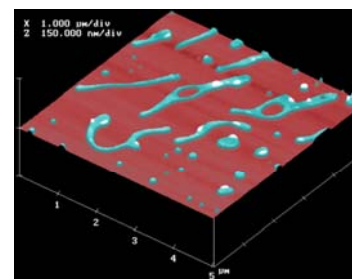
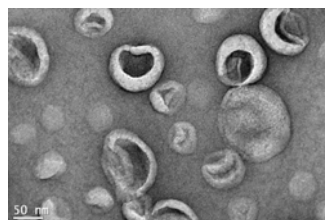
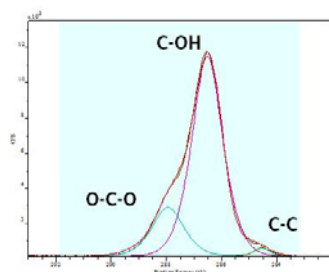
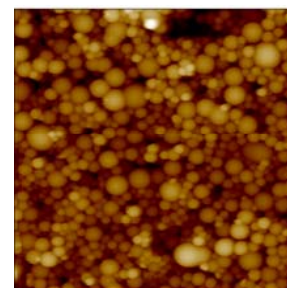
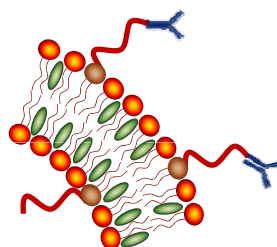
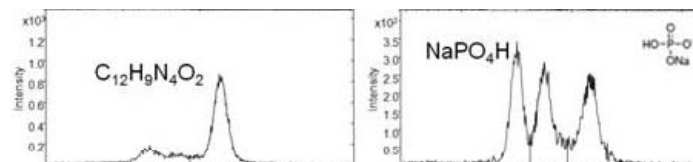
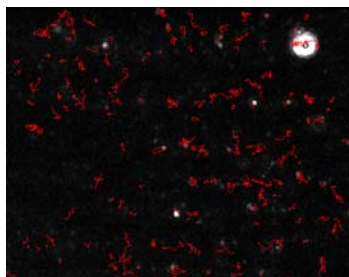


Webinar:

Physicochemical Characterisation of Nanomedicines



Host: Dr Matt Bunker, Molecular Profiles Ltd, UK.
Wednesday 8th December
16:00 CET (15:00 GMT)

This webinar will aim to...

- Provide an introduction to the physiochemical characterisation of nanomedicines
- Raise awareness of different techniques available
- Emphasise practical considerations and relative advantages/disadvantages

Techniques Covered

- Imaging techniques
- Chemical techniques
- Other (X-ray diffraction, particle sizing)

Examples from the **Meditrans project for targeted nanomedicines**

Meditrans is an Integrated Project funded by the European Commission

Consists of a collaboration between 29 academic and industrial partners from 12 different states.

The total budget is €14.8M (€10.7M from EC ,€4.1M from industry)

Chronic inflammatory disorders and cancer are targeted pathologies

<http://www.meditrans-ip.net/>

Imaging Techniques

Scanning electron microscopy

Transmission electron microscopy

Cryo-electron microscopy

Atomic force microscopy

molecular
profiles™

'Field Emission' SEMs can provide high resolution of 1-5 nm or better



Scanning Electron Microscopy

Advantages

Huge range of magnifications

A large area can be imaged at any time

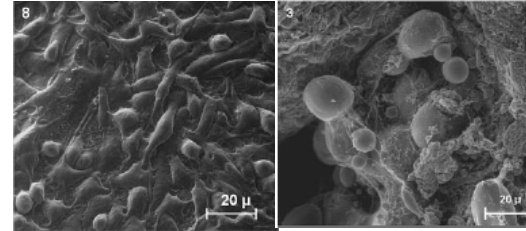
Surface specific

Relatively quick and easy to use

Sample prep can be straightforward

X-ray microanalysis allows elemental identification (spatial resⁿ >1μm)

Environmental SEM allows imaging higher pressure and variable humidity



Disadvantages

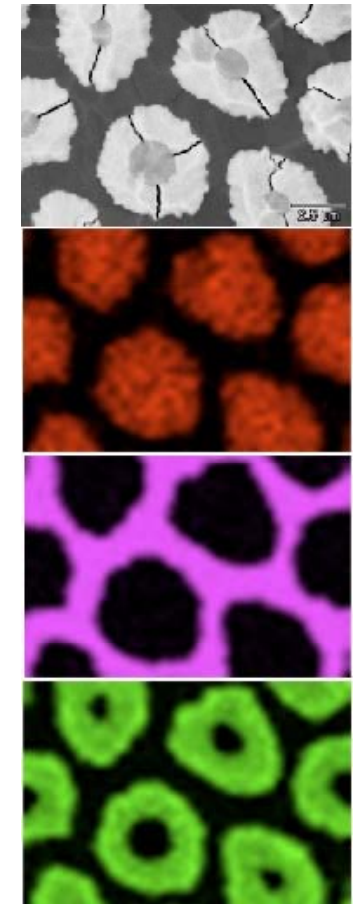
Vacuum technique, so must be dehydrated for conventional SEM

Variable pressure imaging allows imaging of partially hydrated samples but resolution is very much reduced.

Sample considerations

Samples can charge leading to poor image quality, usually need gold coating

Beam damage can cause contamination of the specimen surface and in extreme cases alter or damage structures.

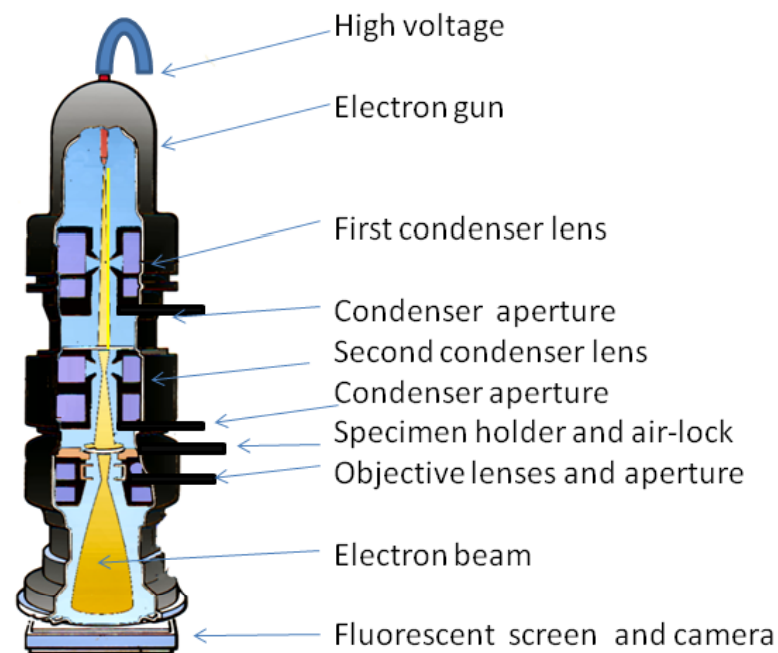


Transmission Electron Microscopy

Principle

A beam of electrons is passed through an ultrathin specimen producing a transmission image.

Electrons may be absorbed or scattered according to the composition of the sample. Electrons which are not scattered hit a fluorescent screen and the resulting image is focussed and magnified.



Transmission Electron Microscopy

Advantages

High resolution

Staining with heavy metals can be used which improves contrast of samples

Hydrated frozen samples may be observed by Cryo-TEM

Disadvantages

High cost

High vacuum

Sample preparation

Sample considerations

Thin samples typically less than 200 nm thick required. Sectioning may be required

Requires dehydrated samples for conventional TEM

Beam damage can cause contamination of the specimen surface and in extreme cases alter or damage structures

Transmission Electron Microscopy

Example PEGylated Liposomes

Samples imaged by TEM following Uranyl acetate staining

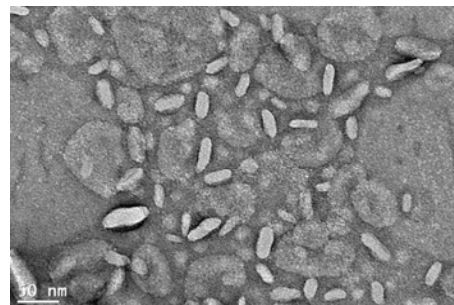
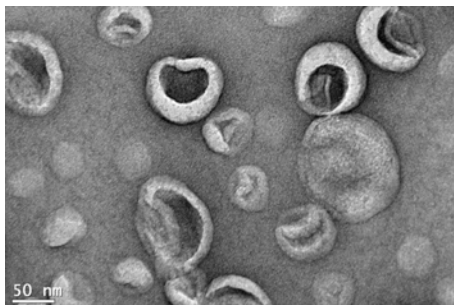
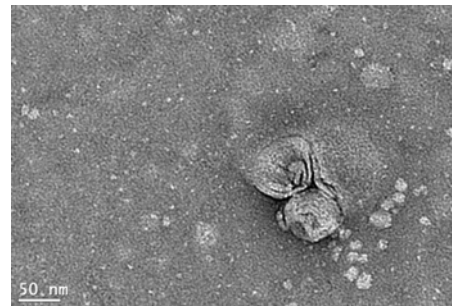
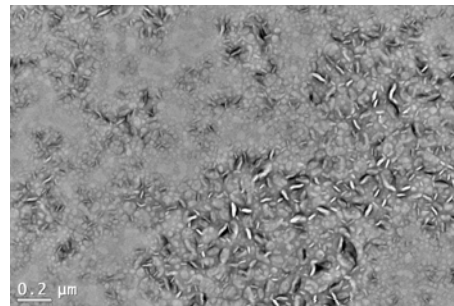
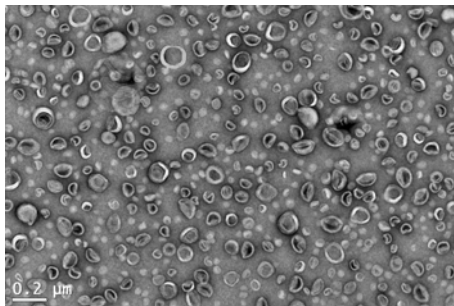
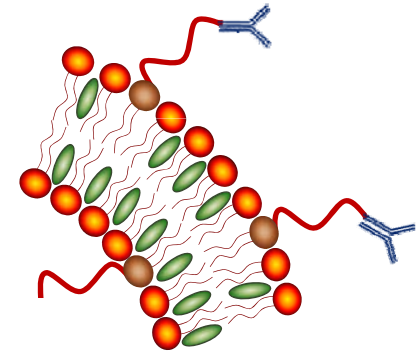
Different PEG concentrations and lipid membranes studied

Different structures observed;

- Spherical shells

- Rounded and flat disks

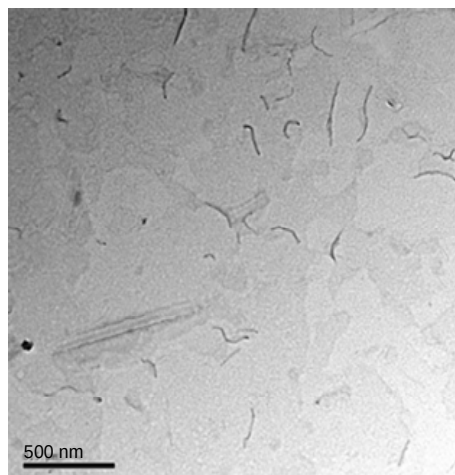
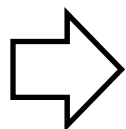
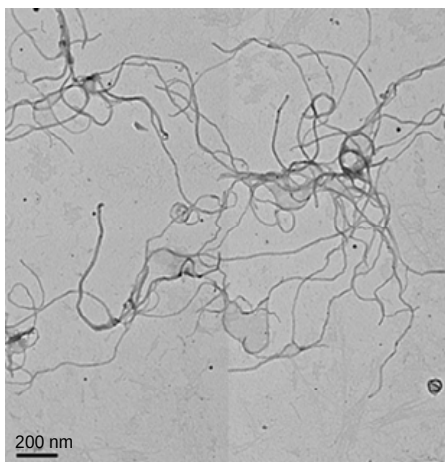
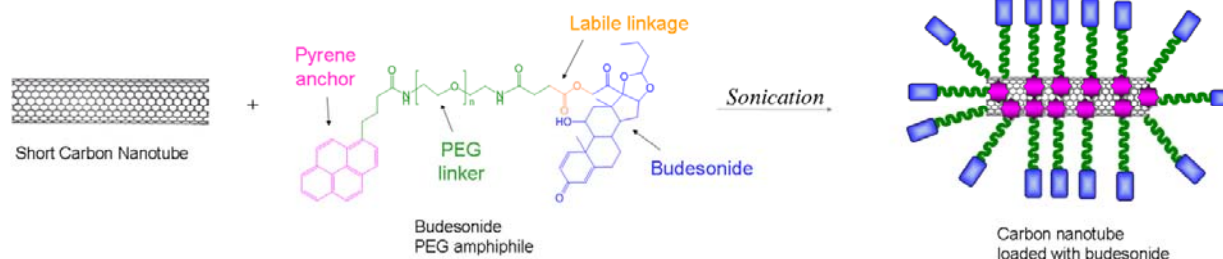
- Multi-lamellar?



Transmission Electron Microscopy

Example - Carbon Nanotubes

- Carbon nanotubes have been successfully functionalised with budesonide, via a pyrene-PEG amphiphilic complex.
- Can be shortened by ultrasonication to 200-300 nm.



