Baxter

Advanced Analytical Techniques for Nano Formulations: Microscopy, Spectroscopy, and Particle Science

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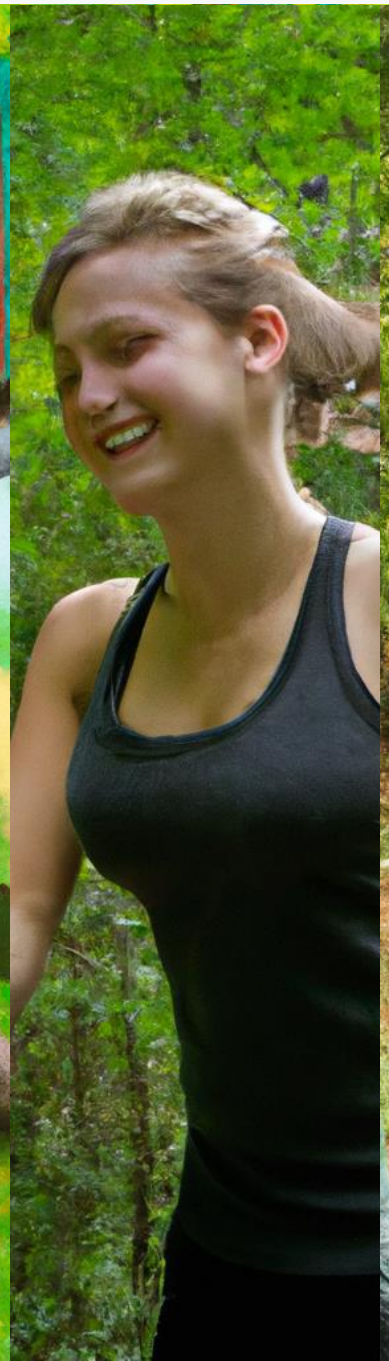
May 19th, 2023

“Two friends running through a forest; one of them blindfolded, being led by the hand by the other” – Interpretations by DALL-E



Overview

- How is nano formulation characterization different?
- What microscopy techniques are used and how do they work?
- How do other spectroscopic analyses play a role – a case study in iron carbohydrates
- Particle size distribution – relation to PK, methodologies, and statistics

How is nano formulation characterization different?

“Traditional” Drug Product –
Small molecule in solution

Identification
Assay / Activity
Impurities
Several standard tests like USP <788>, BET, sterility, pH

How is nano formulation characterization different?

IDENTIFICATION

Change to read:

▲• **SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

ASSAY

• **ANTIBIOTICS—MICROBIAL ASSAYS** ⟨81⟩

Sample solution 1 (where it is represented as being in a single-dose container): Constitute a container of Vancomycin Hydrochloride for Injection in water corresponding to the volume of diluent specified in the labeling. Withdraw all of the withdrawable contents, using a suitable hypodermic needle and syringe. Dilute to an equivalent of 1 mg/mL of vancomycin with water.

Sample solution 2 (where it is packaged for dispensing): Dissolve the contents of 1 container of Vancomycin Hydrochloride for Injection in water, and dilute with water to obtain a solution having a concentration of 1 mg/mL of vancomycin.

Sample solution 3 (where the label states the quantity of vancomycin in a given volume of constituted solution): Constitute a container of Vancomycin Hydrochloride for Injection in water corresponding to the volume of diluent specified in the labeling. Dilute a portion to obtain a final concentration equivalent to 1 mg/mL of vancomycin in water.

Analysis: Proceed as directed in *Antibiotics—Microbial Assays* ⟨81⟩. [NOTE—Use a measured volume of the appropriate *Sample solution*, diluted quantitatively with *Buffer B.4* to yield a *Test Dilution* having a concentration assumed to be equal to the median dose level of the Standard.]

Acceptance criteria: 90.0%–115.0%

PERFORMANCE TESTS

• **UNIFORMITY OF DOSAGE UNITS** ⟨905⟩: Meets the requirements

SPECIFIC TESTS

• **PARTICULATE MATTER IN INJECTIONS** ⟨788⟩: Meets the requirements under small-volume injections

• **BACTERIAL ENDOTOXINS TEST** ⟨85⟩: NMT 0.33 USP Endotoxin Unit/mg of vancomycin

• **STERILITY TESTS** ⟨71⟩: It meets the requirements when tested as directed for *Test for Sterility of the Product to Be Examined, Membrane Filtration*, except to dissolve the specimen in water instead of in *Fluid A*.

• **pH** ⟨791⟩: 2.5–4.5, 50 mg/mL in water

• **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 5.0%

• **INJECTIONS AND IMPLANTED DRUG PRODUCTS** ⟨1⟩, *Specific Tests, Completeness and clarity of solutions*: Meets the requirements at the time of use.

• **CONTENT OF VANCOMYCIN**

How is nano formulation characterization different?

"Traditional" Drug Product –
Small molecule in solution

Identification
Assay / Activity
Impurities
Several standard tests like USP <788>, BET, sterility, pH

Nano formulation –
Emulsion, Liposome, Nanoparticle

Identification (multiple components)
Assay? Multiple components?
Impurities? (HPLC?)
High resolution electron microscopy (STEM, cryoTEM)
Morphology / lamellarity (EM analysis)
Component size/shape (SAXS/SANS/EM)
Crystal structure (Raman, XRD)
Magnetic properties (NMR, EPR, Mössbauer)
Ensemble particle methods (DLS, LD)
Zeta potential
Encapsulation efficiency
Dissolution / in vitro release
Several standard tests like USP <788>, BET, sterility, pH

How is nano formulation characterization different?

IDENTIFICATION

- **A. Iron**

  To 2.5 mL of Injection add 17.5 mL of water and 5 mL of hydrochloric acid. Mix and heat the solution for 5 min in a boiling water bath. Cool, add dropwise 13.5 N ammonium hydroxide until no further precipitation of ferric hydroxide occurs, and filter. Wash the precipitate with water to remove excess ammonium hydroxide, dissolve the precipitate in a minimum volume of 2 N hydrochloric acid, and add sufficient water to make a volume of 20 mL. To 3 mL of the solution add 1 mL of 2 N hydrochloric acid and 1 mL of potassium thiocyanate TS: the resulting solution (Solution 1) is red. To 1 mL of Solution 1 add 5 mL of amyl alcohol or ethyl ether, shake, and allow to stand: the organic layer is pink. To a separate 1-mL aliquot of Solution 1 add 2 mL of mercuric chloride TS: a red color is discharged [iron (III) salts].

- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay* for *Sucrose*.

- **C. Molecular Weight Determination**

ASSAY

- **Sucrose**
- **Iron**

OTHER COMPONENTS

- **Content of Chloride**

IMPURITIES

- **Limit of Iron [Fe(II)]**

SPECIFIC TESTS

- **pH** ⟨791⟩**:** 10.5–11.1 at 20°
- **Absence of Low-Molecular Weight Iron [Fe(II) and Fe(III)] Complexes:** In the polarograms obtained in the test for *Limit of Iron [Fe(II)]*, no additional peaks are found.
- **Turbidity**
- **Alkalinity**
- **Osmolality and Osmolarity** ⟨785⟩
- **Specific Gravity** ⟨841⟩**:** 1.135–1.165 at 20°
- **Particulate Matter in Injections** ⟨788⟩**,** *Method 1 Light Obscuration Particle Count Test*
- **Bacterial Endotoxins Test** ⟨85⟩**:** NMT 3.7 USP Endotoxin Units/mg of iron contained in Injection
- **Other Requirements:** Meets the requirements in *Injections and Implanted Drug Products* ⟨1⟩

How is nano formulation characterization different?

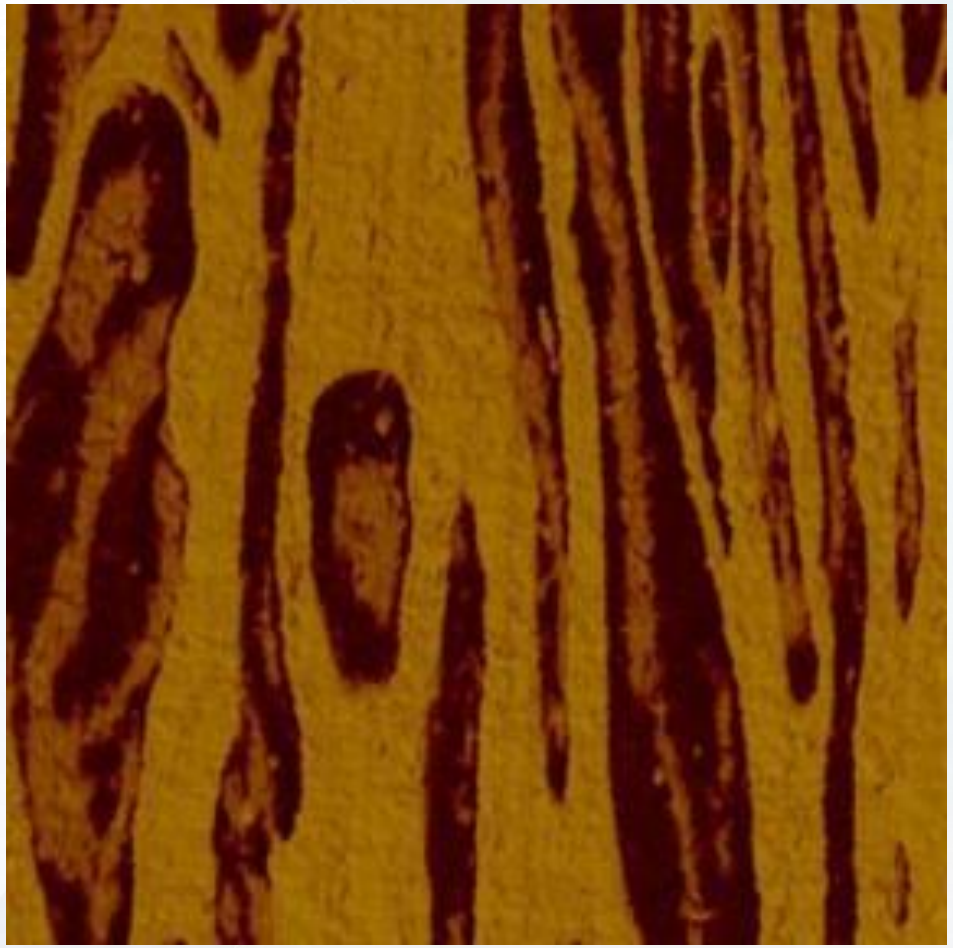
3. Physicochemical properties of the drug product: particle size, surface charge (zeta-potential), colloid molecular size,[5] interaction between ferric oxyhydroxide and sucrose, gluconate; stoichiometric ratios of iron and other components.
4. Ferric oxyhydroxide colloid characterization: ferric oxyhydroxide crystalline structure and environment, magnetic properties, particle morphology, Fe(III) to Fe(II) reduction potential, reduction kinetic and Fe(II) content.
5. Labile iron determination under physiologically relevant conditions. The tests can be performed with an in vitro haemodialysis system,[6] the catalytic bleomycin assay of spiked human serum samples,[6,7] the spectrophotometric measurement of Fe reduction, chelatable iron assay[8] or other methods that are validated for accuracy and precision.

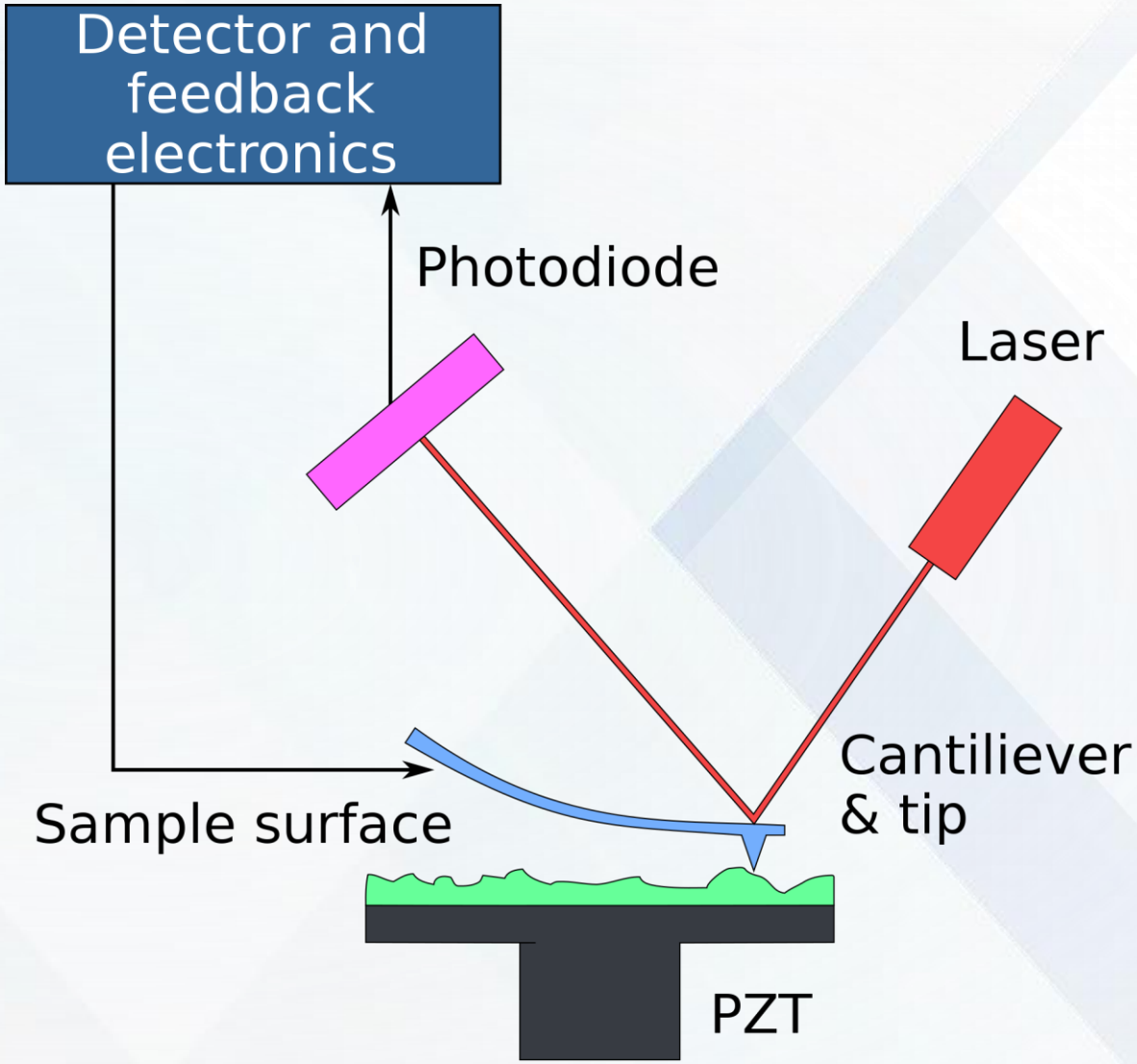
Microscopic techniques for nano formulations

- Atomic force microscopy (AFM)
- Scanning electron microscopy (SEM)
- Scanning transmission electron microscopy (STEM / STEM-in-SEM)
- Transmission electron microscopy (TEM)
- Cryogenic transmission electron microscopy (cryoTEM)

