

# 1 **Biophysical analysis of lipidic nanoparticles.**

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## 7 **Highlights**

- 8 • **Biological nanoparticles play key roles in research and therapeutics.**
- 9 • **A range of techniques are available to study such particles in terms of size and**
- 10 **concentration.**
- 11 • **Each technique has associated advantages and disadvantages.**
- 12 • **Novel approaches allow the analysis of the full range of nanoparticles with a single**
- 13 **instrument.**

## 14 **Abstract**

15 Biological nanoparticles include liposomes, extracellular vesicle and lipid-based discoidal systems.  
16 When studying such particles, there are several key parameters of interest, including particle size and  
17 concentration. Measuring these characteristics can be of particular importance in the research  
18 laboratory or when producing such particles as biotherapeutics. This article briefly describes the major  
19 types of lipid-containing nanoparticles and the techniques that can be used to study them. Such  
20 methodologies include electron microscopy, atomic force microscopy, dynamic light scattering,  
21 nanoparticle tracking analysis, flow cytometry, tunable resistive pulse sensing and microfluidic  
22 resistive pulse sensing. Whilst no technique is perfect for the analysis of all nanoparticles, this article  
23 provides advantages and disadvantages of each, highlighting the latest developments in the field.  
24 Finally, we demonstrate the use of microfluidic resistive pulse sensing for the analysis of biological  
25 nanoparticles.

26 **Keywords:** Nanoparticle, MPRS, nCS1, Extracellular vesicles, Liposomes

## 27 **List of abbreviations**

<b>ACdEV</b>	Apoptotic Cell-Derived Extracellular Vesicle
<b>AFM</b>	Atomic Force Microscopy
<b>An-V</b>	Annexin V
<b>API</b>	Active Pharmaceutical Ingredient
<b>BSA</b>	Bovine Serum Albumin
<b>CCD</b>	Charged-Coupled Device
<b>CSD</b>	Concentration Size Distribution
<b>DDS</b>	Drug Delivery System
<b>DLS</b>	Dynamic Light Scattering
<b>EM</b>	Electron Microscopy
<b>ET</b>	Electron Tomography
<b>EV</b>	Extracellular Vesicle
<b>FCM</b>	Flow Cytometry
<b>FSC</b>	Forward Scatter
<b>HRFCM</b>	High-Resolution Flow Cytometry

<b>HRTEM</b>	High-Resolution Transmission Electron Microscopy
<b>IC</b>	Intracellular Communication
<b>LET</b>	Liposomal Encapsulation Technology
<b>MRPS</b>	Microfluidic Resistive Pulse Sensing
<b>MSP</b>	Membrane Scaffold Protein
<b>MVB</b>	Multivesicular Body
<b>NP</b>	Nanoparticle
<b>NTA</b>	Nanoparticle Tracking Analyser
<b>PS</b>	Phosphatidylserine
<b>PZT</b>	Piezoelectric
<b>RI</b>	Refractive Index
<b>RPS</b>	Repulsive Pulse Sensing
<b>SEM</b>	Scanning Electron Microscopy
<b>SFM</b>	Scanning Force Microscopy
<b>SIOS</b>	Scanning Ion Occlusion Spectroscopy
<b>SMALP</b>	Styrene Maleic Acid Lipid Particle
<b>SSC</b>	Side Scatter
<b>TDFM</b>	Transverse Dynamic Force Microscopy
<b>TEM</b>	Transmission Electron Microscopy
<b>TRPS</b>	Tunable Resistive Pulse Sensing
<b>UC</b>	Ultra-Centrifugation
<b>3-D</b>	3-Dimensional

## 29 **Introduction**

### 30 **Nanoparticles**

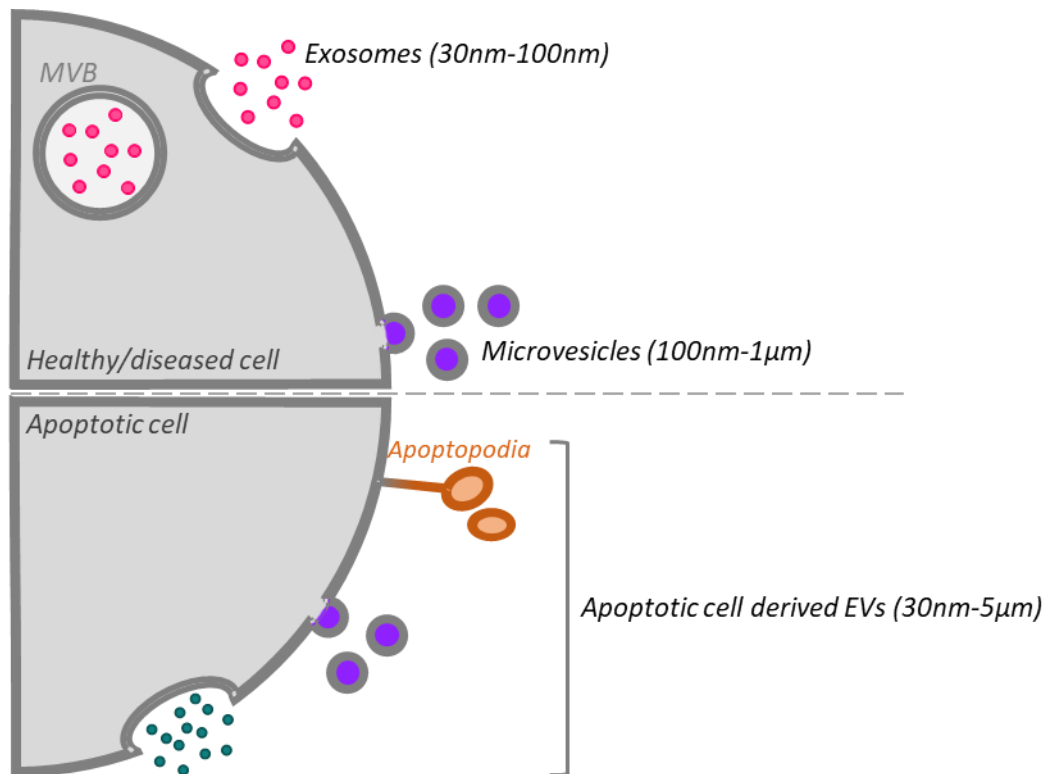
31 Nanoparticle (NP) is a term which encompasses several subpopulations. Nanoparticles can be  
32 naturally occurring, for example, extracellular vesicles (EVs) released by cells across the three main  
33 groups of the phylogenetic tree: Archaea, Bacteria and Eukarya. They can also be engineered, as with  
34 viruses used for vaccines; they can be biological (for example liposomes) or non-biological (such as  
35 metal or metal-derived NPs) or a combination of both. They could also be accidental in their  
36 production, such as exhaust fumes. In this review, we will use the term “nanoparticle” to refer to  
37 extracellular vesicles, liposomes and nanodisc systems, e.g. styrene maleic acid lipid particles  
38 (SMALPs).

39 Whilst the international standards organisation (ISO)[1] defines nanoparticles as ranging in size from  
40 1 to 100nm, it is generally accepted that biological nanoparticles may be larger than this within the  
41 sub-micron range. Due to their size, NPs have a larger surface area to volume ratio when compared  
42 to bulk materials, which give them interesting physical properties. Their size and composition can  
43 make them challenging to study experimentally.

### 44 **Extracellular vesicles**

45 Extracellular vesicles (EVs) are membrane-surrounded structures released by healthy, apoptotic and  
46 diseased cells. They were first described in 1967 by P. Wolf [2] and are found in most biological fluids.  
47 They are a highly heterogeneous population which vary in size, content, and mechanism of formation  
48 [3]. Their origin can also be used to classify them further. The field acknowledges three distinct  
49 subpopulations: apoptotic vesicles (30nm-5 $\mu$ m), microvesicles (100nm-1 $\mu$ m) and exosomes (30nm-  
50 100nm) (Figure 1). Exosomes are initially formed inside multivesicular bodies (MVBs) and are further  
51 released through exocytosis into the extracellular environment. Alternatively, microvesicles and  
52 apoptotic bodies are produced through budding of the plasma membrane.

53 Apoptotic cell-derived EVs (ACdEVs) encompass apoptotic bodies, apoptotic microvesicles and  
54 exosome-like vesicles. Evidence suggests that ACdEV biogenesis seems to be more complex as they  
55 can be formed through membrane protrusions termed apoptopodia [4], or through plasma membrane  
56 blebbing as mentioned previously. However, as little is reported in the literature regarding ACdEVs,  
57 other novel mechanisms of biogenesis are worth investigating.



58

59 **Figure 1 Extracellular vesicles formation and different subpopulations.** Healthy and diseased cells  
 60 release two main forms of EVs named exosomes and microvesicles. They are released through the  
 61 exocytosis of multivesicular bodies (MVBs) and via plasma membrane shedding. Apoptotic cells release  
 62 ACdEVs of various size through membrane protrusion, membrane blebbing and other mechanisms.  
 63 Green vesicles represent exosome-like ACdEVs.

64 It was first believed that EVs were cellular debris, but subsequent research has shown they contain a  
 65 specific subset of proteins, mRNAs, miRNAs, and lipids rather than random cellular components [5]. It  
 66 has been demonstrated that EVs play a major pathophysiological role in various biological processes  
 67 (apoptotic cell clearance, infection, immune response, antigen presentation [6]). They function to  
 68 transport and deliver cargo molecules between cells [7]. This EV-mediated intercellular  
 69 communication (IC) exists in healthy tissues and when altered, can lead to a variety of diseases, namely  
 70 autoimmune diseases and cancer. In the past few decades, EVs have received a great deal of interest  
 71 for their diagnostic and therapeutic potential [8].

## 72 Liposomes

73 Liposomes were first discovered by Bangham and his co-workers in 1961 [9], and can be described as  
 74 spherical artificial vesicles of various lamellarity: usually one or two phospholipid bilayers. Liposomes  
 75 are formed spontaneously, but many different methods of preparation exist. These can be divided  
 76 between passive or active loading techniques. The most common passive techniques are mechanical  
 77 dispersion, solvent dispersion or detergent removal, such as sonication and extrusion [10]. Similar  
 78 structures can also be formed from polymers (sometimes referred to as polymersomes) [11].