Cryo - Electron Microscopy

Principle

A hydrated sample is rapidly frozen by plunging into liquid ethane (or similar)
The frozen sample is then imaged at cryogenic temperature

Advantages

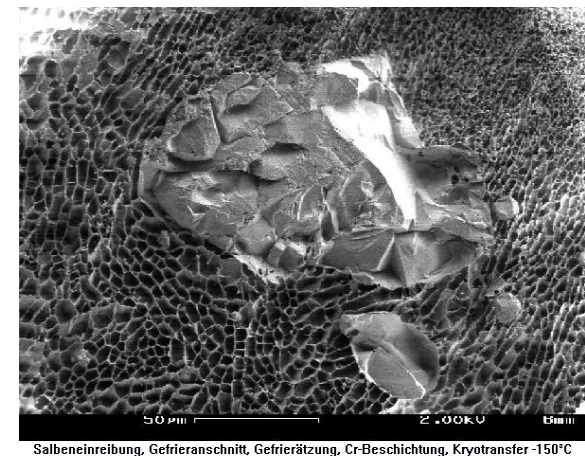
Allows the observation of hydrated samples which have not been fixed or stained in any way
Preserves 'solution state'
Can be only option for samples which can't be dried out (biological samples)

Disadvantages

Sample prep is very difficult!
Potential artefacts from ice crystal damage
Image contrast is very low
More prone to electron beam contamination or damage
Low through put
High cost

Sample considerations

Great care needs to be taken when preparing samples

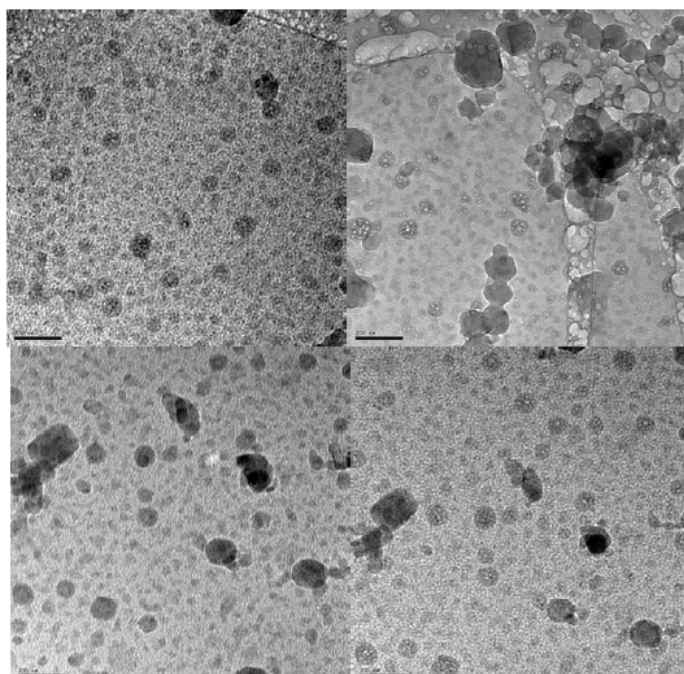
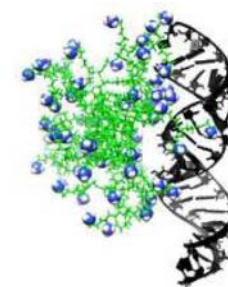


Cryo - Electron Microscopy

Cryo-TEM Example – siRNA polyplexes

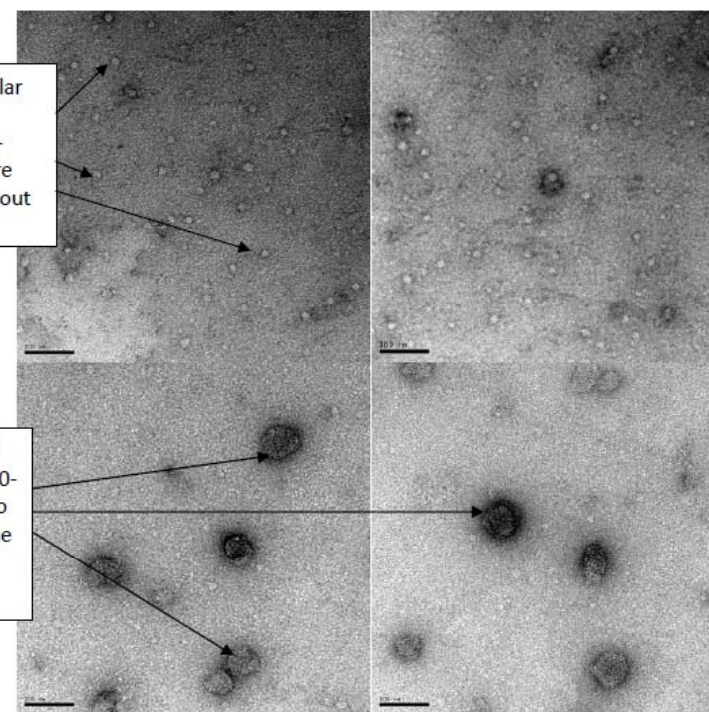
Complexes of siRNA and charged polymers

Different types of feature observed...



Distinct pale circular features of approximately 10-25nm diameter are observed throughout the preparation

Dark features of approximately 40-100nm were also observed in some areas of the preparation



Atomic Force Microscopy

Principle

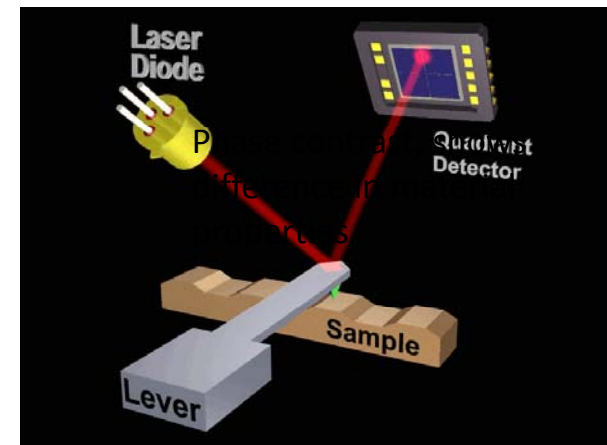
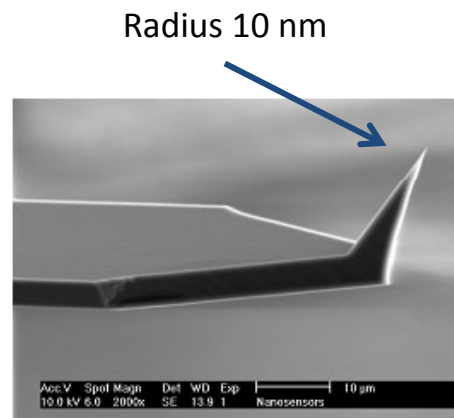
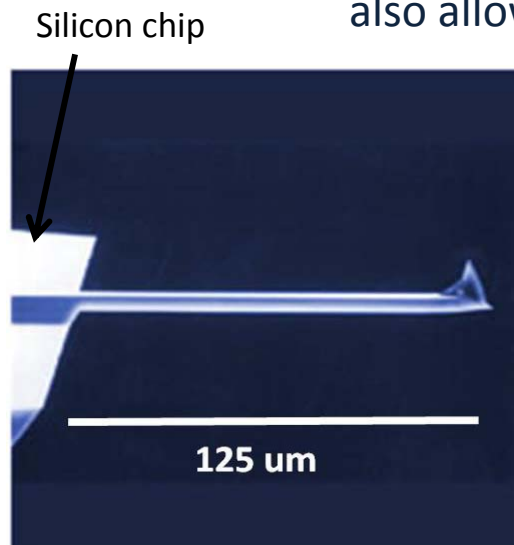
A 'Scanning Probe Microscopy' technique

Based on the deflection of a flexible micro-fabricated cantilever with a sharp probe

Probe is raster scanned across the sample surface

Contact Mode tip in constant contact 'scrapping' sample, can cause damage

Tapping Mode intermittent contact with sample, better for delicate samples
also allows simultaneous collection of topography and *phase* images



Atomic Force Microscopy

Principle

A 'Scanning Probe Microscopy' technique

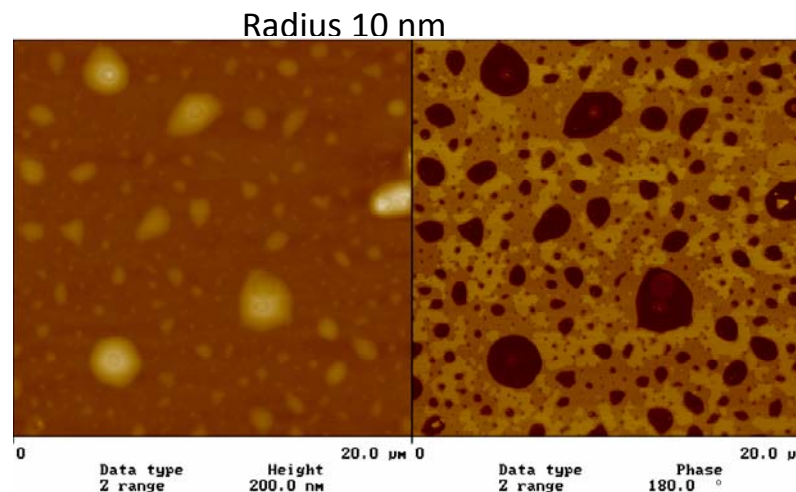
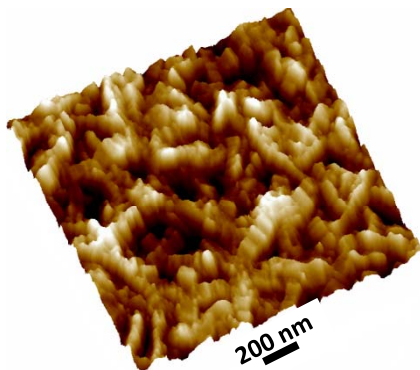
Based on the deflection of a flexible micro-fabricated cantilever with a sharp probe

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Tapping Mode intermittent contact with sample, better for delicate samples
also allows simultaneous collection of topography and **phase images**

Other modes...

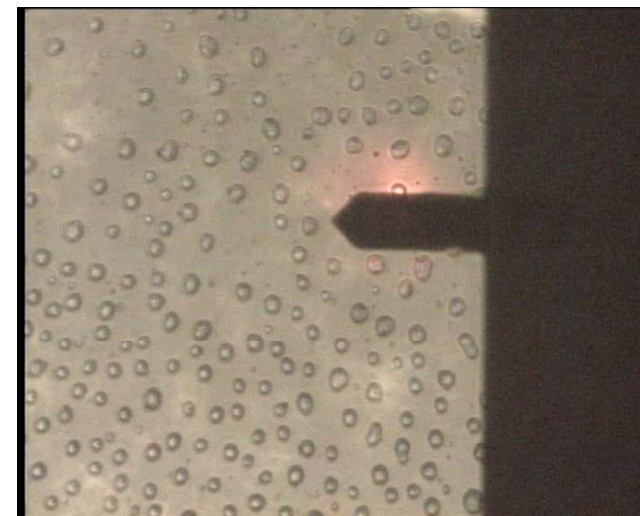
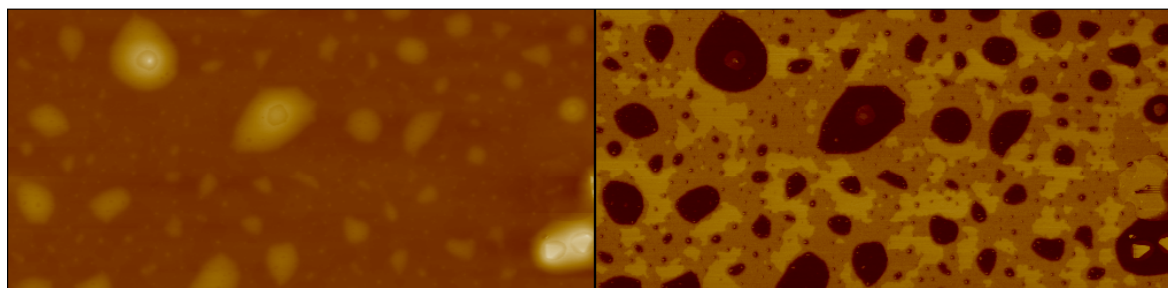


Phase contrast, shows
difference in material
properties

Atomic Force Microscopy

Principle

Image is constructed line-by-line as probe is raster scanned...



0	20.0 μm	0	20.0 μm
Data type	Height	Data type	Phase
Z range	200.0 nm	Z range	180.0 $^{\circ}$

Atomic Force Microscopy

Advantages

High resolution - can visualise nanosized features with relative ease

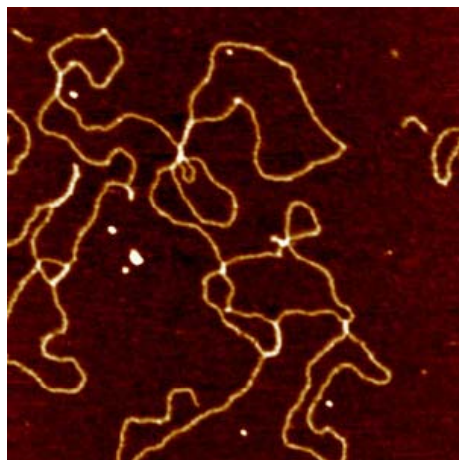
Can operate in variety of environments, liquid, vacuum, variable temperature/RH

Disadvantages

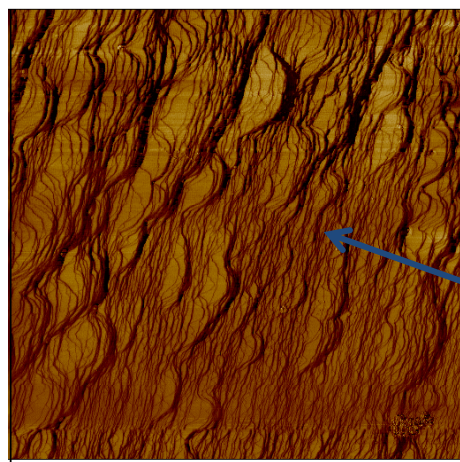
Scanning time

Imaging artefacts

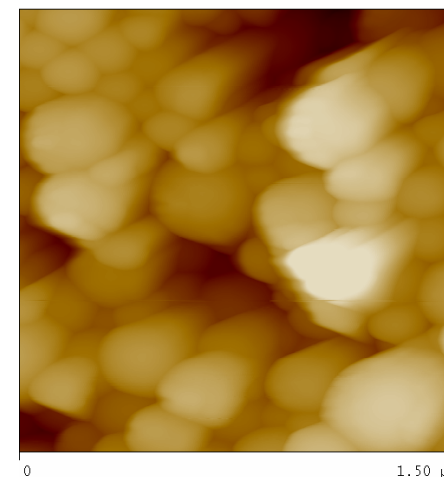
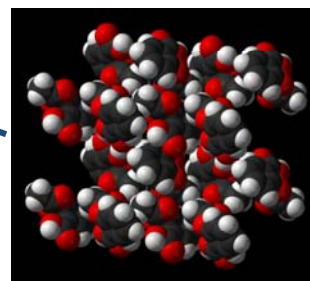
Sample preparation, especially for 'wet' samples



JPK Instruments



0 5.00 μm



0 1.50 μm

Atomic Force Microscopy

Sample considerations

Sample needs to be immobilised – minimise loose material
'flat' – maximum of a few microns in roughness

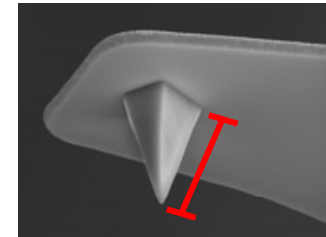
Options for nano-sized samples

Direct immobilisation on flat substrates (silicon, glass)

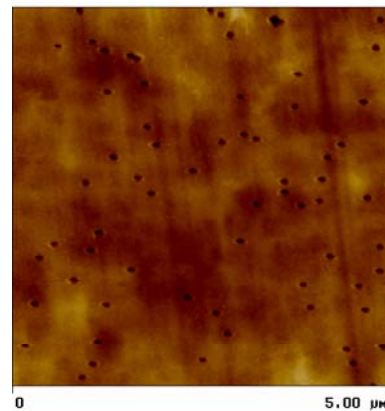
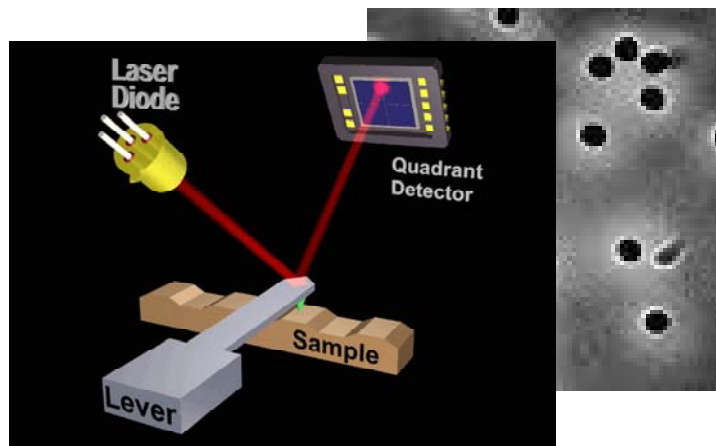
Resins/epoxys (on flat substrates)

Tempfix (waxy resin)

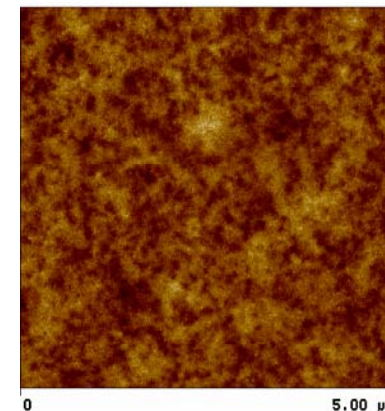
Filters/polymer substrates



Limited by tip height (2-10 μm)
and scanner range (5-20 μm)



Nucleopore filter
(Roughness $\approx$ 10 nm)



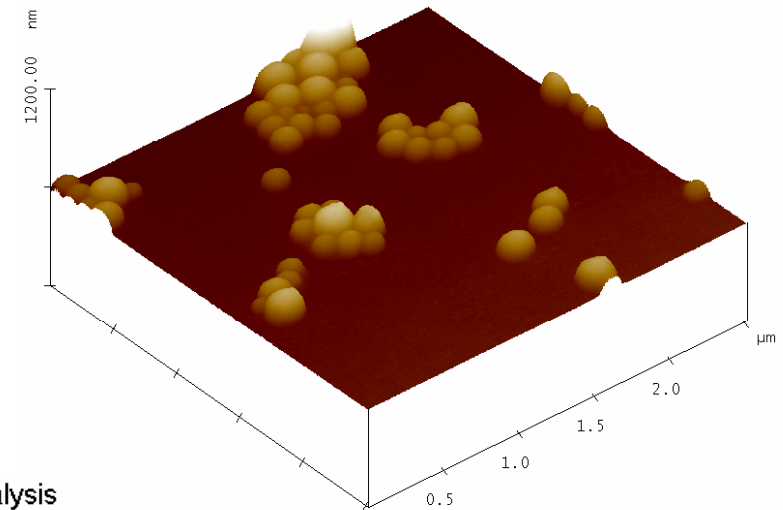
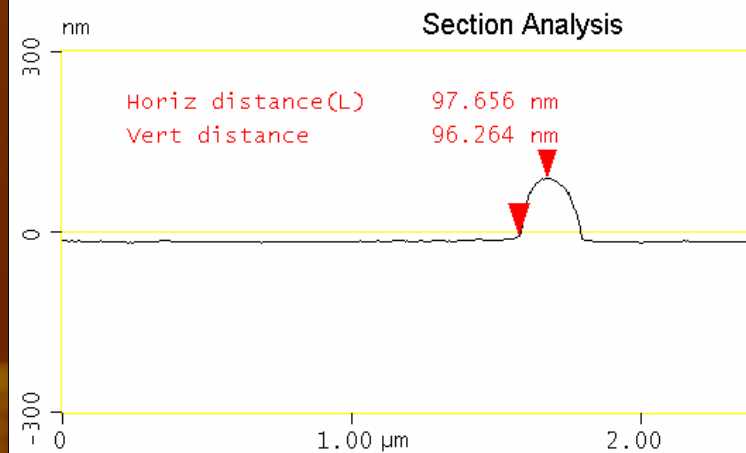
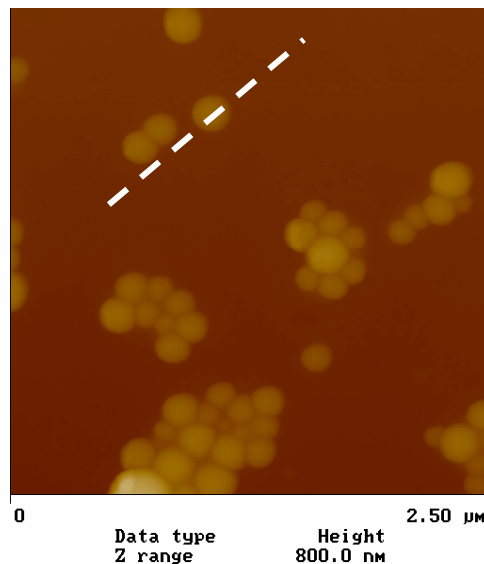
Tempfix resin
(Roughness $\approx$ 1 nm)

Atomic Force Microscopy

Example – PLGA nanoparticles for siRNA delivery

Since the AFM produces 3D images we can measure height

Nanoparticle height 100 nm
width 200 nm
Sample preparation?



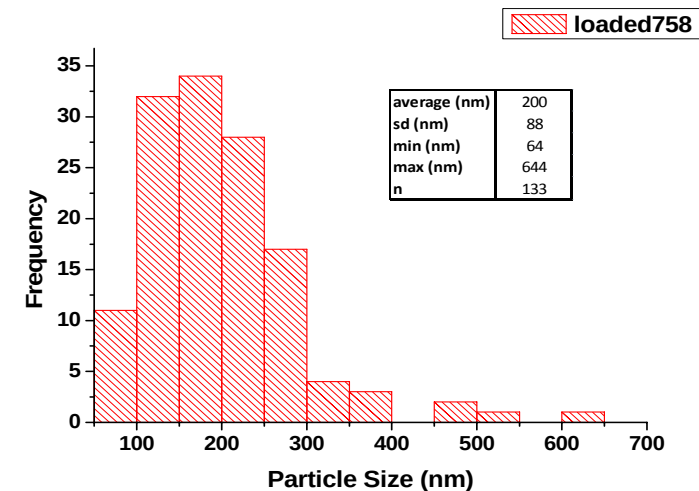
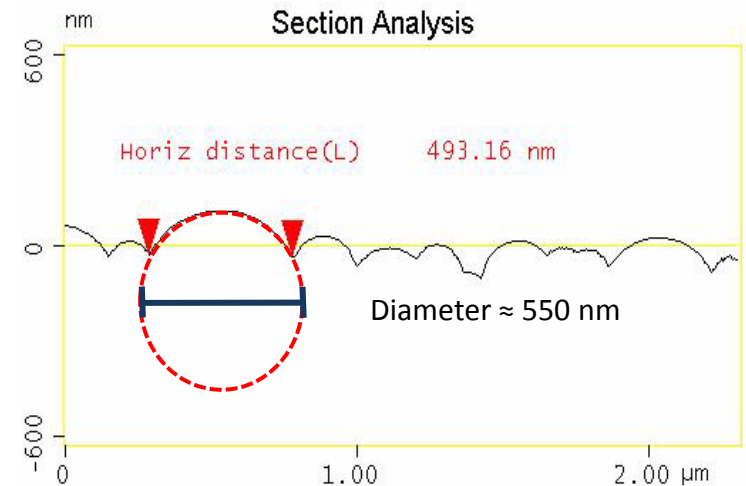
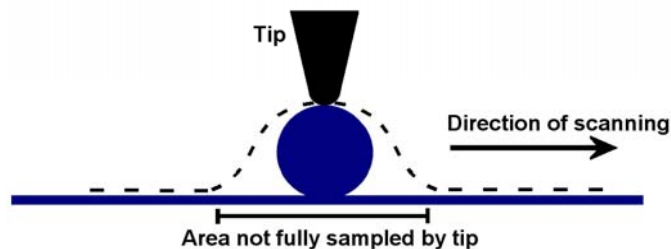
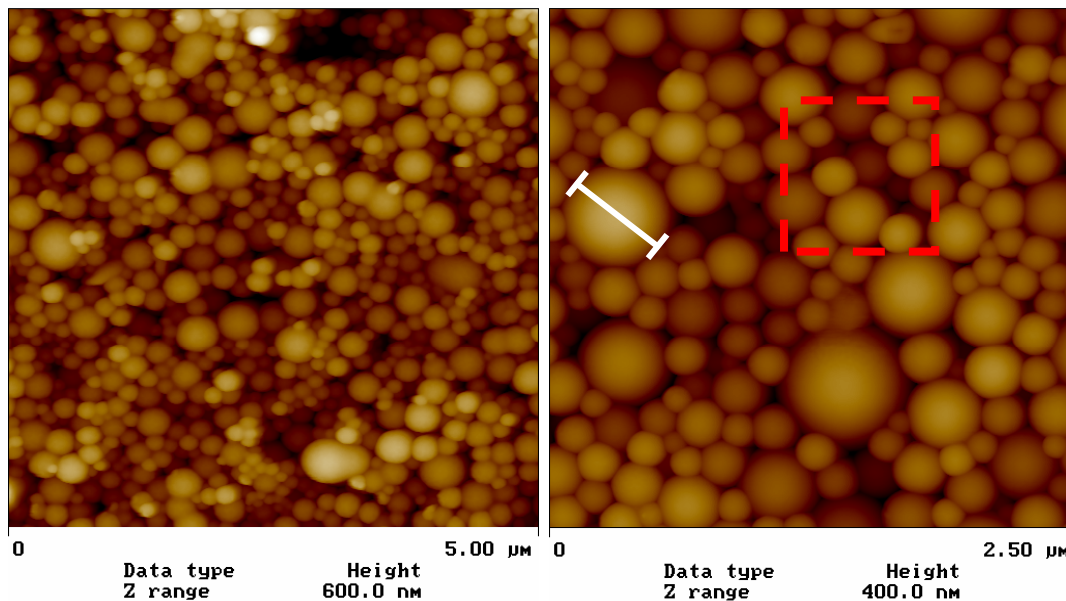
Atomic Force Microscopy

Example – PLGA nanoparticles for siRNA delivery

Imaging a more concentrated sample allows an overview of particle morphology

Can provide an estimate of particle size

be aware of imaging mechanism...