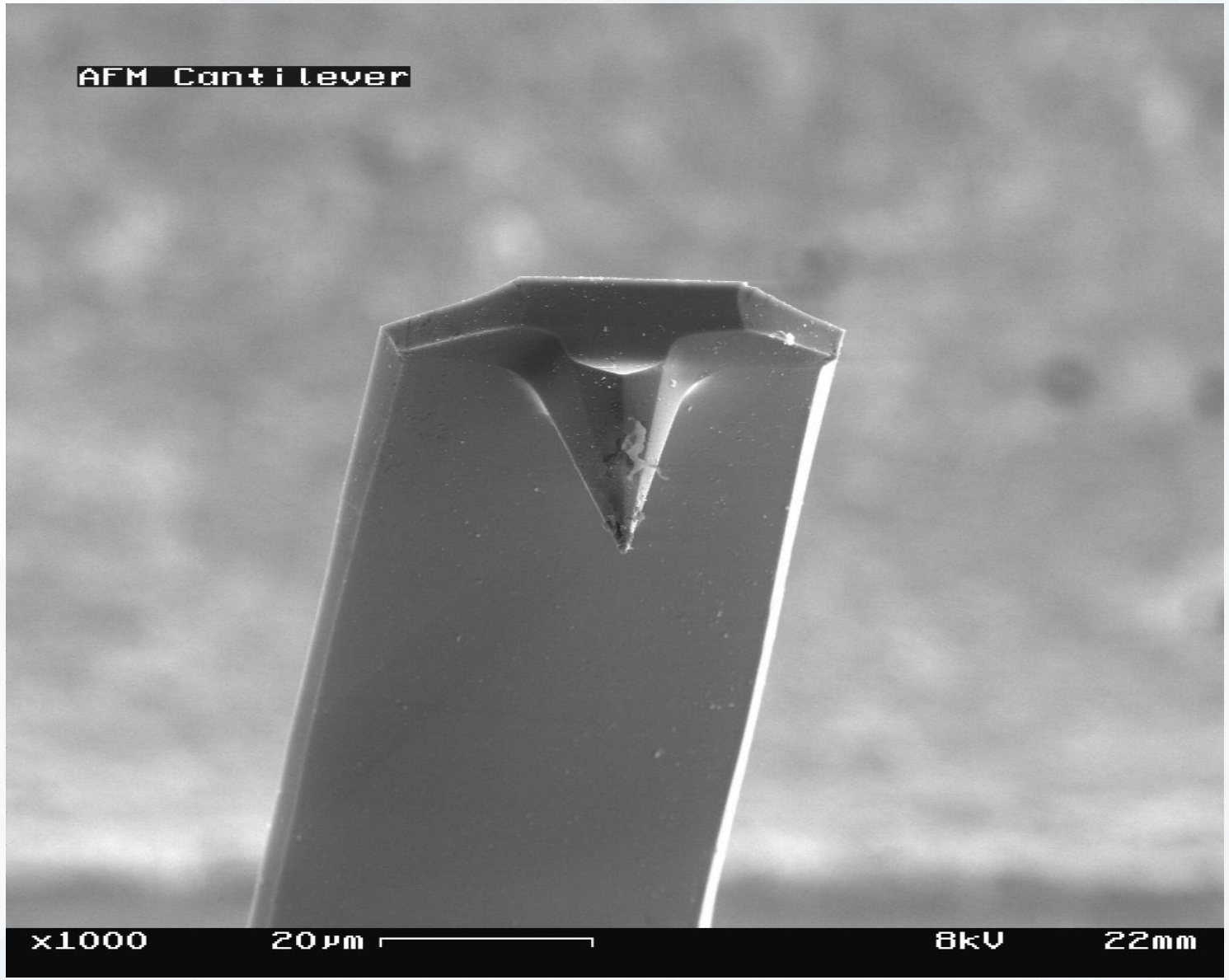
- If a picture is worth a thousand words, how do we extract those words from an image?

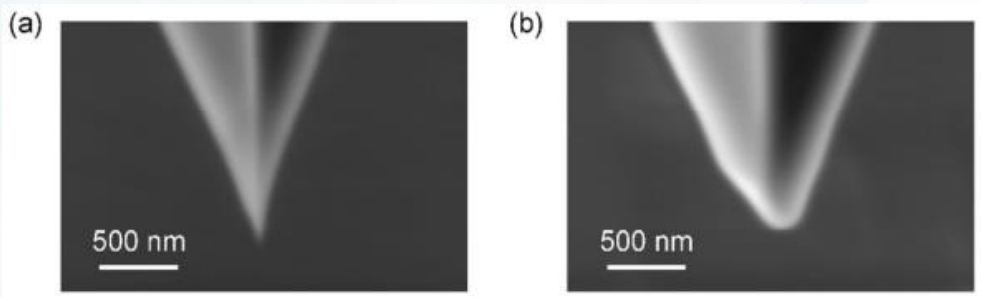
Atomic Force Microscopy

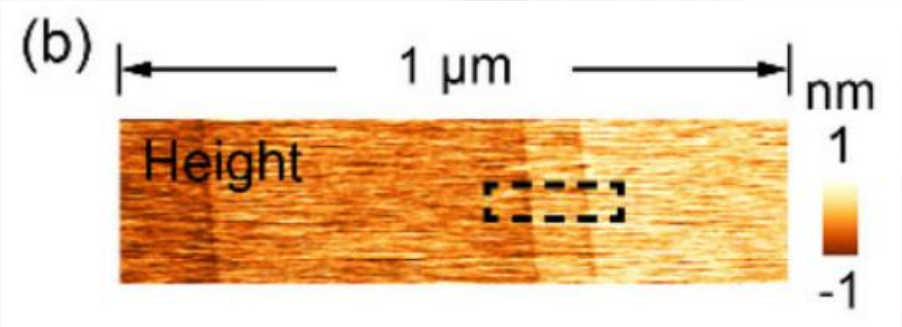




Atomic Force Microscopy





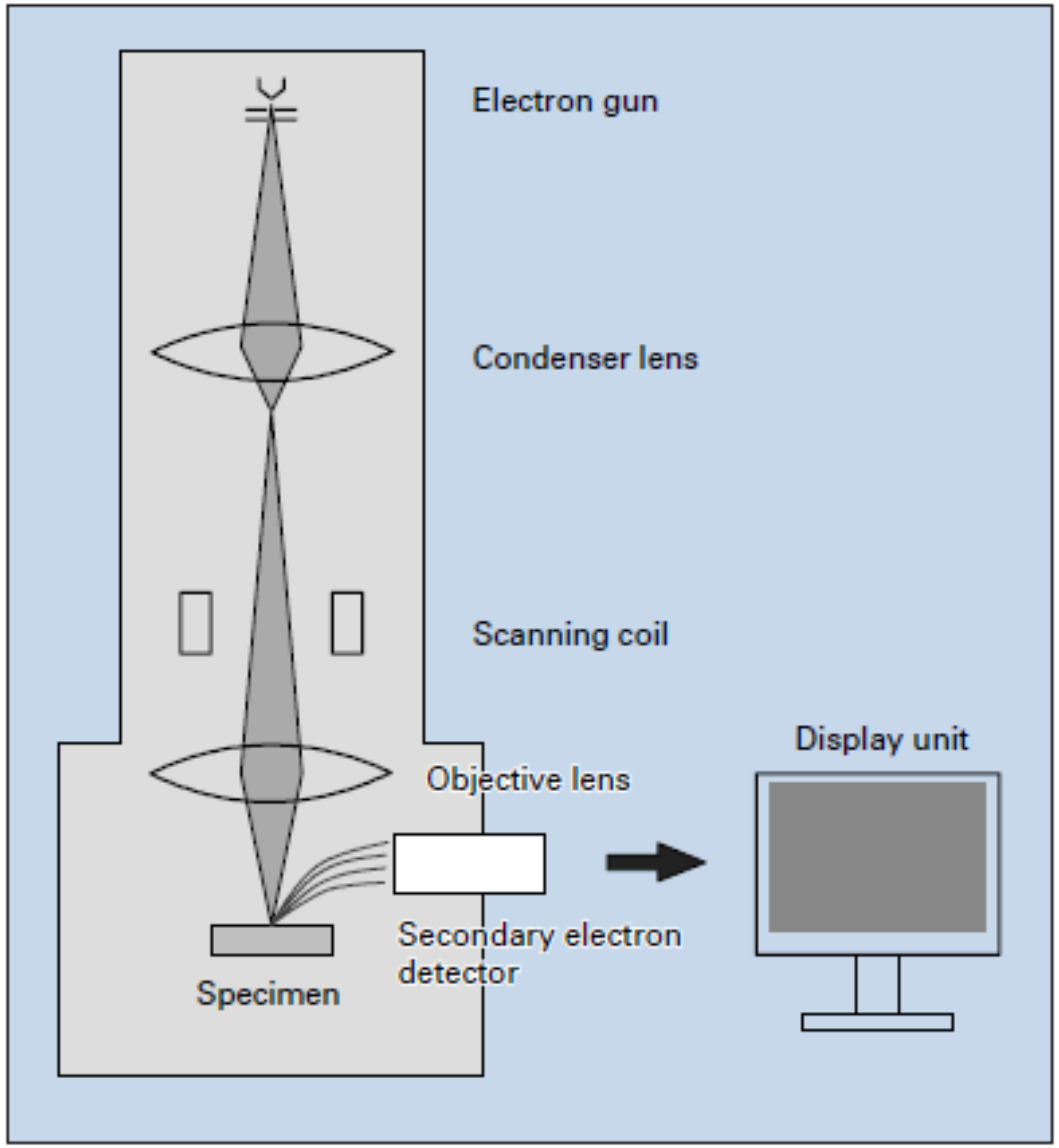


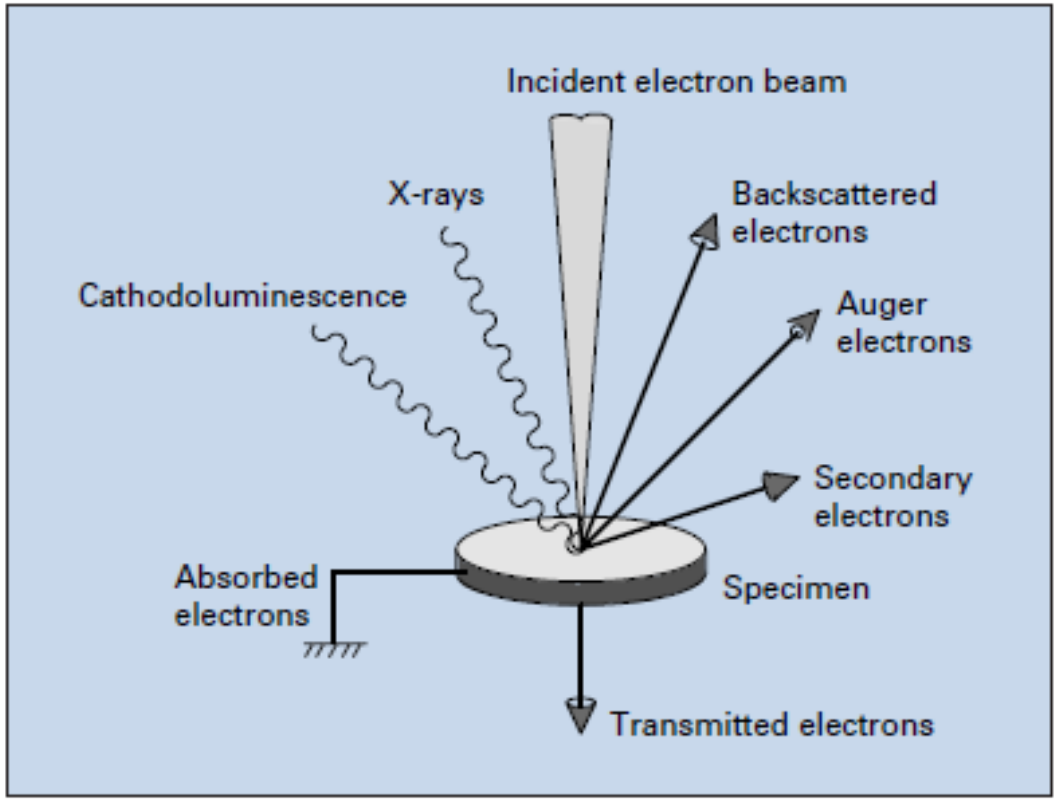
AFM can be used to measure dry or wet samples, but dried samples suffer from aggregation artifacts and wet samples suffer from concentration effects, probe shape effects, and scan mode differences; size measurement may not be accurate.

Wikipedia

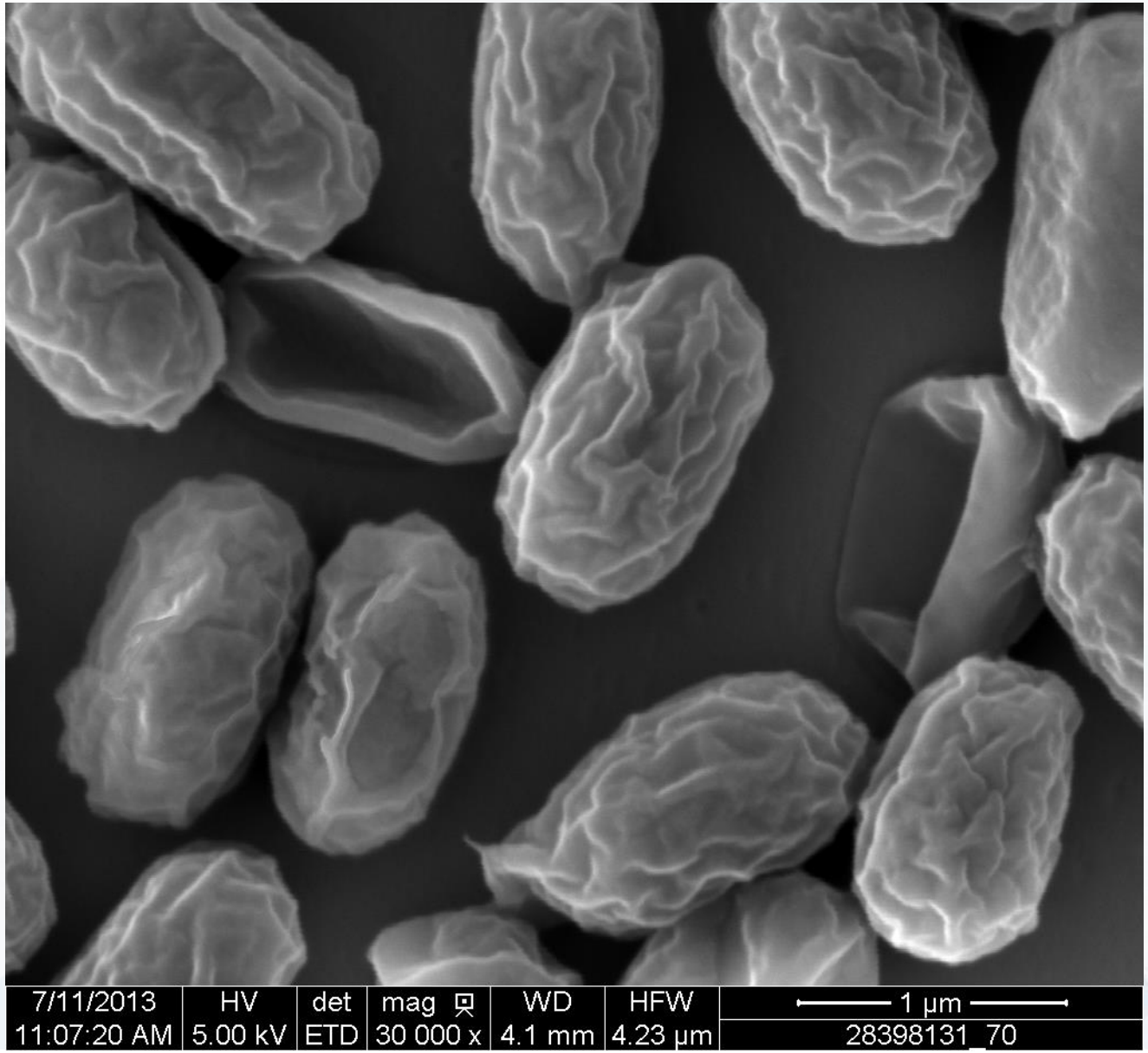
Chen, Kim, Friction 8(4): 802-811 (2020)

