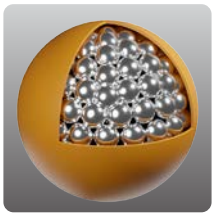
Metallic Nanoparticles



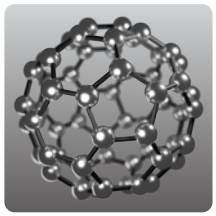
Single and Multi-Walled Carbon Nanotubes



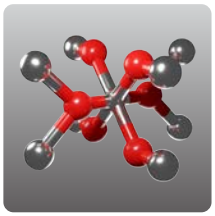
Core-Shell Nanoparticles



Quantum Dots



Fullerenes



Nanocrystals

SIZE/SIZE DISTRIBUTION AND MORPHOLOGY

Size/size distribution is among the most routinely measured nanoparticle attribute, most commonly measured using batch-mode dynamic light scattering (DLS). However, there are multiple other techniques available for size measurement, and it is highly recommended that more than one be incorporated into the characterization portfolio for every nanoparticle formulation.

The techniques used for size measurement will largely depend on the nanoparticle itself, including the platform, its constituents, and its expected size range. For example, formulations expected to contain a population over one micron in diameter should include techniques such as laser diffraction, electron microscopy or others; DLS, in this case, may not be discriminatory for these larger size populations. For formulations with size populations less than approximately 50 nm in diameter, most resistive pulse sensing techniques will be unsuitable.

It is highly recommended to also incorporate flow-mode size distribution measurements into the characterization portfolio. Flow-mode techniques, such as asymmetric-flow field-flow fractionation (AF4), can provide additional insights into the totality of the nanoformulation, unmasking size populations which may not be evident in batch-mode techniques. This is especially important for highly polydisperse formulations, where there could be shifts in the underlying size populations from batch-to-batch, potentially affecting the biological performance of the formulation.

Techniques which allow for visualization of the nanoparticle, for example electron microscopy, are important not only for size distribution but also for assessment of morphology. This is particularly important for nanotech platforms sensitive to morphological variants, for example metallic nanoparticles which could have multiple shapes or liposomal nanoparticles which could include single-, bi-, or multilamellar structures.

Analytical Techniques Used for Size/Size Distribution

- Dynamic light scattering (DLS)
- Multi-angle light scattering (MALS)
- Laser diffraction
- Transmission electron microscopy (TEM)
- Cryogenic-transmission electron microscopy (Cryo-TEM)
- Asymmetric-flow field-flow fractionation (AF4)—MALS/DLS
- Single particle inductively-coupled plasma mass spectrometry (spICP-MS)
- Single particle counting by light scattering
- Resistive pulse sensing
- Tunable resistive pulse sensing

Relevant NCL Publications

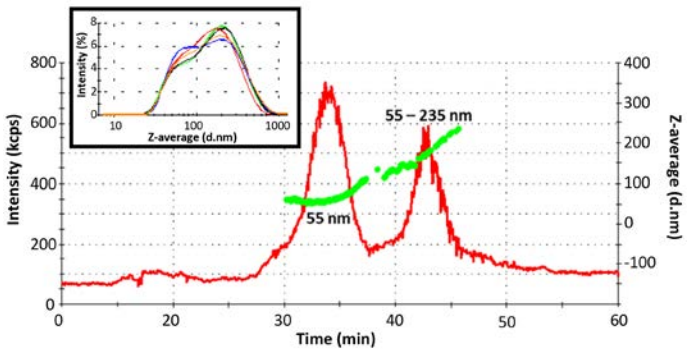
Caputo et al, *J Chromatogr A*, **2021**, 1635, 461767. PMID: 33310281

Hu et al, *Anal Bioanal Chem*, **2020**, 412(2), 425-428. PMID: 31776639

Caputo et al, *J Control Release*, **2019**, 299, 31-43. PMID: 30797868

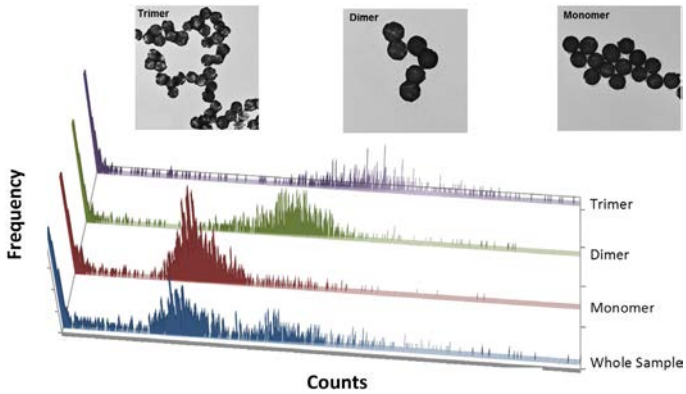
Clogston et al, *Pharmaceutical Research*, **2019**, 37, 6. PMID: 31828540

Batch-Mode vs. Flow-Mode AF4-DLS



Batch-mode (inset) versus flow-mode DLS measurements of a micellar drug formulation. Multiple size populations are observed by both measurements and indicate a polydispersed sample. However, flow-mode DLS (coupled to AF4) can better measure the size distributions of each population. (Adapted from *Anal Bioanal Chem*, **2020**, 412(2), 425–428.)

spICP-MS



Single particle ICP-MS and representative TEM images of PEGylated core-shell nanoparticles. spICP-MS can resolve the different species in a single run and determine the size of each population.

COMPOSITION & PURITY

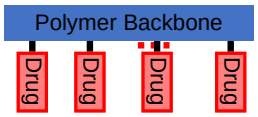
For nanoformulations, composition and purity involves more than simple quantitative measurement of the components in a formulation.

As discussed in the previous size distribution section, nanoformulations are inherently polydispersed. Consequently, in addition to identifying and quantifying the total concentration of individual components, it is also important to quantitate the composition and purity within each size population. Drugs, surface coatings and targeting ligands could all be distributed differently among various size populations in a formulation. This may also be an important aspect for analysis of batch-to-batch consistency as well, as changes in these distributions could have an impact on the biological performance of the nanomedicine.

Furthermore, components in the formulation can exist is several states: covalently nanoparticle-bound, non-covalently nanoparticle-associated, or free in solution. It is imperative to include measurement techniques that can discern these differences, as they too can significantly impact biological performance. Moreover, this distribution can rapidly change in the presence of biological matrix, potentially leading to a fourth population: protein-bound. Therefore, measurements in both buffer and biological matrix are highly recommended. These analyses will not only provide quantitative information about the formulation, but also provide crucial details regarding sample stability (e.g., measurements over time in storage buffer can provide information on shelf-life) and pharmacokinetics (e.g., rapid release of drug in biological matrix can be indicative of similar behavior in vivo).

Analytical Techniques Used for Compositional Analysis

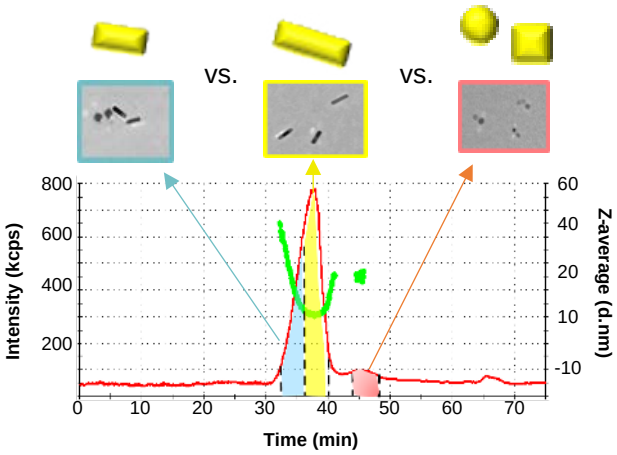
- Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) with UV-vis, fluorescence or charged aerosol detection
- Liquid Chromatography Mass Spectrometry (LC-MS)
- Gas Chromatography Mass Spectrometry (GC-MS)
- Asymmetric-Flow Field-Flow Fractionation (AF4)
- Inductively Coupled Plasma Mass Spectrometry (ICP-MS)
- Thermogravimetric Analysis (TGA)
- Size Exclusion Chromatography-Multi Angle Light Scattering (SEC-MALS)
- Transmission Electron Microscopy with Energy Dispersive X-Ray Spectroscopy (TEM with EDS)
- Elemental CHNOS Analyzer
- Spectroscopy (e.g., UV-Vis, fluorescence)



Polymer-Bound Drug Quantitation

CHN elemental analysis	CHNS elemental analysis	Hydrolysis	SEC-MALS/RI
6.5 ± 0.9%	6.6 ± 0.4%	6.7 ± 0.1% 7.4 ± 0.1%	5.7 ± 3.8%

Several orthogonal techniques to quantitate the percent of drug in a polymeric prodrug formulation. In this example, drug content was determined by elemental analysis (nitrogen and sulfur content), base hydrolysis followed by RP-HPLC separation with UV detection, and size exclusion chromatography coupled with multi-angle light scattering and refractive index detectors (SEC-MALS/RI). (Adapted from *Drug Deliv Transl Res*, **2019**, 9(6), 1057–1066.)