79 Conventional liposomes are commonly made of ester phospholipids such as phosphatidylcholine. A  
 80 new generation of liposomes have modified formulations in order to try to overcome instability issues  
 81 and enhance specificity [12]. For example, archeosomes contain one or more lipids found in  
 82 archeobacterial membranes that offer higher stability because of di-ether linkages [13]. Additionally,  
 83 virosomes are a combination of liposomes with virus-derived envelope proteins, allowing the fusion

84 of the virosomes with the target cell [14]. Finally, novasomes are paucimellar vesicles formed of two  
85 to seven bilayer membranes commonly used in cosmetics [15].

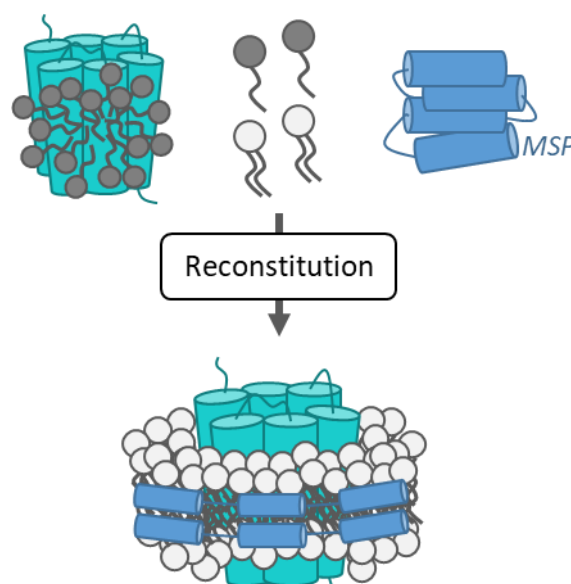
86 Liposomes can be loaded with different drugs and or molecules and used as drug carriers to deliver a  
87 range of active pharmaceutical ingredients (API) to a site of action for therapeutic and diagnostic  
88 purposes [16]. This liposomal encapsulation technology (LET) can involve drug molecules, gene  
89 therapies and bioactive agents. Hydrophobic API can be incorporated into the phospholipid bilayers,  
90 whereas hydrophilic API can be encapsulated inside the aqueous centre. LET has been shown to  
91 increase the stability and decrease the toxicity of the active molecules making it more efficient [17].  
92 Different types of liposomal drug delivery system (DDS) exist [18], for example, by coupling the  
93 liposomes to targeting ligands to increase the specificity. These carriers are most valuable because of  
94 their biocompatibility and non-immunogenicity [19].

95 Another use for liposomes is their relevance as a biological model [20],[21]. They can be used to mimic  
96 both extracellular vesicles and cells, offering a non-invasive alternative to study biological processes  
97 and test drugs. Their composition, size and charge can be tuned to make a highly accurate model.

### 98 **Disc systems**

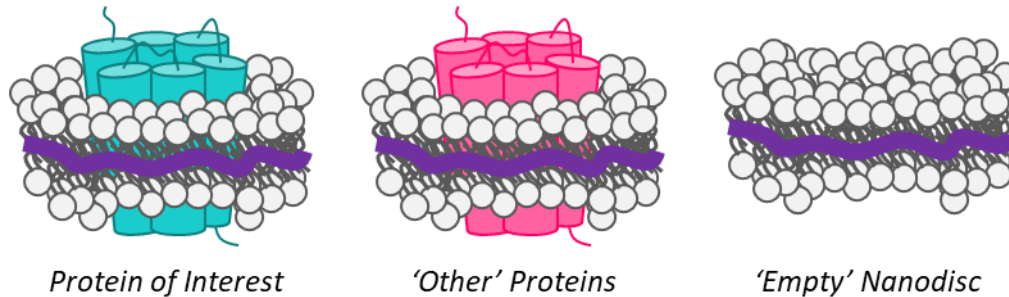
99 In membrane protein research, proteins often have to be isolated from the membrane in order to  
100 study them. This has often resulted in the use of detergents to solubilise the membrane, and therefore  
101 the protein, into detergent micelles. Throughout the past decades, it has become more apparent the  
102 native lipid environment surrounding the protein plays numerous roles in the structural stability and  
103 functionality of the protein. Hence, alternatives to detergents are advantageous [22].

104 Membrane scaffold proteins (MSPs) are one of the alternatives to a purely detergent-based isolation  
105 system. Bayburt et al., 2002 [24] first described the formation of bilayer patches surrounded and  
106 stabilised by a ring of amphipathic helical protein, termed membrane scaffold proteins. The process  
107 of forming these patches was described as a self-assembly mechanism whereby lipids, MSPs, and  
108 detergent were added to protein-detergent micelles forming particles of around 10nm in size [25]  
109 (Figure 2).



110 **Figure 2 Schematic demonstrating the formation of MSP nanodiscs.** A protein is isolated from the  
111 membrane via detergent solubilisation. A mixture of lipids and membrane scaffold proteins (MSPs) are  
112 added to the protein-detergent micelle solution, this results in the self-assembly of MPS encapsulated  
113 protein-lipid nanodiscs.

114 A more recent approach is the use of styrene-maleic acid (SMA) copolymers and derivatives to isolate  
115 membrane proteins in a nanometer-sized disc, known as SMA lipid particles (SMALPs) (Figure 3). The  
116 technique was first described in 2009 by Knowles *et al.* [26] and has the distinct advantage that the  
117 protein is extracted directly from the membrane without the need for detergent, and therefore  
118 maintains a near-native lipid environment, which is beneficial for downstream research [26].



119 **Figure 3 Schematic of SMALP encapsulated nanodiscs.** SMA copolymers punch holes in the membrane  
120 encapsulating anything that is present, i.e. membrane proteins (protein of interest as well as other  
121 proteins) or 'empty' lipid only discs.

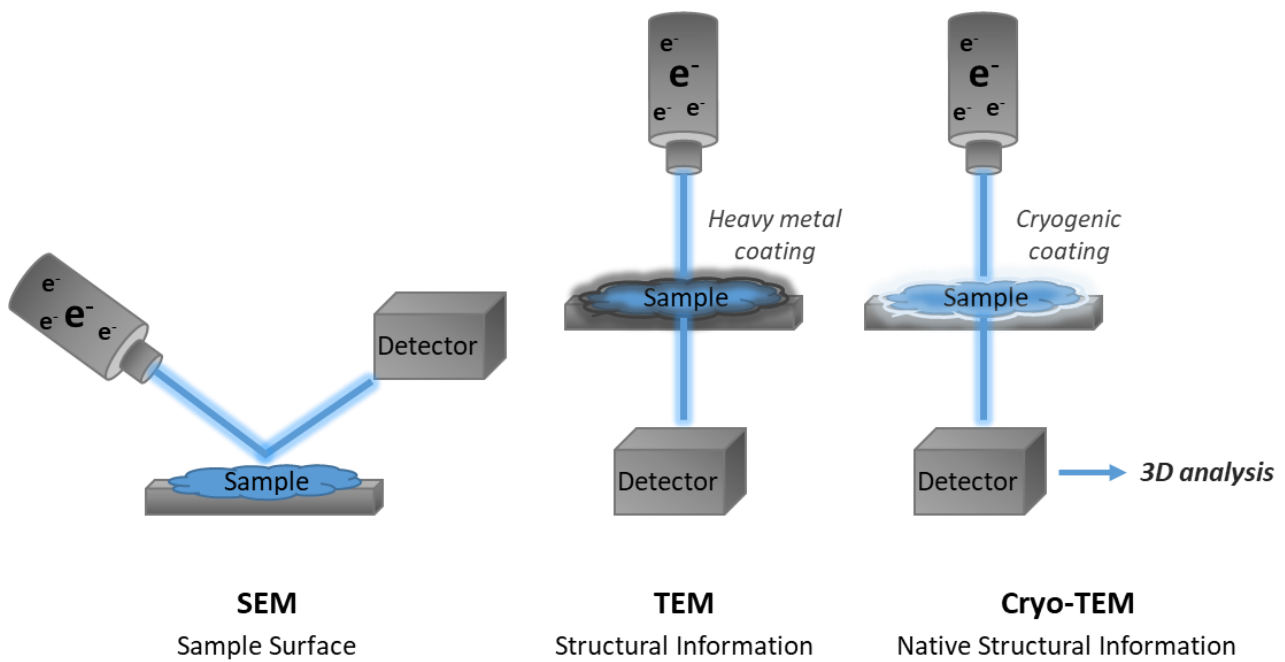
122 The greatest challenge of single NP based isolation and analysis is still the size of the NPs, which are  
123 below the reach of conventional detection methods [27]. In this review, we will present the main  
124 methods for NP analysis and their limitations, focusing on single particle-based quantification and size  
125 profiling. Finally, an advancement in NP analysing technology will be introduced as an alternative to  
126 study NPs with preliminary data presented.

### 127 **Conventional methods for NP analysis**

128 Common isolation methods for NPs include; ultracentrifugation (UC) and precipitation alongside size-  
129 , immunoaffinity capture-, and microfluidic-based techniques [28]. Microfluidic isolation is a promising  
130 field, which offers miniaturisation of conventional techniques, quicker purification times and  
131 improved resolution while enabling a continuous separation of the NPs. Following isolation using any  
132 approach, it is often desirable to analyse the size of the NPs to ensure either efficient separation or to  
133 define the nature of the sample.

### 134 **Electron microscopy (EM)**

135 Conventional optical microscopes cannot be used due to NPs being below the diffraction limit of  
136 visible light (200nm). Traditionally, the only way to visualise NPs via microscopy is by using the shorter  
137 wavelength and higher frequency of an electron microscope. As the wavelength of an electron can be  
138 up to 100,000 times shorter than that of visible light photons, electron microscopes have a higher  
139 resolving power that can reveal the structure of smaller objects, i.e. in the nanoscale. The first electron  
140 microscope was built in 1931 by Ruska and Knoll. Nowadays, two types of electron microscopes exist  
141 Scanning EM (SEM) and Transmission EM (TEM) (Figure 4).



142 **Figure 4 Simple schematic of the most common EM techniques.** Scanning electron microscopy  
 143 determines information about the sample surface. Transmission electron microscopy sends electrons  
 144 through the heavy metal coated sample, acquiring structural information. Cryo-transmission electron  
 145 microscopy utilises freezing of the sample to retain native structural information.