Scanning Electron Microscopy





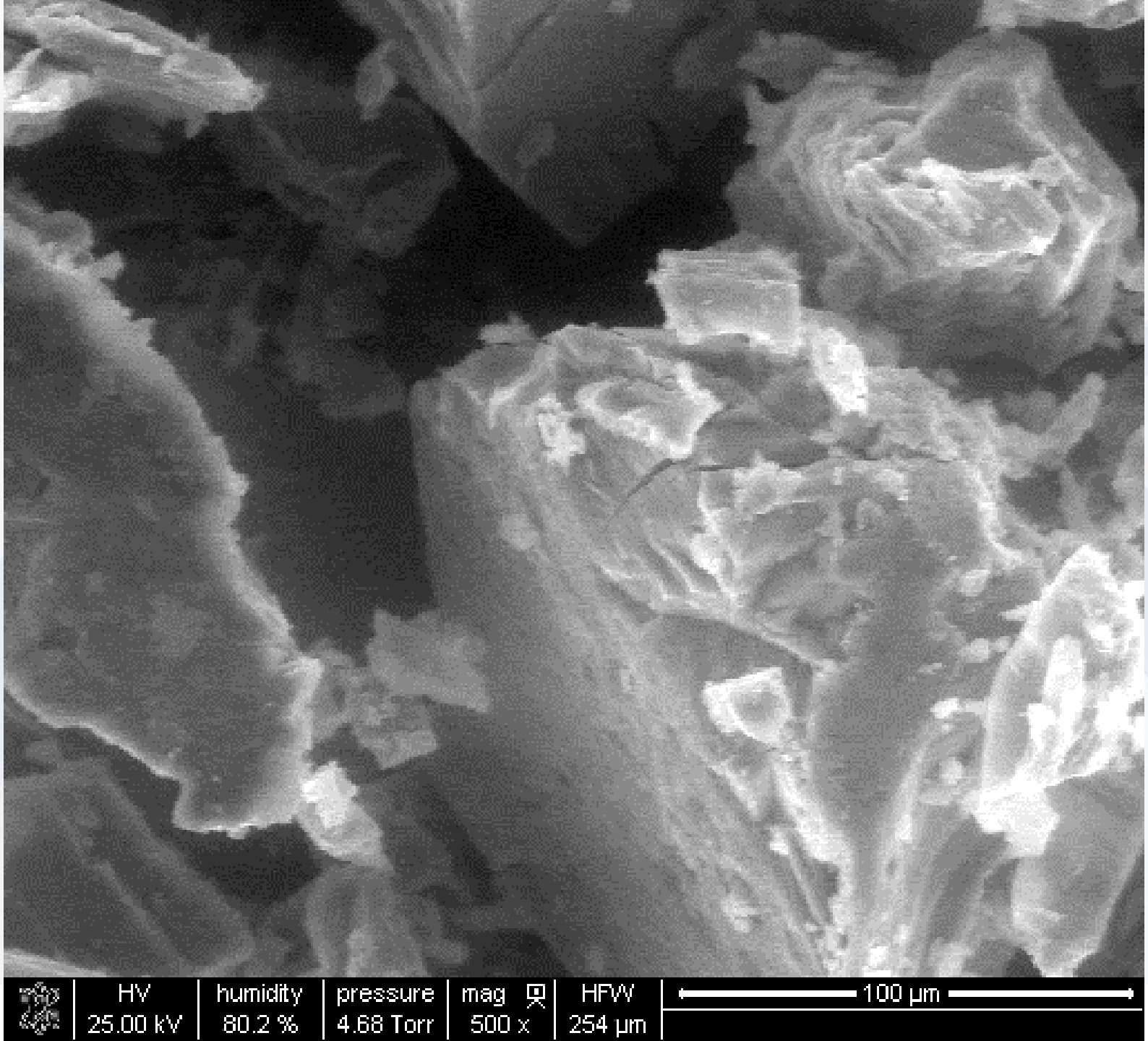
Scanning Electron Microscopy



This technique can be useful for larger particle suspensions such as intramuscular injections but doesn't provide much useful data for liquid nanoscale formulations.

Provides resolution down to ~1nm, but requires dry samples in high vacuum…

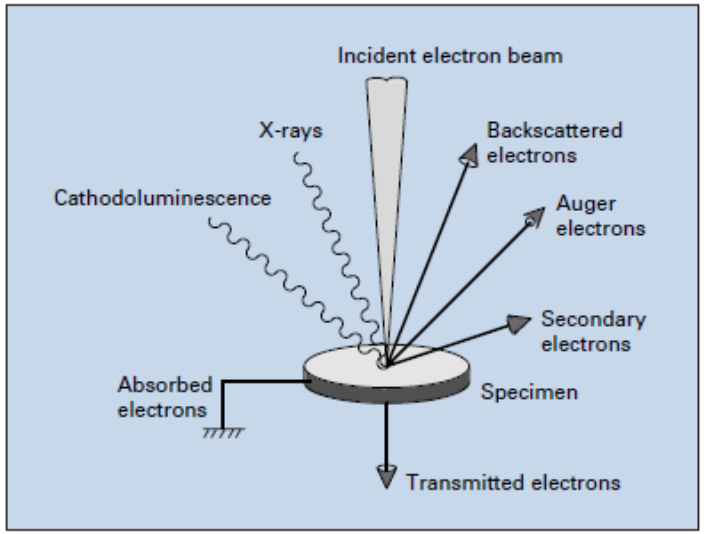
…in most cases. Vacuum trickery can allow you to put some water vapor into the chamber to avoid metal coating, or a LOT of water vapor can be used to actually "observe" liquid samples.


HV
25.00 kV
humidity
80.2 %
pressure
4.68 Torr
mag
500 x
HFW
254 µm
100 µm

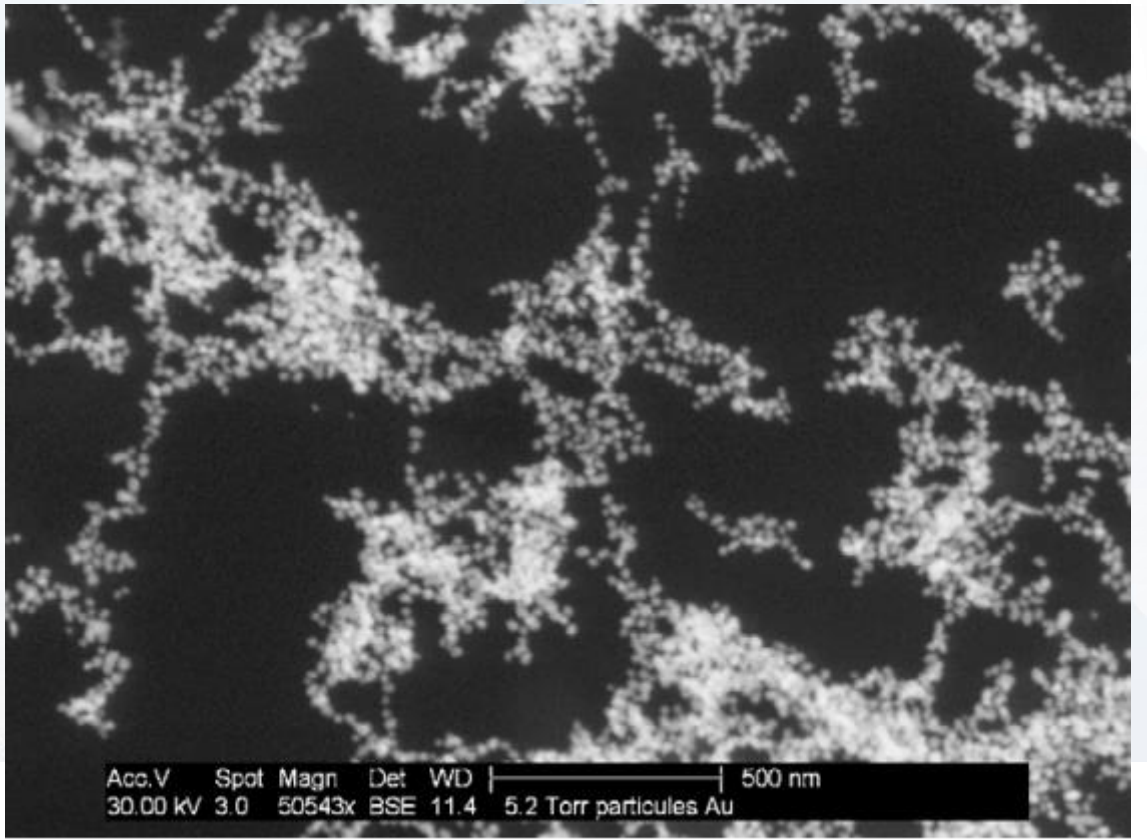
Scanning Transmission Electron Microscopy

- SEMs allow for a range of accelerating voltages, typically from 1 to 30 kV
- STEM-in-SEM detectors capture transmitted electrons and give scattering contrast
- A WetSTEM mode combining the previous technique with STEM is possible

- Much like with AFM, dried samples can produce agglomeration artifacts and collapse any structure that aren't robust, while wet samples require consideration of concentration, heating and scanning artifacts, scattering, etc.



Bogner et al – Micron 38 (2007) 390-401



Scanning Transmission Electron Mic

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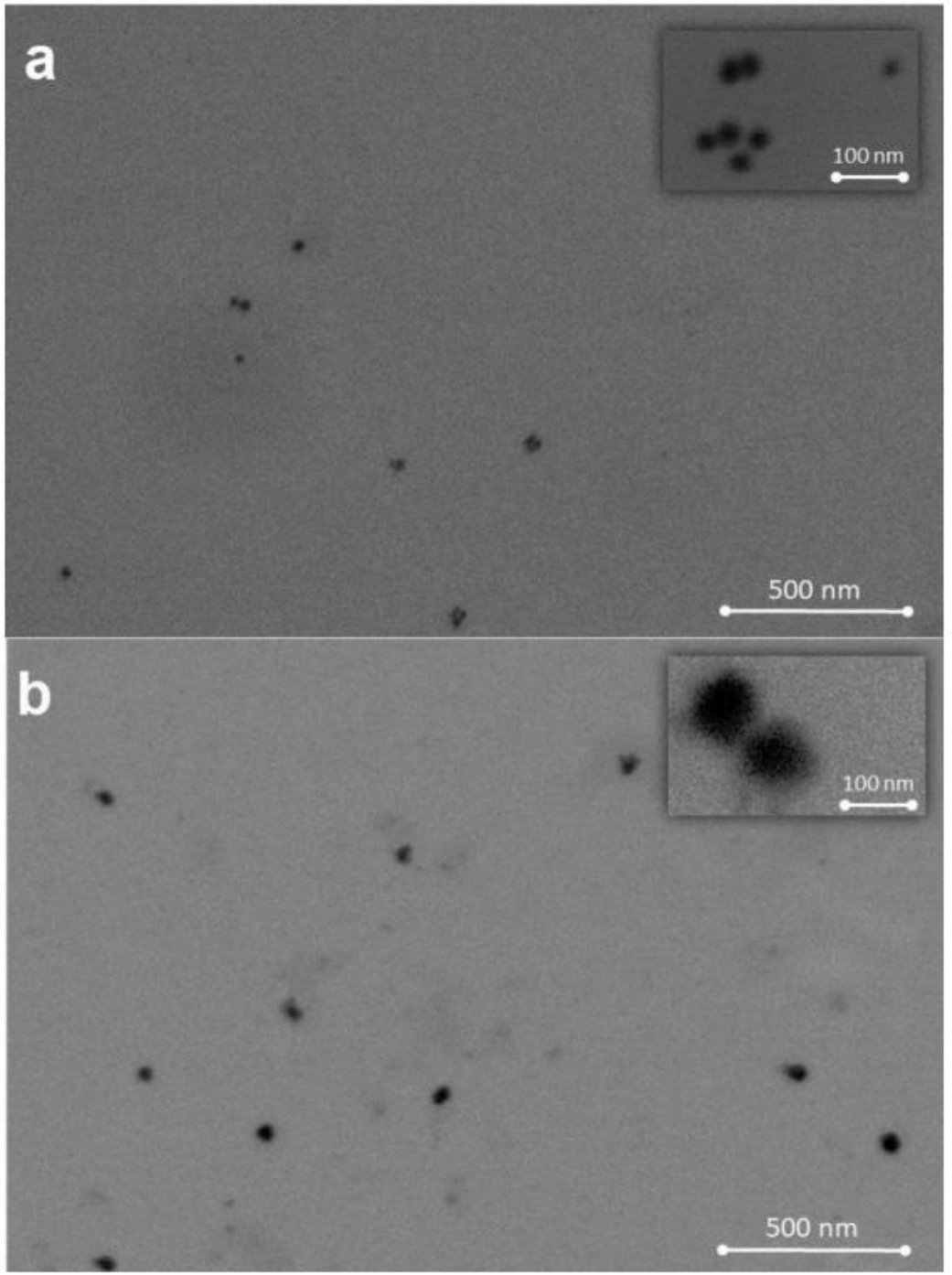
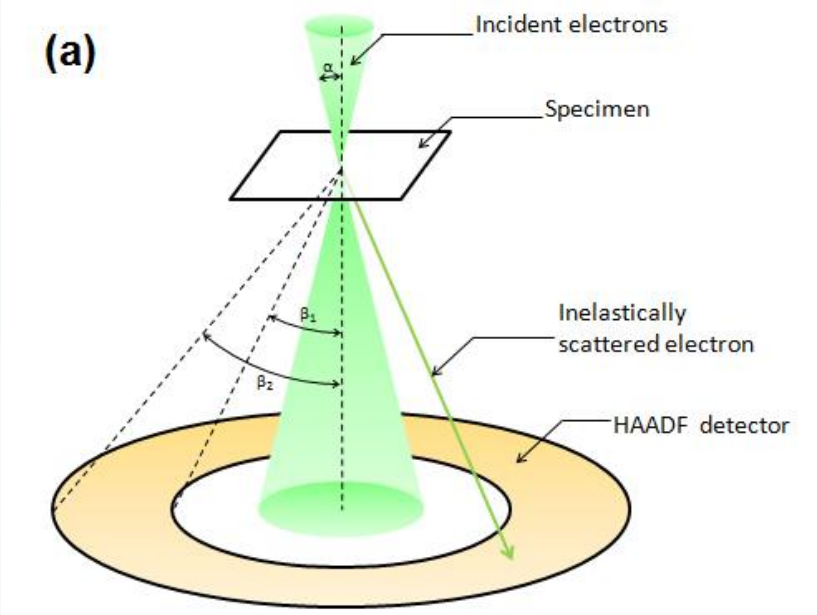


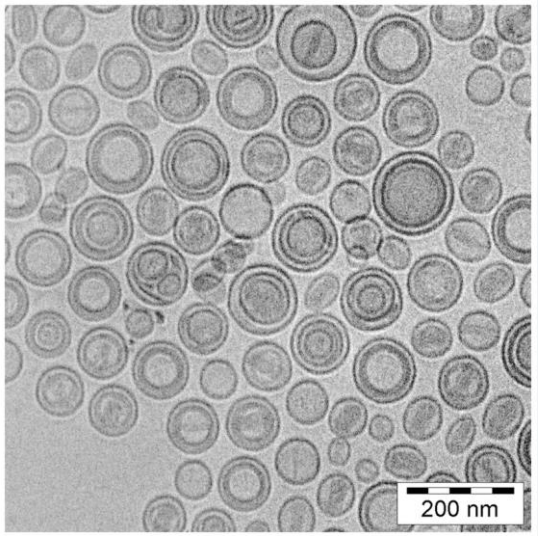
Ozturk et al, J. Mol Struct 1242 (2021) 130846

Transmission Electron Microscopy and CryoTEM

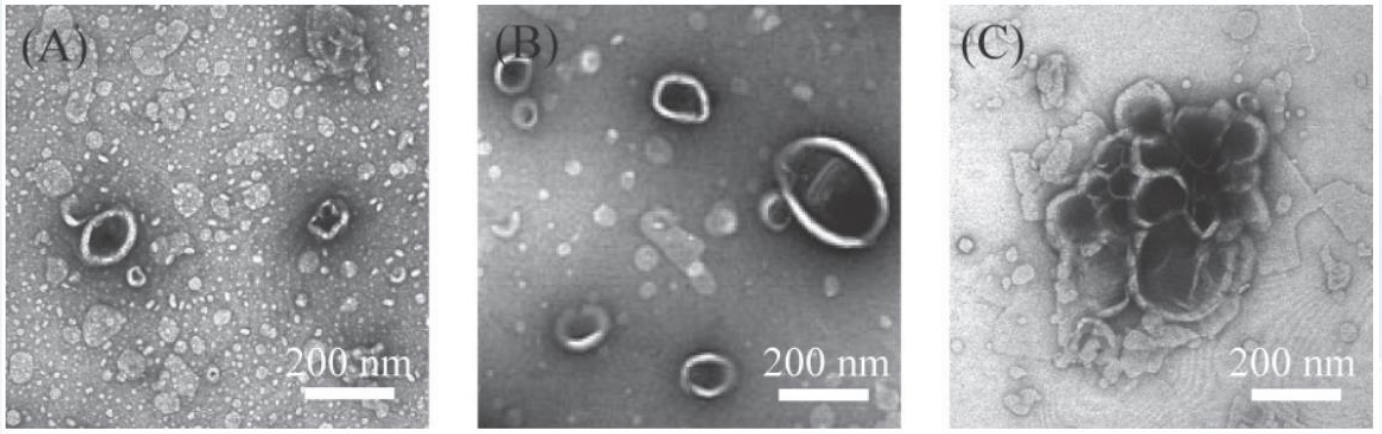
- TEM accelerating voltages typically range from 80-300kV; there are tradeoffs in penetration and contrast
- For heavy element / inorganic nanoparticles that are stable against water surface tension forces, dried samples can be used to examine samples with some artifacts