Atomic Force Microscopy

Example – PLGA nanoparticles for siRNA delivery

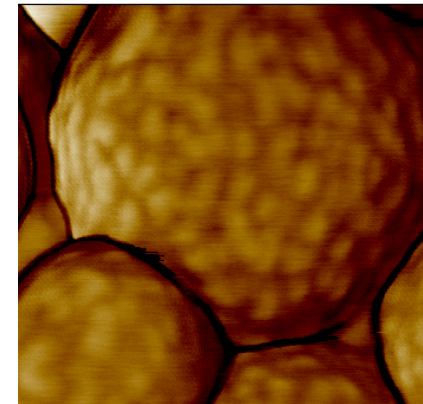
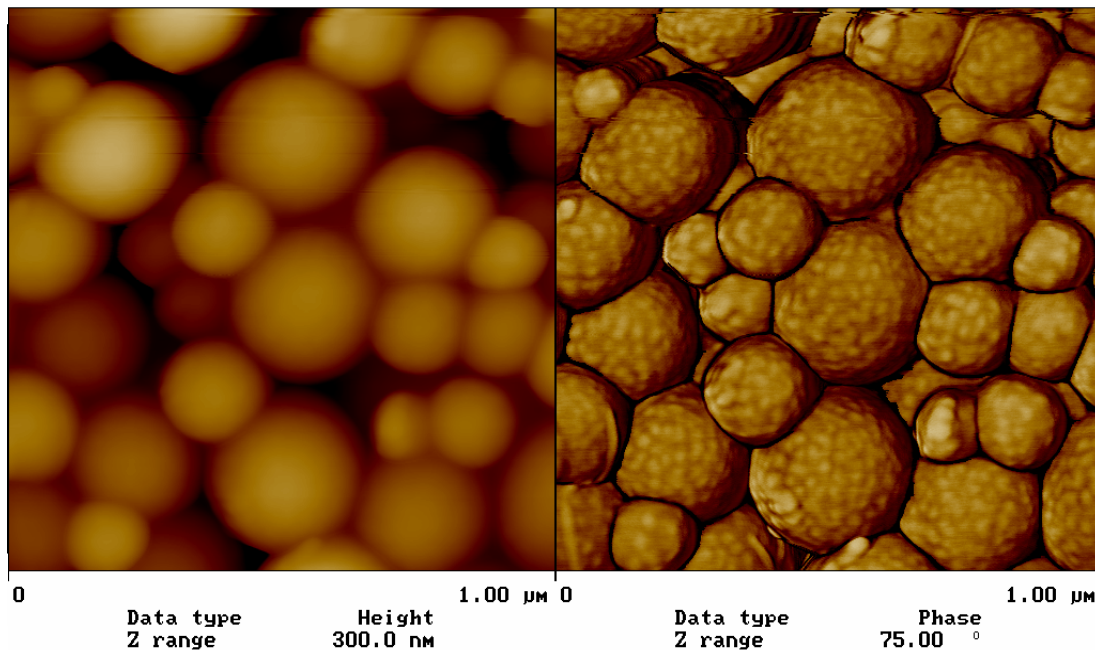
Imaging a more concentrated sample allows an overview of particle morphology

Can provide an estimate of particle size

be aware of imaging mechanism...

Phase imaging can highlight subtle surface detail

Features approx 20-30 nm in width
2-3 nm in height



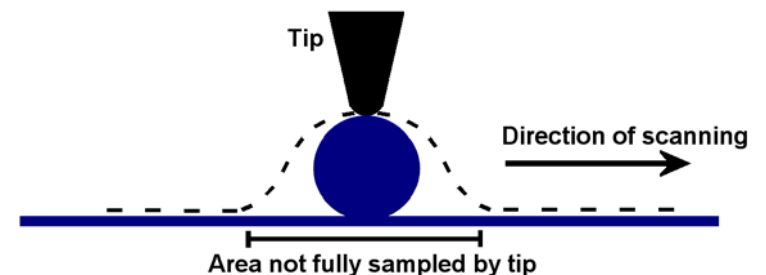
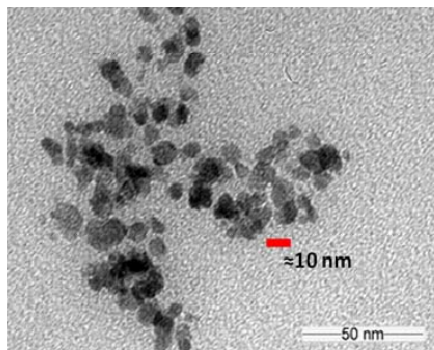
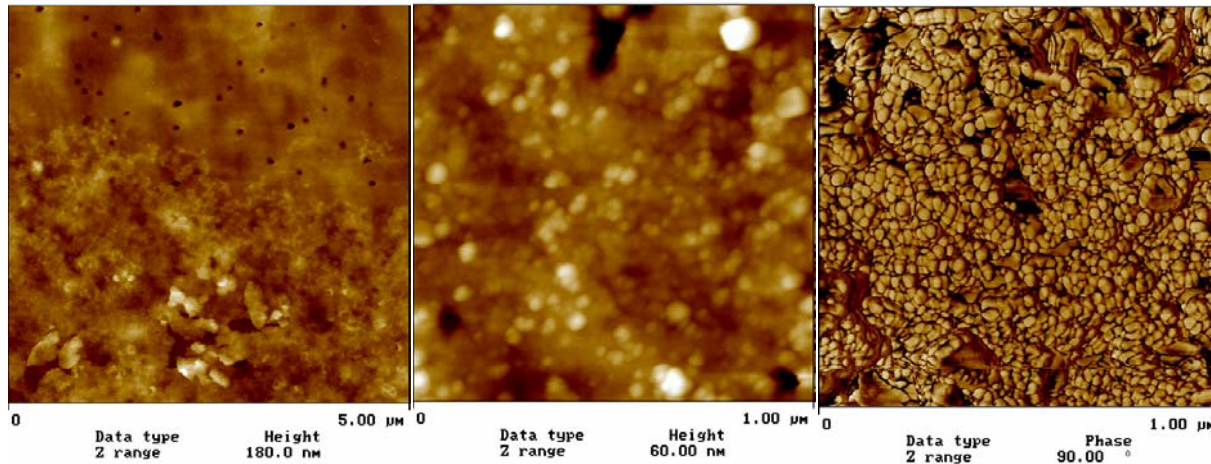
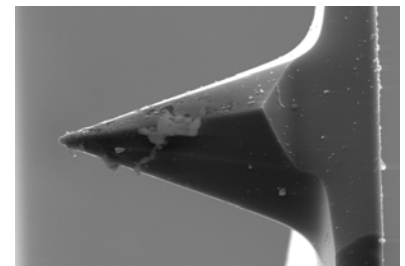
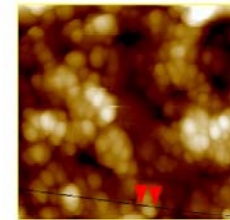
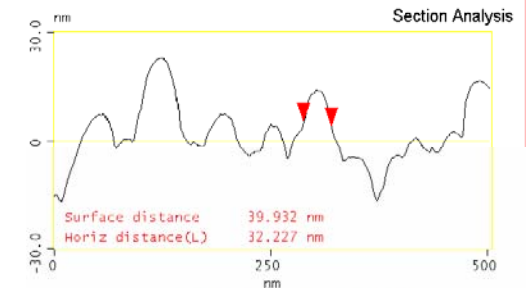
Atomic Force Microscopy

Example – Iron Oxide Nanoparticles

TEM suggests particle size down to 10 nm

Sample prep can be more difficult...

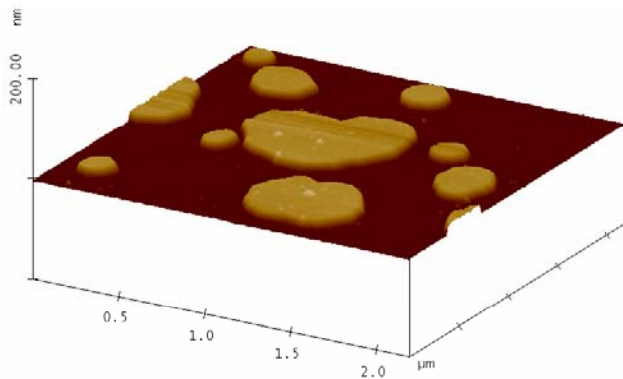
Due to small size influence of probe is much more significant



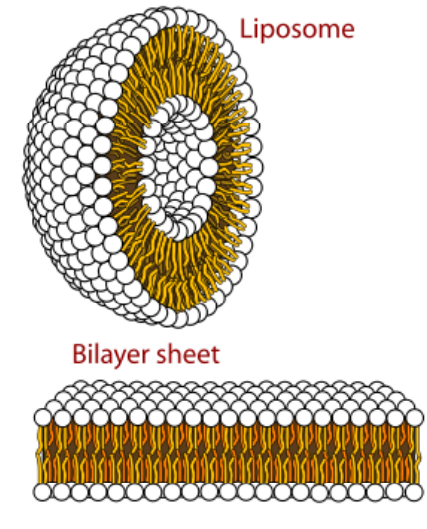
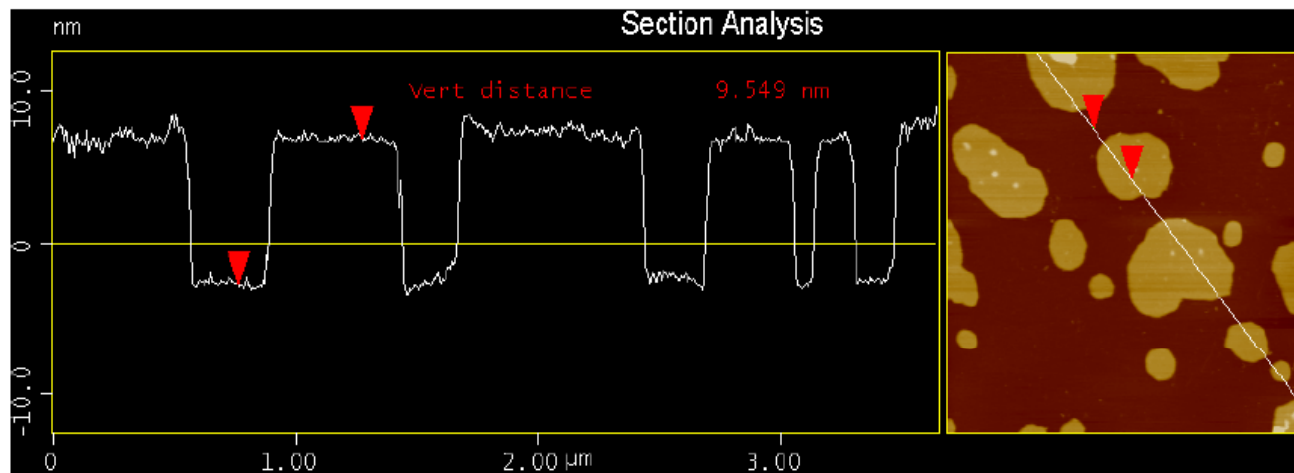
Atomic Force Microscopy

Example – Liposomes

Liposomes in solution can be 'collapsed' onto a substrate for AFM imaging



Can measure the height of the bilayer ≈ 10 nm

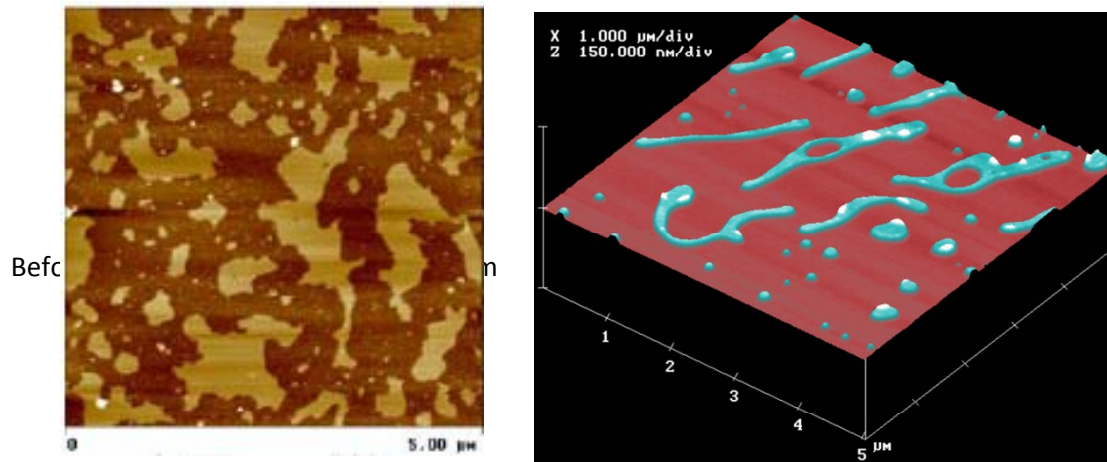
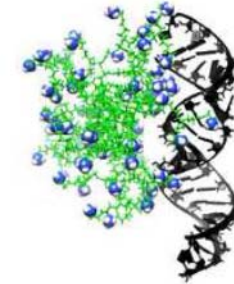


Atomic Force Microscopy

Example – siRNA/polymer polyplexes

Imaging of 'collapsed' polyplexes in liquid environment

Look for changes whilst adding polymer in-situ

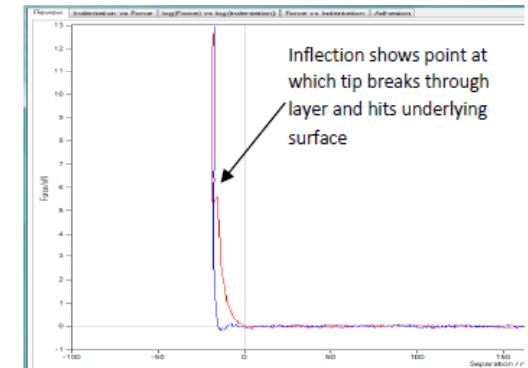


Atomic Force Microscopy

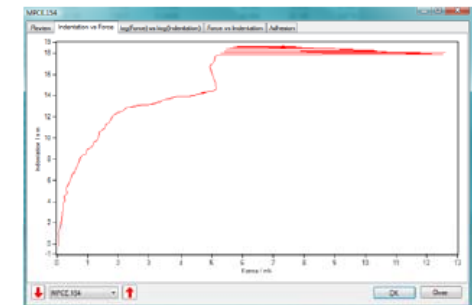
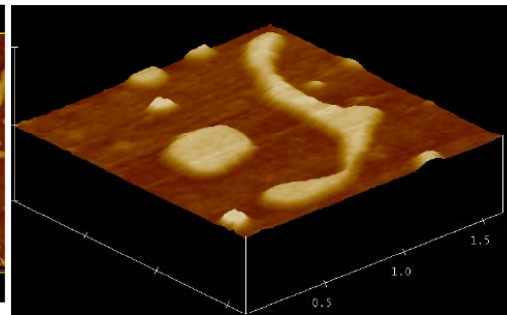
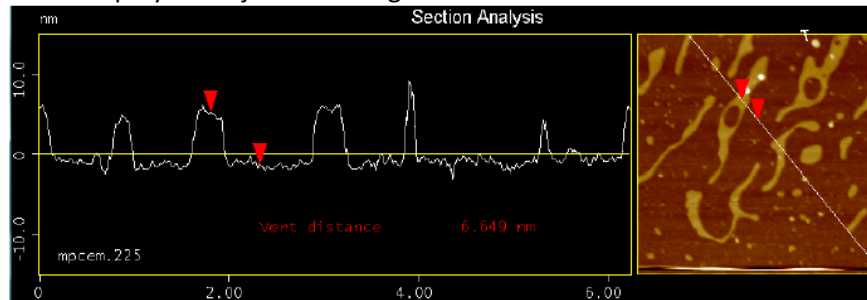
Example – siRNA/polymer polyplexes