146 **SEM** is an electrical conductivity-based EM where the electron beam is focused onto the surface of  
 147 the samples, scanning it line by line. A detector simultaneously counts the scattered secondary  
 148 electrons. The images acquired by SEM only allow visualisation of the surface of the specimen, but  
 149 SEM can also provide 3-dimensional (3D) images. However, this method offers a lower resolution than  
 150 TEM [29].

151 **TEM** is an electron emission-based EM for thin samples (~100nm). Here, the beam of accelerated  
 152 electrons passes through the samples. The detector is below the sample retrieving the electron to  
 153 provide structural information. The entire electron path through the column must be under vacuum.  
 154 High-resolution TEM (HRTEM) is now able to provide a resolution below 0.5 angstroms [30]. However,  
 155 this resolution is limited to non-biological samples, regarding lipidic nanoparticles the resolution of  
 156 ~1.5 angstroms is more accurate [31]. TEM can have variants such as immune-EM, Cryo-EM (single-  
 157 particle and tomography), 3D-EM and conventional TEM.

158 For conventional TEM, the thin sections are coated with heavy metals in order to obtain a better  
 159 contrast and to visualise the lipid bilayer [32]. Depending on where the section was made, the  
 160 diameter can differ. The morphology of the whole particle thus remains uncertain. Fixatives can be  
 161 added to improve the sample retention. This method is a rapid way of confirming the presence of NPs  
 162 in a sample.

163 The least invasive technique is cryo-TEM, where the sample is frozen using a cryogen (liquid ethane or  
 164 propane) (Figure 4). Upon rapid freezing, a thin liquid film of the buffer is transformed into an  
 165 amorphous solid, allowing the object beneath to be observed in the native frozen-hydrated state. The  
 166 immediate freezing of the sample allows no time for crystal formation, removing the need to use  
 167 fixative and heavy metals. The sample is then transferred in a cryo-electron microscope and observed  
 168 at low temperature (-170 °C). The lipid bilayer appears as two thin dark lines because of the  
 169 phosphorous atoms (the heaviest) scattering more electrons [33].

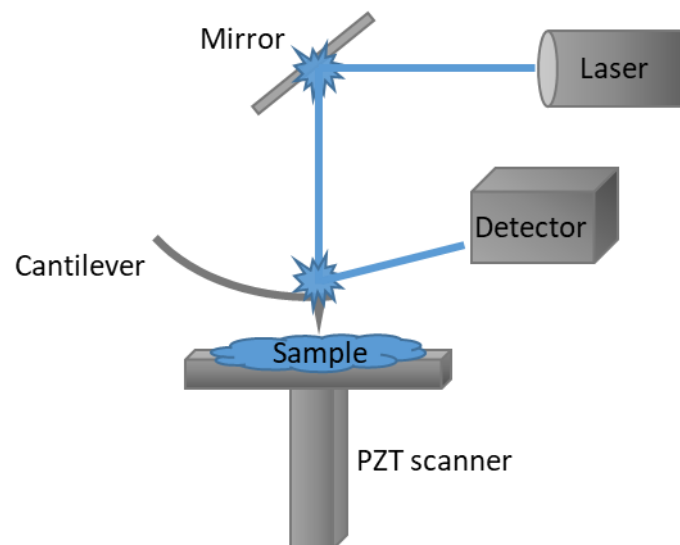
170 Phenotyping of EVs can be performed using cryo-TEM combined with immune-gold-labelling. Gold  
171 particles provide high contrast in EM thanks to their large electron scattering properties (i.e. plasma  
172 EVs exposing phosphatidylserine (PS) labelled with annexin V (An-V) conjugated with gold particles)  
173 [34]. Additionally, electron tomography (ET) can be used for 3D reconstruction based on 2-  
174 dimensional pictures taken at many different angles creating a tilt series. This can be combined with  
175 cryo-TEM by creating electronic slices as thin as 1nm through the reconstructed 3-D volume [35]. The  
176 details are much finer compared to cryo-EM alone and allow discrimination of vesicles spatially below  
177 or inside another one. The angles of the image do not yet permit a fully 3D structure due to an artefact  
178 that is referred to in the literature as “the missing wedge” [28, 29].

179 TEM has some potential disadvantages for particle analysis including possible radiation damage to the  
180 sample such as re-conformation and de-crystallisation to the breaking of atom bonds, removal of side-  
181 chains and in general a loss of mass [38].

### 182 Atomic force microscopy (AFM)

183 Atomic force microscopy (AFM) is a type of scanning force microscopy (SFM) developed in the 1980s  
184 by Binning, Quate, and Gerber [39]. Typically, a near-horizontal cantilever is used with a “nanofiber”  
185 tip facing towards the sample. Lasers are then reflected off the back of the cantilever to track the  
186 change in its displacement, which depending on its mode of operation, provides different information  
187 (Figure 5).

188



189 **Figure 5 Schematic representation of Atomic Force Microscopy.** The cantilever is in contact with the  
190 sample and the sample stage (piezoelectric (PZT) scanner) moves the x, y, and z position. A laser  
191 monitors the position of tip with feedback via a photodiode to the piezoelectric scanner to move the  
192 stage z-position to build a topological image at atomic resolution.

193 There are three modes of AFM; the first mode is contact-based, where the sample stage is positioned  
194 to provide a small upwards deflection of the tip when contact occurs. The sample is then moved along  
195 the x- and y-axis. As the topology of the sample changes so does the level of cantilever deflection,  
196 some operate by simply tracking the height of the cantilever in absolute terms and generating the  
197 topology from this [40]. In others, the system will for each x and y co-ordinate aim to deliver a  
198 consistent level of deflection, and the required changes in the samples z position will generate the  
199 topological map.



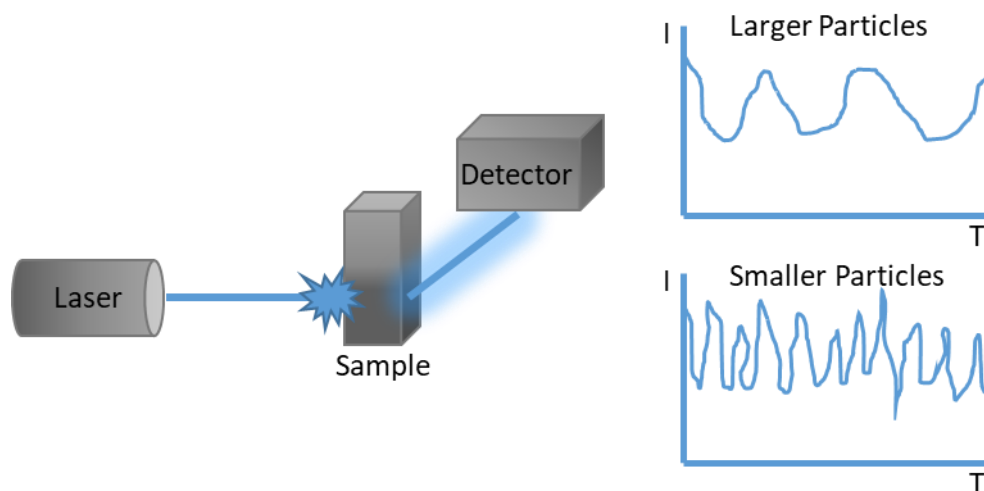
200 The second mode is non-contact. Here instead of an upwards deflection, a downwards deflection is  
201 observed. This is due to atomic forces, e.g. Van der Waals on this scale interacting with the cantilever,  
202 pulling it towards the sample [41]. Again, the concept is to maintain a consistent deflection and  
203 generate a topological map through record stage positioning.

204 There is a third non-contact mode. This involves the cantilever being oscillated at very high  
205 frequencies. As the tip gets close to the sample, either atomic forces or physical interactions, often  
206 with a layer of moisture present, leads to a dampening of the oscillation frequency. A consistent level  
207 of damping is required to generate the topological map [42].

208 Some advantages are that AFM can be operated in ambient conditions and often requires no special  
209 preparation of the sample. The sample sizes are often small, e.g. <200nm squared. Advances in  
210 piezoelectrics and control systems allow for near real-time scanning to generate videos, particularly  
211 useful for studying biological samples [43],[44]. Improved methods are always being developed, e.g.  
212 Transverse dynamic force microscopy (TDFM) which uses a vertical cantilever [45].

### 213 **Dynamic light scattering (DLS)**

214 Dynamic light scattering (DLS) is one of the most common and simple techniques used in the analysis  
215 of particle size distribution [46]. DLS is a measure of time-dependent fluctuations in the scattered light  
216 of particles undergoing Brownian motion (diffusion caused by random collisions with solvent  
217 molecules). A particle's hydrodynamic diameter is calculated as a function of the diffusion coefficient.  
218 Where the diffusion coefficient is inversely proportional to the particle size according to the Stokes-  
219 Einstein equation (Figure 6). This calculation assumes that all particles are spherical, reporting an  
220 equivalent particle diameter which can be disadvantageous for studying discoidal systems.



221 **Figure 6 Schematic of Particle Size Analysis by Dynamic Light Scattering.** A laser illuminates particles  
222 undergoing Brownian motion in a sample. A photodetector measures the fluctuations in the intensity  
223 of scattered light over a time period. Stokes-Einstein's equation is used to calculate the hydrodynamic  
224 diameter of the particles.