JEOL



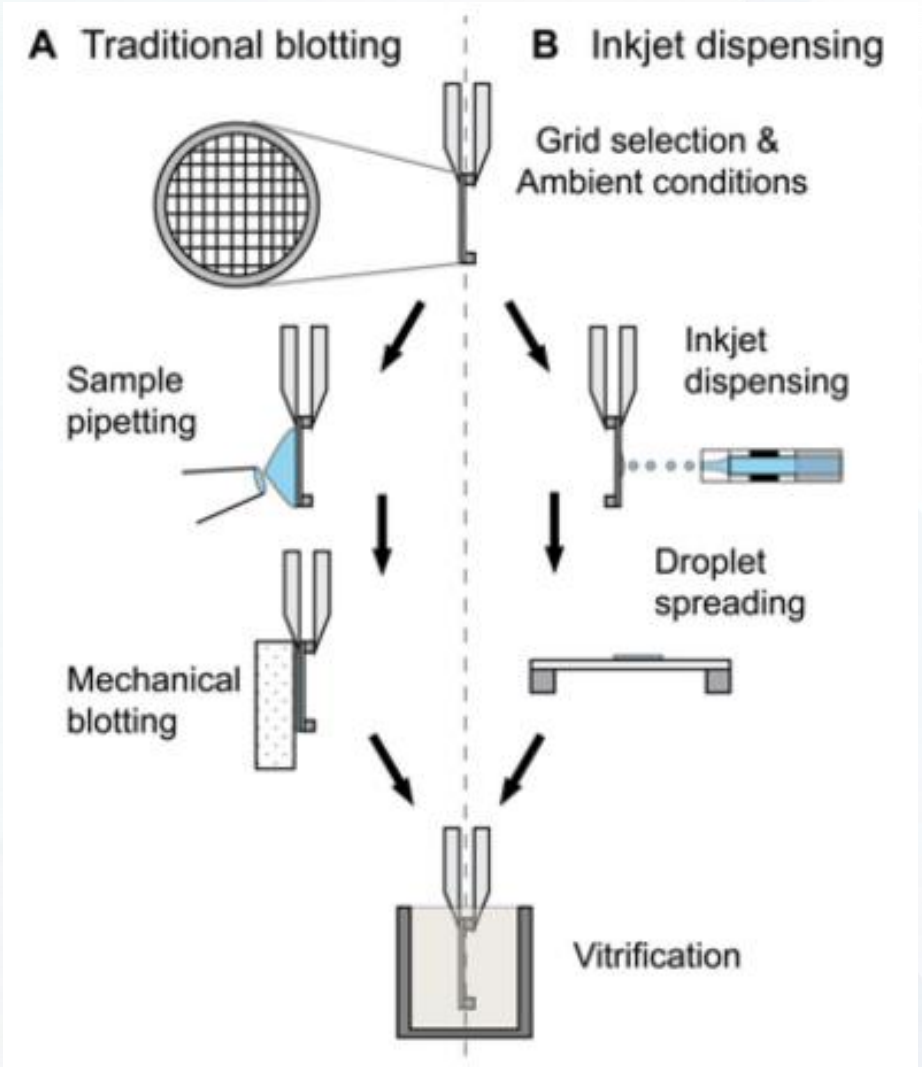
Jerzy et al PLoS ONE 9, 3 (2014)



Furuike et al J Encap and Adsorpt Sci 5, 144-154 (2015)

Transmission Electron Microscopy and CryoTEM

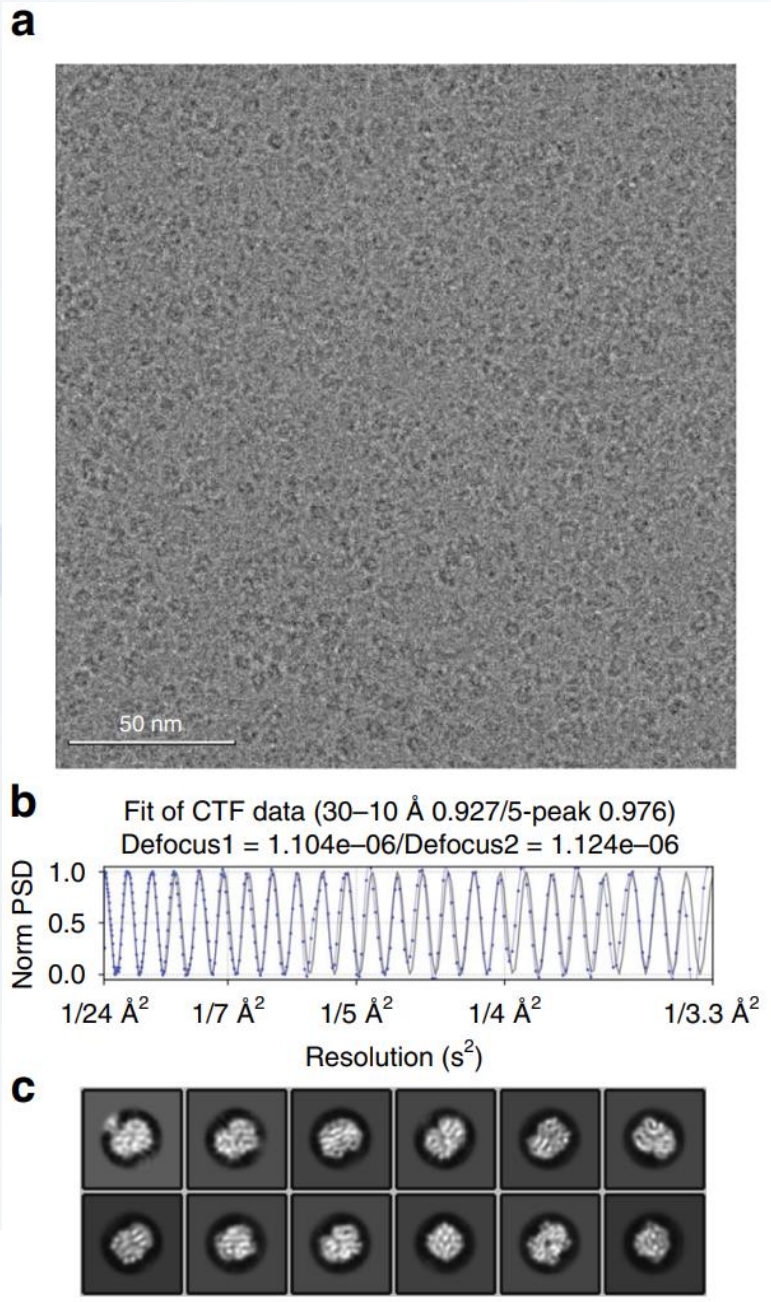
- CryoTEM is considered the gold standard for high magnification observation of nanostructures that preserves their native structure in solution
- Sample prep and imaging are an art as much as a science requiring a skilled and experienced operator
  - Vitreous ice / sample handling in the context of formulation excipients, defocus, dose and exposure to avoid ice

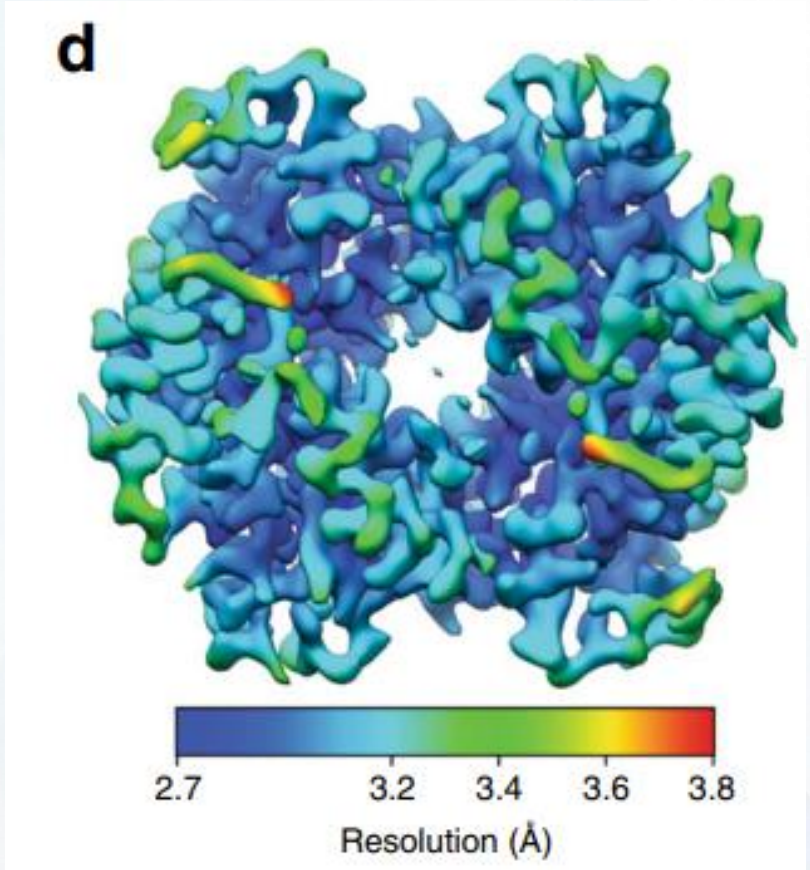


Jain et al J Struct Biol. 2012, 179(1), 68-75

Transmission Electron Microscopy and CryoTEM

- Class averaging approaches work extremely well for a monotypic population e.g. antibodies, proteins; crystal structures can even be derived

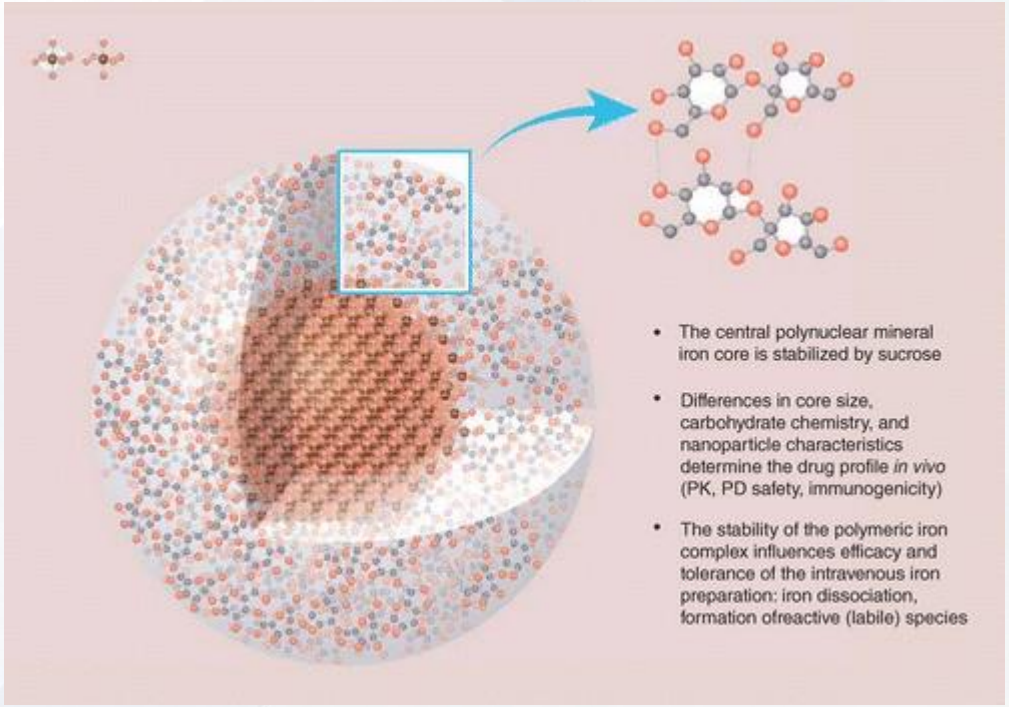




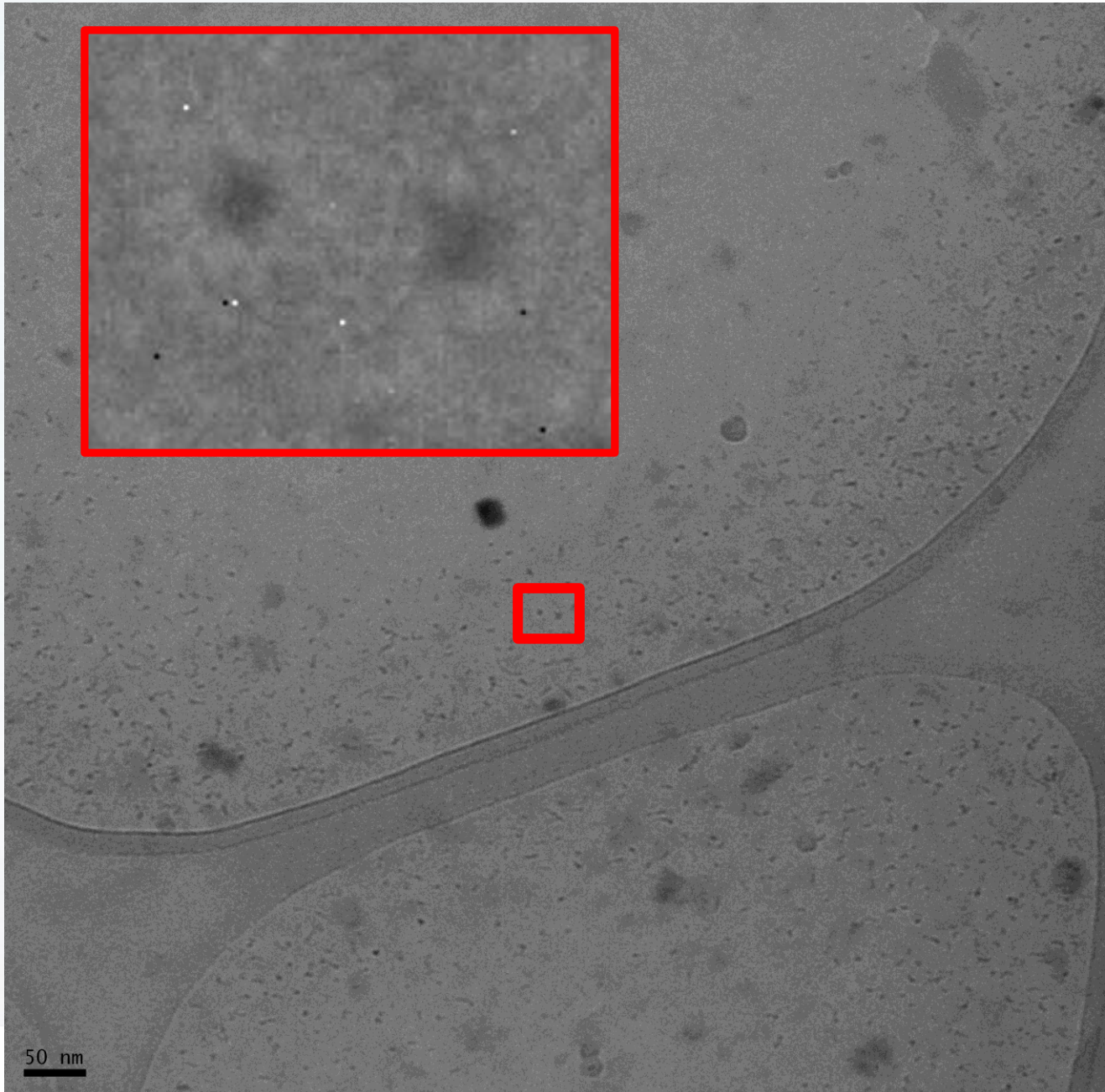
Herzik et al, Nature Comm (2019)10:1032

Transmission Electron Microscopy and CryoTEM

- Other samples with more variable structures may require a more manual approach…