Imaging of 'collapsed' polyplexes in liquid environment

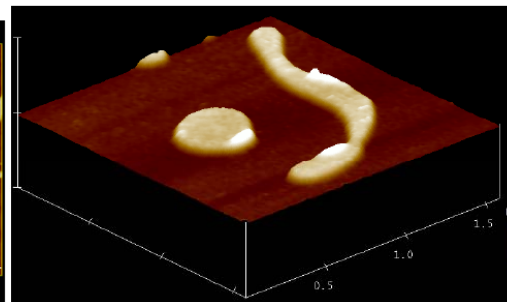
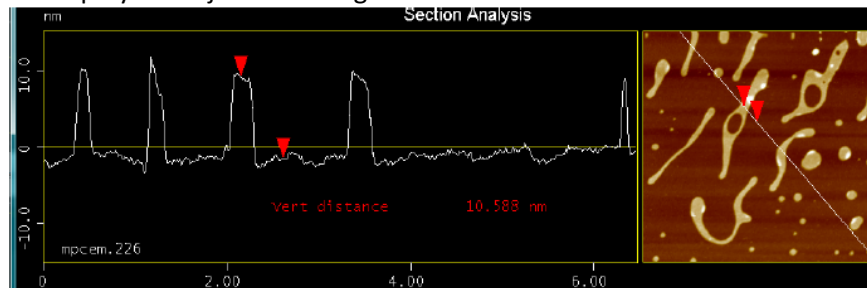
Look for changes whilst adding polymer in-situ



Before polymer injection – height = 7 nm



After polymer injection – height = 11 nm



Surface Chemical Techniques

X-ray photoelectron spectroscopy

Time of flight secondary ion mass spectroscopy

Particularly applicable to nanometre scale studies

Other available, Raman, IR etc,

X-ray Photoelectron Spectroscopy

Principle

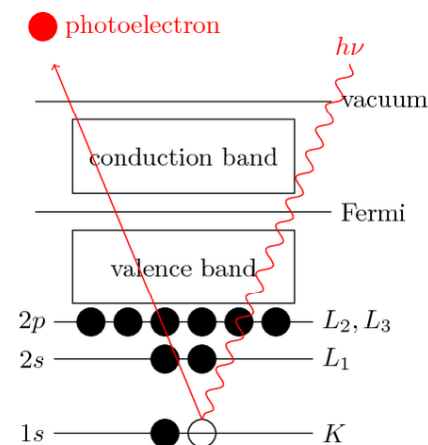
Photoelectric effect; X-rays in – electrons out

Kinetic energy of emitted
electron (measured)

Binding energy of emitted
electron (calculated)

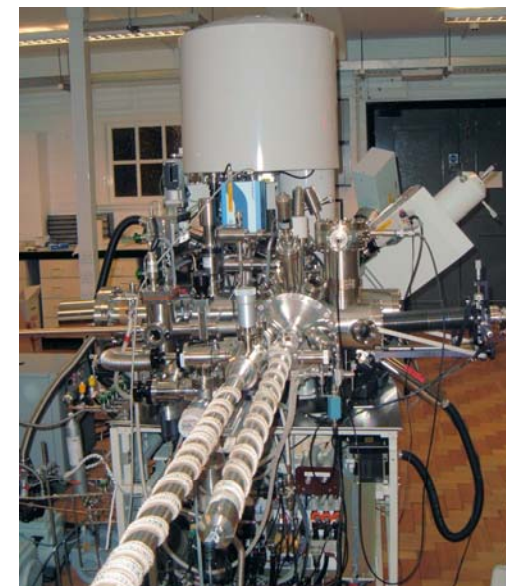
$$KE = h\nu - BE$$

Energy of photon in
(known)



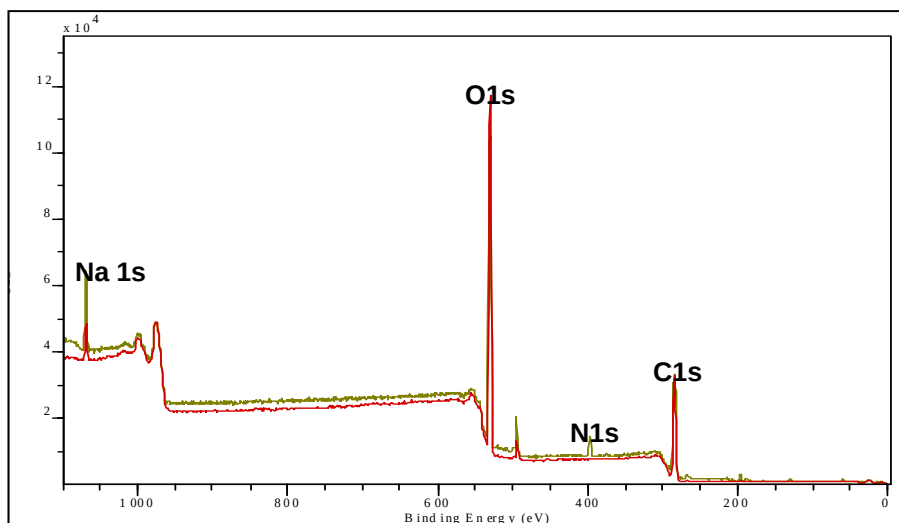
Binding energies are characteristic of elements

- Ultra high vacuum
- Sample the top 5-10nm of a surface
- Elemental detection ≈ 0.1 at.%
- Quantitative elemental composition
- Detailed information on functional groups



X-ray Photoelectron Spectroscopy

Example data



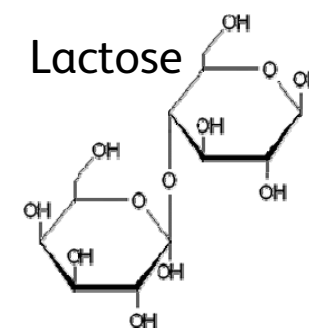
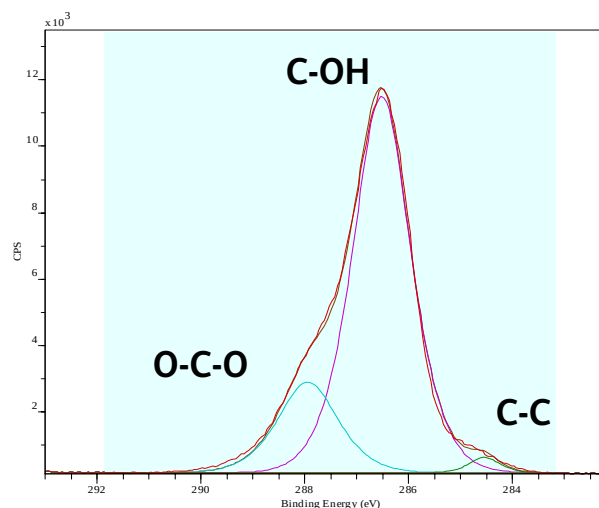
Survey 'wide scan' spectra of microparticle raft

Red Trace: Polymer only
Green Trace: Protein loaded

Presence of Nitrogen shows protein at the surface

Core line 'high res scan' of lactose

Three different carbon functionalities resolved in the molecule



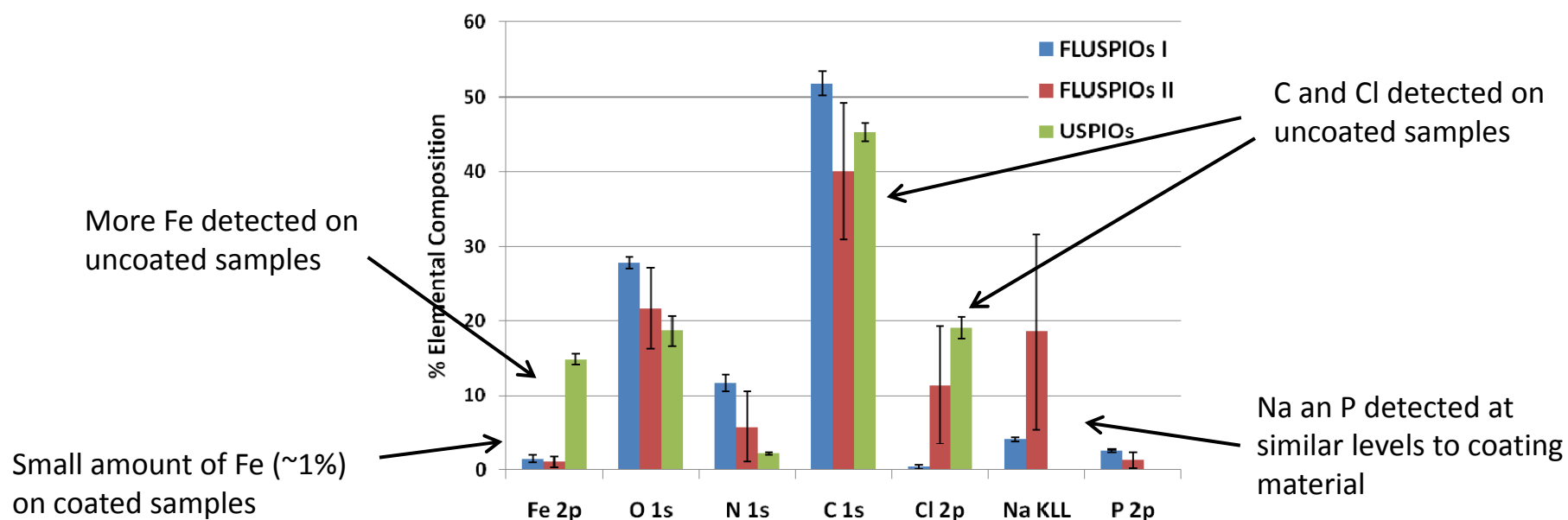
X-ray Photoelectron Spectroscopy

Examples – Iron Oxide nanoparticles

Characterising the surface composition of Iron Oxide nanoparticles;

Coated with an organic material (blue and red)

Uncoated (green)



X-ray Photoelectron Spectroscopy

Advantages

Provides elemental chemical information

Surface Specific $\approx 5\text{-}10\text{ nm}$

Provides information on chemical environments of elements

Has mapping/imaging capabilities

Can be performed at cryo temperatures

Disadvantages

Ultra high vacuum

Spatial resolution limited

Sensitive to $\approx 0.1\text{at}\%$

Expensive

Sample considerations

'Vacuum proof'

Immobilise on a relatively flat substrate

At least $0.1\text{ at}\%$ as a marker for molecule of interest

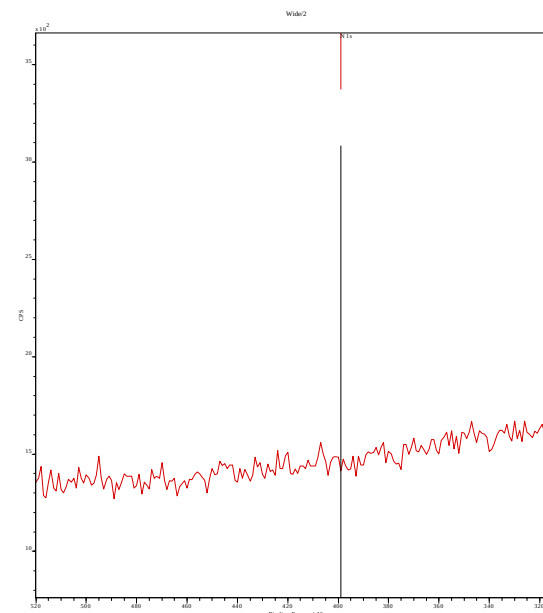
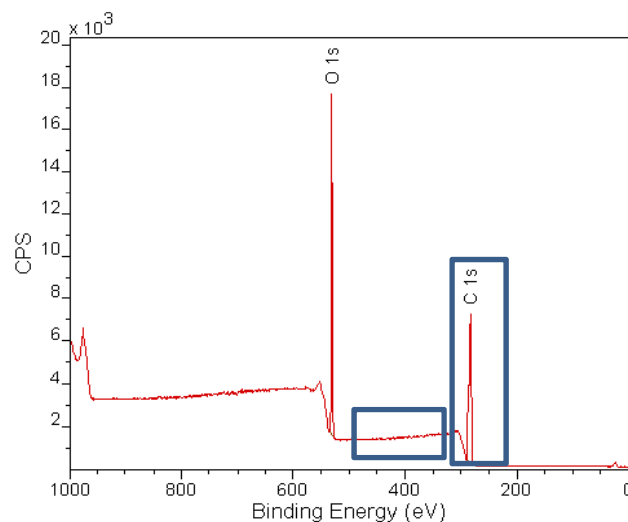
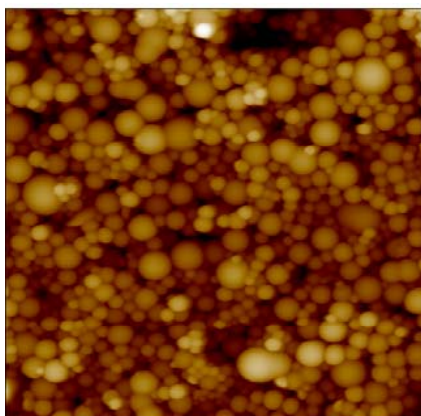
X-ray Photoelectron Spectroscopy

Examples – PLGA nanoparticles for siRNA delivery

siRNA loading of $\approx 2 \mu\text{g/mL}$ polymer- on the surface?