225 DLS can analyse particles ranging from 0.5 to 10,000nm dependent on the instrument and can analyse  
226 as little as 45µL of the sample, although common practice is to use a standard size cuvette which  
227 requires around 500µL for analysis. DLS also uses Rayleigh's approximation, where the intensity, I, of  
228 scattered light is proportional to the sixth power of the particle diameter, d:  $I \propto d^6$  (i.e. a particle of  
229 100nm will scatter light with a million times the intensity of a 10nm particle)[46],[47]. This means that

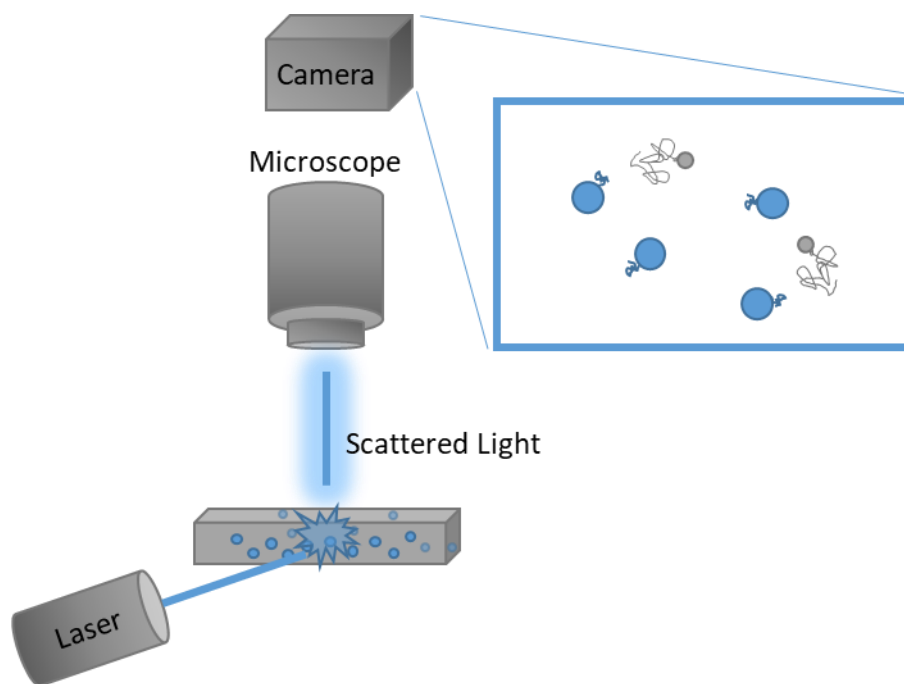
230 DLS has an inherent bias towards larger particles and therefore, a tendency to oversize particles in a  
231 polydisperse mixture and as such, it is not suitable for highly polydisperse samples.

### 232 Nanoparticle tracking analyser (NTA)

233 Nanoparticle tracking analysis (NTA) can be used for various applications, including but not limited to  
234 vesicles, exosomes, and proteins [48]. NTA uses the same underlying principle as DLS as it measures  
235 time-dependent fluctuations in scattered laser light of particles undergoing Brownian motion to  
236 determine their hydrodynamic radius. Where NTA differs is how it detects the scattered light. NTA  
237 uses a laser beam to illuminate particles, and the scattered light is easily visualised with a conventional  
238 microscope equipped with a 20x objective lens. Particle movement, i.e. scattered light, is then  
239 recorded with a light-sensitive charged-coupled device (CCD) or CMOS camera, arranged at a 90° angle  
240 to the irradiation plane [49]. The camera operates at 30 frames per second (fps), capturing a video file  
241 of the particles moving under Brownian motion. The software tracks many particles individually and  
242 using the Stokes-Einstein equation calculates their hydrodynamic diameters (Figure 7) [50]. As NTA is  
243 capable of tracking individual particles, the concentration of particles within a sample can be  
244 calculated. However, this requires calibration with standards of a known size and concentration.

245

246



247

248 **Figure 7 Schematic Representation of Particle Size Analysis by Nanoparticle Tracking Analysis.** A  
249 laser illuminates particles undergoing Brownian motion in a sample. Particle movement i.e. scattered  
250 light is then recorded with a light-sensitive charged-coupled device (CCD) or CMOS camera. Software  
251 tracks individual particles and uses the Stokes-Einstein equation calculates their hydrodynamic  
252 diameters.

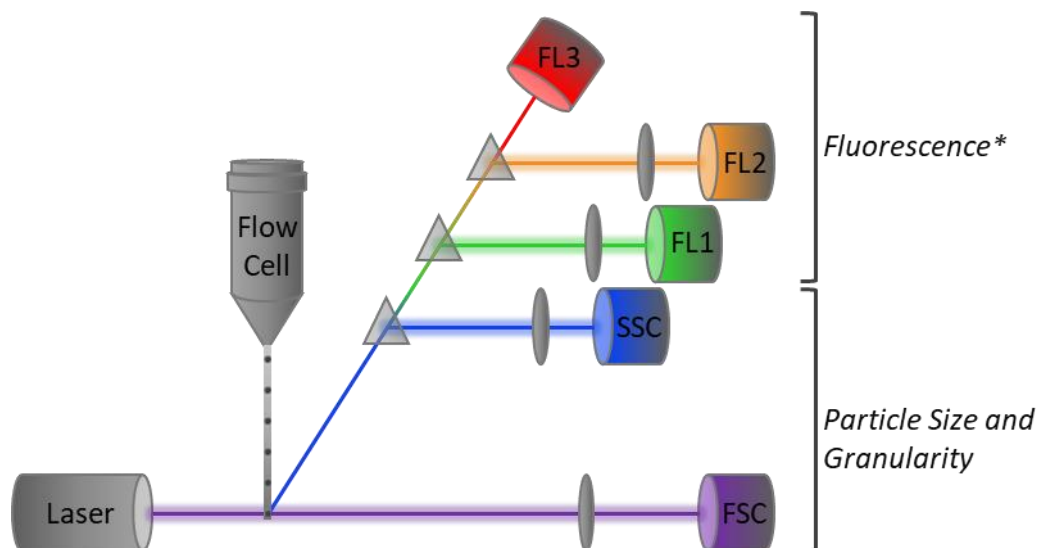
253 NTA is reported to size particles between 10 and 1000nm at a concentration range of 1E+06 to 1E+09  
254 particles.mL<sup>-1</sup>; however, this is sample and system configuration dependent [50]. For samples (such as  
255 EVs), where the refractive index (RI) of the particle is close to the RI of the electrolyte it is suspended  
256 in, the detection resolution is compromised, resulting in a restricted size analysis range and unreliable

257 concentration determination [51]. Like DLS, NTA also has restricted resolution for polydisperse  
258 samples due to Rayleigh's approximation, making it difficult to detect and track small particles [46].

### 259 **Conventional Flow Cytometry (FCM)**

260 Flow cytometry (FCM) is a laser-based technology developed for high throughput, multiparametric  
261 analysis of individual particles (e.g. cells). It allows the analysis of thousands of cells per second.  
262 However, in order to be detected, particles need to scatter light and/or induce fluorescence. NPs are  
263 much smaller than the cells for which flow cytometry was designed. Conventional FCM has a lower  
264 detection limit for polystyrene beads between 200 and 500nm [52]; consequently, the detection of  
265 NPs remains a challenge. Recently, dedicated small particle high-resolution flow cytometry (HRFCM)  
266 can offer a better resolution down to 40nm [53].

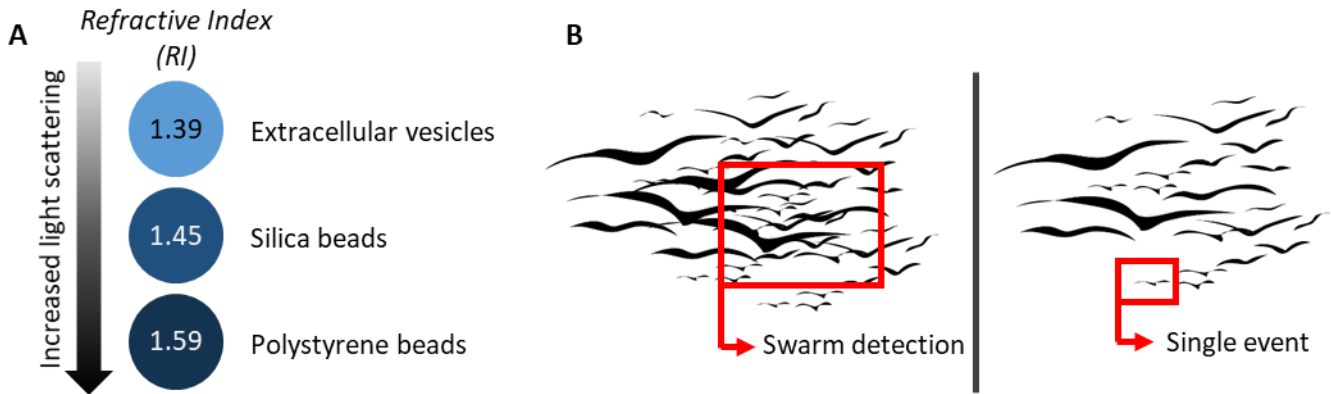
267 Cells and NPs are guided through a laser beam. The first detector is parallel to this beam and measures  
268 forward scattered light (FSC). The other detector is perpendicular to the beam and measures the side  
269 scattered light (SSC). FSC is used to look at the specimen size, whereas SSC is used to look at its  
270 granularity (Figure 8). The smaller the particle, the less the light scatters and the more background.  
271 FSC depends on the radius, the illumination wavelength, the refractive index and the light collection  
272 angle [54]. Thus, for an identical size, particles can have various refractive indices, resulting in a  
273 different scattering (Figure 9A). For the analysis of EVs, it is better to use silica beads for calibration,  
274 as EVs refractive index is closer to that of silica beads than polystyrene beads.



275 **Figure 8 Schematic Representation of Particle Analysis by Flow Cytometry.** A particle suspension  
276 enters the flow cell where the particles are aligned into a single stream and pass through a focused  
277 laser-beam. The resultant signal provides information on their size/granularity based on their forward  
278 and side scatter and the fluorescent intensities (\*if fluorescently labelled) of each particle.

279 Fluorescent detection of surface proteins can also be achieved as long as the labelling of the NP is  
280 bright enough to discriminate NPs from the optical and electronic noise. To do so, the light scatter  
281 threshold can be modified, or the excess of label can be removed using a density gradient. Focusing  
282 on EVs, the fluorescence trigger can be used as long as the antigen is abundant enough to allow  
283 detection. This can be an issue for smaller particles with fewer copies of the antigen of interest [55].  
284 Thus, sensitivity limits should be kept in mind when using fluorescently-labelled antibodies.

285 The concentration of the particles can have an important impact on both scatter and fluorescence  
 286 [56]. If the concentration is too high, it will result in a swarm detection (Figure 9B). This occurs when  
 287 two or more particles are in the measurement volume when the data acquisition is triggered, resulting  
 288 in multiple NPs counted as a single event [57]. In order to have a better single NP detection, the  
 289 samples need to be carefully diluted to an appropriate particle concentration. FCM offers a great  
 290 possibility for single NP based analysis as long as there is a standardisation of the sample preparation  
 291 as well as the appropriate instrument calibration.