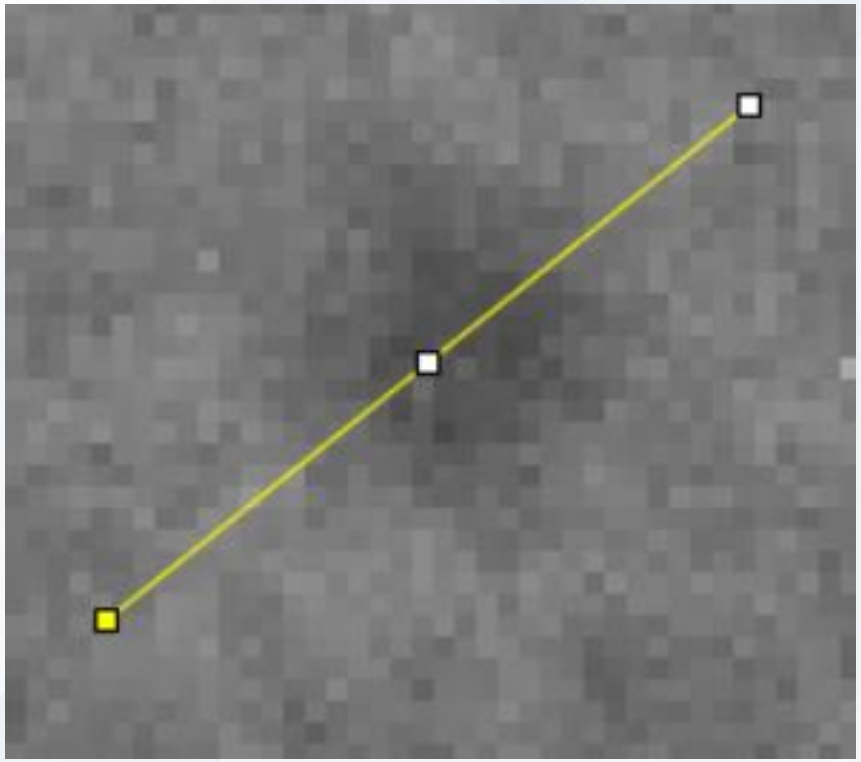


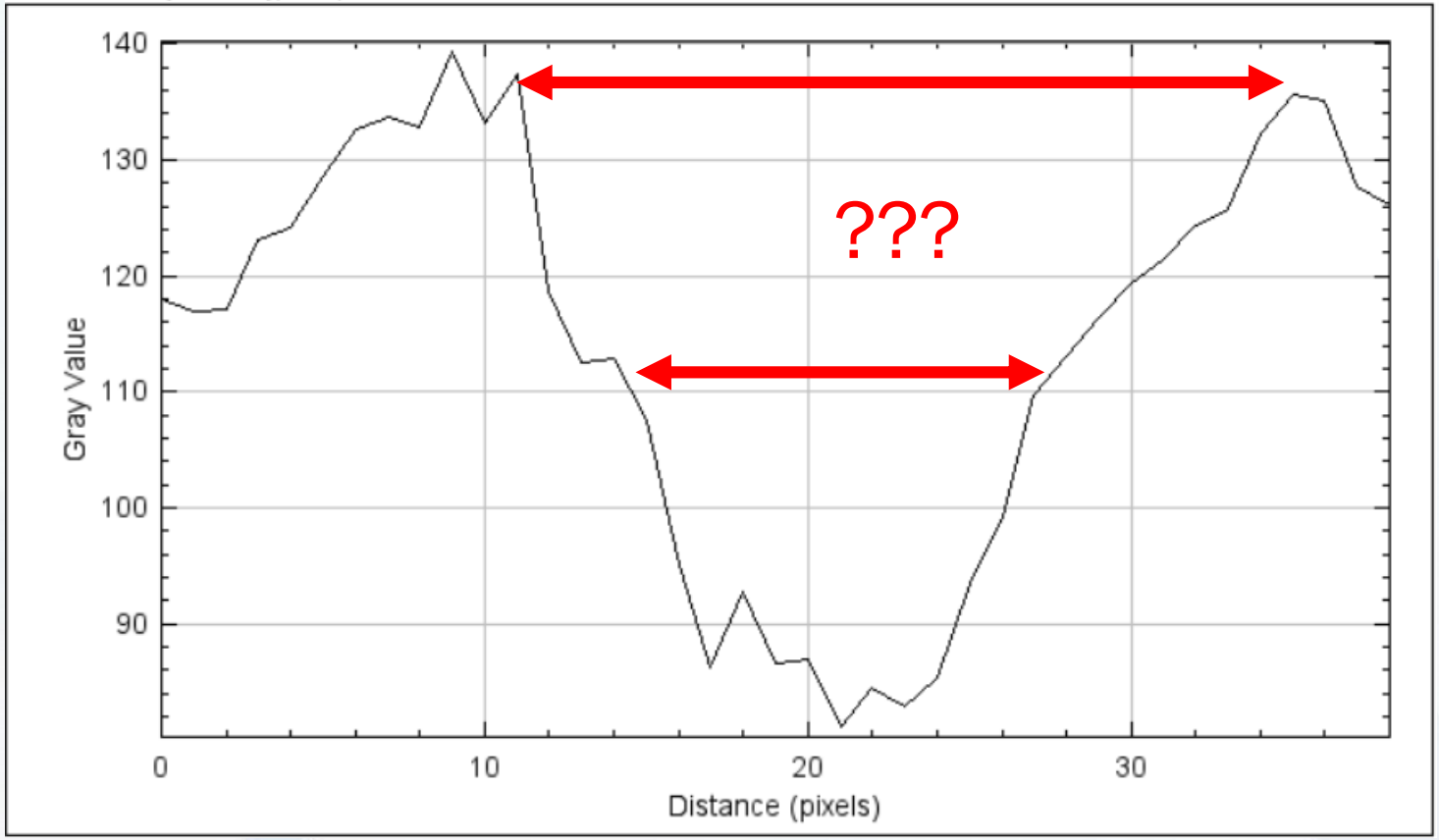
Mühlebach et al Nanomedicine Vol 10 No 4 (2015)



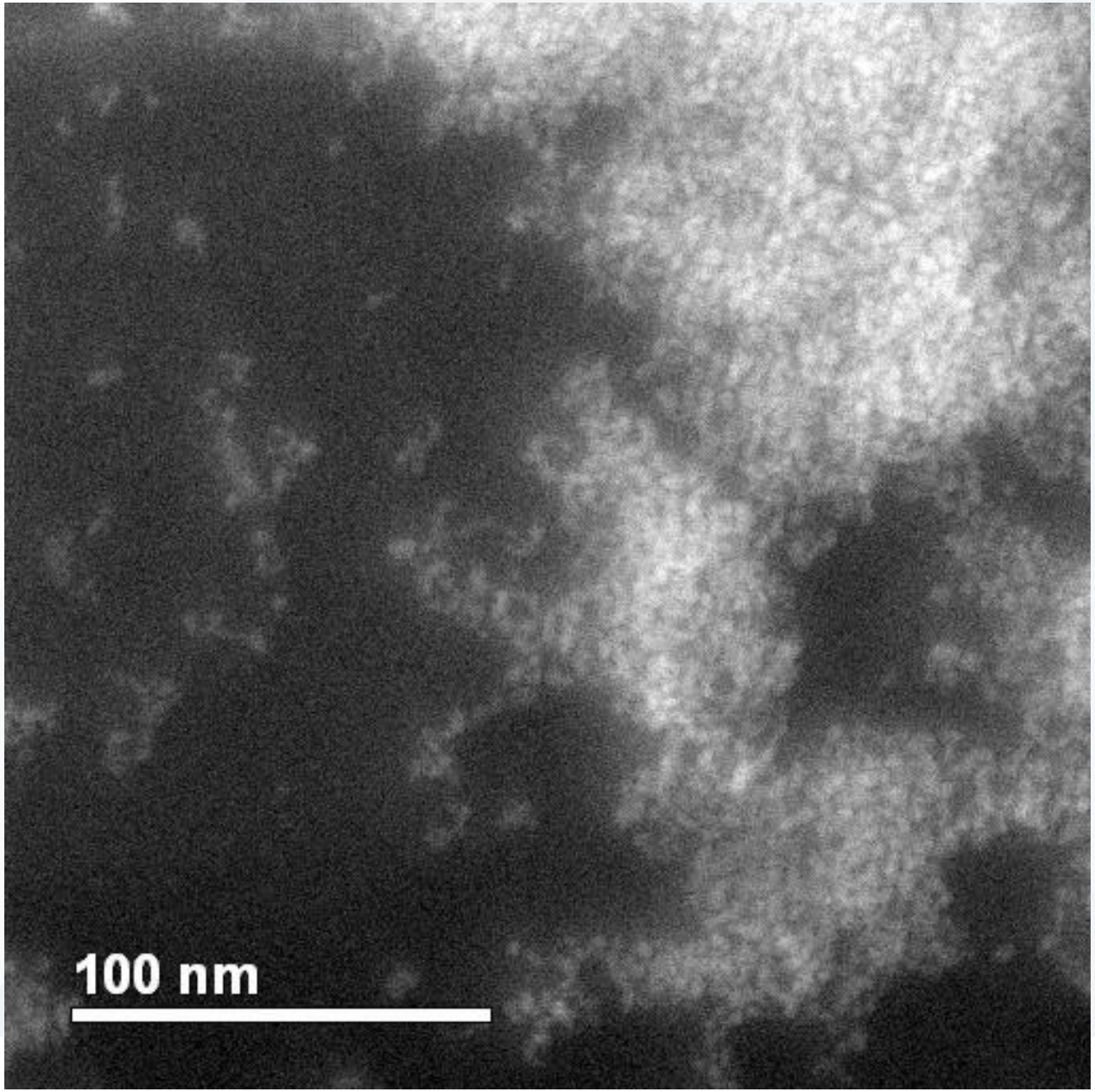
Transmission Electron Microscopy and CryoTEM

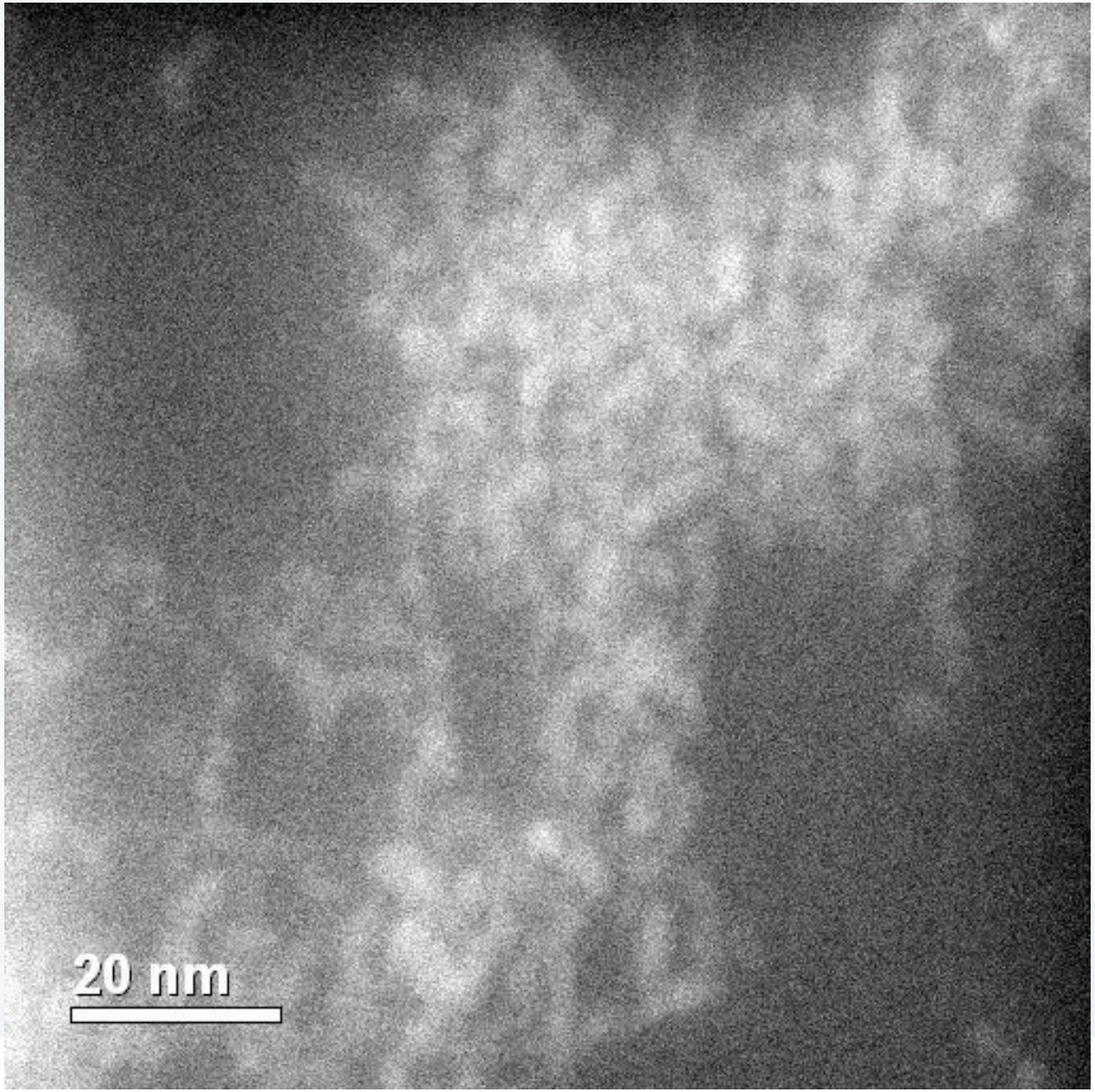
- Other samples with more variable structures may require a more manual approach, but there are complications





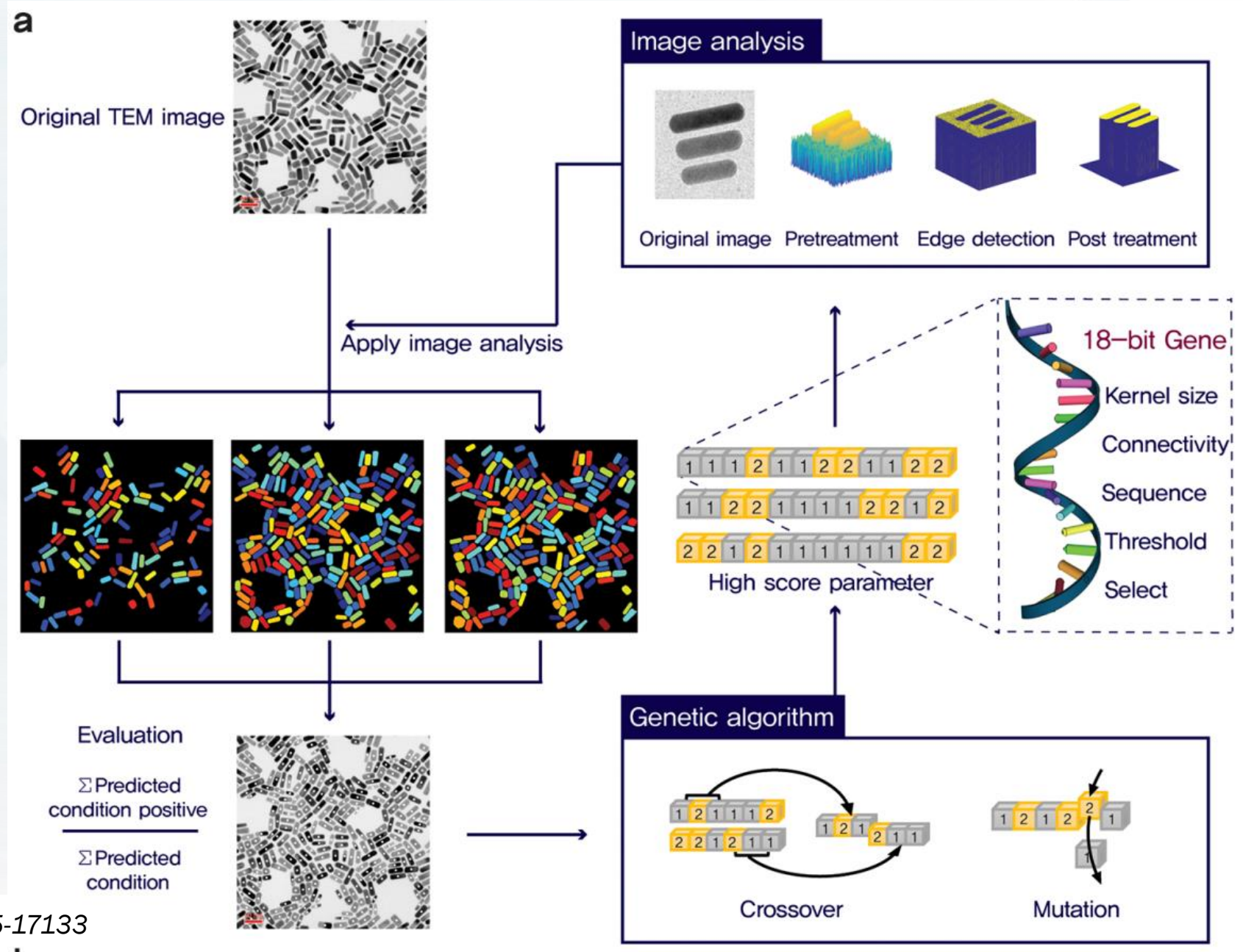
Transmission Electron Microscopy and CryoTEM