Purity assessment of PEGylated gold nanorods as defined by the presence of non-rod shaped particles. Particles were separated by AF4-DLS, and collected fractions were analyzed by TEM to confirm shape. (Adapted from *Anal Bioanal Chem*, **2020**, 412(2), 425–428.)

Relevant NCL Publications

Wu et al, *J Pharm Biomed Anal*, **2019**, 165, 41–46. PMID: 30502551
Hu et al, *Drug Deliv Transl Res*, **2019**, 9(6), 1057–1066. PMID: 31119521
Hansen & Clogston, *Molecular Biology*, Vol. 1628, **2018**, p. 37–47. PMID: 29039091
Dongargaonkar & Clogston, *Methods in Molecular Biology*, Vol. 1628, **2018**, p. 57–63. PMID: 29039093

SURFACE PROPERTIES

Surface characteristics have been notably challenging to quantitatively assess. Although, in recent years many new technologies and methodologies have allowed for a much more rigorous examination of these properties.

While zeta potential estimation of surface charge is considered a staple of nanoparticle surface analysis, many other parameters are equally important. Size (molecular weight) of surface lipids, degree of surface coverage, orientation and conformation are all essential elements of the characterization repertoire for surface properties.

In addition to characterization of the nanoparticle's surface-bound ligands, assessing protein binding to the nanoparticle is equally important. This can typically be achieved though incubation in plasma followed by analysis using techniques such as AF4 and QCM-D.

Analytical Techniques Used for Analysis of Surface Properties

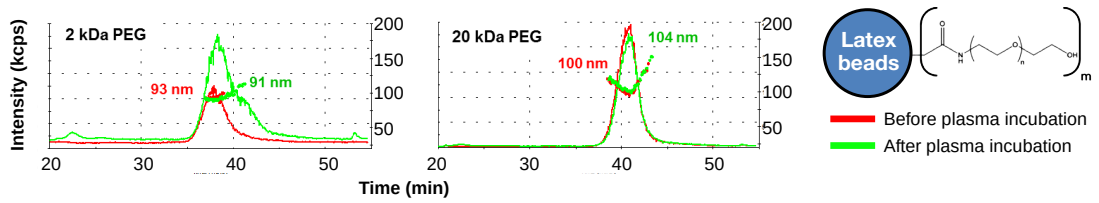
- Zeta Potential
- IZON Q Nano
- Asymmetric-Flow Field-Flow Fractionation (AF4)
- Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D)
- Reversed-Phase High Performance Liquid Chromatography (RP-HPLC)
- Liquid Chromatography Mass Spectrometry (LC-MS)
- Thermogravimetric Analysis (TGA)
- Size Exclusion Chromatography-Multi Angle Light Scattering (SEC-MALS)
- UV-vis Spectroscopy
- Fluorescence Spectroscopy

Relevant NCL Publications

Hu et al, *Anal Bioanal Chem*, **2020**, 412(2), 425–428. PMID: 31776639
Smith et al, *Molecular Biology*, Vol. 1628, **2018**, p. 49–55. PMID: 29039092
Smith et al, *Anal Bioanal Chem*, **2017**, 409(24), 5779–5787. PMID: 28762066
Smith et al, *Anal Bioanal Chem*, **2015**, 407(13), 3705–3716. PMID: 25749798

AF4-DLS demonstration of protein binding

Polystyrene beads with 2 kDa and 20 kDa PEG surface functionalization. After incubation in human plasma, there is a significant broadening of the size distribution for the 2 kDa PEG NP, indicating protein binding to the surface. In contrast, there is no significant change after human plasma incubation for the 20 kDa PEG NP, indicating no/minimal protein binding to the surface. This highlights the important relationship between surface ligands and protein binding.



STABILITY

Stability of a nanoformulation is assessed under a variety of conditions, the most common of which are outlined in the adjacent box.

When assessing stability, it is important to monitor several different physical and chemical properties to adequately identify potential effects on the formulation. Size/size distribution is often monitored as a means of determining constraints under which the formulation degrades and/or aggregates. It is a relatively simple and quick technique and provides a wealth of information on the products. However, it should not be the only parameter monitored.

Often, measurements will be selected dependent upon the formulation. For example, hydrolysis of a prodrug in a polymer formulation, release of free metal ions in metallic nanoparticles, lipid hydrolysis and degradation in lipid-based formulations, and degradation/release of surface coatings or targeting ligands may also be important measurements. Drug release and drug degradation products, both in storage buffer and in physiological matrix, is also an important measure of stability.

Parameters for Assessment of Stability

- Short- and long-term storage; shelf-life
- Temperature and freeze-thaw
- pH
- Light sensitivity
- Solvents and excipients
- Lyophilization
- Centrifugation
- Filtration

LOT-TO-LOT REPRODUCIBILITY

Similar to stability, assessment of lot-to-lot variability of a formulation should be monitored using a variety of techniques, which will often be dependent upon the specific nanoformulation under investigation. And, as noted for stability assessment, size distribution should not be the only parameter monitored.

Many other physical and chemical features can contribute to the biological performance of a nanoparticle. Ideally, well-designed experiments should be conducted to identify the formulation's critical quality attributes (CQA), that is, those physical and chemical properties which directly influence biological performance, identifying upper and lower limits of acceptability.

With the CQA properly identified, a refined list of assays and techniques can be used to quickly screen lots prior to use.

Analytical Techniques Used for Assessment of Reproducibility

- Dynamic Light Scattering (DLS)
- Zeta Potential
- Reversed-Phase High Performance Liquid Chromatography (RP-HPLC)
- Liquid Chromatography Mass Spectrometry (LC-MS)
- Asymmetric-Flow Field-Flow Fractionation (AF4)
- Inductively Coupled Plasma Mass Spectrometry (ICP-MS)
- Thermogravimetric Analysis (TGA)
- Size Exclusion Chromatography-Multi Angle Light Scattering (SEC-MALS)
- Transmission Electron Microscopy with Energy Dispersive X-Ray Spectroscopy (TEM with EDS)
- Elemental CHNOS Analyzer

Relevant NCL Publications

Hu et al, *Anal Bioanal Chem*, **2020**, 412(2), 425–428. PMID: 31776639

STARTING MATERIAL CHARACTERIZATION

Prior to formulation, starting materials should be analyzed for purity, degradation products and a variety of other traits. This can be especially important when purchasing a new synthetic lot, switching suppliers, or changing parameters in the production process. Establishing a rigorous protocol for screening starting materials prior to formulation not only helps to ensure batch-to-batch reproducibility of the formulation's physicochemical attributes, but may also help avoid uncharacteristic results when moving into biological screening of the formulation.

For example, small molecule drugs may contain epimers, which could vary in concentration from lot to lot, thus influencing the effective loading of the active component.

Polymers can also vary from lot-to-lot with respect to molar mass distribution, purity, polydispersity and, where applicable, degree of functionalization. Lipids could have varied fatty acid content and degradation products.

Before functionalization of colloidal metal nanoparticles, the particles should be screened for size distribution, shape irregularities, and free metal concentration, all of which could influence the biological outcomes.

Additionally, water and other solvents and buffers should be screened for unwanted chemical (e.g., total organic content) and biological (e.g., endotoxin, microbes) contaminants.

Parameters for Assessment of Starting Materials

- **Drugs**: structure, degradation products, impurities, epimerization
- **Polymers**: molar mass, polydispersity, purity, degree of functionalization
- **Lipids**: fatty acid distribution, free fatty acids, lysophospholipids
- **Colloidal metal NP**: size, shape, free metal concentration, purity (e.g., other residual metals)
- **Water, solvents & buffers**: chemical and biological impurities

Relevant NCL Publications

Grossman et al, *AAPS J*, **2017**, 19(1), 92–102. PMID: 27612680

MEET THE EXPERT

Dr. Jeffrey D. Clogston, PhD
Head of Physicochemical Characterization

About Dr. Clogston

Dr. Clogston is Head of the NCL's Physicochemical Characterization Section and has been with the lab for over 15 years. During this time, Jeff has helped to establish many protocols for nanoparticle characterization, working collaboratively with experts at the National Institute of Standards and Technology (NIST) and standards organizations such ASTM International and International Organization for Standardization (ISO). Throughout his tenure, Jeff has published nearly 50 manuscripts describing new analytical techniques for various nanoparticle platforms, keeping pace with the quick evolution of the nanomedicine field. His areas of expertise include physicochemical characterization of and in vitro release from lipid- and polymer-based drug delivery systems, development of novel analytical methodologies to address characterization shortcomings, and protein and lipid biochemistry.

Jeff received his Ph.D. in Chemical Engineering from The Ohio State University. His research dissertation was on the application of the lipidic cubic phase for drug delivery, wastewater remediation, and membrane protein crystallization.

Additional Resources

- Select protocols for assays described here can be downloaded for free on the NCL's website:
  https://ncl.cancer.gov/resources/assay-cascade-protocols
- Publications on these topics are available here:
  https://ncl.cancer.gov/resources/ncl-scientific-bibliography
- Particle specific handouts detailing NCL capabilities for select platforms:
  - Liposomes
  - Polymeric Formulations
  - Colloidal Metal Nanoparticles

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For questions or further discussion on any of the topics highlighted here, please feel free to reach out to Dr. Clogston via email.

Frederick National Laboratory
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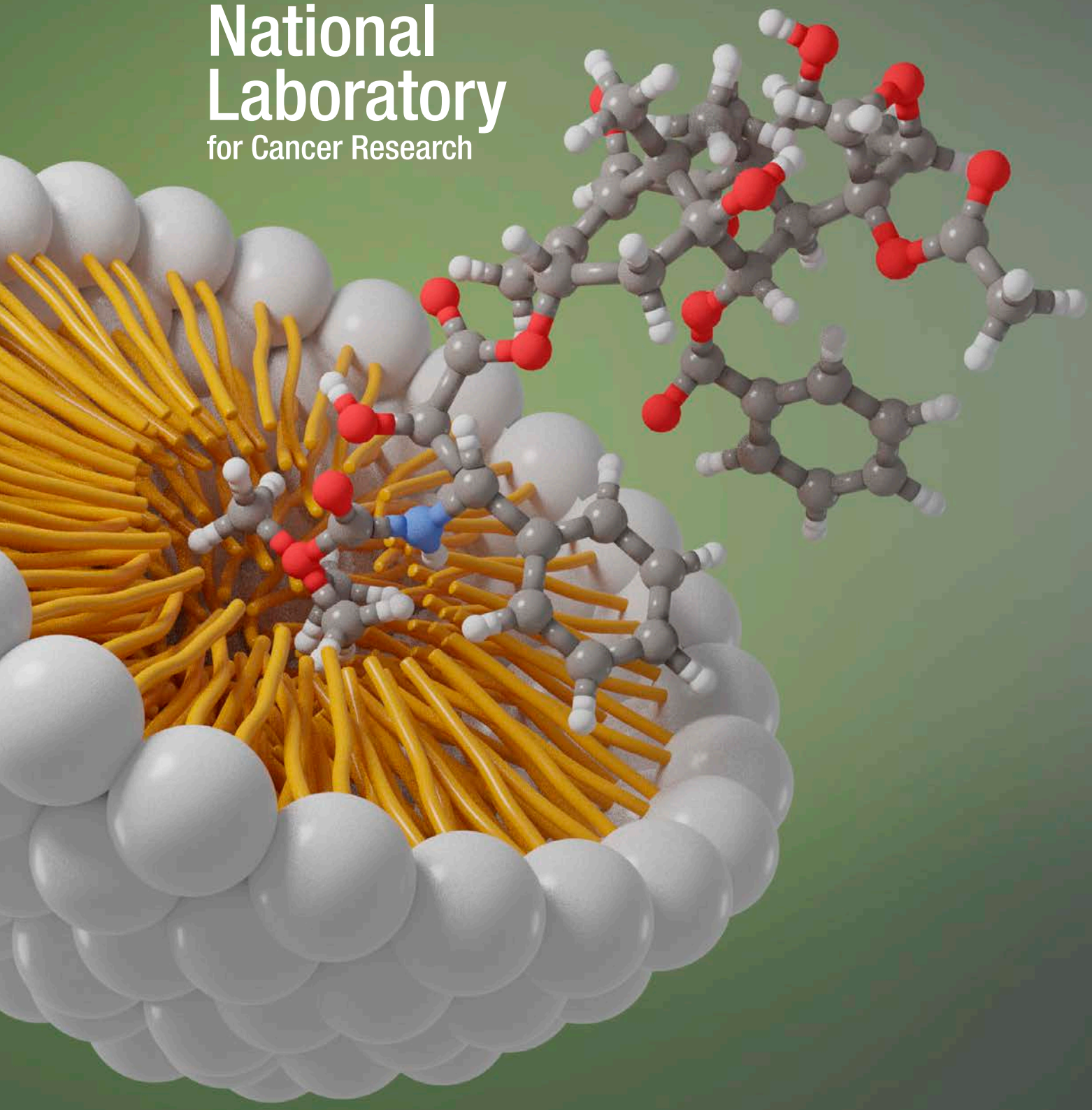

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