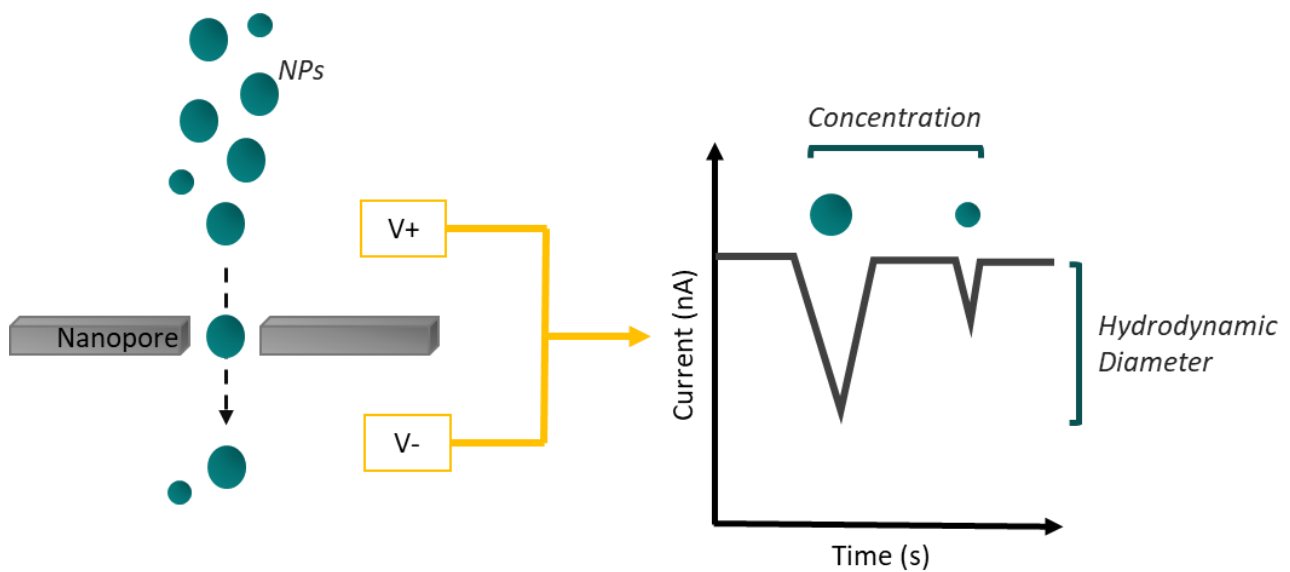


292 **Figure 9 Principles of flow cytometry for NP analysis** A. Effect of the refractive index (RI) of different  
 293 nature of nanoparticles on the light scattering. B. Representation of particle flowing: swarm detection  
 294 in opposition to a single event detection. Individual seagull representing an individual particle.

### 295 Tunable resistive pulse sensing (TRPS)

296 Tunable resistive pulse sensing (TRPS), previously known as scanning ion occlusion spectroscopy (SIOS)  
 297 [58], is suitable for biological and inorganic NP size, concentration and charge analysis. The equipment  
 298 most largely associated with TRPS is produced by Izon Science Ltd and can be referred to as qNano.

299 TRPS requires a nanosized stretchable pore that separates two conductive fluid compartments. After  
 300 applying a voltage across the nanopore, a current is established which is disrupted by the movement  
 301 of particles through the nanopore. When a particle moves through the opening, it decreases the flow  
 302 of ions through the nanopore, causing a transient current decrease known as a blockade event [59].  
 303 The amplitude of this event provides information for the size profiling of the particle. The number of  
 304 blockades throughout time gives information about the concentration (Figure 10). NP measurement  
 305 using TRPS requires an initial run with a calibration sample of known diameter and concentration,  
 306 usually polystyrene beads of a modal size appropriate to the range of interest [41,42]. Samples and  
 307 calibration should ideally be performed in the same buffer, which can cause issues with biological  
 308 samples. Spiking the sample directly with calibration beads offers an alternative calibration method.



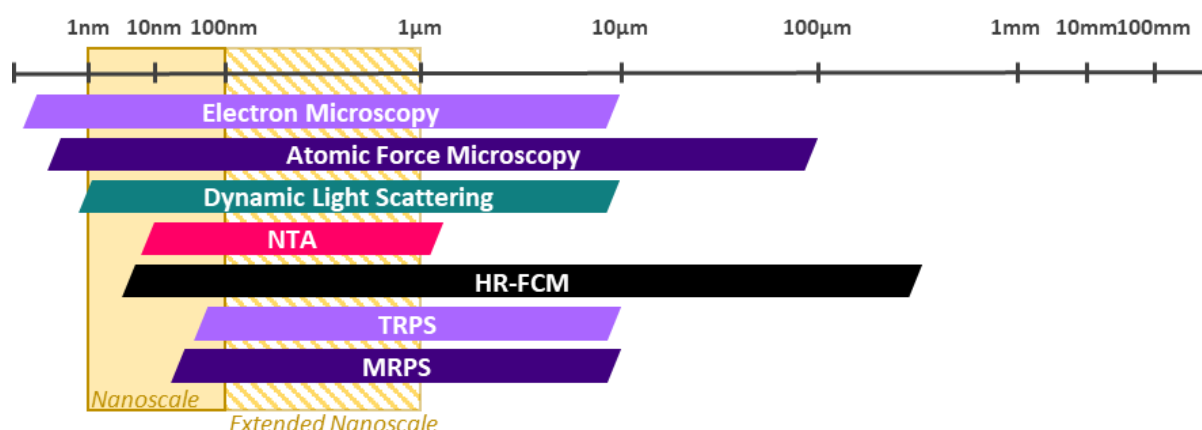
309 **Figure 10 Principle of the TRPS technique to measure the diameter and concentration of**  
 310 **nanoparticles.** The magnitude of the blockade event gives information on the hydrodynamic diameter,  
 311 whereas the number of blockades over time gives information on the concentration.

312 The size range of the nanopores can be tuned depending on the NPs of interest. Currently, the smallest  
 313 nanopore is the NP100 that has a size range of 70-200nm. However, the full range of manufactured  
 314 tunable pores extends from 70nm to 10 $\mu$ m. The measurement settings are flexible between each user.  
 315 Different parameters can be adjusted in order to have the best measurement conditions, such as the  
 316 nanopore stretch, the applied voltage and the pressure. The same parameters must be used between  
 317 measurements to ensure the reproducibility of the data. A total of 500 particles or more is then  
 318 counted per measurement. The TRPS technique provides a particle-by-particle analysis of the size and  
 319 concentration using tunable nanopores. However, it can be challenging due to the size heterogeneity  
 320 of the samples that can often lead to the blockage of the nanopore; this can be especially true for  
 321 biological samples.

TECHNIQUE	ADVANTAGES	DISADVANTAGES
<b>EM</b>	High resolution Morphology 3D Contrast	Expensive equipment Specific training and expertise Radiation damage Sample preparation Invasive/destructive technique Highly time consuming
<b>AFM</b>	Atomic resolution Morphology/ surface roughness Sample preparation	Expensive equipment Specific training and expertise Highly time consuming
<b>DLS</b>	Quick Easy to use Relatively inexpensive High throughput	Low resolution in polydisperse samples Tendency to oversize particles due to Rayleigh's approximation of scattered light
<b>NTA</b>	Quick Easy to use Relatively inexpensive High throughput	Data bias dependent on user settings Dependent on refractive index Low resolution in highly concentrated polydisperse samples due to Rayleigh's approximation. Moderately time consuming
<b>FCM</b>	Full NP diameter range High throughput Multiparametric analysis	Low resolution Risk of swarm detection Low sensitivity no information about morphology Moderately time consuming
<b>TRPS</b>	Inexpensive Reusable pore Single particle-based analysis High throughput	Easily clogged nanopores Calibration required Moderately time consuming Required standardisation

322

323 *Table 1 Comparison of the conventional techniques used to characterize the size and concentration*  
 324 *of NPs.*



325

326 *Figure 11 Size ranges for particle analysis of discussed techniques.*

327

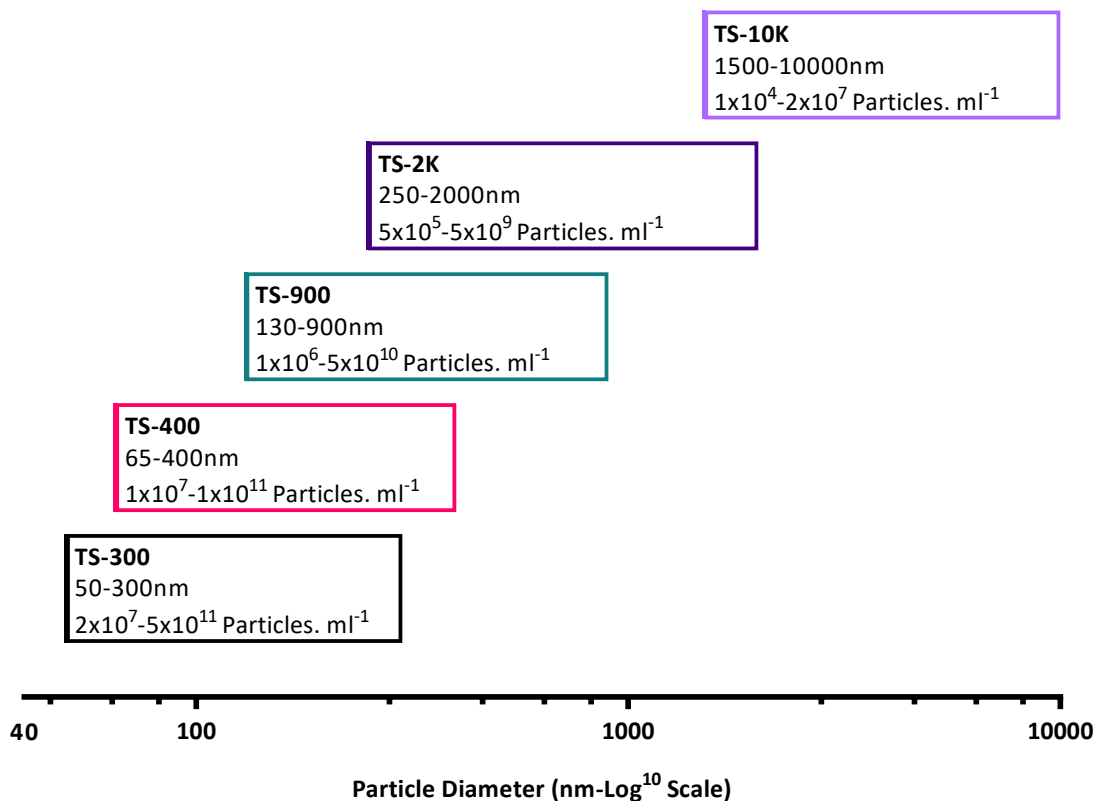
328 Each technique for NP characterisation has different limitations and advantages (Table 1 and Figure  
329 11) [62]. Thus, it is often recommended to combine several techniques instead of choosing one  
330 method, as there is no standard procedure for the characterisation of NPs to date.