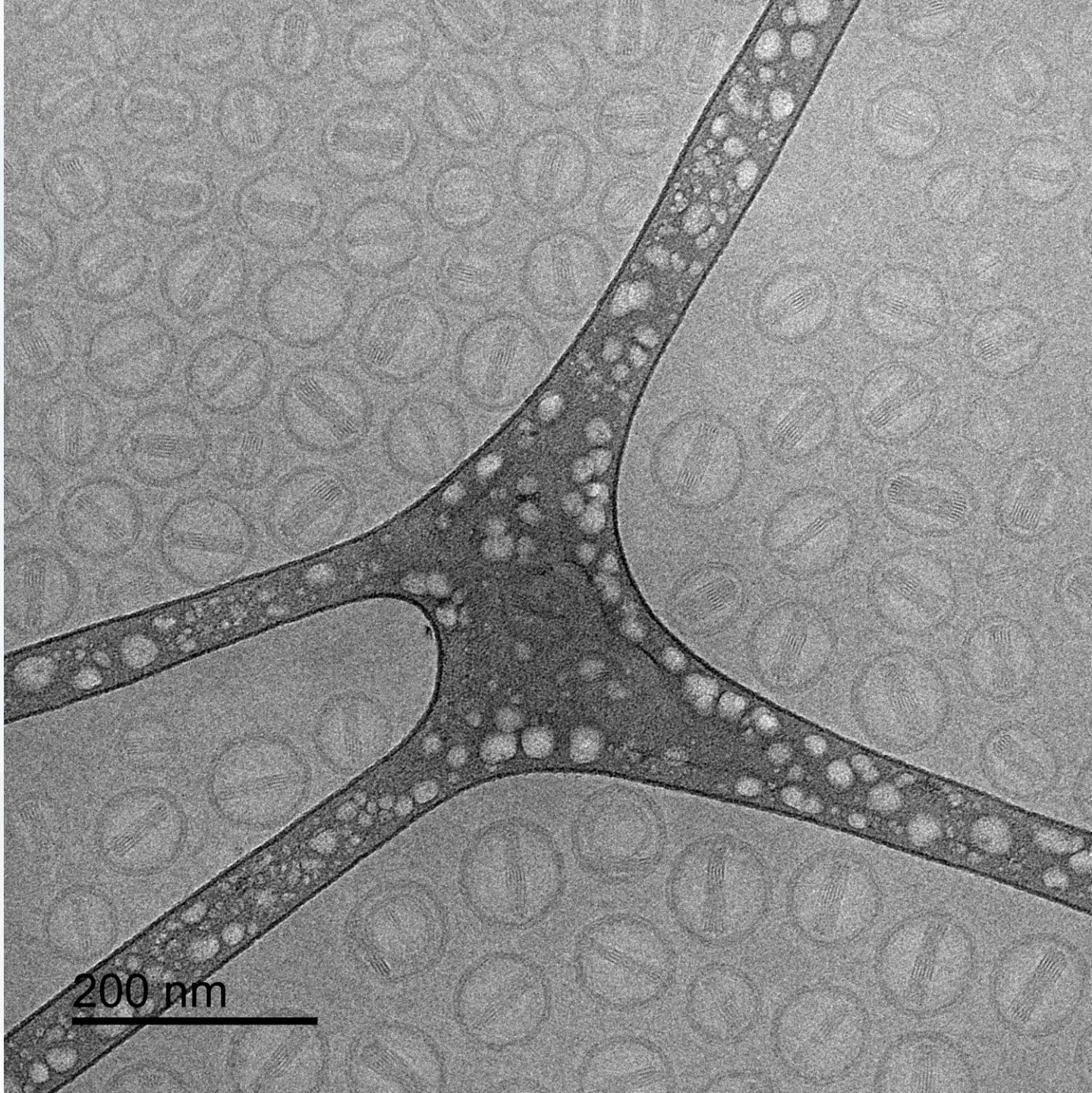
Transmission Electron Microscopy and CryoTEM

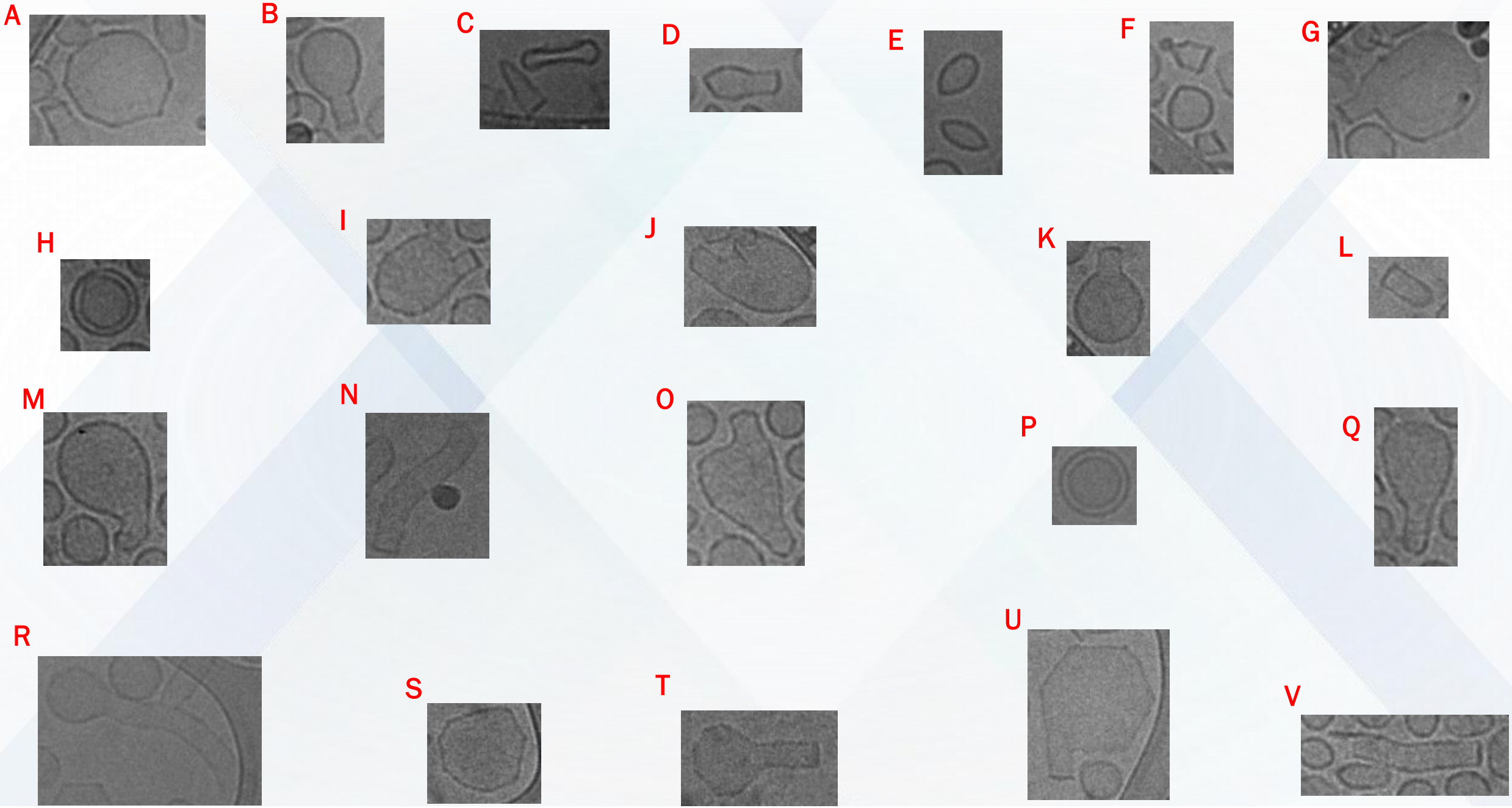
- Machine learning algorithms can be used, but work best with high quality source data that is consistent from micrograph to micrograph and sample to sample
- This is a very active area of research!



Lee et al, ACS Nano 2020, 14, 17125-17133

Some structures are well-behaved...

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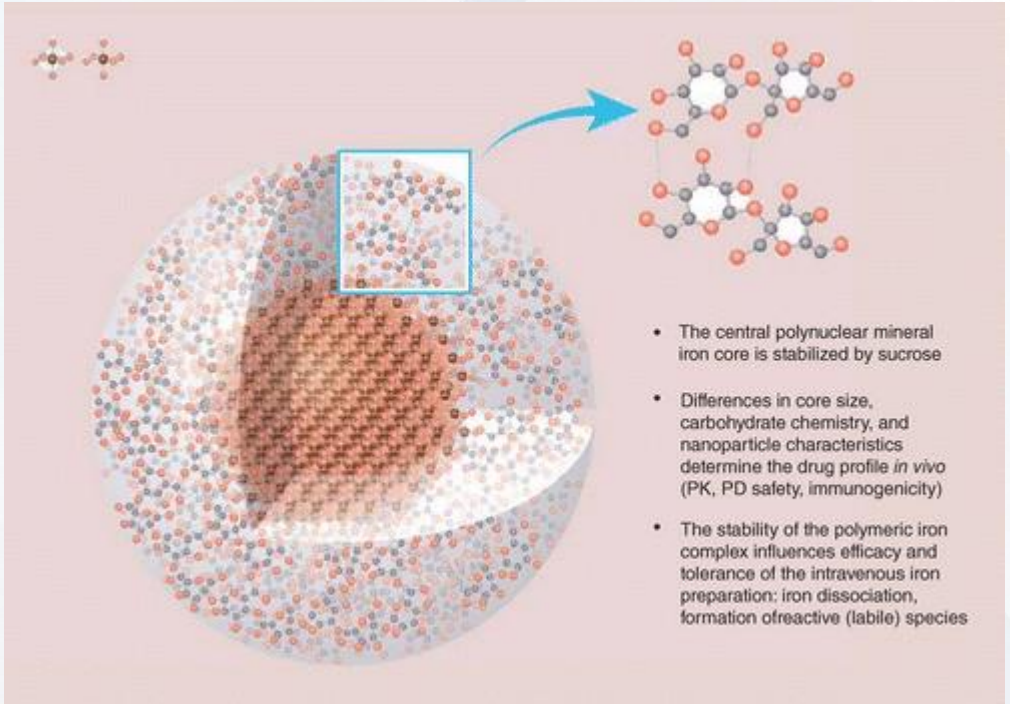
Spectroscopy – A case study of iron carbohydrates

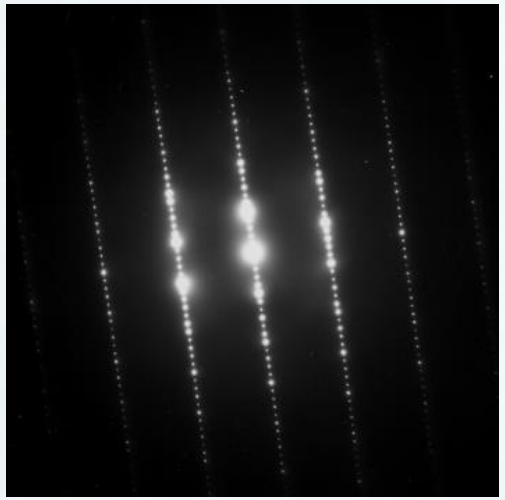
- Iron carbohydrates treat iron deficiency anemia. Nanoparticles of iron oxyhydroxide break down over time, releasing atoms of labile iron which can be bound by transferrin and contribute to populations of biologically useful iron (e.g. storage as ferritin).
- The rate of labile iron liberation depends on numerous parameters:
  - Surface area to volume ratio, size, and shape of the core
  - Crystal structure of the core
  - Iron oxidation state / magnetic parameters as a surrogate
  - Sugar chemistry
    - Degree of branching
    - Intermolecular associations to the core
    - Molecular weight
    - Hydrodynamic radius
    - State of aggregation
  - pH of the formulation, zeta potential
- How do we establish physicochemical sameness?



Mühlebach et al Nanomedicine Vol 10 No 4 (2015)

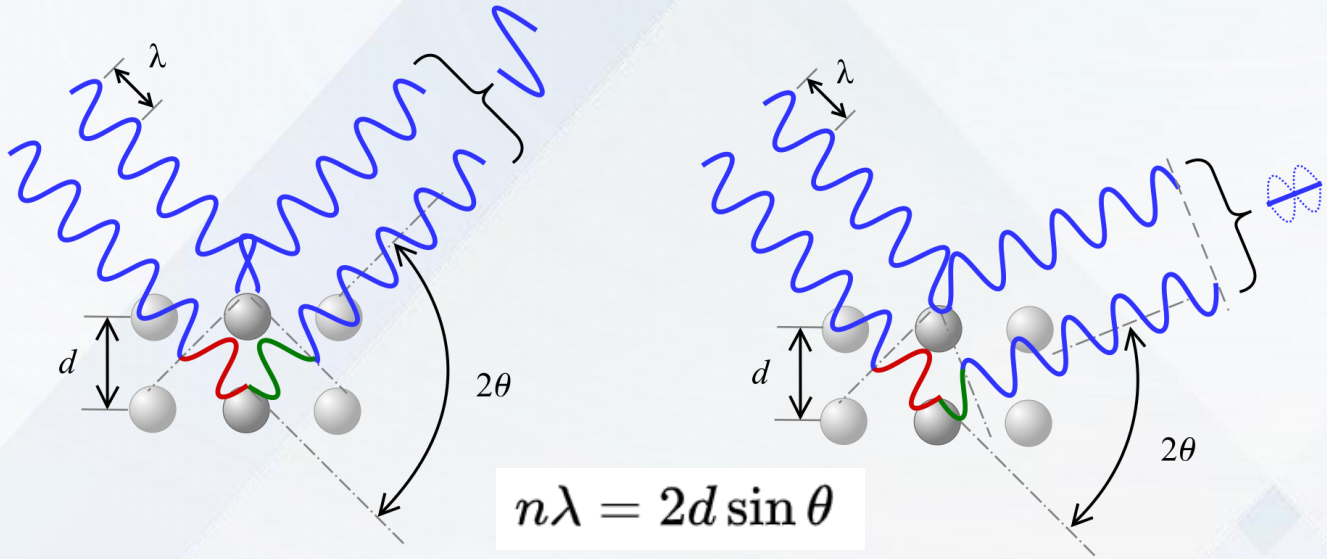
Spectroscopy – A case study of iron carbohydrates

- The crystal structure can be probed in the TEM by SAED or in bulk by x-ray diffraction
- Bragg peak width can estimate size