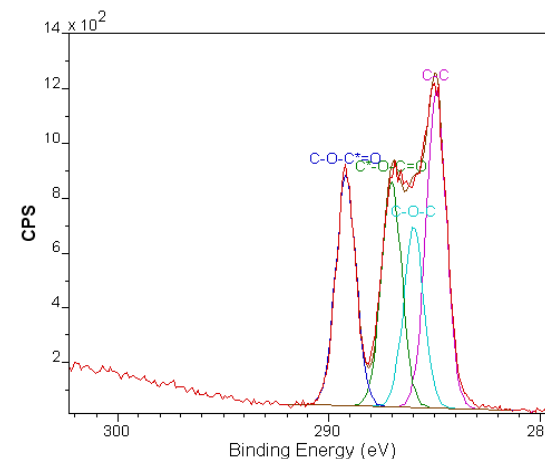
The siRNA has N, the polymer only C and O

Use N as a marker



Not sensitive enough to pick up siRNA...

Lots of chemical information on the carbon $\rightarrow$

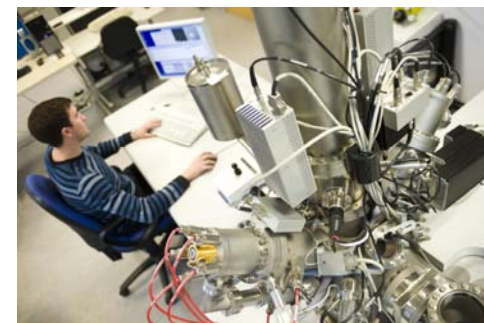
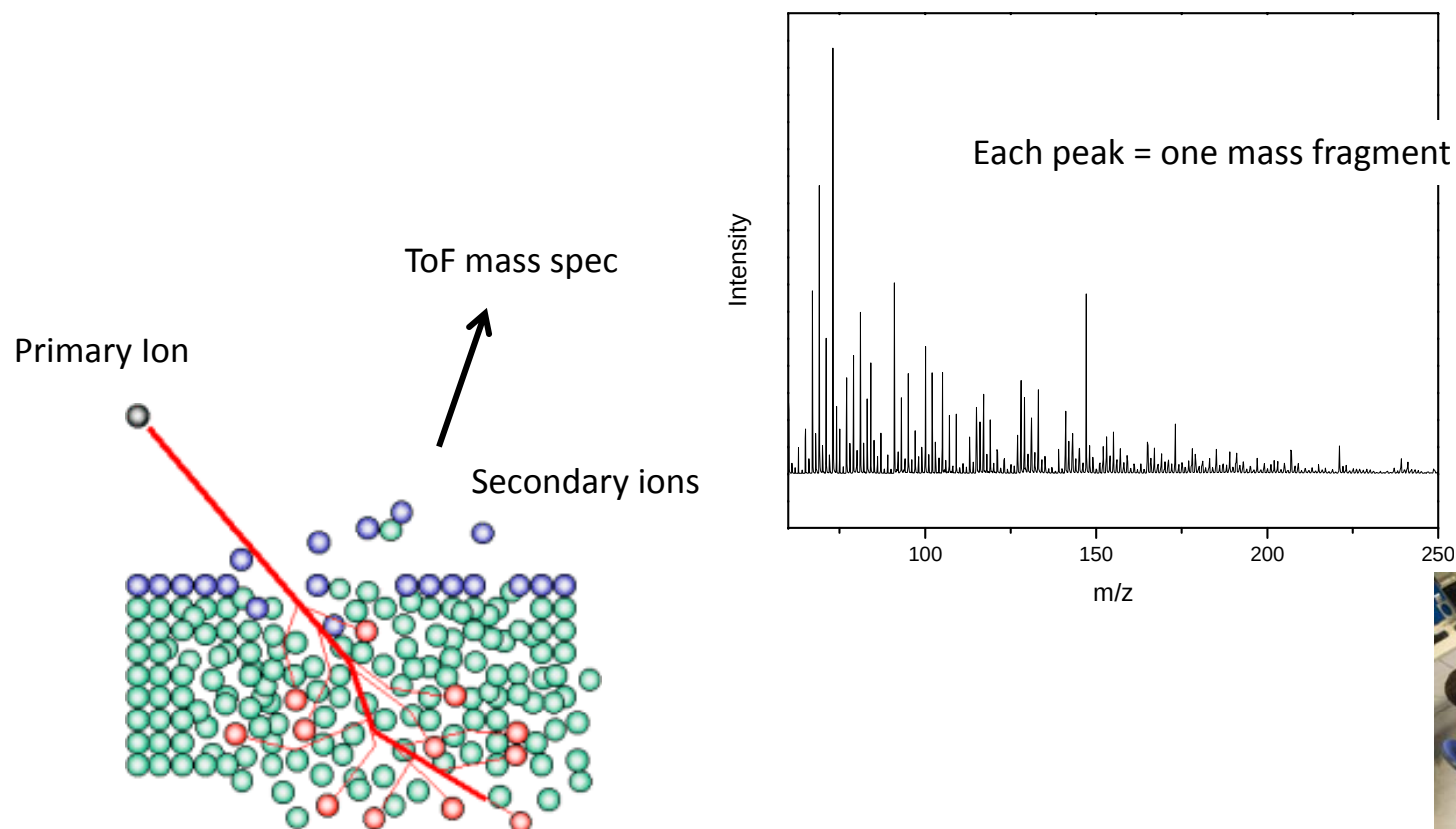


Time of Flight Secondary Ion Mass Spectrometry

Principle

A primary ion gun breaks up the surface into fragments (secondary ions) which are measured by mass spectroscopy

Highly surface specific process (1-2 molecular layers)



Time of Flight Secondary Ion Mass Spectrometry

Advantages

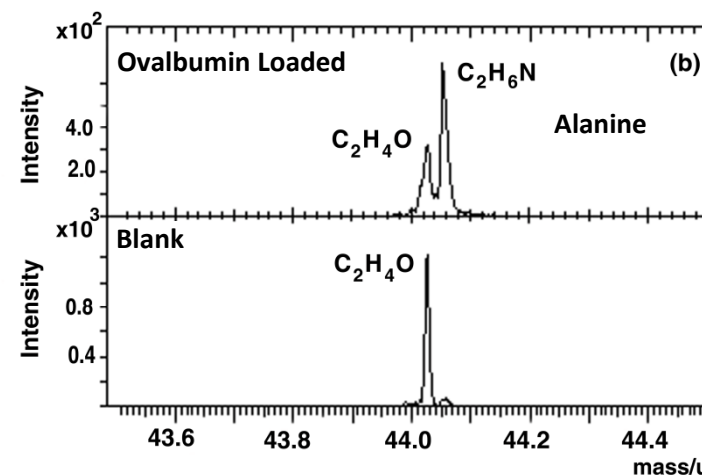
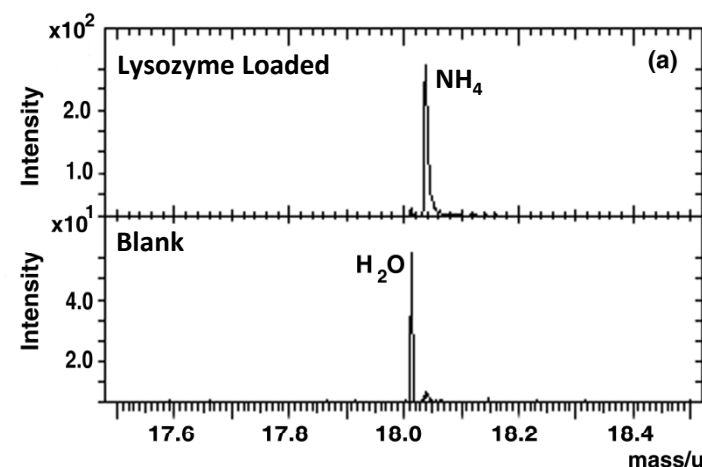
- Extremely surface sensitive
- Very high mass resolution →
- Good quality mapping
- Depth profiling
- Can be performed at cryo temperatures

Disadvantages

- Ultra high vacuum
- Sample preparation and handling
- Extremely sensitive to contamination
- Not quantitative
- Interpretation tricky

Sample considerations

- Contamination – careful handling required
- Immobilise on a relatively flat substrate

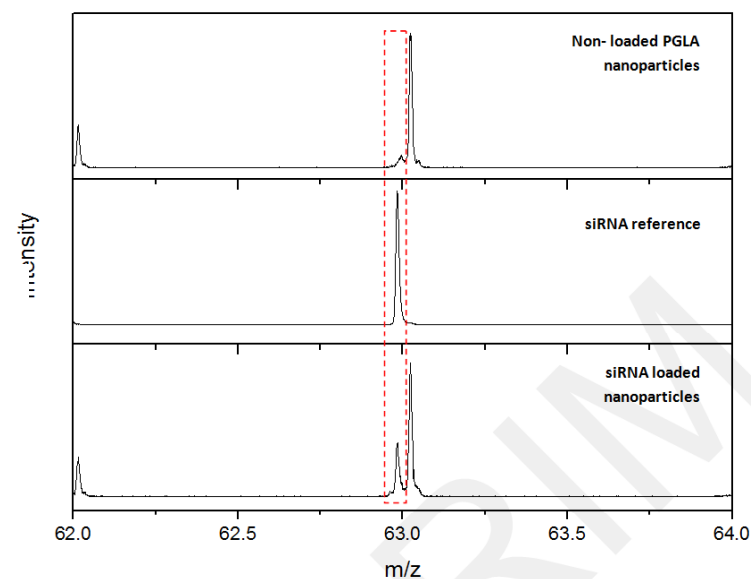
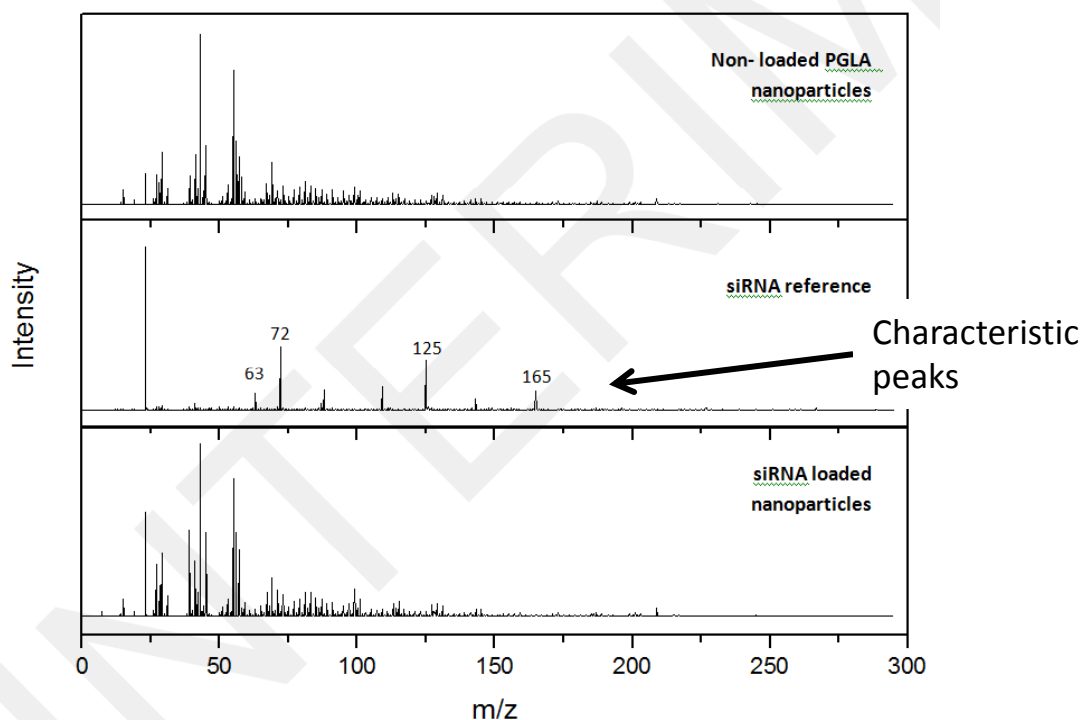
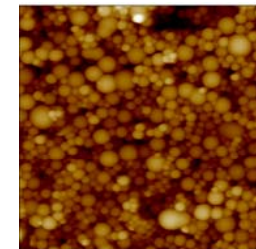