### 331 Recent Advances in Nanoparticle Analysis

#### 332 Microfluidic resistive pulse sensing

333 Microfluidic resistive pulse sensing (MRPS) is a promising advancement in the resistive pulse sensing  
334 techniques originally described in the Coulter Principle over half a century ago [63]. The last decade  
335 has seen advancement in the form of TRPS (see the previous section). Although TRPS has pushed the  
336 limits of size detection of the resistive pulse sensing (RPS) technique, it still has limitations which need  
337 to be addressed for research to progress. In recent years there has been rapid development in the  
338 manufacturing of microfluidic chips and devices. An American company, Spectradyne, has utilised this  
339 research to develop an RPS instrument with patented microfluidic technology, the nCS1 [64].

340 The microfluidic technology in question is a series of pre-calibrated, single-use, low volume  
341 microfluidic cartridges. Each cartridge has a distinct size and concentration range (Figure 12). This has  
342 the benefits of making the technique an absolute method, i.e. no need for calibration material, the  
343 disposable microfluidic cartridges also dramatically reduce the risk of sample cross-contamination,  
344 with the added advantage of only requiring 3µL of sample to gain reliable, reproducible, statistically  
345 significant results.



346

347 *Figure 12 nCS1 Cartridge map denoting the size and concertation ranges.*

348

349

350 The principle of the technique is essentially the same as that described by Wallace Coulter [63],  
351 whereby, a voltage is applied across an aperture (hole) submerged in an electrolyte, as a particle  
352 passes through the aperture (also termed a nano-constriction) the displacement of the electrolyte  
353 causes a voltage drop which is proportional to the size/volume of the particle, enabling its  
354 hydrodynamic diameter to be calculated [63],[65],[66]. This allows the user to obtain analytical data  
355 for individual particles making the technique highly precise.

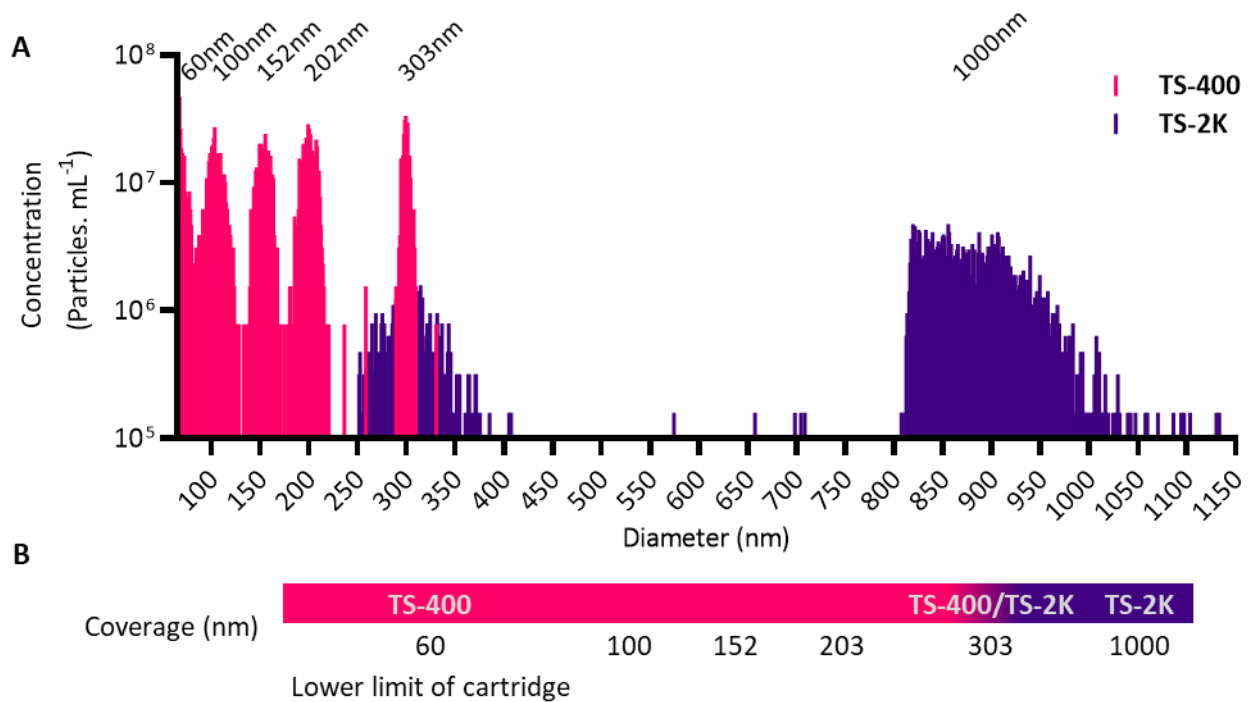
356 The microfluidic cartridge of Spectradyne's nCS1 also significantly reduces one of the major issues of  
357 the RPS technique, which is aperture blockages by incorporating a sample pre-filter embedded within  
358 the microfluidic cartridge. The only drawback with the incorporation of this pre-filter is overcoming  
359 the surface tension in the smallest (TS-300/-400) size cartridges. Spectradyne recommends the  
360 addition of a surfactant (polysorbate-20/ Tween-20) at 1%(v/v) to the sample or 0.1%(w/v) bovine  
361 serum albumin (BSA) as a wetting agent where surfactant cannot be used (i.e. with lipidic samples).

362 The technology also overcomes some of the more technical aspects of the technique, such as  
363 distinguishing agglomerated particles from monodisperse particles. It does this in the form of filters  
364 applied to the data with parameters specific to each of the different sized cartridges, such as transit  
365 time, the time it takes the particle to pass through the aperture, and symmetry.

#### 366 **Analysis of nanoparticles using the nCS1**

367 To test the capabilities of the instrument, a polydisperse sample of polystyrene size standards  
368 (Nanospheres) were analysed (Figure 13A). A broad range of Nanospheres were used (see Figure 13B)  
369 this was to test the accuracy of the instrument over multiple size cartridges. The Nanospheres were  
370 used at an approximate concentration of  $4.2E+8$  particles.mL<sup>-1</sup>, i.e. a total concentration of  $2.52E+9$   
371 particles.mL<sup>-1</sup> in the polydisperse sample, this is in line with the acceptable concentration range for  
372 both the TS-400 and TS-2K cartridges used in the analysis. Approximately 2500 particles were analysed  
373 with default cartridge filters applied (Table S1); the subsequent data was analysed graphically by  
374 determining size ranges for Gaussian analysis/fit (Table S2).



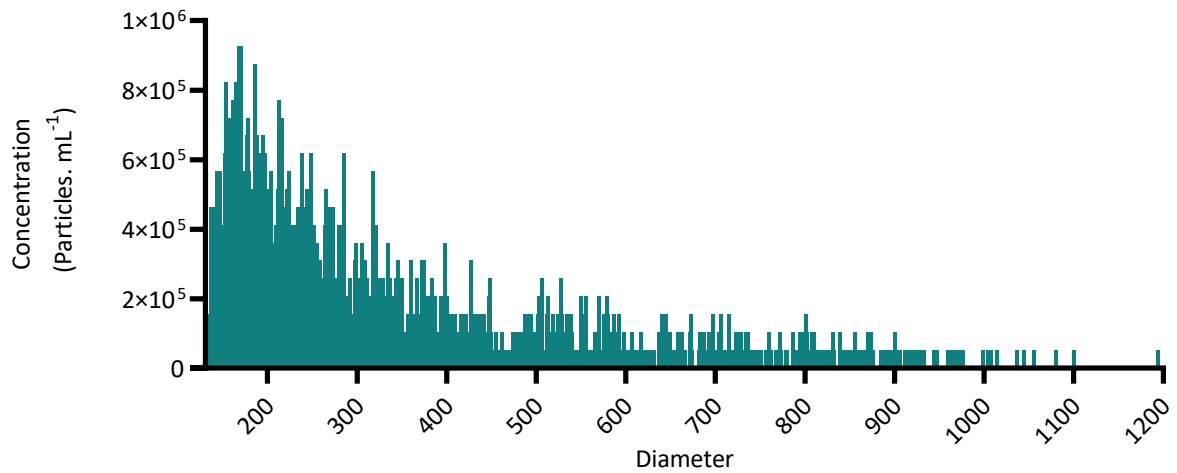


375 **Figure 13 Analysis of a polydisperse polystyrene size standard sample using the nCS1.** A. Size  
 376 distribution data demonstrating clear separation of peaks corresponding to the nominal size range of  
 377 the polystyrene standards, the TS-2K cartridge perfectly overlays with the TS-400 cartridge at 303nm  
 378 however demonstrates a broader distribution. B. Schematic demonstrates the size standards used in  
 379 the polydisperse mix and the theoretical coverage of the cartridges.