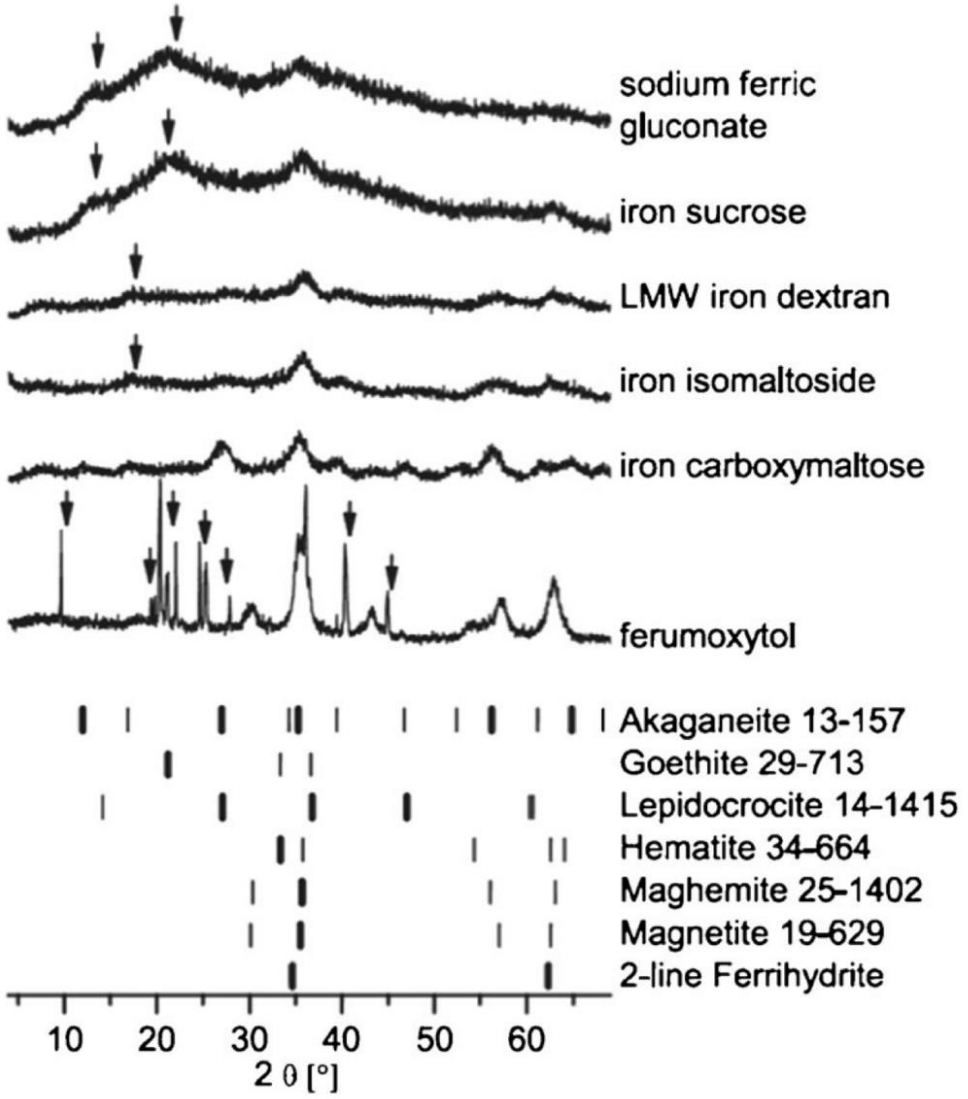
$$\tau = \frac{K\lambda}{\beta \cos\theta}$$

By Oysteinp at the English-language Wikipedia, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=3193481



$$n\lambda = 2d\sin\theta$$

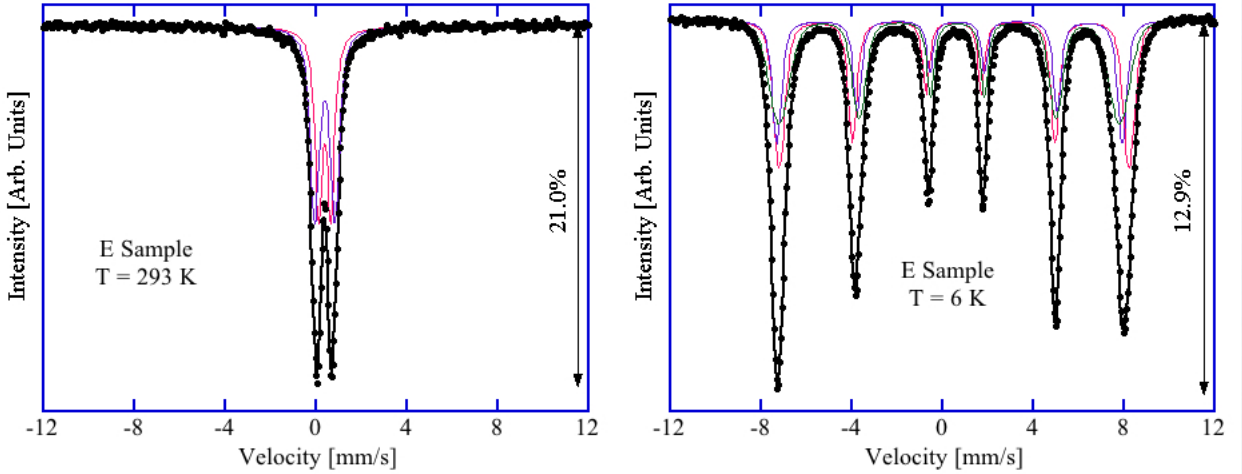
By Loi_de_bragg.png: User:Cdangderivative work: Gregors (talk) - Loi_de_bragg.png, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=14524146

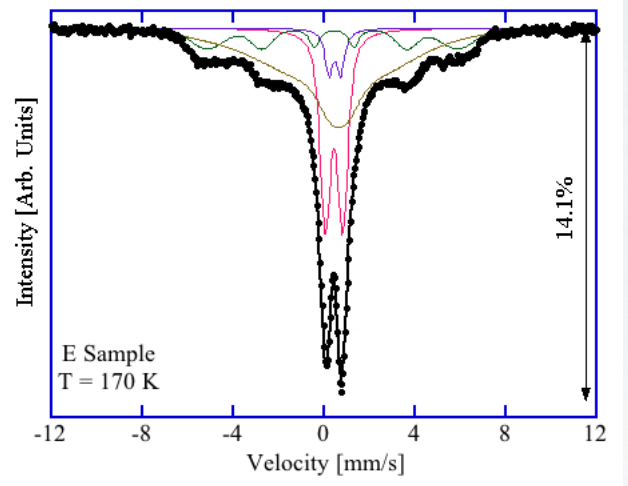


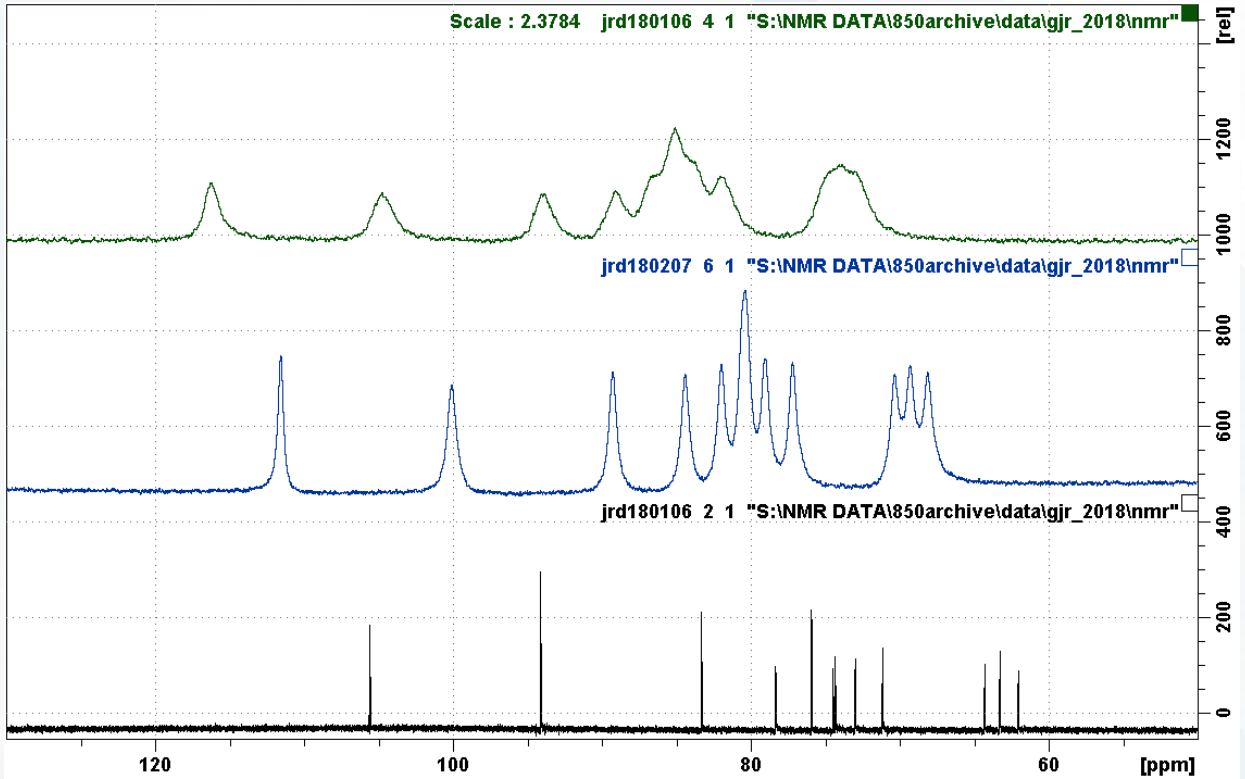
Zou et al AAPS J Vol. 19, No. 5, Sep 2017

Spectroscopy – A case study of iron carbohydrates

- NMR, EPR, and Mössbauer give magnetic properties of the core and interactions between the core and ligands, which are indirectly correlated to PK

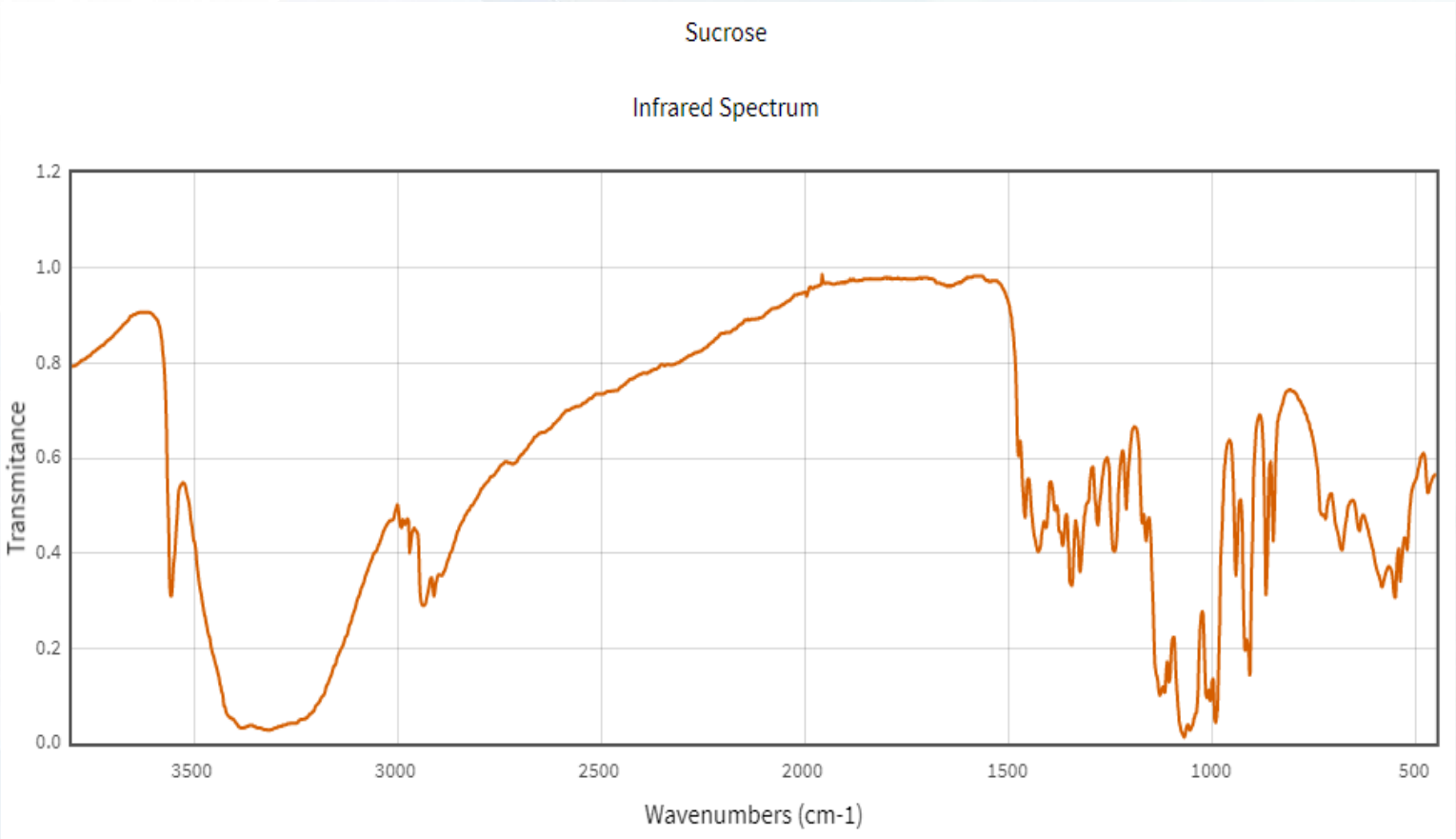


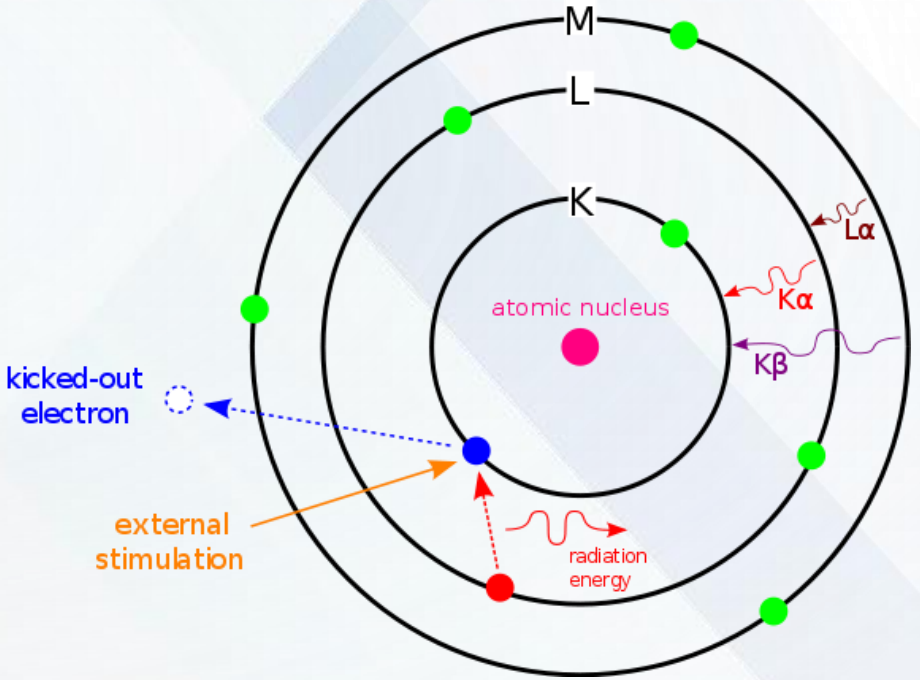




Spectroscopy – A case study of iron carbohydrates

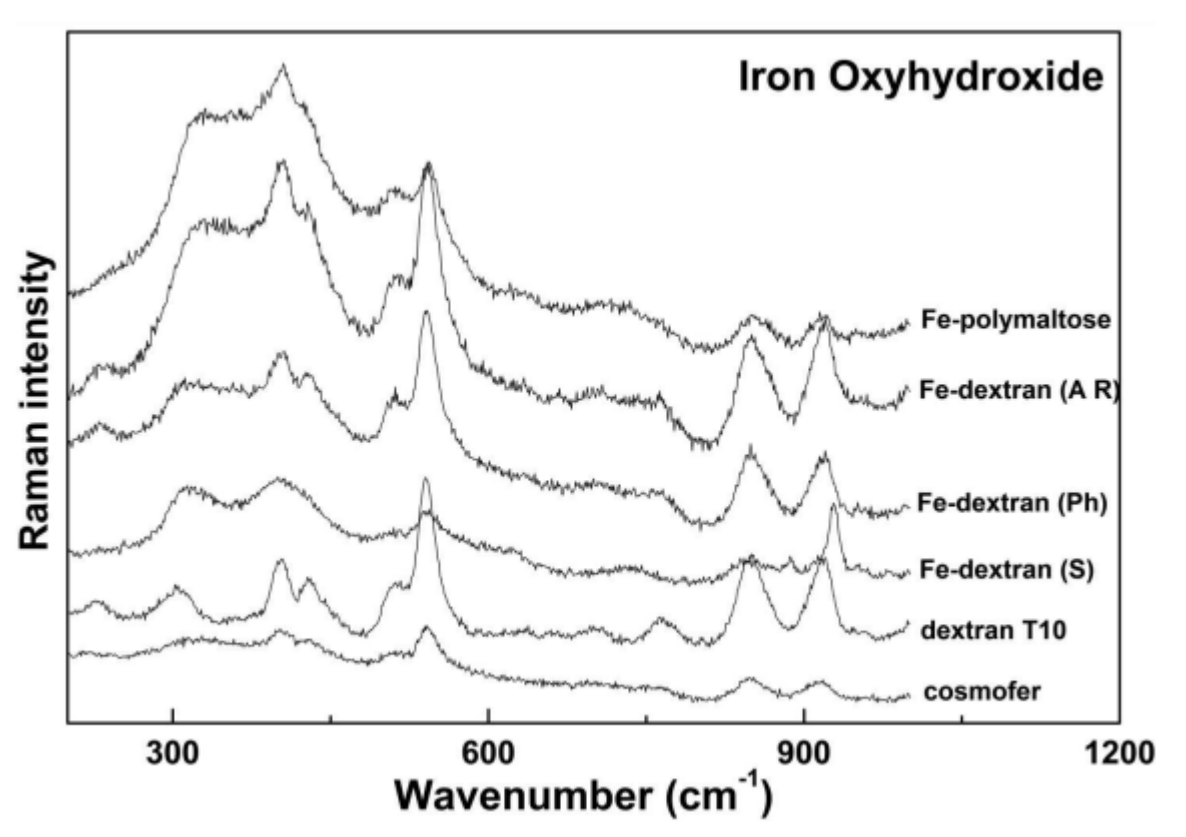
- FT-IR spectroscopy gives general chemical bonding information and typically just shows a strong carbohydrate signal.
- This can be complemented with CHN elemental analysis, SEM-EDXS, and TGA.





Spectroscopy – A case study of iron carbohydrates

- Raman gives similar information (scattering of laser light vs. absorption of IR depending on dipole moment), but also yields information on crystallinity at low wavenumber
- Peaks can be seen after dialysis of excess sugars that correspond to Fe-O symmetric bending (307), Fe-O-Fe symmetric stretching (385), and Fe-OH asymmetric stretching (533/619/725)



Szybowicz et al Acta Physica Polonica 127 (2015)

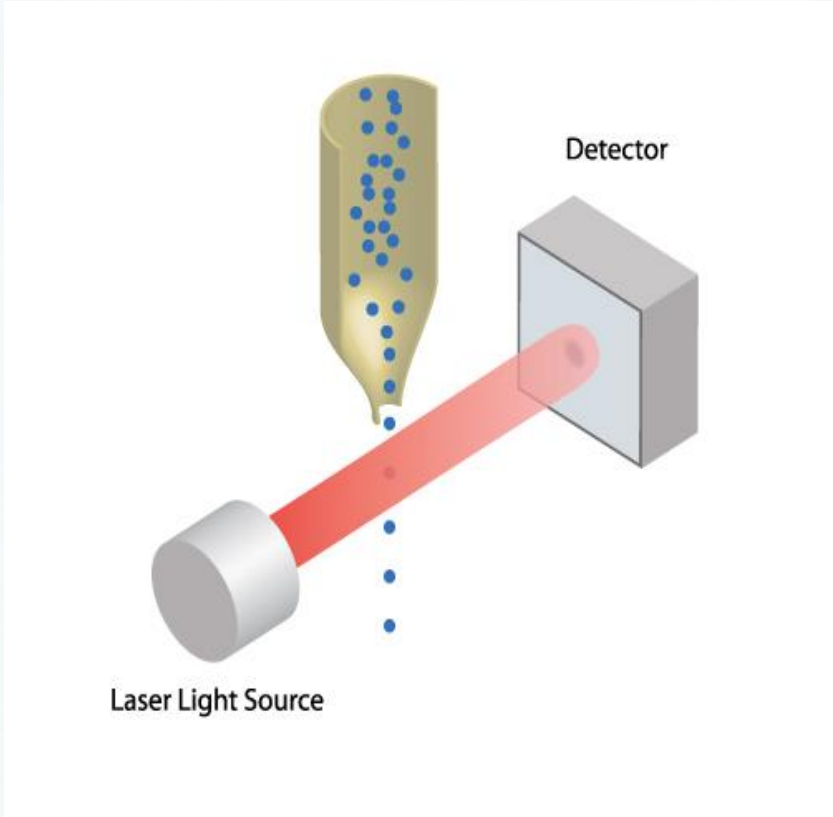
Spectroscopy – A case study of iron carbohydrates

- Combining multiple spectroscopic techniques can yield correlating information about core size, atomic arrangements / crystal structure, sugar chemistry near and far away from the core
- Orthogonal techniques – you can derive estimations of core size from cryoTEM, XRD, Mössbauer

Szybowicz et al Acta Physica Polonica 127 (2015)

Particle Size Distribution – Ensemble Methods

- Light obscuration counting is used for particulate matter testing per <788>; many nano formulations are not clear/colorless and require dilution
- The resolution of discerning particles is related to the diffraction limit of light, 0.6 microns at best and more practically, 2 and up



Run No.	Particle Size(µm)	Cumulative Count	Differential Count	Cumulative Counts/mL	Differential Counts/mL
Run 1	2.000	16862.00	12031.00	3372.40	2406.20
	5.000	4831.00	4413.00	966.20	882.60
	10.000	418.00	418.00	83.60	83.60
	20.000	0.00	0.00	0.00	0.00
	25.000	0.00	0.00	0.00	0.00

Nanoscale Particle Size Distribution

- Laser diffraction measures variation in the intensity of scattered light versus the scattering angle; the motion of the particles doesn't matter because the intensity of scattering is correlated to size directly
- The range of sizes is approximately 10nm to 1mm, so it can be useful for larger objects but single digit nanometer complexes are not measurable
- Dynamic light scattering instead looks at fluctuations in the intensity of scattered light based on the speed of Brownian motion fluctuations and can measure from approximately 1nm to 10µm

$$d(H) = \frac{kT}{3\pi\eta D}$$

where:-

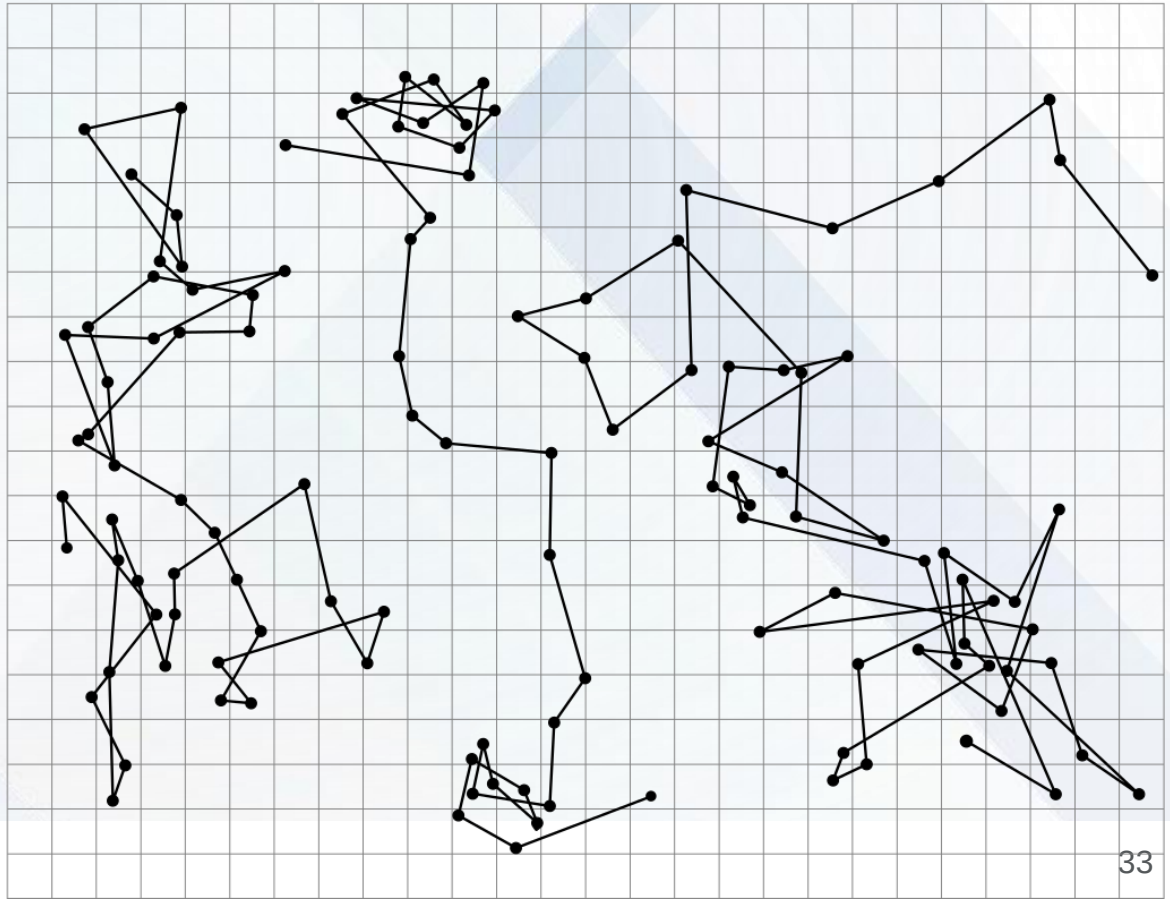
$d(H)$ = hydrodynamic diameter

D = translational diffusion coefficient

k = Boltzmann's constant

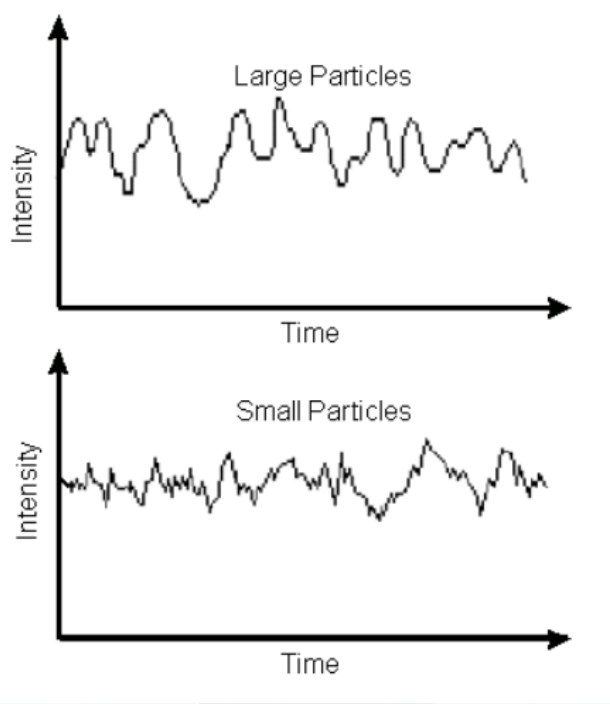
T = absolute temperature

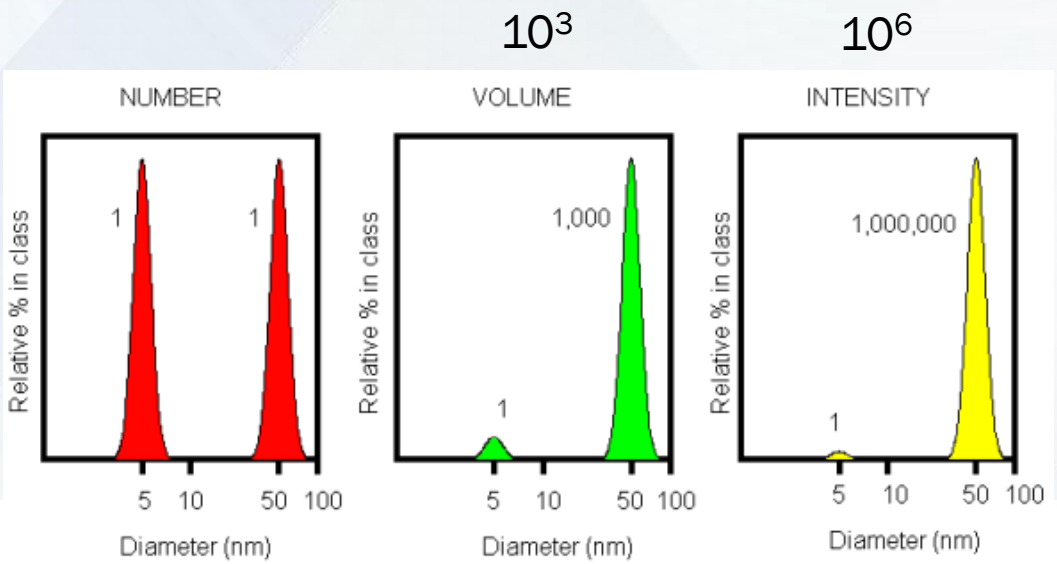
η = viscosity



Nanoscale Particle Size Distribution

- The viscosity of the solution is an important input parameter – measure it separately!
- The size you get is the Equivalent Spherical Hydrodynamic Size for non-spherical particles
- Outputs (and corresponding requirements for establishing population bioequivalence) are either:
  - D_{10}, D_{50}, D_{90} and SPAN $(D_{90}\text{-}D_{10})/D_{50}$
  - Z_{ave} and PdI (distribution width^2 / mean)





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where:-

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Nanoscale Particle Size Distribution

Statistical Approaches to Establishing Bioequivalence

Guidance for Industry

DRAFT GUIDANCE

December 2022
Biopharmaceutics

1. Population Bioequivalence

One of the recommended statistical approaches for evaluating in vitro BE is population BE (PBE). To test for PBE, the null and alternative hypotheses are given as follows:

$$H_0: \theta \geq \theta_P \quad \text{vs.} \quad H_a: \theta < \theta_P$$

where $\theta = \frac{(\mu_T-\mu_R)^2+\sigma_T^2-\sigma_R^2}{\sigma_R^2}$ if the estimated $\sigma_R > \sigma_0$ or $\theta = \frac{(\mu_T-\mu_R)^2+\sigma_T^2-\sigma_R^2}{\sigma_0^2}$ if the estimated $\sigma_R \leq \sigma_0$.

Here, μ_T and μ_R are the population means, σ_T^2 and σ_R^2 are the population variances of the log-transformed measure for T and R products, respectively; σ_0^2 is a regulatory constant for variance; and θ_P is the PBE limit. The concept of PBE is to compare the difference of the T and R products with that of the reference versus reference itself. This comparison can be denoted in terms of the population difference ratio as follows:

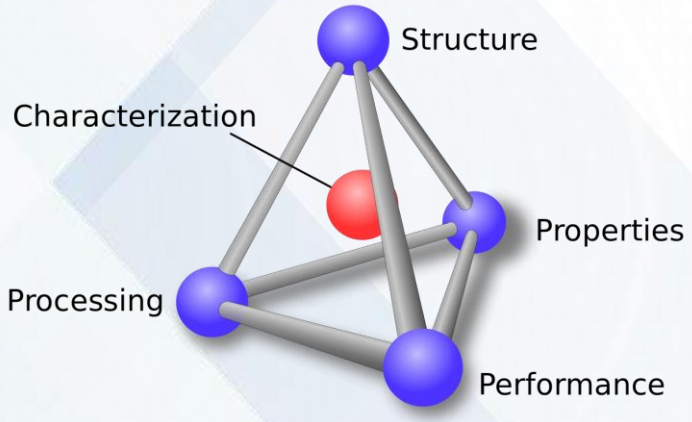
$$\sqrt{\frac{E(Y_T - Y_R)^2}{E(Y_R - Y_R')^2}} = \sqrt{\frac{(\mu_T - \mu_R)^2 + \sigma_R^2 + \sigma_T^2}{2\sigma_R^2}} = \sqrt{\frac{\theta}{2} + 1}.$$

The regulatory constant variance, σ_0^2, is set based on the following considerations. Due to the low variability of in vitro measurements, this guidance recommends that the ratio of geometric means should fall within 0.90 and 1.11. As a result, an upper BE limit of 1.11 is recommended for the average BE limit for in vitro data. Assuming $\sigma_R^2 = \sigma_T^2 = \sigma_0^2$, $\mu_T - \mu_R = \ln 1.11$ and the maximum allowable limit for population difference ratio is 1.25, this leads to the recommended choice of $\sigma_0^2 = 0.01$.

Summary

- Seeing is believing, but every imaging technique has its limitations and caveats and extracting numerical data can be a challenge

- The key to understanding is comparison to the reference product and the use of multiple orthogonal techniques
  - Core size can be estimated from AFM, cryoTEM, Mössbauer, XRD…
  - Magnetic properties are derived from NMR, EPR, Mössbauer…
  - Crystalline structure can be studied with SAED, XRD, Raman…
  - Sugar chemistry is assessed through DLS, FT-IR, TGA…



- Creative non-standard methodologies that consider the biology of the situation can have good composite predictive power

- Ensemble particle methods are standardized tools and can be very useful in establishing processing-property relationships