Time of Flight Secondary Ion Mass Spectrometry

Example – PLGA nanoparticles for siRNA delivery

XPS did not show any siRNA on the surface... try ToF-SIMS

Using a reference sample of pure siRNA, we can find characteristic peaks



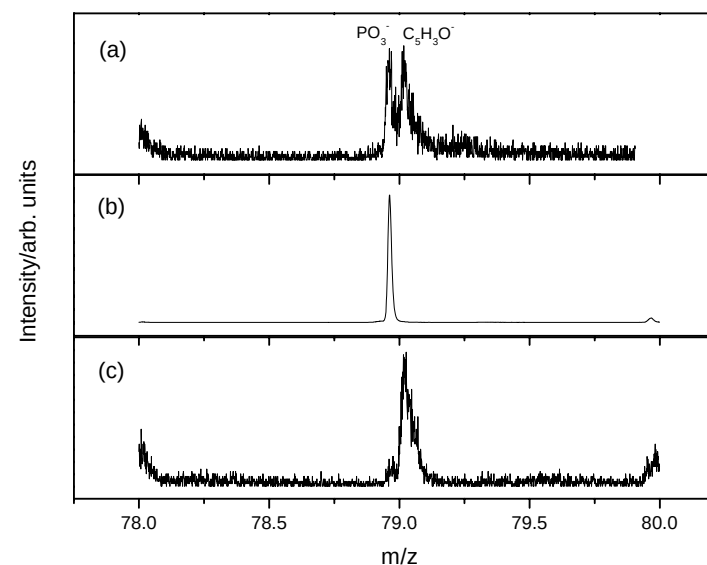
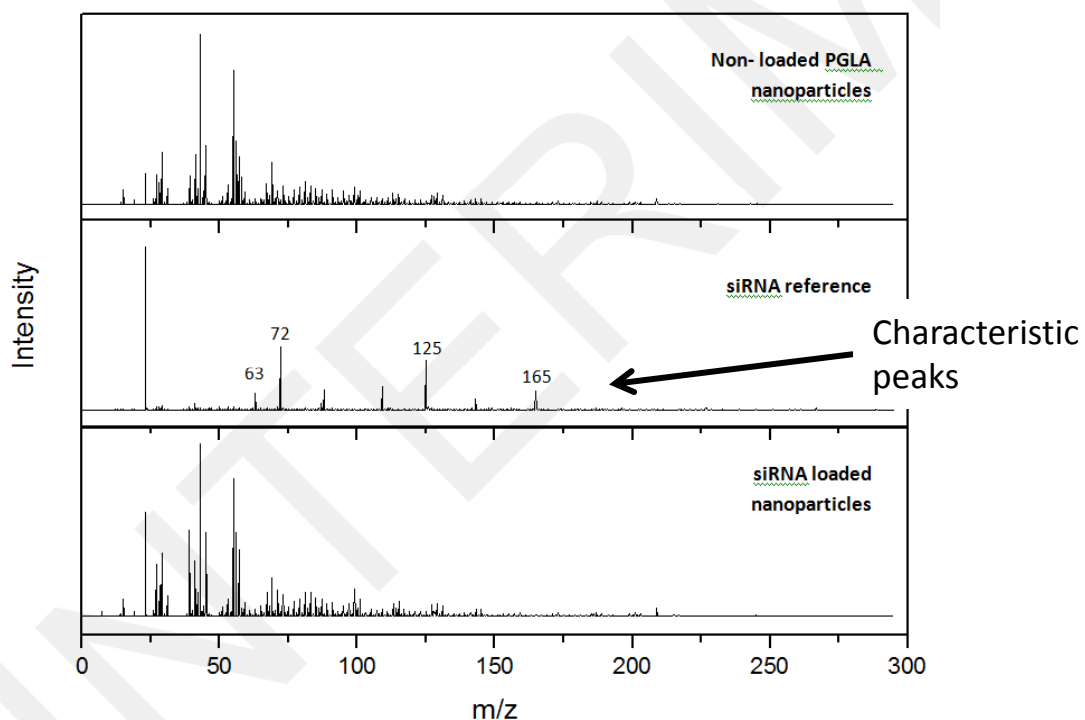
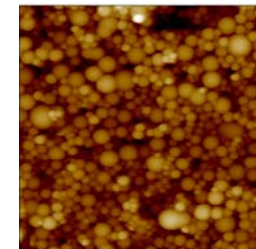
Our reference peak present on the loaded sample, missing on the placebo

Time of Flight Secondary Ion Mass Spectrometry

Example – PLGA nanoparticles for siRNA delivery

XPS did not show any siRNA on the surface... try ToF-SIMS

Using a reference sample of pure siRNA, we can find characteristic peaks



Our reference peak present on the loaded sample, missing on the placebo

Presence of phosphate groups from siRNA shown

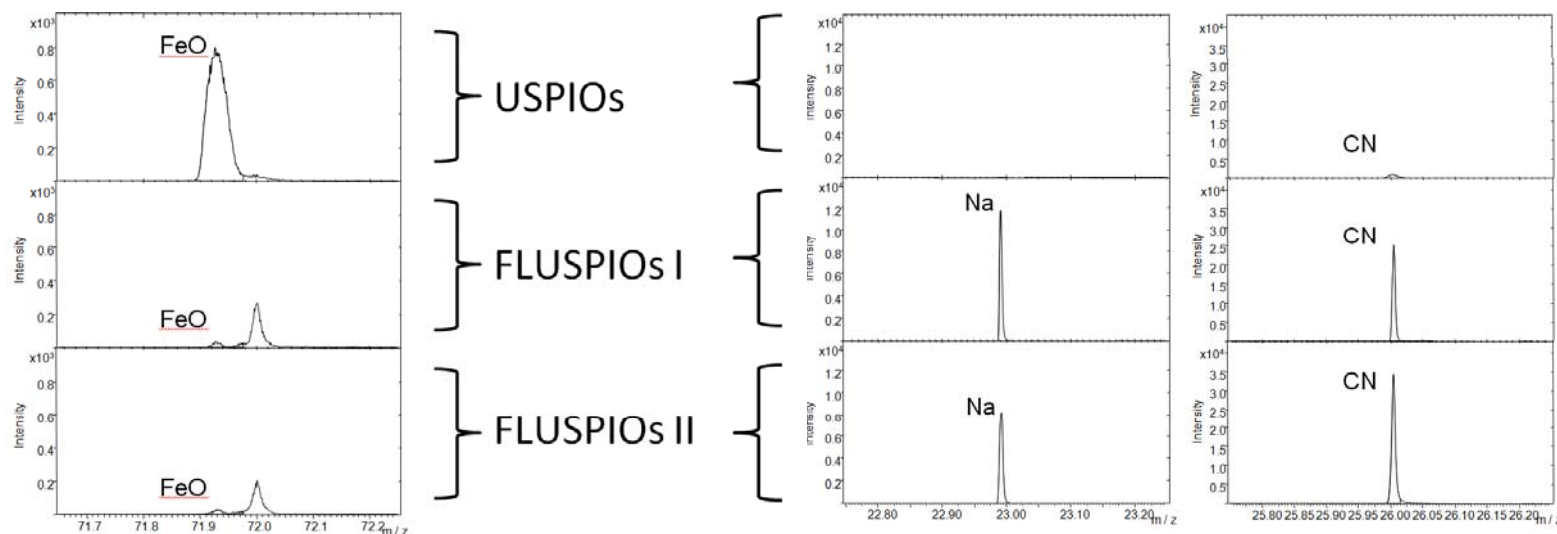
Time of Flight Secondary Ion Mass Spectrometry

Examples – Iron Oxide nanoparticles

Characterising the surface composition of Iron Oxide nanoparticles;

Coated with an organic material (FLUSPIOs)

Uncoated (USPIOs)



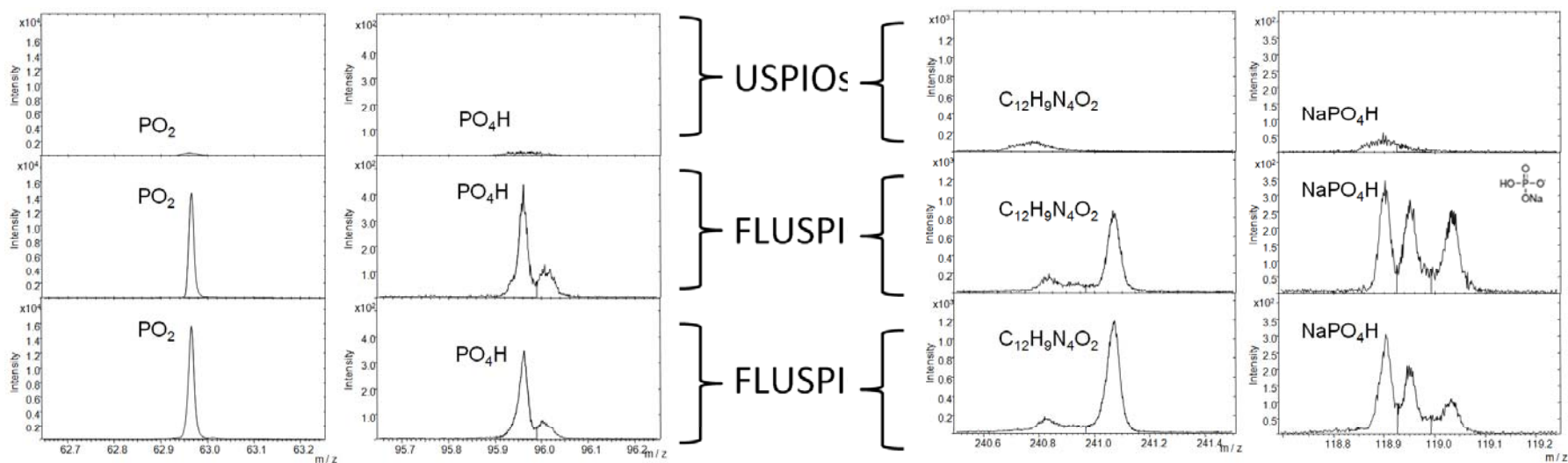
Time of Flight Secondary Ion Mass Spectrometry

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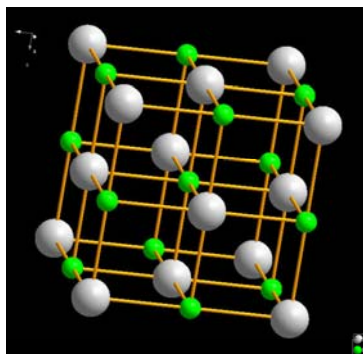
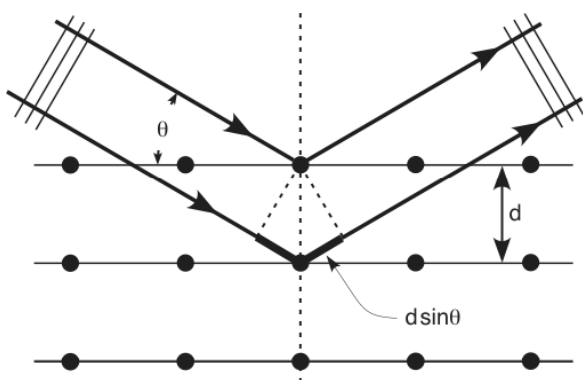
X-ray diffraction

Principle

XRD can be used to examine the crystallographic structure of materials

The diffraction of X-rays from a crystalline material is governed by Bragg's law

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



X-ray diffraction

Principle

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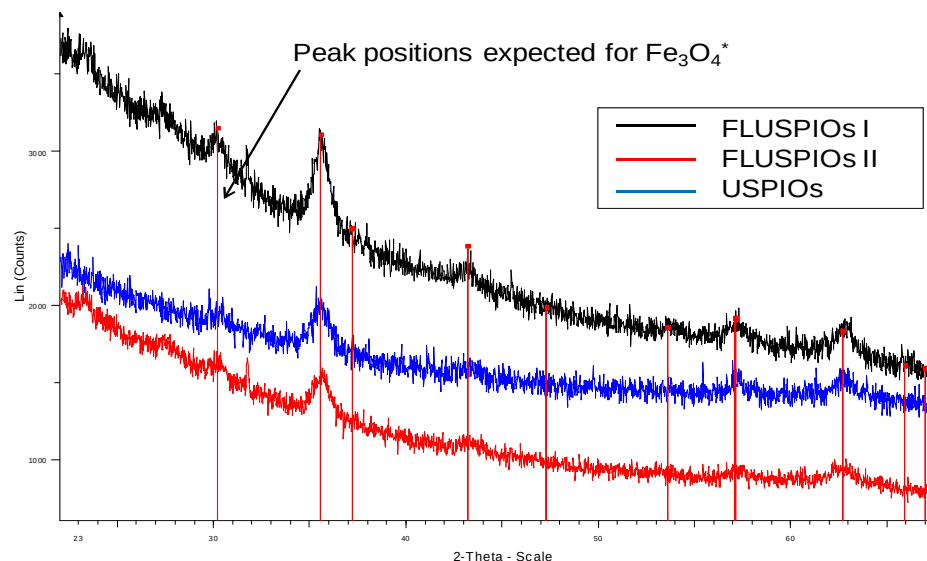
$$2d \sin \theta = n\lambda$$

Example – FeO nanoparticles

XRD allows determination of the crystal structure of the nanoparticles...

And an estimate of crystallite size (not particle size)

Sample	Fe ₃ O ₄ Crystallite Size (nm)	Rwp* (%)
USPIOs	9	2.8
FLUSPIOs I	10	2.1
FLUSPIOs II	10	2.9



* Acta Crystallographica B (1996), 53, 762-6

Particle size – PCS/DLS

Principle

The sample is placed in a suitable cuvette and illuminated by a laser.

The intensity of the laser source is adjusted

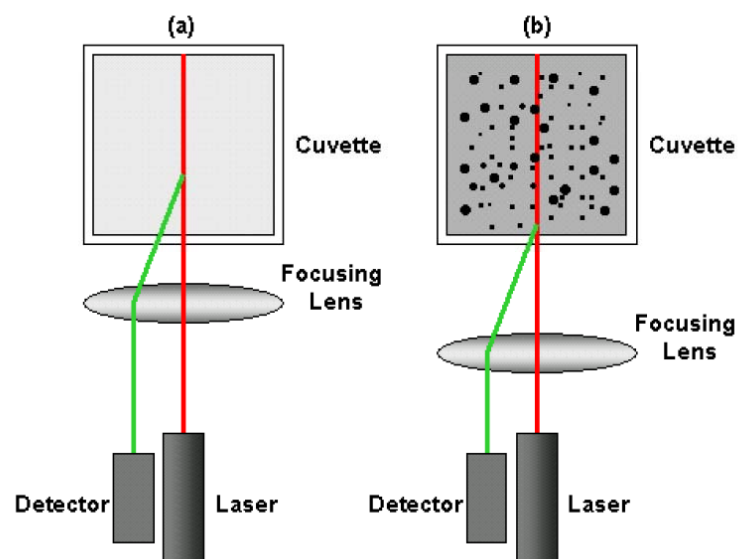


Figure 10: Schematic diagram showing the measurement position for (a) small, weakly scattering samples and for (b) concentrated, opaque samples. The change in measurement position is achieved by moving the focusing lens accordingly

The scattering intensity is compared at successive time intervals (photon correlation" function)

Particle size is determined by the software from the rate at which the intensity is changing

Theory

Brownian Motion- the random movement of particles due to the bombardment by the solvent molecules around them.

The larger the particle the slower the motion

Hydrodynamic Diameter- The diameter of a sphere which has the same translational diffusion coefficient (D) as the particle

The Stokes- Einstein Equation

$$r = \frac{k_B T}{6\pi\eta D}$$

Where r is the hydrodynamic radius, k_B is Boltzmann's constant, T is temperature and η is solvent viscosity.

Particle size – PCS/DLS



Advantages

Fast and relatively simple to operate. Settings automatically adjusted

Wide range of sample concentrations

Observation in natural hydrated state

Disadvantages

Smallest detectable size might be only 25-35 nm for particles of low refractive index.

Small particles may not be detected in polydisperse samples (low peak resolution)

Sample considerations

Sample volume

Particle size range 1 nm - 6 μ m depending on particle type

Translational diffusion coefficient is also affected by:

- Ionic strength of solvent

- Surface structure of the particles- a layer which is sticking out will reduce the speed more than one that is lying flat on the surface.

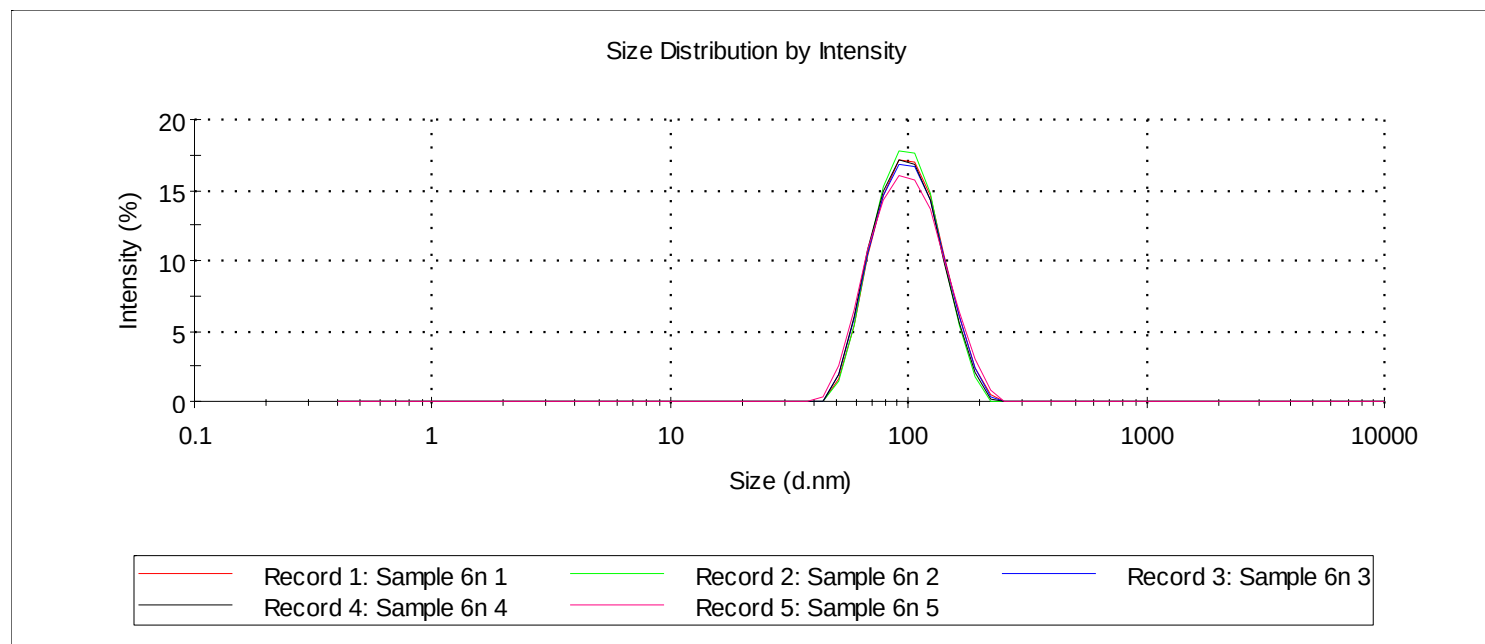
- Shape of the particles

Particle size – PCS/DLS

Example – PEGylated liposomes

Particle size of a monodisperse liposome suspension.

Five sample measurements with a z-average mean of 94nm and a polydispersity index (Pdl) of 0.093



Particle size – Nanosight™

Principle

Nanoparticle Tracking Analysis - particle-by-particle sizing by direct visualisation and image analysis (using Brownian motion /Stokes- Einstein Equation)

Advantages

Observation in natural hydrated state

Direct real-time analysis

Ability to resolve differently sized particles

Relatively cheap

Low concentrations (down to 10^7 particles/ml)

Small sample volume

Can estimate concentration



Particle size – Nanosight™

Disadvantages

Not particularly high throughput? (matter of opinion)

Sample dilution necessary within a narrower range than DLS

Relatively new method

Skilled operator required for several optimisation steps (DLS can adjust automatically)

Sample considerations

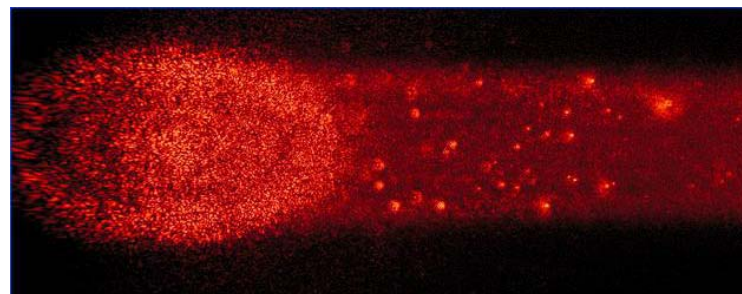
Sample volume

Particles between 10 nm -1 μ m

Need appropriate concentration

(dependent on the sample and particle size)

- too low, not enough data
- too high, particles hard to distinguish



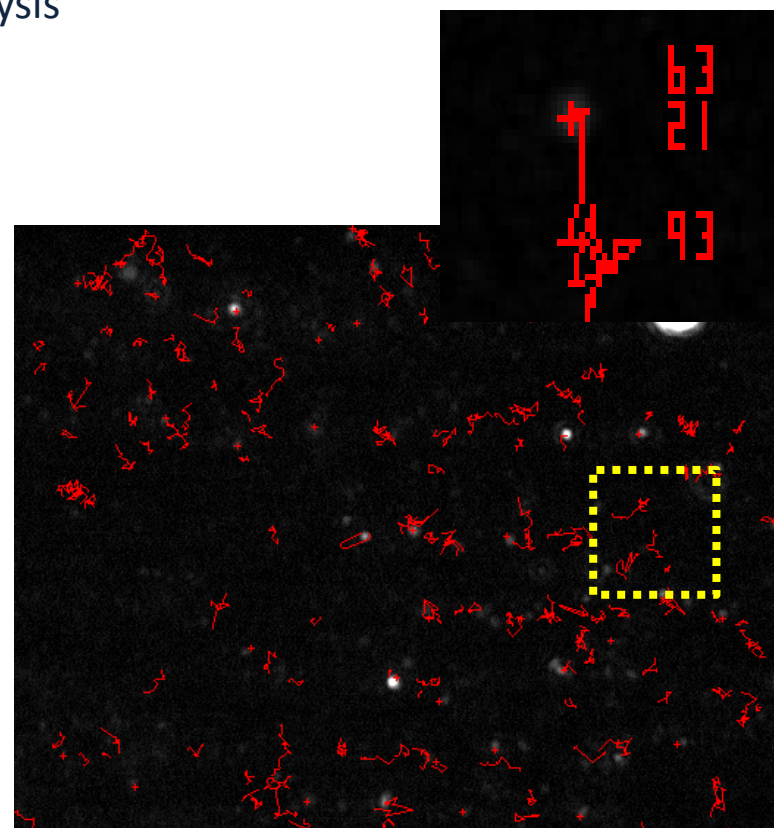
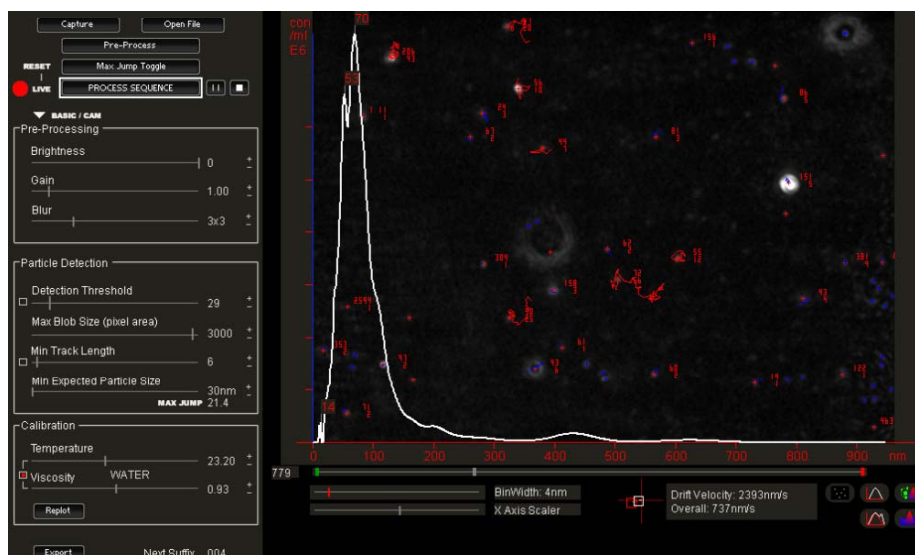
Particle size – Nanosight™

A laser beam is passed through a prism edged optical flat.

The beam refracts at the liquid sample prism interface and passes through the sample volume.

A microscope is used to visualise the particles moving under Brownian motion and video is captured (typically 1- 2 minutes is sufficient)

The movement of each particle is tracked by image analysis



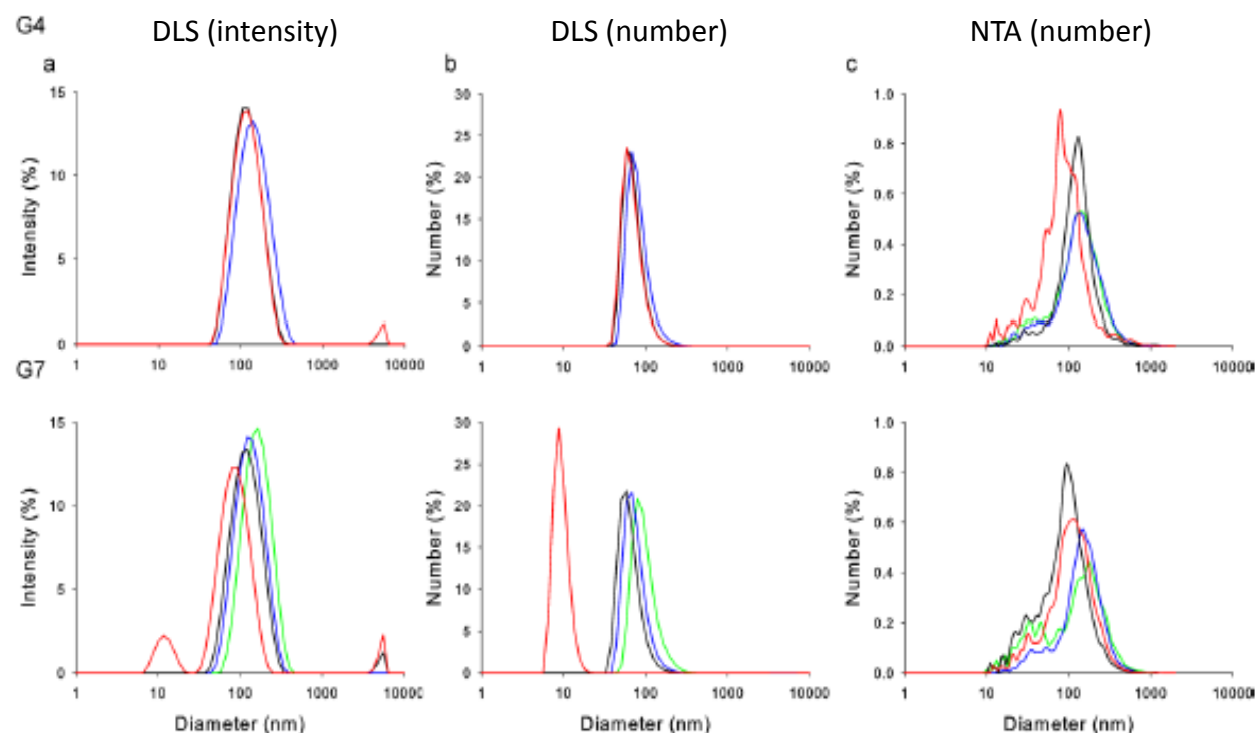
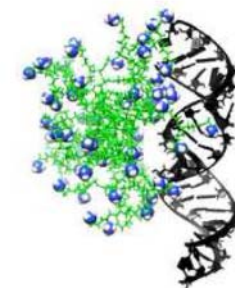
Particle size – Nanosight™

Example – siRNA dendriplexes

Better peak resolution compared to DLS

Aggregates revealed below 80 nm

Influence of molar ratios and dendrimer generation can be studied.



Acknowledgements



From Meditrans...

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Shellie Rigby-Singleton

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Ian Portman (University of Warwick)

Thanks for listening!

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