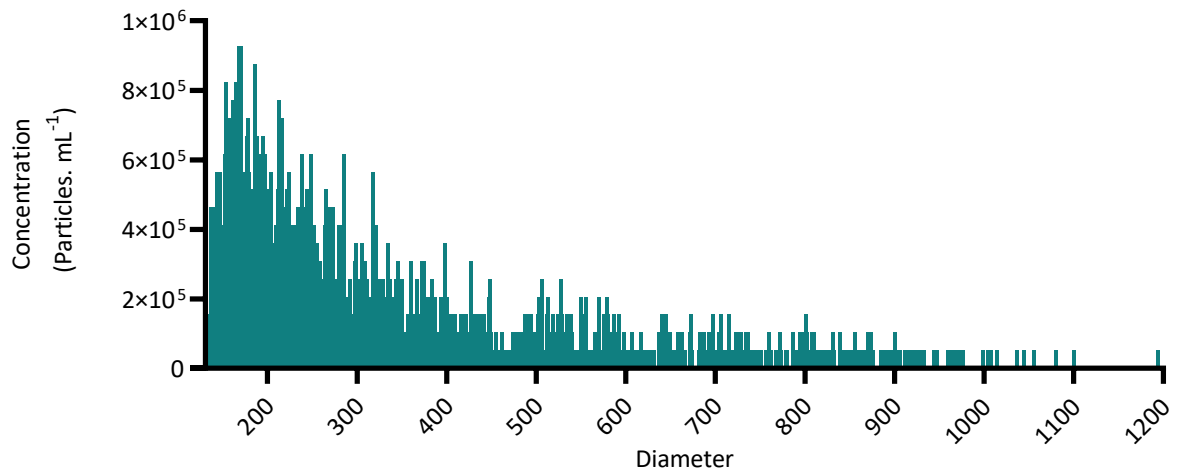
380 The data demonstrates that the nCS1 is capable of discerning multiple peaks in a single polydisperse  
 381 sample. The TS-400 coverage range starts at 65nm; therefore, the resolution of the 60nm Nanosphere  
 382 is hindered, resulting in a slight oversizing of the particle (Nanosphere™ NIST traceable certified  
 383 diameter of 60±4nm), these data would be best captured using a TS-300 cartridge which has a lower  
 384 detection threshold of 50nm. The other peak that demonstrated variability was the 1000nm peak; this  
 385 showed a broad peak with a mean of 873.3nm (Figure 13, Table S1). The remaining discernible peaks  
 386 fall within their certified mean diameter (provided on the safety data sheet), demonstrating the  
 387 accuracy/resolution of the nCS1.

### 388 **Analysis of Extracellular vesicles using the nCS1**

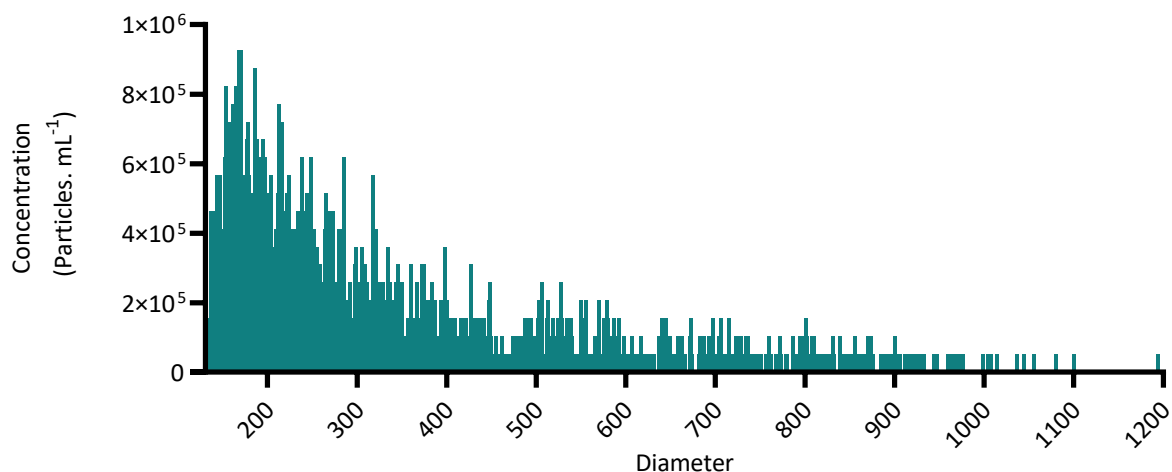
389 Following analysis of the polystyrene size standards, we sought to test the instrument's capabilities at  
 390 detecting a biological sample. Extracellular vesicles derived from apoptotic Jurkat cells were analysed  
 391 for both size range and concentration. The apoptotic cell-derived EVs (ACdEVs) were isolated using  
 392 centrifugation at 2000xg, and therefore it was suspected that the EVs could vary greatly in size; as  
 393 such analysis using both a TS-300 and TS-900 was conducted. The analysis was conducted without the  
 394 recommended surfactant (polysorbate-20) over concern that it could cause damage/ change the  
 395 integrity of the EVs. Acquisition with the TS-900 demonstrated a continuous size distribution with a  
 396 higher concentration of EVs at the lower size limit; this is in line with what was expected (



397  
 398 Figure 14). Analysis across the entire TS-900 detection range (130-1000nm) reported a total of 2146  
 399 particles analysed in a time period of three minutes 30 seconds, demonstrating the high throughput  
 400 capacity of the instrument. The concentration of particles across this range was calculated at  $1.1E+08$   
 401 particle. $mL^{-1}$ , with a D10, D50, and D90 (corresponding to 10%, 50%, and 90% of particles in the  
 402 concentration size distribution (CSD), see



403  
 404 Figure 14 for CSD) of 164.8, 269.2, and 669.4nm respectively.  
 405



406  
 407 **Figure 14 ACdEV analysis using the nCS1.** *3 $\mu$ l of ACdEVs (Jurkat cells) in phenol-red free RPMI-1645*  
 408 *were analysed using a TS-900 cartridge on the nCS1. Approximately 2000 events/particles were*  
 409 *analysed and demonstrate a size distribution with a higher concentration of EVs at the lower detection*  
 410 *threshold.*

411 ACdEV analysis using the TS-300 cartridge was relatively unsuccessful with only a few (<100)  
 412 discernible particles (data not shown). This did not acquire enough data for confident reporting of the  
 413 size distribution. The lack of apparent particulate is surmised to be due to one of two reasons; the lack  
 414 of surfactant in the sample and therefore a lack of wetting agent for the cartridge prefilter, or, the  
 415 sheer concentration of particles/EVs larger than the aperture being retained in the prefilter and  
 416 saturating it, and therefore severely reducing the flow of smaller particles. These hypotheses form  
 417 part of ongoing investigations to identify an alternative to detergent as a wetting agent and to  
 418 determine if pre-filtering for broad distributions is required. Nevertheless, MRPS shows great promise  
 419 for the analysis of lipidic nanoparticles.

## 420 **Conclusions**

421 There are a wide variety of techniques for studying the size and concentration of lipidic NPs. Each  
 422 approach has advantages and disadvantages, so careful thought is required to determine the correct  
 423 technique for a particular sample. This is particularly true when a sample is highly polydisperse and  
 424 may even contain particles outside of the nano range. This article illustrates the benefits and  
 425 drawbacks of the most commonly-used approaches for analysing NPs and highlights the promising  
 426 new technique of MRPS. It is nearly always advantageous to apply multiple approaches to a particular  
 427 sample to enable the most complete analysis to be performed.

## 428 **Material and methods**

### 429 **Cell lines and culture**

430 Jurkat T cell line, derived from an acute T cell leukaemia patient, were cultured at a density of 5E+05  
 431 cells.mL<sup>-1</sup> in RPMI 1640 medium (Sigma, Aldrich, UK) supplemented with 10% (v/v) FBS, 100  $\mu$ g.mL<sup>-1</sup>  
 432 penicillin/streptomycin, and 2mM glutamine. Cells were passaged every two to three days and  
 433 maintained at 37°C, 5% v/v CO<sub>2</sub>.

### 434 **EVs production**

435 Jurkat cells were washed with PBS and resuspended in serum-free and phenol red-free RPMI-1640  
 436 (Sigma, Aldrich, UK) at a density of 4E+06 cells.mL<sup>-1</sup>. Cells were then irradiated with a UV-B dose of 30

437 mJ.(cm<sup>2</sup>)<sup>-1</sup> using the UVP chromato-vue C71 cabinet. Analysis of apoptosis was performed using  
438 annexin V-FITC / propidium iodide (PI) staining kit (BioLegend, San Diego) to confirm the cell death by  
439 flow cytometry (CytoFLEX S, Beckman Coulter, USA).

#### 440 **Measurement on nCS1**

##### 441 • **Nanosphere analysis**

442 National Institute of Standards and Technology (NIST) traceable Nanosphere™ size standards  
443 (ThermoScientific) were suspended in Isoton-II solution with 1% Tween-20, filtered to 0.22 μm using  
444 a PES syringe filter, to create a polydisperse mixture (60, 100, 202, 303, and 1000nm) with a final  
445 concentration of ~2.52E+09 particles.mL<sup>-1</sup>. 3μL of sample was loaded into two different cartridges, TS-  
446 400 and TS-2K, in order to visualize the different sized nanospheres.

##### 447 • **ACdEV analysis**

448 3μL of ACdEVs suspended in phenol-red free RPMI (Sigma, Aldrich, UK) was loaded into TS-300 and  
449 TS-900 cartridges and loaded into the machine for analysis.

450 The cartridge mould I.D. and date has to be inserted into the software, this provides the auto analysis  
451 engine with the parameters of the cartridge loaded, the machine then automatically primes the  
452 cartridge and collects the data. The machine will collect and analyse 10-second analysis windows until  
453 you either manually stop the runs or enter stop acquisition parameters, such as number of particles,  
454 in the auto-analysis run window.

#### 455 **ACKNOWLEDGEMENTS**

456 We are grateful for funding from the European Union's Horizon 2020 research and innovation  
457 programme under Marie Skłodowska-Curie grant agreement No 847419 (MemTrain) and from  
458 Innovate UK in the form of a Knowledge Transfer Partnership between Aston University and Meritics  
459 Ltd (KTP 11605).

460

461 **REFERENCES**

- 462 [1] ISO/TS 80004-2:2015(en), Nanotechnologies — Vocabulary — Part 2: Nano-objects.  
463 <https://www.iso.org/obp/ui/#iso:std:iso:ts:80004:-2:ed-1:v1:en>.
- 464 [2] Wolf P. The nature and significance of platelet products in human plasma. *Br J Haematol*.  
465 1967. DOI:10.1111/j.1365-2141.1967.tb08741.x
- 466 [3] Gyorgy B, Szabo TG, Pasztoi M, et al. Membrane vesicles, current state-of-the-art: emerging  
467 role of extracellular vesicles. *Cell Mol Life Sci*. 2011;68(16):2667-2688. DOI:10.1007/s00018-  
468 011-0689-3
- 469 [4] Atkin-Smith GK, Tixeira R, Paone S, et al. A novel mechanism of generating extracellular  
470 vesicles during apoptosis via a beads-on-a-string membrane structure. *Nat Commun*.  
471 2015;6:7439. DOI:10.1038/ncomms8439
- 472 [5] Zaborowski MP, Balaj L, Breakefield XO, Lai CP. Extracellular Vesicles: Composition, Biological  
473 Relevance, and Methods of Study. *Bioscience*. 2015;65(8):783-797.  
474 DOI:10.1093/biosci/biv084
- 475 [6] Caruso S, Poon IKH. Apoptotic cell-derived extracellular vesicles: More than just debris. *Front*  
476 *Immunol*. 2018;9(JUN). DOI:10.3389/fimmu.2018.01486
- 477 [7] Nieuwland R, Sturk A. Why do cells release vesicles? *Thromb Res*. 2010;125 Suppl:S49-51.  
478 DOI:10.1016/j.thromres.2010.01.037
- 479 [8] Lotvall J, Hill AF, Hochberg F, et al. Minimal experimental requirements for definition of  
480 extracellular vesicles and their functions: a position statement from the International Society  
481 for Extracellular Vesicles. *J Extracell Vesicles*. 2014;3:26913. DOI:10.3402/jev.v3.26913
- 482 [9] Bangham AD, Horne RW. Negative staining of phospholipids and their structural modification  
483 by surface-active agents as observed in the electron microscope. *J Mol Biol*. 1964.  
484 DOI:10.1016/S0022-2836(64)80115-7
- 485 [10] Akbarzadeh A, Rezaei-Sadabady R, Davaran S, et al. Liposome: classification, preparation, and  
486 applications. *Nanoscale Res Lett*. 2013;8(1):102. DOI:10.1186/1556-276X-8-102
- 487 [11] Zhang X, Zhang P. Polymersomes in Nanomedicine - A Review. *Curr Nanosci*. 2016.  
488 DOI:10.2174/1573413712666161018144519
- 489 [12] Melis Çağdaş ADS and SB. Liposomes as Potential Drug Carrier Systems for Drug Delivery. In:  
490 *Application of Nanotechnology in Drug Delivery*. Ali Demir. IntechOpen. DOI:10.5772/58459
- 491 [13] Patel GB, Agnew BJ, Deschatelets L, Fleming LP, Sprott GD. In vitro assessment of  
492 archaeosome stability for developing oral delivery systems. *Int J Pharm*. 2000;194(1):39-49.  
493 DOI:10.1016/s0378-5173(99)00331-2
- 494 [14] Kaneda Y. Virosomes: evolution of the liposome as a targeted drug delivery system. *Adv Drug*  
495 *Deliv Rev*. 2000;43(2-3):197-205. DOI:10.1016/s0169-409x(00)00069-7
- 496 [15] Anupama Singh RM and PKS. Novasome-A Breakthrough in Pharmaceutical Technology a  
497 Review Article. *Adv Biol Res (Rennes)*. 2011;5(4):5.
- 498 [16] Hofheinz RD, Gnad-Vogt SU, Beyer U, Hochhaus A. Liposomal encapsulated anti-cancer drugs.  
499 *Anticancer Drugs*. 2005;16(7):691-707. DOI:10.1097/01.cad.0000167902.53039.5a
- 500 [17] Gill SE, Savage K, Wysham WZ, Blackhurst DW, Winter WE, Puls LE. Continuing routine cardiac  
501 surveillance in long-term use of pegylated liposomal doxorubicin: Is it necessary? *Gynecol*

- 502 *Oncol.* 2013;129(3):544-547. DOI:10.1016/j.ygyno.2013.03.012
- 503 [18] Sercombe L, Veerati T, Moheimani F, Wu SY, Sood AK, Hua S. Advances and Challenges of  
504 Liposome Assisted Drug Delivery. *Front Pharmacol.* 2015;6:286.  
505 DOI:10.3389/fphar.2015.00286
- 506 [19] van Rooijen N, van Nieuwmegen R. Liposomes in immunology: Multilamellar  
507 phosphatidylcholine liposomes as a simple, biodegradable and harmless adjuvant without  
508 any immunogenic activity of its own. *Immunol Invest.* 1980;9(3):243-256.  
509 DOI:10.3109/08820138009065997
- 510 [20] Bangham AD, Hill MW, Miller NGA. Preparation and Use of Liposomes as Models of Biological  
511 Membranes. In: *Methods in Membrane Biology.* ; 1974. DOI:10.1007/978-1-4615-7422-4\_1
- 512 [21] Routledge SJ, Linney JA, Goddard AD. Liposomes as models for membrane integrity. *Biochem*  
513 *Soc Trans.* 2019;47(3):919-932. DOI:10.1042/BST20190123
- 514 [22] Wright J, Muench SP, Goldman A, Baker A. Substrate polyspecificity and conformational  
515 relevance in ABC transporters: New insights from structural studies. *Biochem Soc Trans.*  
516 2018;46(6):1475-1484. DOI:10.1042/BST20180146
- 517 [23] Bayburt TH, Grinkova Y V., Sligar SG. Self-Assembly of Discoidal Phospholipid Bilayer  
518 Nanoparticles with Membrane Scaffold Proteins. *Nano Lett.* 2002;2(8):853-856.  
519 DOI:10.1021/nl025623k
- 520 [24] Bayburt TH, Grinkova Y V., Sligar SG. Self-Assembly of Discoidal Phospholipid Bilayer  
521 Nanoparticles with Membrane Scaffold Proteins. *Nano Lett.* 2002;2(8):853-856.  
522 DOI:10.1021/nl025623k
- 523 [25] Goddard AD, Dijkman PM, Adamson RJ, Dos Reis RI, Watts A. Reconstitution of Membrane  
524 Proteins: A GPCR as an Example. In: *Methods in Enzymology.* ; 2015.  
525 DOI:10.1016/bs.mie.2015.01.004
- 526 [26] Knowles TJ, Finka R, Smith C, Lin YP, Dafforn T, Overduin M. Membrane proteins solubilized  
527 intact in lipid containing nanoparticles bounded by styrene maleic acid copolymer. *J Am Chem*  
528 *Soc.* 2009;131(22):7484-7485. DOI:10.1021/ja810046q
- 529 [27] Clogston JD, Hackley VA, Prina-Mello A, Puri S, Sonzini S, Soo PL. Sizing up the Next  
530 Generation of Nanomedicines. *Pharm Res.* 2019;37(1):6. DOI:10.1007/s11095-019-2736-y
- 531 [28] Li P, Kaslan M, Lee SH, Yao J, Gao Z. Progress in exosome isolation techniques. *Theranostics.*  
532 2017;7(3):789-804. DOI:10.7150/thno.18133
- 533 [29] Miranda K, Girard-Dias W, Attias M, de Souza W, Ramos I. Three dimensional reconstruction  
534 by electron microscopy in the life sciences: An introduction for cell and tissue biologists. *Mol*  
535 *Reprod Dev.* 2015. DOI:10.1002/mrd.22455
- 536 [30] Kisielowski C, Freitag B, Bischoff M, et al. Detection of single atoms and buried defects in  
537 three dimensions by aberration-corrected electron microscope with 0.5-Å information limit.  
538 *Microsc Microanal.* 2008. DOI:10.1017/S1431927608080902
- 539 [31] Matsumoto S, Ishida S, Araki M, Kato T, Terayama K, Okuno Y. Extraction of Protein Dynamics  
540 Information Hidden in Cryo-EM Map Using Deep Learning. February  
541 2020:2020.02.17.951863. DOI:10.1101/2020.02.17.951863
- 542 [32] Stegmayr B, Brody I, Ronquist G. A biochemical and ultrastructural study on the endogenous  
543 protein kinase activity of secretory granule membranes of prostatic origin in human seminal  
544 plasma. *J Ultrastruct Res.* 1982;78(2):206-214. DOI:10.1016/s0022-5320(82)80024-5

- 545 [33] Efremov RG, Gatsogiannis C, Raunser S. Lipid Nanodiscs as a Tool for High-Resolution  
546 Structure Determination of Membrane Proteins by Single-Particle Cryo-EM. In: *Methods in*  
547 *Enzymology.* ; 2017. DOI:10.1016/bs.mie.2017.05.007
- 548 [34] Arraud N, Linares R, Tan S, et al. Extracellular vesicles from blood plasma: determination of  
549 their morphology, size, phenotype and concentration. *J Thromb Haemost.* 2014;12(5):614-  
550 627. DOI:10.1111/jth.12554
- 551 [35] Höög JL, Lötvall J. Diversity of extracellular vesicles in human ejaculates revealed by cryo-  
552 electron microscopy. *J Extracell Vesicles.* 2015;4(1):28680. DOI:10.3402/jev.v4.28680
- 553 [36] Midgley PA, Weyland M. 3D electron microscopy in the physical sciences: The development  
554 of Z-contrast and EFTEM tomography. In: *Ultramicroscopy.* ; 2003. DOI:10.1016/S0304-  
555 3991(03)00105-0
- 556 [37] Deng Y, Chen Y, Zhang Y, Wang S, Zhang F, Sun F. ICON: 3D reconstruction with ‘missing-  
557 information’ restoration in biological electron tomography. *J Struct Biol.* 2016.  
558 DOI:10.1016/j.jsb.2016.04.004
- 559 [38] Grubb DT. Radiation damage and electron microscopy of organic polymers. *J Mater Sci.*  
560 1974;9(10):1715-1736. DOI:10.1007/BF00540772
- 561 [39] Binnig G, Quate CF, Gerber C. Atomic force microscope. *Phys Rev Lett.* 1986;56(9):930-933.  
562 DOI:10.1103/PhysRevLett.56.930
- 563 [40] Liu S, Wang Y. Application of AFM in microbiology: a review. *Scanning.* 2010;32(2):61-73.  
564 DOI:10.1002/sca.20173
- 565 [41] Martin Y, Williams CC, Wickramasinghe HK. Atomic force microscope-force mapping and  
566 profiling on a sub 100-Å scale. *J Appl Phys.* 1987;61(10):4723-4729. DOI:10.1063/1.338807
- 567 [42] Milhiet PE, Dosset P, Godefroy C, et al. Nanoscale topography of hepatitis B antigen particles  
568 by atomic force microscopy. *Biochimie.* 2011;93(2):254-259.  
569 DOI:10.1016/j.biochi.2010.09.018
- 570 [43] Dufrêne YF. Using nanotechniques to explore microbial surfaces. *Nat Rev Microbiol.*  
571 2004;2(6):451-460. DOI:10.1038/nrmicro905
- 572 [44] