NCI Alliance for Nanotechnology in Cancer

Physicochemical Characterization of Nanomedicines

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NATIONAL CANCER INSTITUTE®

Frederick National Laboratory for Cancer Research

Nanotechnology Characterization Lab (NCL)

NCL Nanotechnology Characterization Laboratory | NCI Alliance for Nanotechnology in Cancer

NCL was established in 2004 as a collaboration among the NCI, NIST and FDA, with 4 primary objectives:

Characterize nanoparticles using standardized methods

Facilitate regulatory review of nanotech constructs

Conduct structure activity relationship (SAR) studies

Engage in educational and knowledge sharing efforts

Assay Cascade is a free service

Visit https://ncl.cancer.gov/

NCL has 10+ years of knowledge and expertise in nanoparticle characterization, and utilizes this to *help accelerate the translation of promising nanotech drugs and diagnostics.*

NCL Assay Cascade – 50+ Standardized Protocols for Nanotech

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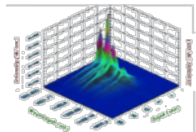


Physicochemical Characterization

Size/Size Distribution
- Dynamic Light Scattering (DLS)
- Electron Microscopy (TEM, SEM, cryo)
- Atomic Force Microscopy (AFM)
- Field Flow Fractionation (FFF), SEC-MALLS

Composition
- TEM with EDS
- Inductively coupled plasma-mass spec. (ICP-MS)
- Spectroscopy (NMR, CD, Fluorescence, IR, UV-vis)

Purity
- Chromatography
- Capillary Electrophoresis

Surface Chemistry
- Biacore
- Zeta Potential

Stability
- Stability can be measured with any number of instruments with respect to time, temperature, pH, etc.

In Vitro Characterization

Sterility
- Bacterial/Viral/Mycoplasma
- Endotoxin

Cell Uptake/Distribution
- Cell Binding/Internalization
- Targeting

Hematology
- Hemolysis
- Platelet Aggregation
- Coagulation
- Complement Activation
- Plasma Protein Binding

Immune Cell Function
- Cytokine Induction
- Chemotaxis
- Phagocytosis
- Leukocyte Proliferation
- Leukocyte Procoagulant Activity

Toxicity
- Cytotoxicity
- Autophagy

In Vivo Characterization

Pharmacology
- Clinical Tx cycle
- NP Quantitation methods
- PK Parameters

Immunotoxicity
- Local lymph node proliferation assay
- T-cell dependent antibody response
- Adjuvanticity
- Rabbit pyrogen test

Single and Repeat Dose Toxicity
- Blood Chemistry
- Hematology
- Histopathology (42 tissues)
- Gross Pathology
- Immunogenicity

Efficacy
- Therapeutic
- Imaging

NCL testing links physicochemical properties to biological outcomes.

Protocols Available Online: https://ncl.cancer.gov/resources/assay-cascade-protocols

NCL Collaborators in Clinical Trials

NCL Supports:

- Preclinical Characterization
- Regulatory Concerns
- Clinical Characterization
- Exploring Alternate Indications
- Next-Generation Nanoparticles

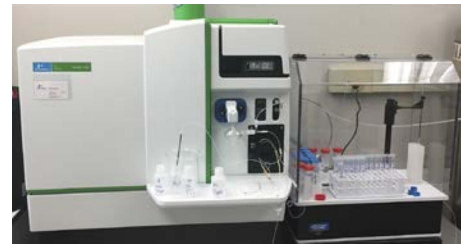
14 Collaborators in clinical trials with novel nanomedicine therapies.

Visit https://ncl.cancer.gov/

Physicochemical Characterization

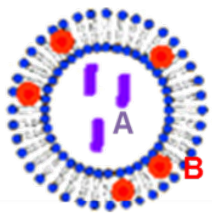
Physicochemical characterization boils down to analytical instrumentation and development of new methods

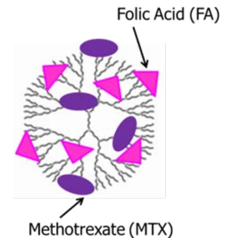
- Dynamic Light Scattering (DLS)
- Static Light Scattering (MALS)
- Laser Diffraction
- Electron Microscopy (TEM, SEM, cryo-TEM, EDS)
- Atomic Force Microscopy (AFM)
- Resistive Pulse Sensing (RPS)
- Zeta Potential
- Chromatography (RP-HPLC, SEC, AF4, FPLC)
- Liquid chromatography–mass spectrometry (LC-MS)
- Inductively coupled plasma-mass spectrometry (ICP-MS)
- CHNOS Elemental Analysis
- Spectroscopy (UV-Vis, Fluorescence, IR, Raman)
- Thermal Analysis (TGA, DSC)
- Quartz Crystal Microbalance with Dissipation monitoring (QCM-D)

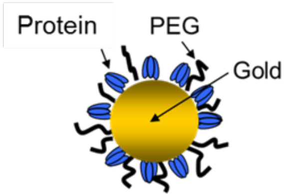


Leveraging over 13 years of experience, NCL has identified the key PCC parameters and methodology needed

- Liposomal Products
- Polymeric Nanoparticles
- Colloidal Metal Nanoparticles







Liposome Drug Products

Development of new methods to address FDA questions

Liposome Drug Products

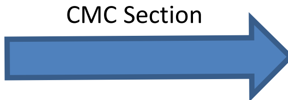
Chemistry, Manufacturing, and Controls; Human Pharmacokinetics and Bioavailability; and Labeling Documentation

Guidance for Industry

Additional copies are available from:
Office of Communications, Division of Drug Information
Center for Drug Evaluation and Research
Food and Drug Administration
10001 New Hampshire Ave., Hillandale Bldg., 4th Floor
Silver Spring, MD 20993-0002
Phone: 855-543-3784 or 301-796-3400; Fax: 301-431-6353
Email: druginfo@fda.hhs.gov
http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htm

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)

April 2018
Pharmaceutical Quality/CMC



I. INTRODUCTION
II. BACKGROUND
III. DISCUSSION
A. Chemistry, Manufacturing, and Controls
1. *Description and Composition*
2. *Physicochemical Properties*
3. *Critical Quality Attributes*
4. *Description of Manufacturing Process and Process Controls*
5. *Control of Lipid Components*
6. *Drug Product Specification*
7. *Stability*
8. *Postapproval Changes in Manufacturing*
B. Human Pharmacokinetics: Bioavailability and Bioequivalence
1. *Clinical Pharmacology Studies*
2. *Biopharmaceutics*
C. Labeling
IV. REFERENCES

Liposomal Products
Parameters, Methods, and Considerations

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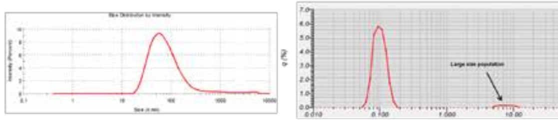
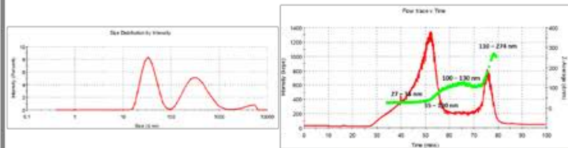
Size/Size Distribution

- Dynamic Light Scattering (DLS)
- Multi-Angle Light Scattering (MALS)
- Laser Diffraction
- Cryogenic-Transmission Electron Microscopy (Cryo-TEM)
- Resistive Pulse Sensing
- Asymmetric-Flow Field-Flow Fractionation (AF4) – MALS/DLS



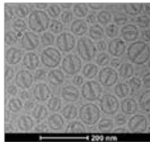
Size and size distribution measurements of a protein/lipid nanovesicle using two different techniques: batch-mode DLS (left panel) and laser diffraction (right panel). The smaller size population (85 -100 nm) was consistent by both techniques, whereas the larger size population (7.7 μm) was more accurately measured by laser diffraction.



Batch-mode (left panel) versus flow-mode (right panel) DLS measurements of dual-drug loaded liposomes. Multiple size populations are observed by both techniques and indicate a polydispersed sample. However, flow-mode DLS (coupled to AF4) can better resolve the size distributions of each population.

Morphology

- Cryo-TEM

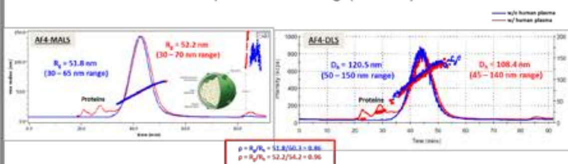


Representative cryo-TEM image of PEGylated liposomal doxorubicin. Cryo-TEM can be used to evaluate:

- Size distribution
- Liposome morphology
- Internal liposome volume
- Bilayer thickness
- Number of lamellae
- Drug appearance/state

Surface Characteristics

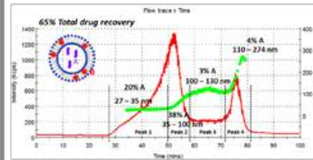
- Zeta Potential
- Protein Binding Assessment by AF4-MALS/DLS, Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D)



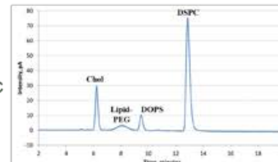
Flow-mode AF4-MALS (left panel) and AF4-DLS (right panel) of PEGylated irinotecan liposomes before and after incubation in human plasma. The increase in the ratio of the measured MALS and DLS sizes (ρ) after human plasma incubation suggests protein binding to the surface of the liposomes.

Composition

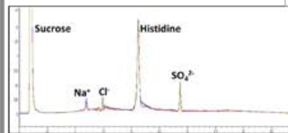
- Drug Concentration: Total, Free, and Encapsulated
- Drug Distribution as a Function of Size
- Targeting Ligand Concentration: Total, Bound, and Unbound
- Individual Lipid Concentrations
- Counterion Concentrations: Interior and Exterior
- Excipient Concentrations
- Particles per mL Concentration
- Osmolality, Viscosity Measurements



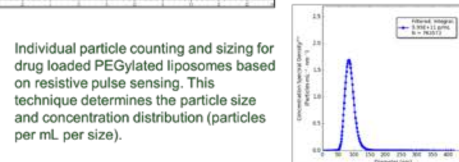
Flow-mode DLS (coupled to AF4) of dual-drug loaded liposomes. Collected fractions (denoted by dashed vertical lines) are assayed for drug content by RP-HPLC with UV detection to determine the drug distribution.



Individual lipid concentration for liposomes containing four lipid components is measured by RP-HPLC with charged aerosol detection (CAD).



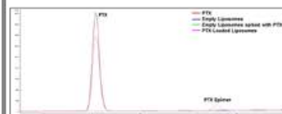
Counterion and excipient concentrations for PEGylated liposomal doxorubicin are measured by RP-HPLC with charged aerosol detection (CAD).



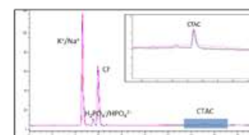
Individual particle counting and sizing for drug loaded PEGylated liposomes based on resistive pulse sensing. This technique determines the particle size and concentration distribution (particles per mL per size).

Purity

- Drug Impurities
- Lipid Impurities
- Free Drug/Lipid/Targeting Ligand Concentrations
- Residual Solvents and Surfactants



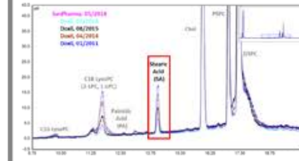
Purity assessment of PEGylated liposomal paclitaxel (PTX) as defined by the presence of drug impurities. The drug epimer concentration was measured by RP-HPLC and its identity confirmed by mass spectrometry.



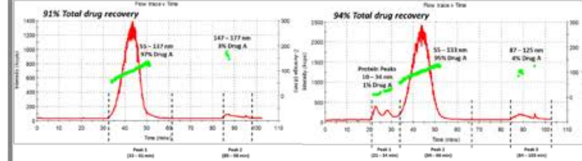
Residual surfactant assessment of liposomes encapsulating a metalloid nanoparticle. Surfactant concentration was measured by RP-HPLC with charged aerosol detection (CAD).

Stability

- Size/Size Distribution (aggregation)
- Drug Leakage and Degradation
- Hydrolysis of Lipids
- Drug Release in Plasma
- Solvent, Thermal, pH, Photo, Freeze-Thaw, Lyophilization, Centrifugation, Filtration
- Storage Conditions / Shelf-Life



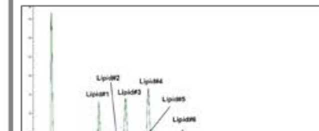
Stability assessment of PEGylated liposomal doxorubicin as defined by the hydrolysis of phospholipids. The formation of free fatty acids and lysophospholipids of several batches with varying expiration dates were measured by RP-HPLC with charged aerosol detection (CAD).



Stability assessment of drug-loaded liposomes in the presence of human plasma. The flow-mode DLS (coupled to AF4) before (left panel) and after (right panel) incubation with human plasma gives the size distribution. Collected fractions (denoted by dashed vertical lines) are assayed for drug content by RP-HPLC with fluorescence detection to determine the drug distribution. The high total drug recovery suggests drug stability in the presence of proteins.

Batch-to-Batch Consistency

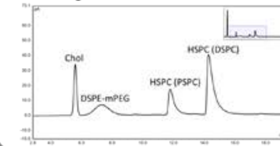
- Assessed by choosing relevant parameters (i.e. lot release criteria) that relate to a desired in vivo outcome



Batch-to-batch consistency for lipid nanoparticles with siRNA was assessed by lipid composition. Six individual lipid concentrations for three batches were determined by RP-HPLC with charged aerosol detection (CAD).

Starting Material Characterization

- Drug Identity: structure
- Drug Purity: degradation products
- Lipid Composition: structure, fatty acid distribution
- Lipid Purity: free fatty acid, lysophospholipids
- Storage Conditions / Shelf-Life



Lipid composition (identity and individual lipid concentrations) and purity (presence of free fatty acid and lysophospholipids) were determined by RP-HPLC with charged aerosol detection (CAD) for a commercially available lipid mix.

Polymeric Nanoparticles
Parameters, Methods, and Considerations

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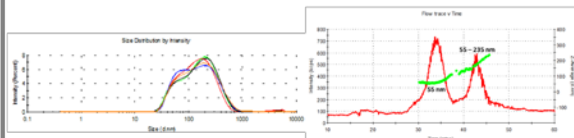
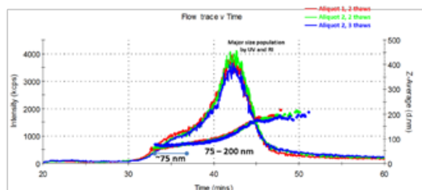
Size/Size Distribution

- Dynamic Light Scattering (DLS)
- Multi-Angle Light Scattering (MALS)
- Laser Diffraction
- Transmission Electron Microscopy (TEM)
- Resistive Pulse Sensing
- Asymmetric-Flow Field-Flow Fractionation (AF4) – MALS/DLS



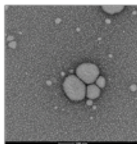
Batch-mode (left panel) versus flow-mode (right panel) DLS measurements of a micellar drug formulation. Multiple size populations are observed by both measurements and indicate a polydispersed sample. However, flow-mode DLS (coupled to AF4) can better measure the size distributions of each population.



Flow-mode DLS (couple to AF4) measurements of a polymeric micelle to test vial-to-vial consistency as well as freeze-thaw stability.

Morphology

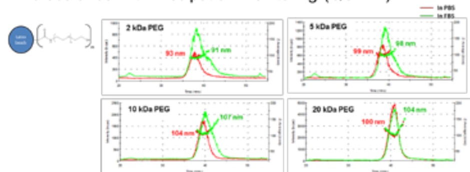
- TEM



Representative TEM image of PEGylated oil-filled prodrug nanocapsules. In addition to determining the size distribution, TEM can be used to evaluate morphology and purity assessment. Here, the presence of residual surfactant forming smaller micelles are observed in the background.

Surface Characteristics

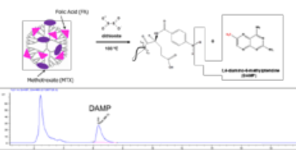
- Zeta Potential
- Protein Binding Assessment by AF4-MALS/DLS, Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D)



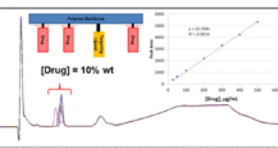
Flow-mode DLS, coupled to AF4, of polystyrene beads conjugated to various lengths of PEG before and after human plasma incubation. The decrease in the polydispersity with increasing PEG length after human plasma incubation suggests minimal protein binding when 20kDa PEG is used.

Composition

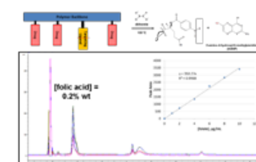
- Drug Concentration: Total, Free, and Encapsulated
- Prodrug Drug Content
- Drug/Prodrug Loading Distribution (as a function of size)
- Targeting Ligand Concentration
- Individual Polymer Concentrations
- Excipient Concentrations
- Particles per mL Concentration
- Osmolality, Viscosity Measurements



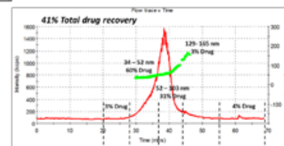
Methotrexate content of methotrexate-conjugated folate-targeted Generation 5 PAMAM dendrimers was determined by dithionite reduction followed by RP-HPLC separation and fluorescence detection.



Drug content of a targeted polymer drug conjugate was determined by base hydrolysis followed by RP-HPLC separation and UV detection.



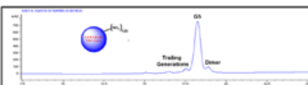
Folate content of a targeted polymer drug conjugate was determined by dithionite reduction followed by RP-HPLC separation and fluorescence detection.



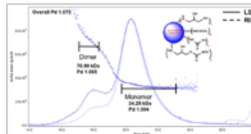
Flow-mode DLS (coupled to AF4) of a micellar drug formulation. Collected fractions (denoted by dashed vertical lines) are assayed for drug content by RP-HPLC with fluorescence detection to determine the drug distribution.

Purity

- Drug/Prodrug Impurities
- Free Drug/Prodrug Concentration
- Polymer Impurities and Degradation
- Polymer Functionality (end-group analysis)
- Residual Solvents and Reagents



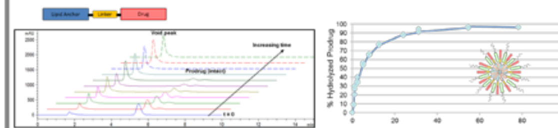
Purity assessment of Generation 5 amine-terminated dendrimers as defined by the presence of dimers and trailing generations measured by RP-HPLC-UV.



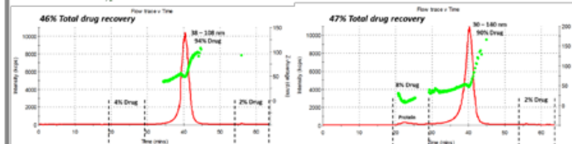
Purity assessment of drug-conjugated and targeted Generation 5 PAMAM dendrimers by SEC-MALS-RI. The molecular mass distribution and polydispersity for the monomer and dimer are based on the bracketed molar mass distribution.

Stability

- Size/Size Distribution (aggregation)
- Drug Leakage and Degradation
- Polymer Degradation
- Drug/Prodrug Release in Plasma
- Solvent, Thermal, pH, Photo, Freeze-Thaw, Lyophilization, Centrifugation, Filtration
- Storage Conditions / Shelf-Life



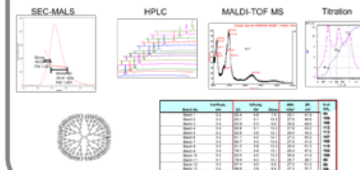
Stability assessment of prodrug micelles in the presence of human plasma. The hydrolysis of the intact prodrug was measured by RP-HPLC (left panel) and used to construct the hydrolysis versus time graph (right panel), which can be used to determine $t_{1/2}$.



Stability assessment of micellar drug formulation in the presence of human plasma. The flow-mode DLS (coupled to AF4) before (left panel) and after (right panel) incubation with human plasma gives the size distribution. Collected fractions (denoted by dashed vertical lines) are assayed for drug content by RP-HPLC. The low total drug recovery suggests release of drug upon exposure to proteins.

Batch-to-Batch Consistency

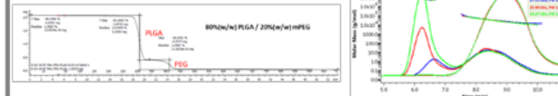
- Assessed by choosing relevant parameters (i.e. lot release criteria) that relate to a desired in vivo outcome



Batch-to-batch consistency for G5 amine-terminated dendrimers was assessed by several techniques. In this case, HPLC and titration were the relevant characterization methods.

Starting Material Characterization

- Drug/Prodrug Identity (structure)
- Drug/Prodrug Purity (degradation products)
- Polymer Composition (molecular mass and polydispersity index)
- Polymer Purity and Functionality
- Storage Conditions / Shelf-Life



Polymer composition and molar mass distribution of mPEG-PLGA were determined by thermogravimetric analysis (left panel) and SEC-MALS-RI (right panel), respectively.

Colloidal Metal Nanoparticles Parameters, Methods, and Considerations

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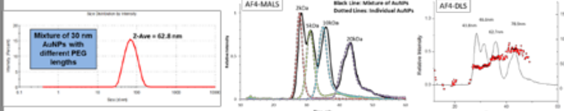
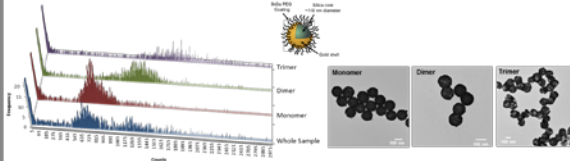
Size/Size Distribution

- Dynamic Light Scattering (DLS)
- Multi-Angle Light Scattering (MALS)
- Laser Diffraction
- Transmission Electron Microscopy (TEM)
- Resistive Pulse Sensing
- Asymmetric-Flow Field-Flow Fractionation (AF4) – MALS/DLS
- Single Particle Inductively-Coupled Plasma Mass Spectrometry (spICP-MS)



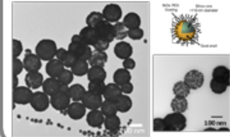
Batch-mode (left panel) versus flow-mode AF4-MALS (center panel) and AF4-DLS (right panel) measurements of a mixture of 30 nm colloidal gold nanoparticles grafted with 2k, 5k, 10k, and 20kDa PEG. Batch-mode DLS is unable to resolve the individual species. However, individual peaks can be resolved using AF4, giving size measurements for each of the individual components.



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

Morphology

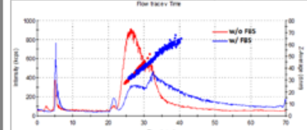
- TEM



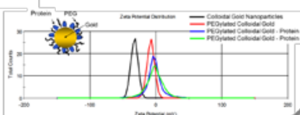
Representative TEM image of gold-core silica-shell nanoparticles. In addition to determining the size distribution, TEM can be used to evaluate morphology and purity assessment. Here, smaller gold nanoparticles and incomplete gold coating were observed. Elemental composition was confirmed by energy dispersive spectroscopy (EDS).

Surface Characteristics

- Zeta Potential
- Protein Binding Assessment by AF4-MALS/DLS, Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D)



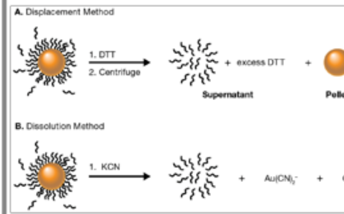
Flow-mode AF4-DLS of drug-conjugated silica nanoparticles before and after incubation in fetal bovine serum (FBS). The increase in size and polydispersity after FBS incubation suggests protein binding to the surface of the nanoparticles.



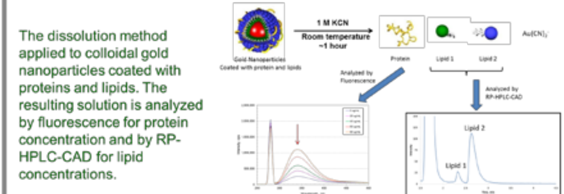
Monitoring the reaction progress of PEG and protein grafting to the surface of colloidal gold nanoparticles by zeta potential. Measurement conditions were identical for all samples.

Composition

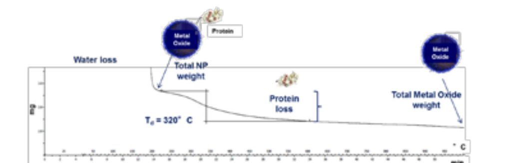
- Metal Concentration: Total and Free
- Coating (Polymer, Protein, Lipid) Concentrations: Total and Free
- Targeting Ligand Concentration: Total and Free
- Excipient Concentrations
- Particles per mL Concentration
- Osmolality, Viscosity Measurements



Two methods to quantitate the amount of PEG on colloidal gold nanoparticles. The displacement method (A.) uses dithiothreitol to displace PEG from the surface. The dissolution method (B.) uses potassium cyanide to dissolve the gold nanoparticles. Both methods use RP-HPLC with charged aerosol detection (CAD) to quantitate total PEG.



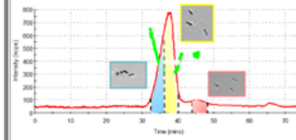
The dissolution method applied to colloidal gold nanoparticles coated with proteins and lipids. The resulting solution is analyzed by fluorescence for protein concentration and by RP-HPLC-CAD for lipid concentrations.



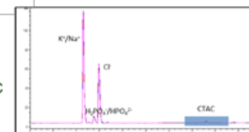
Thermogravimetric analysis (TGA) of protein-coated metal oxide nanoparticles to determine the amount of protein coating and total metal oxide weight.

Purity

- Metal Impurities
- Shape Distribution: spheres versus rods
- Coating Impurities
- Residual Solvents and Reagents



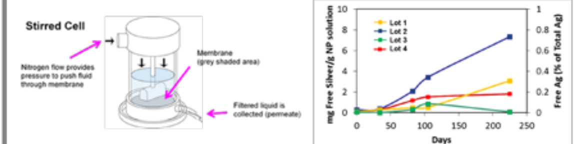
Purity assessment of PEGylated gold nanorods as defined by the presence of non-nanorod particles measured by AF4-DLS. Collected peak fractions were analyzed by TEM to confirm shape.



Residual surfactant assessment of mesoporous metal nanoparticles coated with a lipid bilayer. Surfactant concentration was measured by RP-HPLC with charged aerosol detection (CAD).

Stability

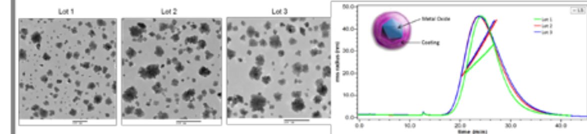
- Size/Size Distribution: aggregation
- Free Metal Ion Release
- Coating Release
- Release of Coating and Metal in Plasma
- Solvent, Thermal, pH, Photo, Freeze-Thaw, Lyophilization, Centrifugation, Filtration
- Storage Conditions / Shelf-Life



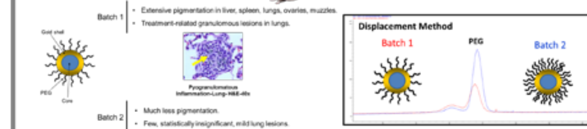
Stability assessment of four batches of colloidal silver nanoparticles as determined by free silver concentrations. Stirred cell filtration was used to separate free silver from nanoparticle silver. The permeate silver concentration was measured by ICP-MS.

Batch-to-Batch Consistency

- Assessed by choosing relevant parameters (i.e. lot release criteria) that relate to a desired in vivo outcome



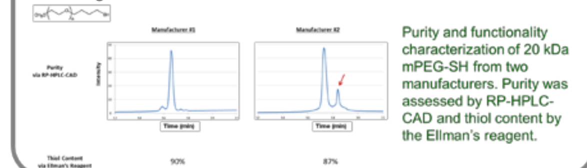
Representative TEM images (left panel) versus flow-mode AF4-MALS (right panel) measurements for three batches of a polymer coated metal oxide formulation. In this case, AF4-MALS was better suited in assessing the size distribution and differences between these three lots.



Batch-to-batch consistency for gold-core silica-shell nanoparticles assessed by total PEG concentration. A 14-day ADME-Tox study of two batches resulted in different outcomes due to differences in the amount of PEG bound to the nanoparticle surface.

Starting Material Characterization

- Colloidal Metal Nanoparticles: size, shape, composition, and purity
- Coating Composition: molecular mass and polydispersity index
- Coating Purity and Functionality
- Storage Conditions / Shelf-Life

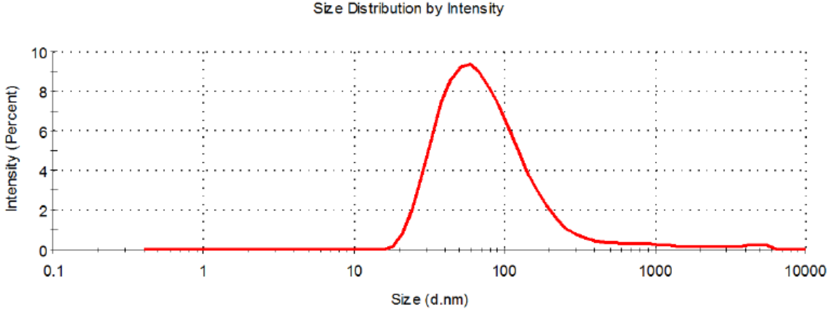


Purity and functionality characterization of 20 kDa mPEG-SH from two manufacturers. Purity was assessed by RP-HPLC-CAD and thiol content by the Ellman's reagent.

Sizing by Different Techniques

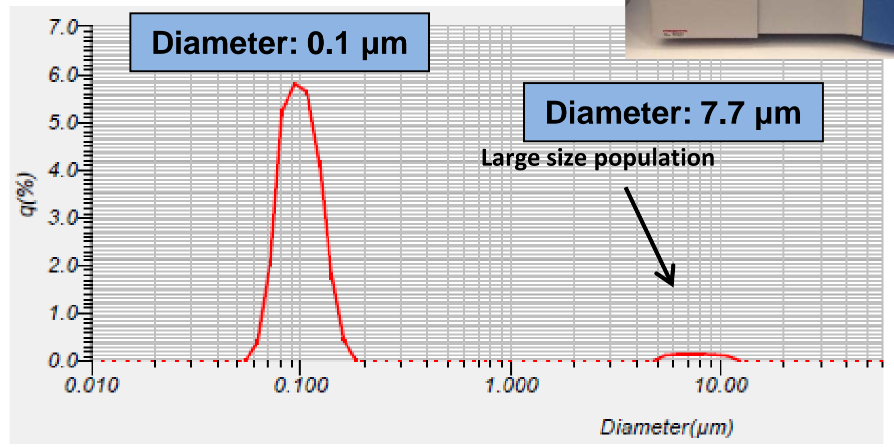
Lipid-Protein Nanovesicles

Dynamic Light Scattering (DLS)

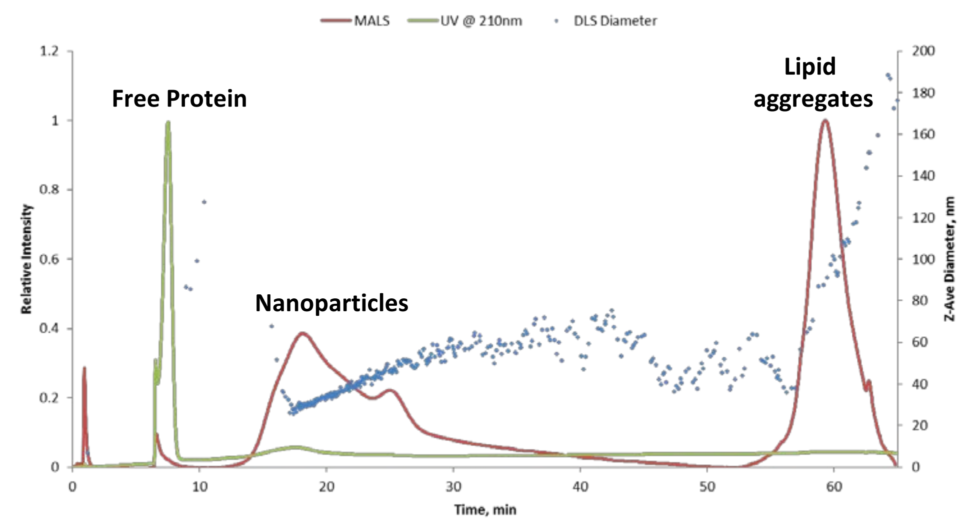


DLS not suitable for >1 micron particles

Laser Diffraction



Asymmetric-Flow Field-flow Fractionation (AF4)



- **Contains >5 µm particles**
- **Free protein present**
- **Polydispersed size population**
- **Lipid aggregates present**

Orthogonal methods highly recommended!

Cryo-TEM
PEGylated Liposomal Doxorubicin

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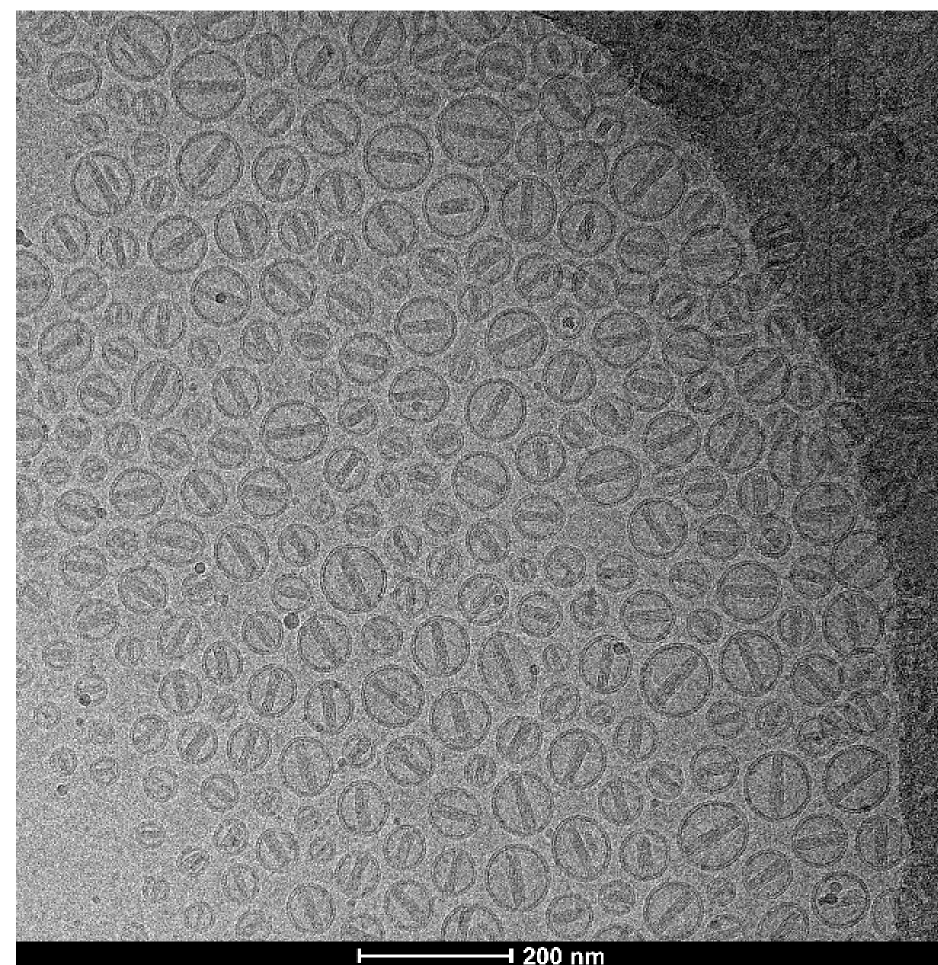
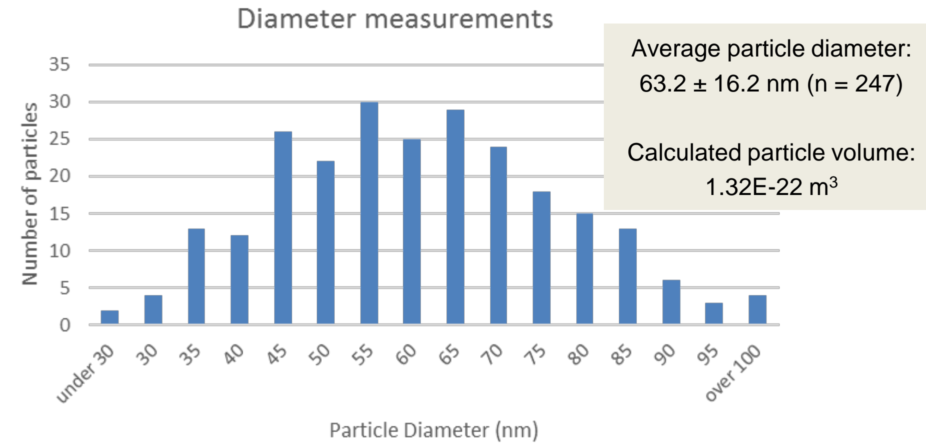
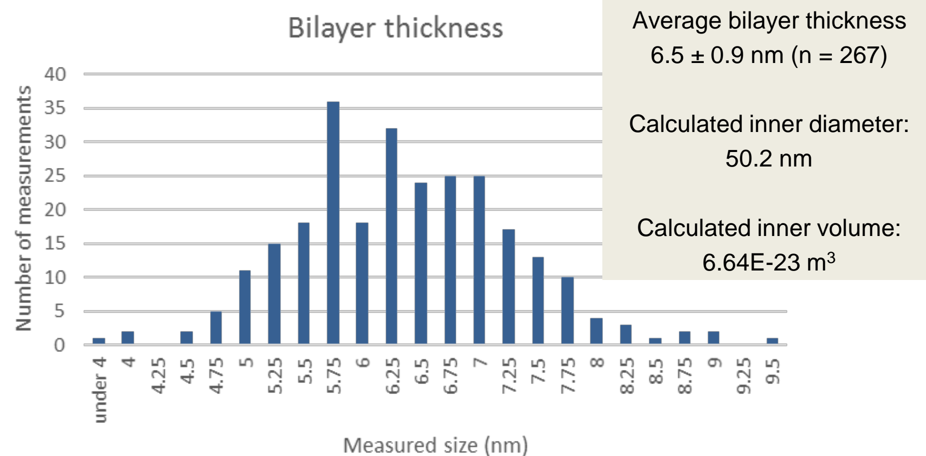
Stock solution (2.5 µL) was applied to a cryo-TEM carbon film grid (Electron Microscopy Sciences). The grid was flash-frozen in ethane using a Vitribot apparatus. Images were taken using a Titan Krios (FEI) equipped with a high-brightness X-FEG gun at 120 V acceleration voltage. Particle size analysis was performed using ImageJ (https://imagej.nih.gov/ij/).







Particle Size and Concentration

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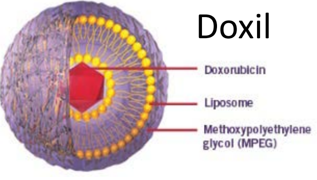
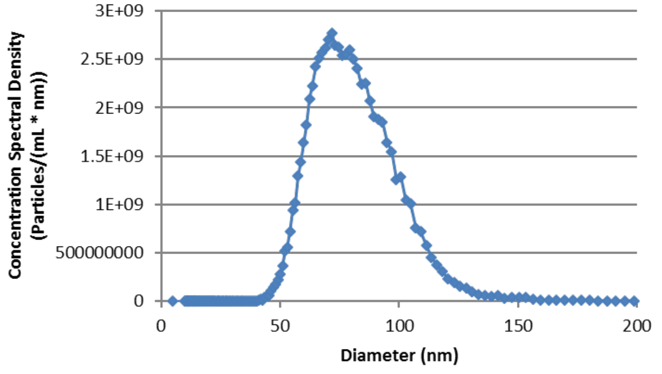
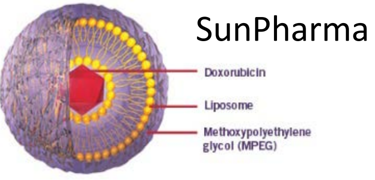
Spectradyne nCS1 (Resistive Pulse Sensing)
Running Buffer: Samples diluted 100-fold in running buffer (PBS + 1% Tween)

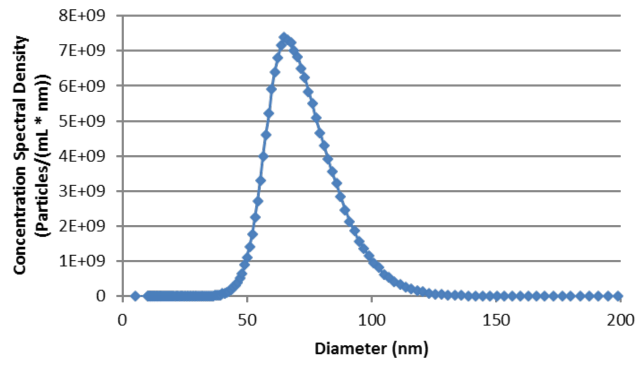




Average size: 78.0 nm
Approximate range: 45-125 nm

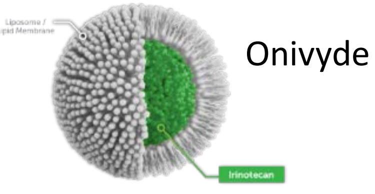
Integrated concentration: 1.15E11 particles/mL
Sample concentration: 1.15E13 particles/mL

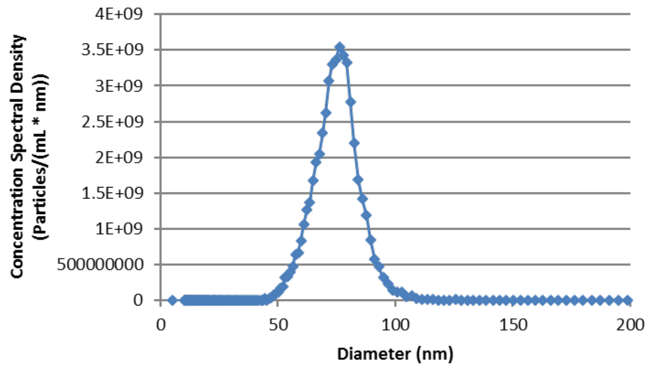




Average size: 69.7 nm
Approximate range: 40-110 nm

Integrated concentration: 2.24E11 particles/mL
Sample concentration: 2.24E13 particles/mL





Average size: 74.1 nm
Approximate range: 50-100 nm

Integrated concentration: 7.55E10 particles/mL
Sample concentration: 7.55E12 particles/mL

Zeta Potential

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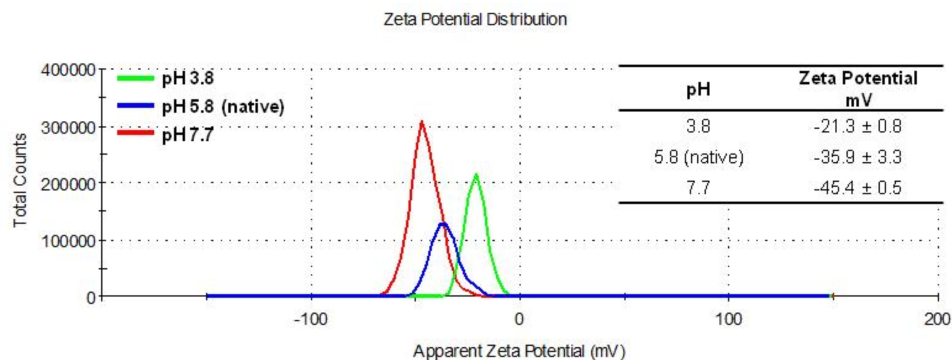
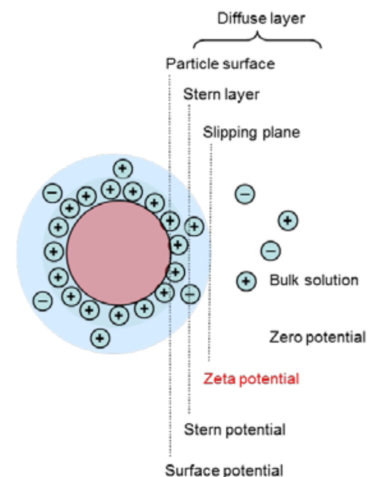
We know zeta potential depends on:

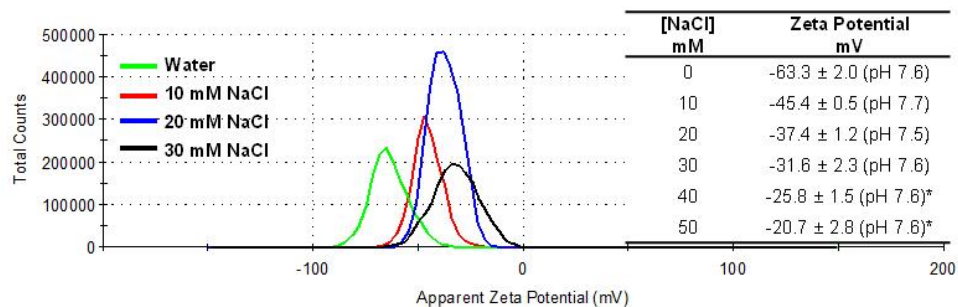
pH



pH	Zeta Potential mV
3.8	-21.3 ± 0.8
5.8 (native)	-35.9 ± 3.3
7.7	-45.4 ± 0.5

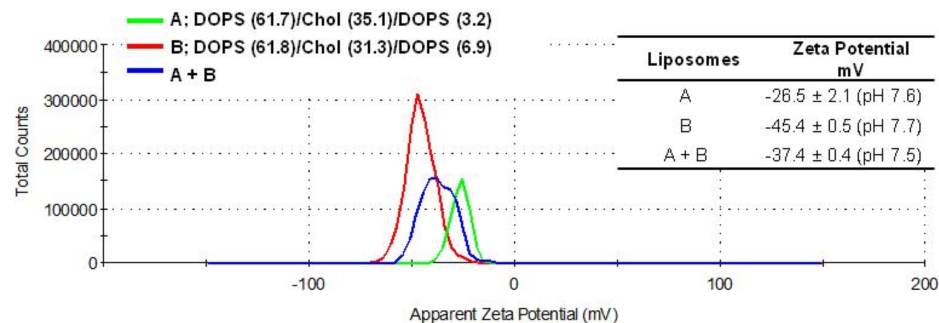


Ionic strength of dispersing media



[NaCl] mM	Zeta Potential mV
0	-63.3 ± 2.0 (pH 7.6)
10	-45.4 ± 0.5 (pH 7.7)
20	-37.4 ± 1.2 (pH 7.5)
30	-31.6 ± 2.3 (pH 7.6)
40	-25.8 ± 1.5 (pH 7.6)*
50	-20.7 ± 2.8 (pH 7.6)*

And has limitations in resolving power



Liposomes	Zeta Potential mV
A	-26.5 ± 2.1 (pH 7.6)
B	-45.4 ± 0.5 (pH 7.7)
A + B	-37.4 ± 0.4 (pH 7.5)

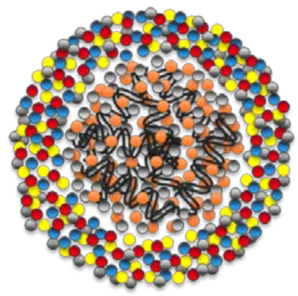
Smith MC, Crist RM, Clogston JD, McNeil SE. Zeta potential: a case study of cationic, anionic, and neutral liposomes. Anal Bioanal Chem. 2017, 409(24), 5779-5787.

Lipid Composition Characterization

Lipid composition to assess batch-to-batch consistency.

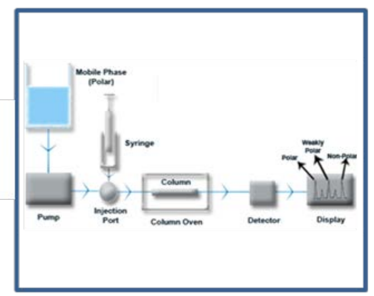
Formulation contains 6 different lipids

- Quantitate all six lipids
- Unique elution profile to separate lipids
- No unique spectral properties for detection
- Minimize number of HPLC runs



Lipid Nanoparticles with siRNA

Reversed-phase High performance liquid chromatography (RP-HPLC)



Separation by RP-HPLC, detection by charged aerosol detector (CAD)

Lipid#1
Lipid#2
Lipid#3
Lipid#4
Lipid#5
Lipid#6

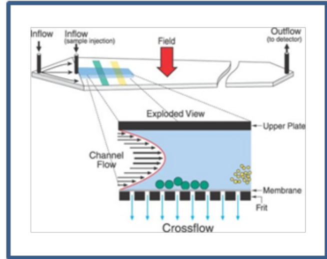
pA: 0, 25, 50, 75, 100, 125, 150, 175
min: 0, 5, 10, 15, 20, 25

New method developed to separate and quantitate six lipids in two runs.

Assessing Protein Binding

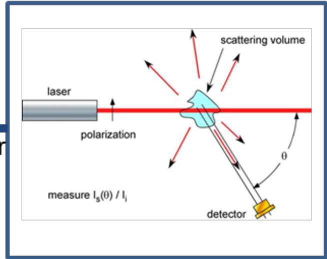
AF4-MALS/DLS method developed to assess protein binding to liposomes.

Asymmetric-flow field-flow fractionation (AF4)



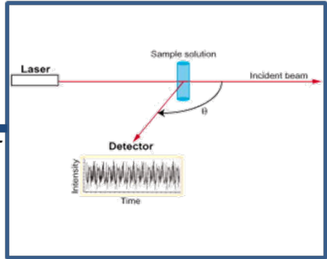
in-line detector

Multiple-angle light scattering (MALS)



in-line detector

Dynamic light scattering (DLS)



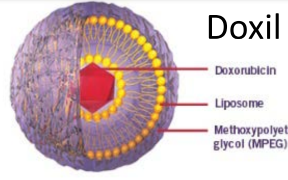
Separates NP, proteins

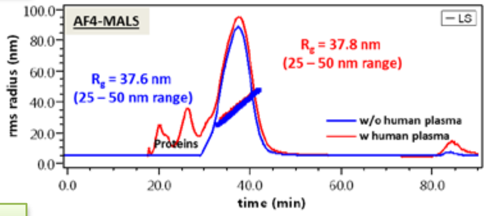
Measures radius of gyration

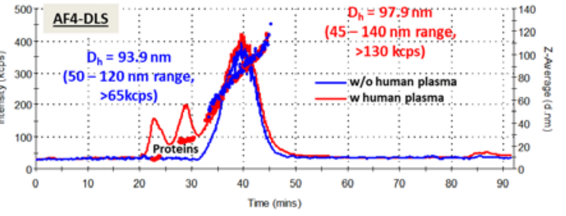
Measures hydrodynamic size

HSPC : Cholesterol : mPEG-DSPE 55 : 40 : 5

Doxil



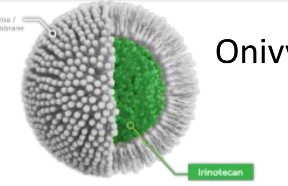


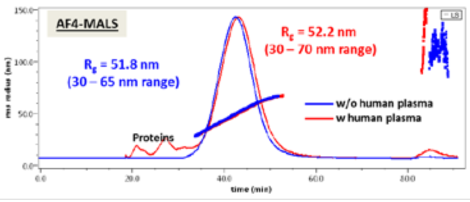


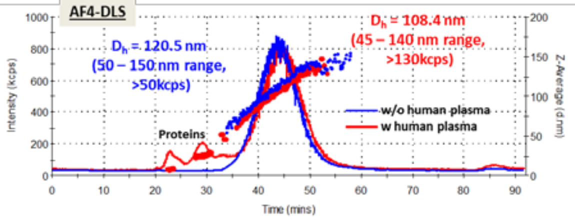
$\rho = R_g/R_h = 0.77$
(was 0.80)
No shift in mass distribution indicating minimal protein binding.

DSPC : Cholesterol: mPEG-DSPE 59.8 : 39.9 : 0.3

Onivyde



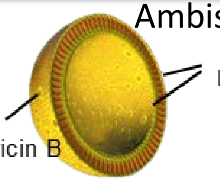


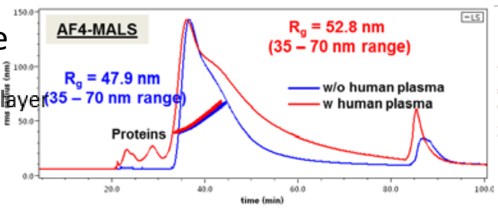


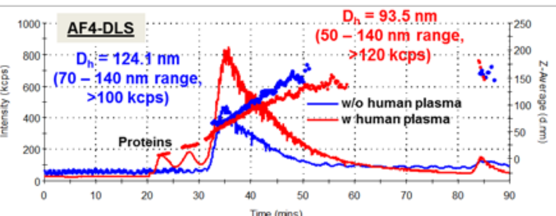
$\rho = R_g/R_h = 0.96$
(was 0.86, 11.6% increase)
Mass distribution shift to the surface, indicating protein binding due to relatively low percentages of PEGylation.

HSPC : Cholesterol : DSPG 53 : 26 : 21 (no PEGylation)

Ambisome







$\rho = R_g/R_h = 1.13$
(was 0.77, 46.8% increase)
Mass distribution shifts to the surface and increased peak intensity/width indicate substantial amount of protein binding.

Ion Quantitation in Liposomal Products

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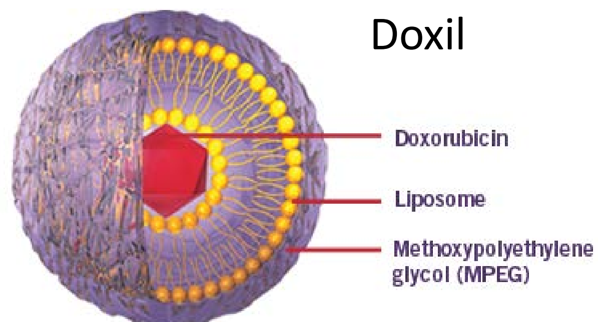
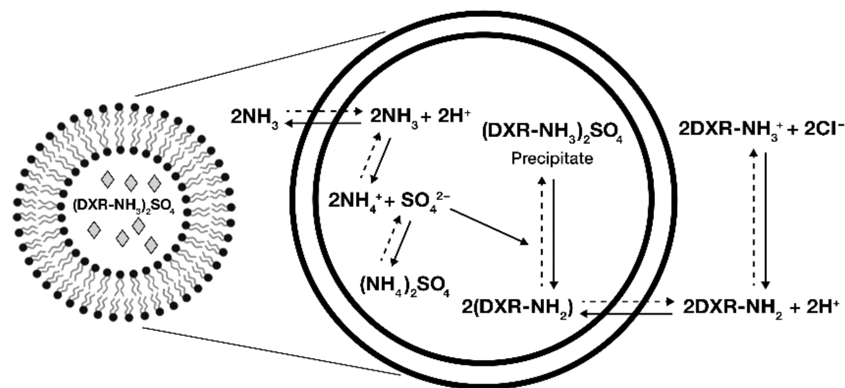
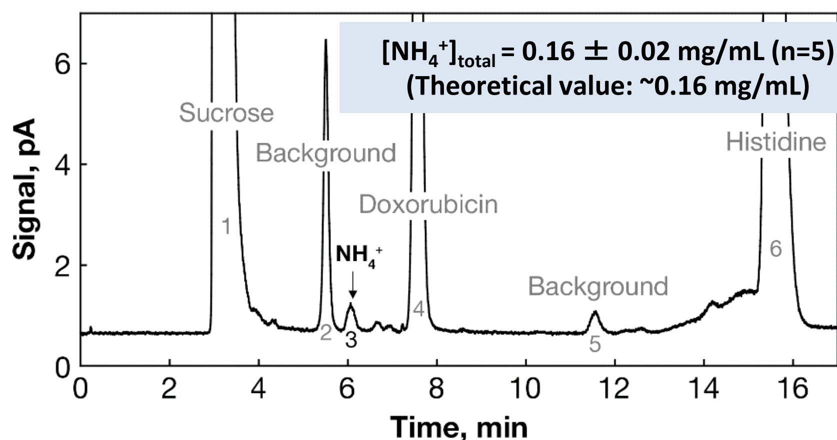
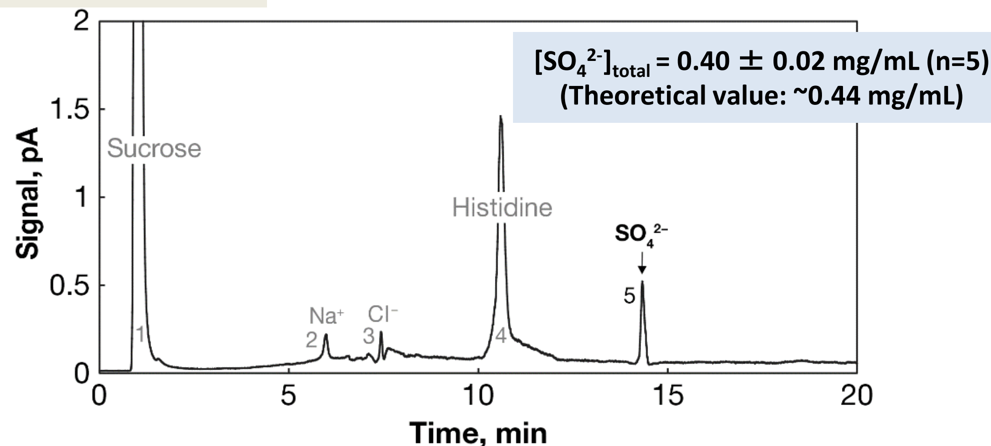




RP-HPLC with CAD detection





- **New method developed to measure total $[NH_4^+]$ and $[SO_4^{2-}]$.**
- **Using microcon centrifugal filters, external $[NH_4^+]$ and $[SO_4^{2-}]$ can be measured.**
- **Mass balances allow for internal $[NH_4^+]$ and $[SO_4^{2-}]$.**
- ***Supports the FDA regulatory guidance for doxorubicin hydrochloride liposomal injection products.***
- **Method can be used to compare batch-to-batch consistency and Doxil-analogs.**
- **Method can also be used for other counterions (Ca^{2+}, CH_3COO^-, $MeSO_3^-$) as well as buffer ingredients (sucrose, histidine, Na^+, Cl^-).**

Wu J, Crist RM, McNeil SE, Clogston JD. Ion quantification in liposomal drug products using high performance liquid chromatography. J Pharm Biomed Anal. 2019, 165, 41-46.

Impurities and Stability Assessment of Liposomal Products

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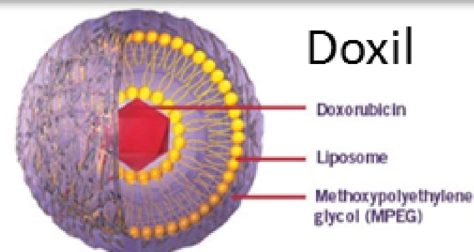
Components

HSPC = DSPC (major component) + PSPC/SPPC
DSPE-mPEG
Cholesterol

HSPC : Cholesterol : mPEG-DSPE
55 : 40 : 5

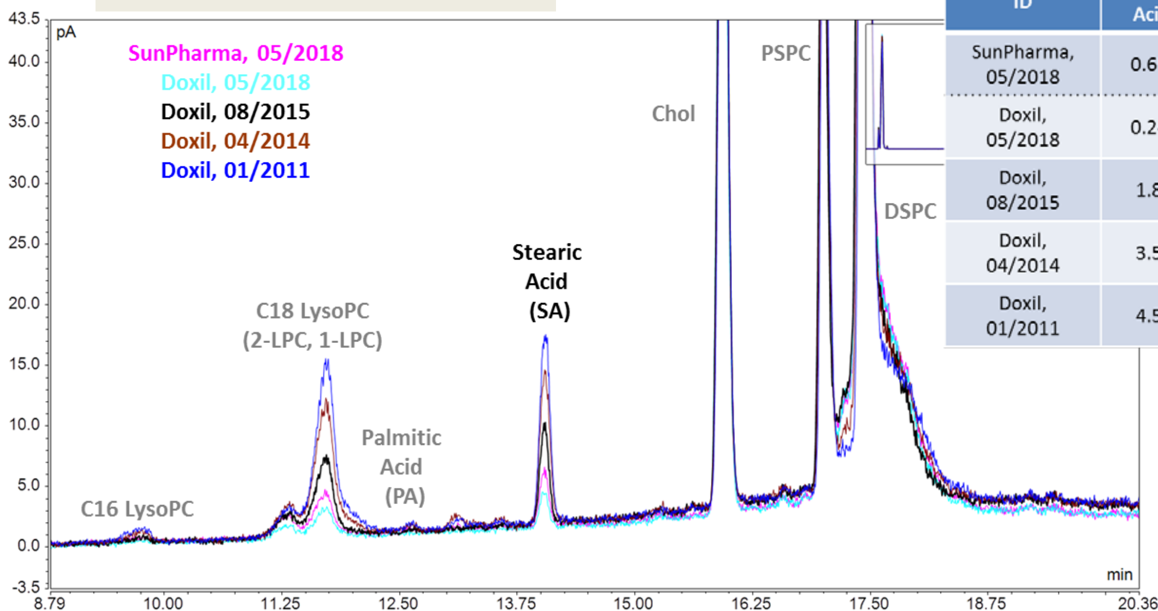


Hydrolysis Products

DSPC → SA + C18 LysoPC (1-LPC, 2-LPC)
DSPE-mPEG → SA + C18 LysoPC-mPEG (1-LPC-mPEG, 2-LPC-mPEG)
PSPC/SPPC → SA + C16 LysoPC (1-LPC, 2-LPC)
*P*SPC/S*P*PC → *PA* + C18 LysoPC (1-LPC, 2-LPC)

SA = stearic acid
PA = palmitic acid

RP-HPLC with CAD detection



ID	%Stearic Acid*
SunPharma, 05/2018	0.65%
Doxil, 05/2018	0.24%
Doxil, 08/2015	1.8%
Doxil, 04/2014	3.5%
Doxil, 01/2011	4.5%

RP-HPLC method developed to monitor degradation products in liposomes.

Method can also be used to assess stability/shelf-life.

* Normalized to theoretical total lipid concentration, 15.96 mg/mL ([HSPC] = 9.58 mg/mL, [Chol] = 3.19 mg/mL, [DSPE-mPEG] = 3.19 mg/mL)

Drug Loading Quantification of Prodrugs

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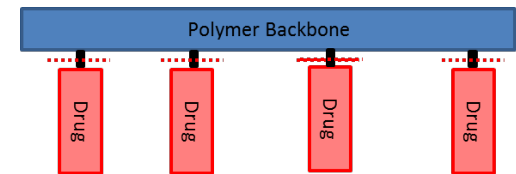
- **Drug loading is one of the most important critical quality attributes (CQAs) of prodrugs.**
- **Quantification of chemically conjugated drugs in polymeric prodrugs is difficult.**
  - ➢ **Development of novel orthogonal method**

Polymer with Conjugated Drug

- Drug absorbs at a unique wavelength but UV-Vis detection not sensitive enough
- Wavelength shift observed for conjugated drug
- Chemical method needed

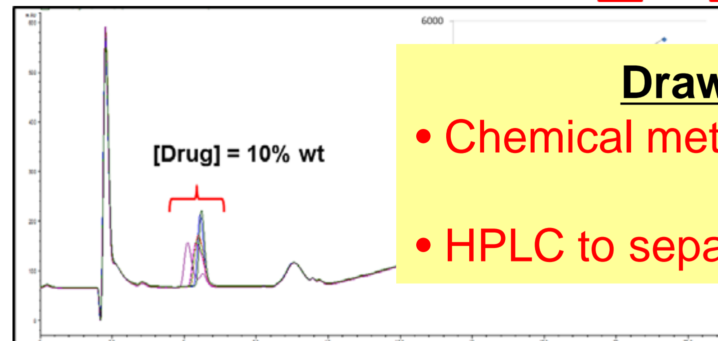


Hydrolysis Method

1) 20 µL sample (in 50 %(v/v) ACN) + 20 µL 1 M NaOH.
2) Incubate overnight at room temperature.
3) Add 20 µL 1 M HCl to neutralize.

followed by RP-HPLC separation

4) Assayed by RP-HPLC with UV detection

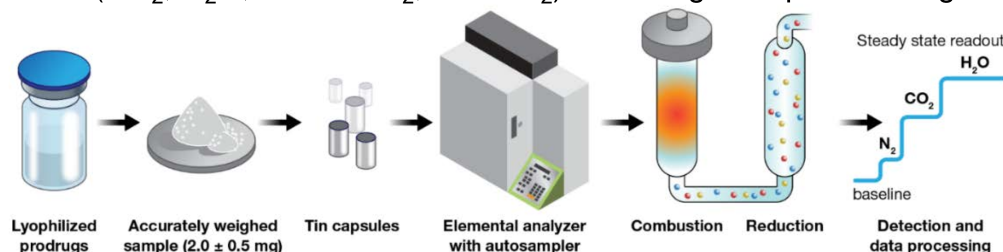


Drawbacks

- Chemical method optimization
- HPLC to separate components

Elemental analyzer – the application of combustion analysis

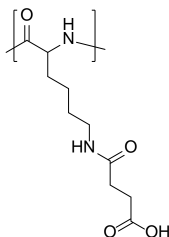
- Determine the elemental composition by combusting the sample under certain conditions
- Only elements of carbon (C), hydrogen (H), nitrogen (N), and sulfur (S) as combustion of materials are used to convert to their oxidized form (CO_2, H_2O, NO or NO_2, and SO_2) under high temperature high oxygen conditions



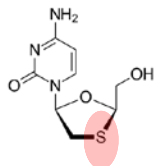
http://www.perkinelmer.com/product/2400-chns-o-series-ii-system-100v-n2410650

Drug Loading Quantification of Prodrugs

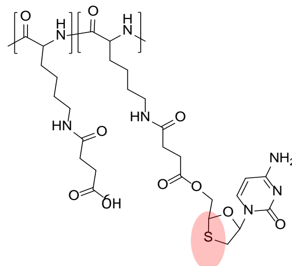
NCI Alliance for Nanotechnology in Cancer — NCL Nanotechnology Characterization Laboratory



Poly L-lysine succinylated (PLS)



Lamivudine (LAM)



PLS-LAM

Sample	%S	%N
Poly L-lysine succinylated	0.46	12.17
Lamivudine	14.17	18.44
PLS-LAM	1.37	12.58

$$\%WT_{LAM} = \frac{\%S_{prodrug} - \%S_{PLS}}{\%S_{LAM} - \%S_{PLS}} \times 100\%$$

$$\%WT_{LAM} = 6.6 \pm 0.4\%$$

$$\%WT_{LAM} = \frac{\%N_{prodrug} - \%N_{PLS}}{\%N_{LAM} - \%N_{PLS}} \times 100\%$$

$$\%WT_{LAM} = 7.0 \pm 0.9\%$$

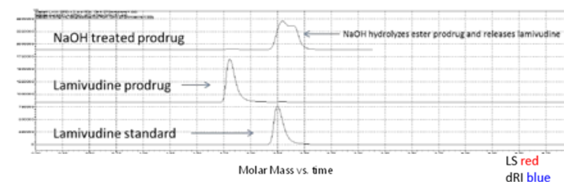
Orthogonal methods comparison

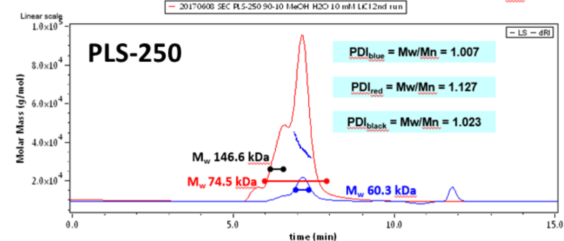
Prodrug	CHNS elemental analysis	CHN elemental analysis	Hydrolysis & RP-HPLC	SEC-MALS
PLS-LAM	6.6 ± 0.4%	7.0 ± 0.9%	6.7 ± 0.1% 7.4 ± 0.1%	5.7 ± 3.8%

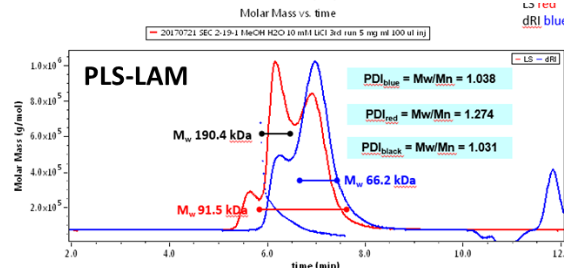
Advantages

- ✓ Robust: no method development required
- ✓ Fast: approximately 5 min/sample in CHN mode; 7 min/sample in CHNS mode
- ✓ ~2 mg sample (powder) needed
- ✓ Accurate: sample-to-sample consistency, validated by other methods

New method to quantitate polymer-bound drug







Drug Release of Prodrugs in Human Plasma

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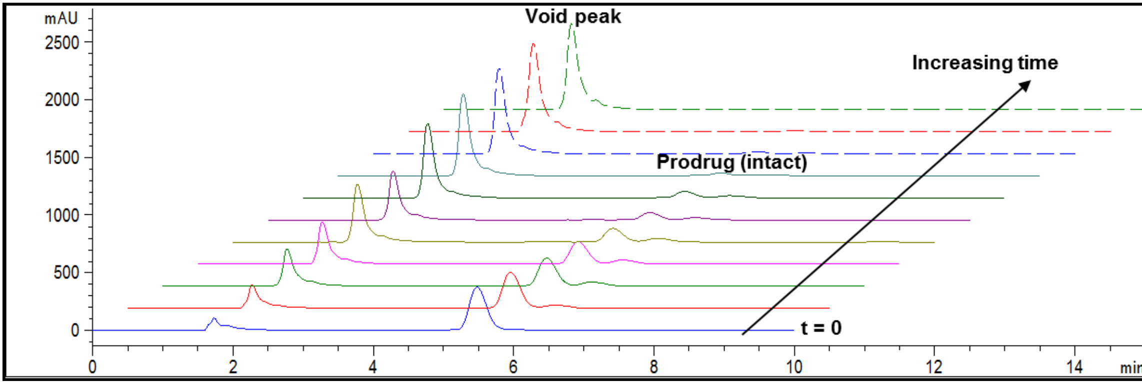
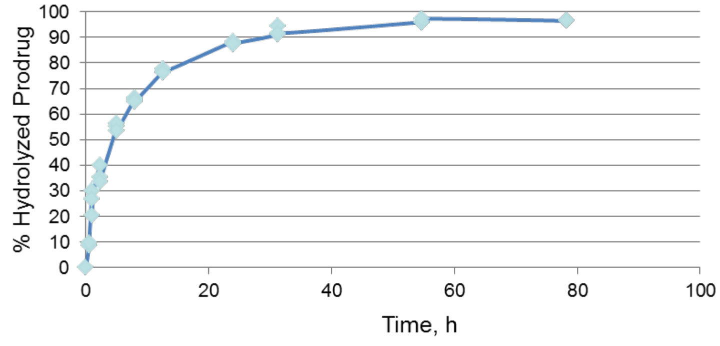
Prodrug loaded micelles

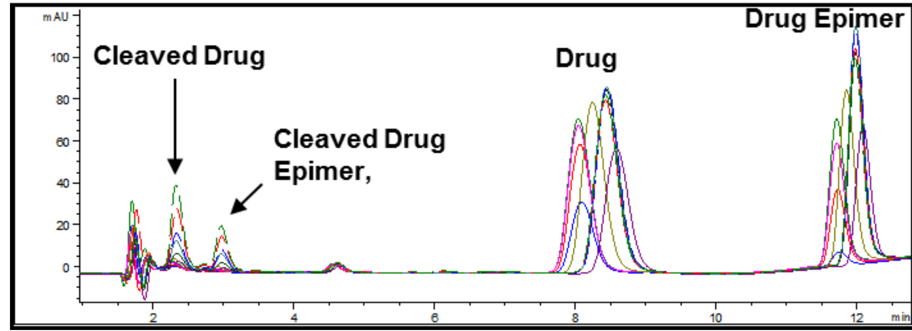
- Does it cleave? How fast does it cleave?
- Cleavage site?
- Stability of drug?

HPLC





LC/MS



- HPLC to separate components of formulation and plasma
- LC/MS to determine cleavage site and drug stability

HPLC = high performance liquid chromatography; LC/MS = liquid chromatography–mass spectrometry

Drug Stability of Polymeric NPs by AF4-DLS-HPLC

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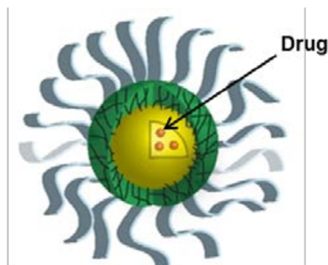
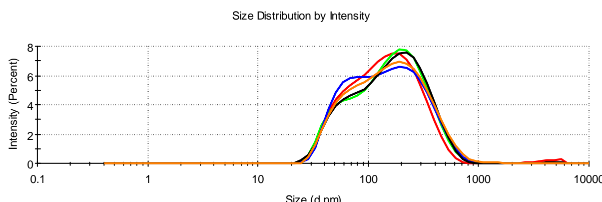


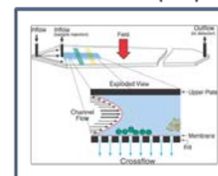
Polymeric micelles

Batch-mode DLS

Where is the drug located?



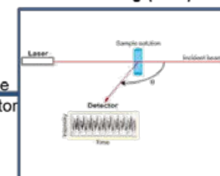
Asymmetric-flow field-flow fractionation (AF4)



Separates NP, plasma

in-line detector

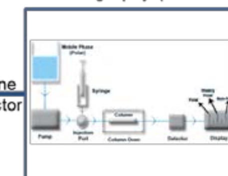
Dynamic light scattering (DLS)



Measures hydrodynamic size

off-line detector

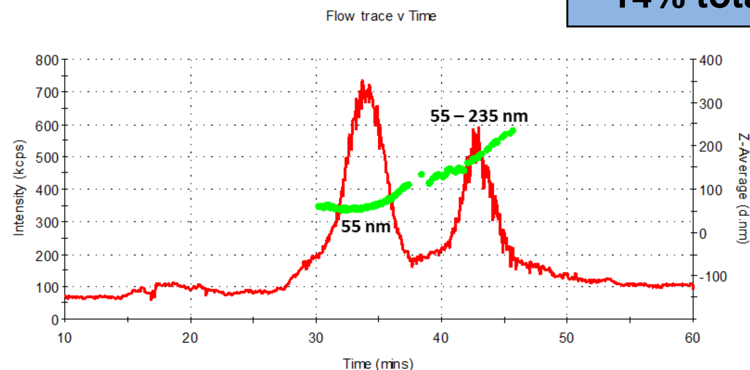
Reversed-phase High performance liquid chromatography (RP-HPLC)



Measures drug concentration

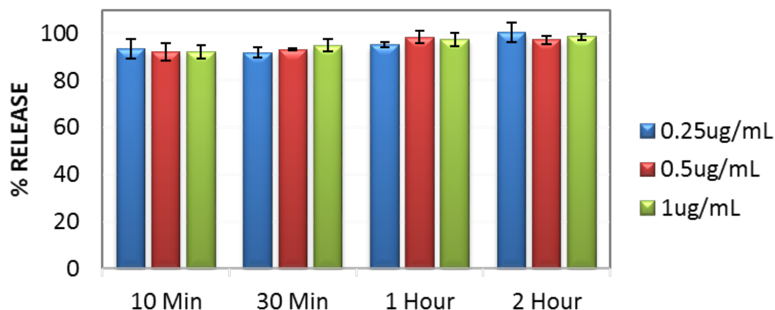
Flow-mode DLS

14% total drug Recovered



- **AF4 membrane passivated with BSA**
- **Drug partitions to BSA; has more affinity for protein than NP**

85-100% released drug



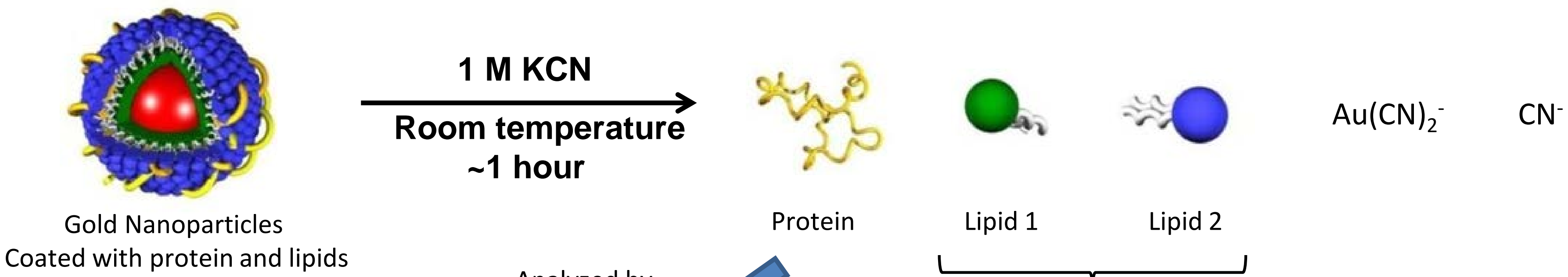
Findings are similar to stable isotope method.

A new method to assess drug stability.

Tested on several micellar and liposomal formulations with consistent results.

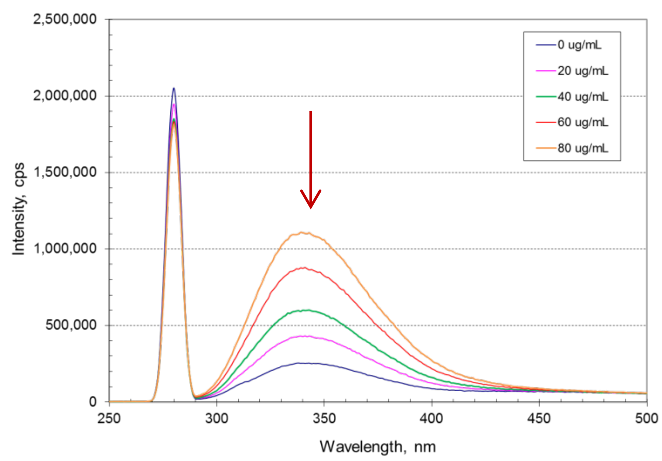
Protein and Lipid Coating Quantification

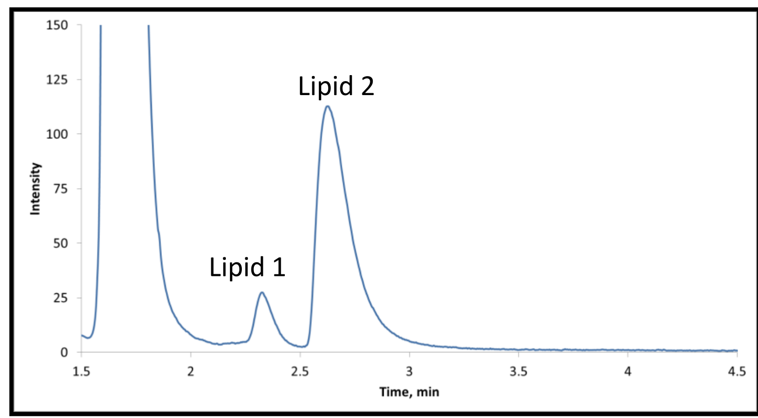
Dissolution Method



Analyzed by Fluorescence

Analyzed by RP-HPLC-CAD





Bound protein and lipids concentration on colloidal gold nanoparticles can now be determined .

Batch-to-Batch Consistency by Tunable Resistive Pulse Sensing (TRPS)

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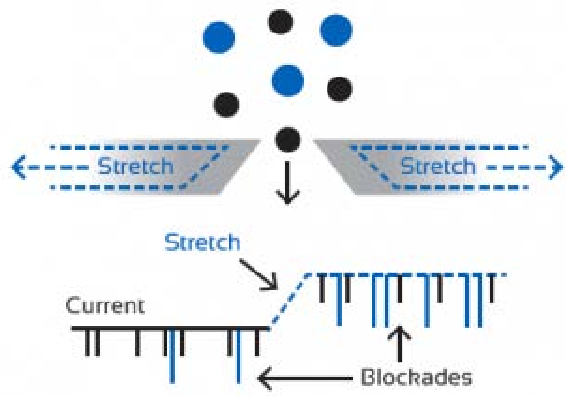
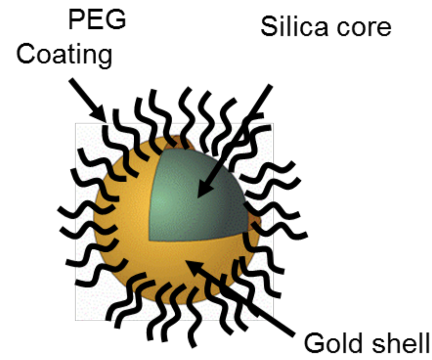
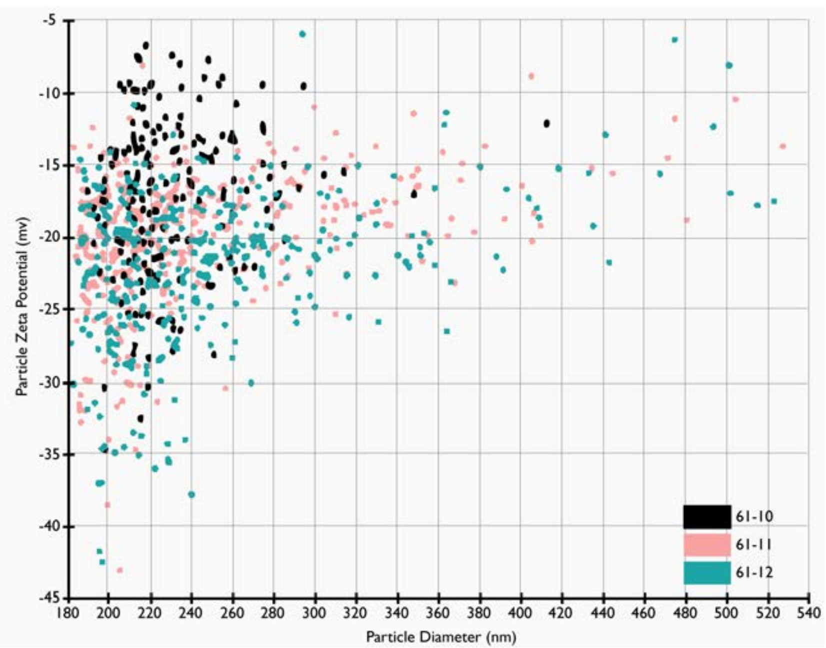


http://www.izon.com/products/capabilities/particle-sizing/



Addition of charge distribution capability…



	Diameter, nm	Particle Count	Zeta Potential, mV
Lot 1	220	180	-13.4
Lot 2	210	362	-18.4
Lot 3	209	344	-20.5

Examine size and charge on a per particle basis. Assess batch-to-batch consistency.

Batch-to-Batch Consistency by AF4-MALS

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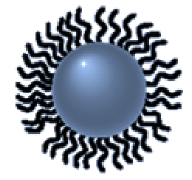
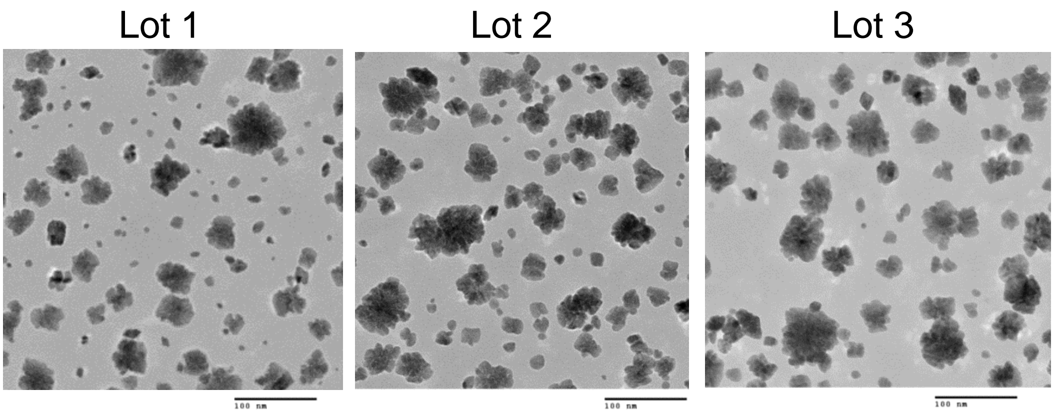
PEGylated metal oxide nanoparticles



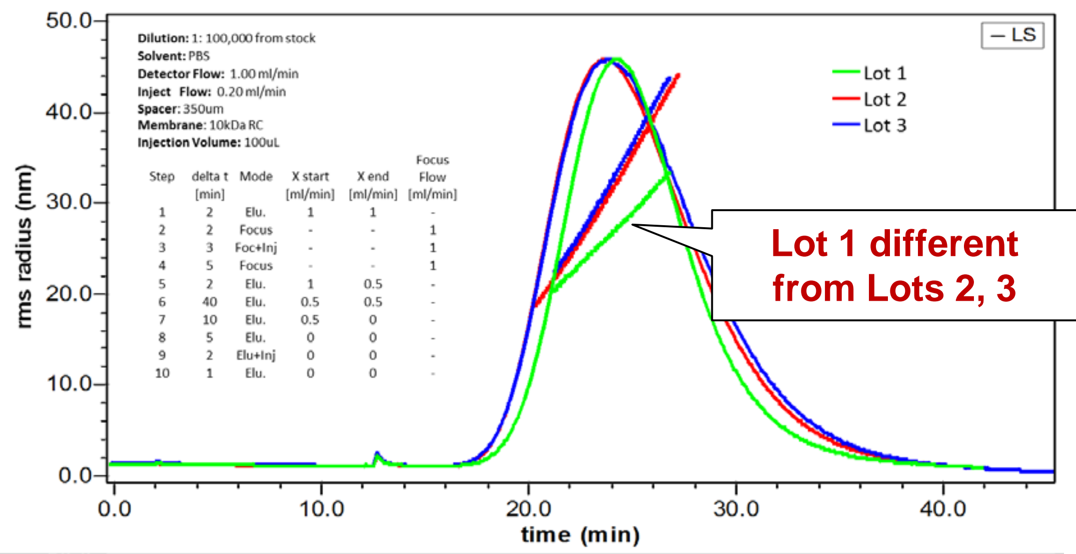


TEM initially performed to assess batch-to-batch consistency.

Lot	n	Average Diameter (nm)
1	661	20 ± 17
2	465	22 ± 16
3	690	23 ± 17

No difference in size by TEM

AF4-MALS



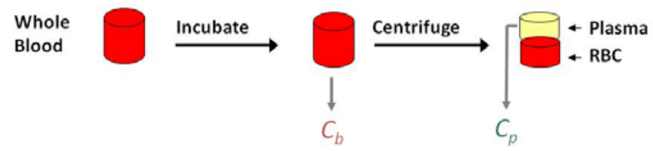
Step	delta t [min]	Mode	X start [ml/min]	X end [ml/min]	Focus Flow [ml/min]
1	2	Elu.	1	1	-
2	2	Focus	-	-	1
3	3	Foc+Inj	-	-	1
4	5	Focus	-	-	1
5	2	Elu.	1	0.5	-
6	40	Elu.	0.5	0.5	-
7	10	Elu.	0.5	0	-
8	5	Elu.	0	0	-
9	2	Elu+Inj	0	0	-
10	1	Elu.	0	0	-

Flow-mode detects differences batch-mode cannot.

AF4-MALS better suited in terms of throughput and ease for assessing batch-to-batch consistency in this case.

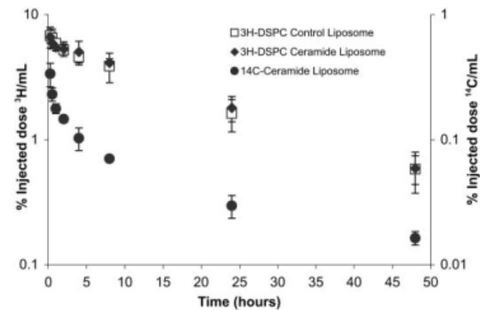
NCL Methods for Measuring Drug Release

- Blood partitioning assay



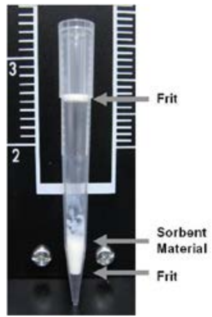
Zolnik et al., Drug Metab Dispos. 2008, 36(8):1709-15.

- Dual labeling/complementary analysis in vivo

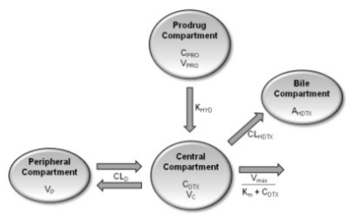


Zolnik et al., Drug Metab Dispos. 2008, 36(8):1709-15.

- Extraction methods to separate free and encapsulated

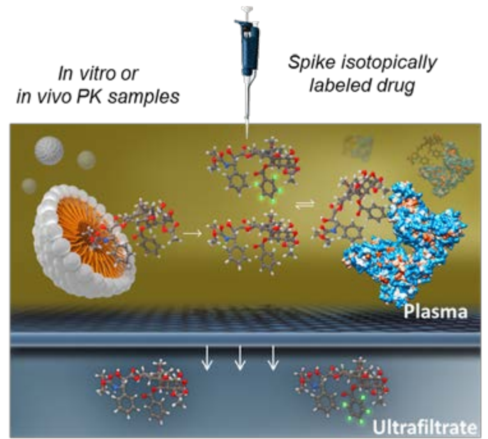


- Metabolite modeling to predict free drug



Stern ST et al., J Control Release, 2013, 172(2), 558-567.

- Measurement of fraction unbound to quantify drug encapsulated/released

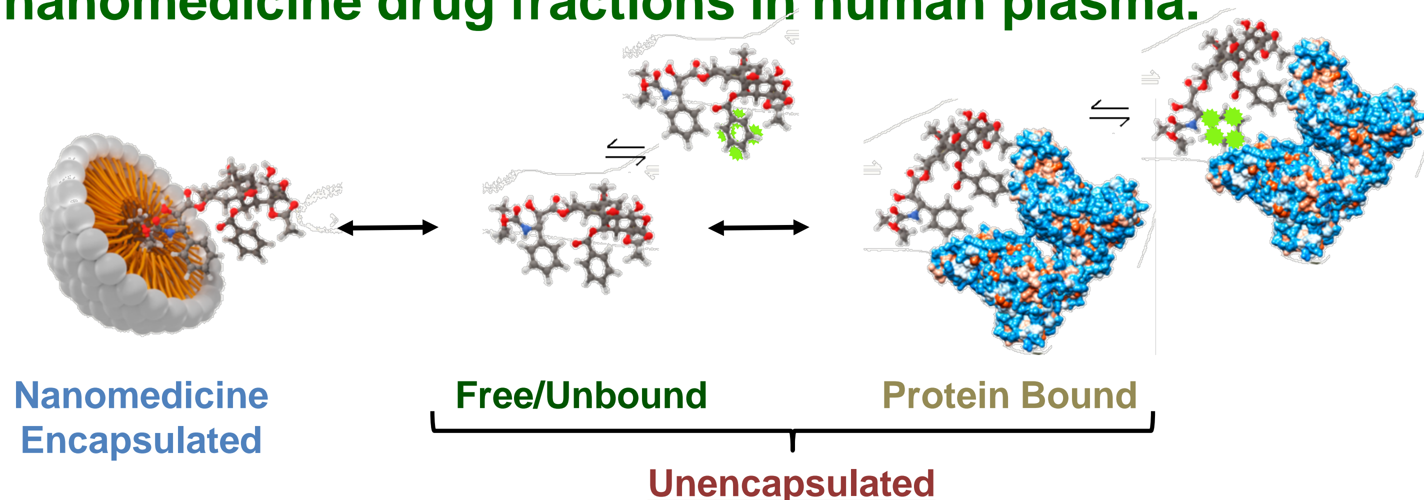


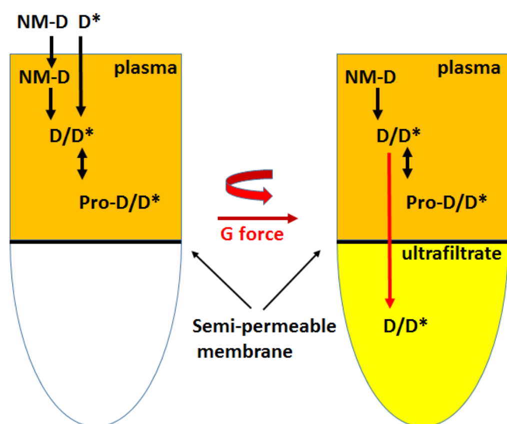
Stern et al. J Control Release. 2015, 220(PtA), 169-174

Novel Stable Isotope Tracer Method to Measure Nanomedicine Drug Fractions

NCL Nanotechnology Characterization Laboratory | NCI Alliance for Nanotechnology in Cancer

Stable Isotope Tracer Ultrafiltration Assay (SITUA) can assess nanomedicine drug fractions in human plasma.





- Stable isotopically labeled drug (**D***) equilibrates with protein and unlabeled, normoisotopic drug (**D**) released from nanomedicine (NM) formulation.
- % **D*** bound estimation gives reliable prediction of %**D** bound.

$$\text{\%Bound} = \frac{([\text{Total D*}] - [\text{Ultrafilterable D*}]) * 100}{[\text{Total D*}]}$$

$$[\text{Unencapsulated D}] = \frac{[\text{Ultrafilterable D}]}{(1-(\text{\%Bound D*}/100))}$$

$$[\text{Encapsulated D}] = [\text{Total D}] - [\text{Released D}]$$

For more information:
ncl.cancer.gov/working-ncl/technical-services
Contact us:
ncl@mail.nih.gov

Skoczen SL, McNeil SE, Stern ST J Control Release. 2015; 220 (Pt A): 169-174.
Skoczen SL, Stern ST. Methods Mol Biol 2018; 1682: 223-239.

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NCL Nanotechnology Characterization Laboratory | NCI Alliance for Nanotechnology in Cancer

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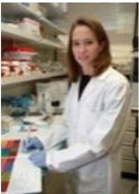

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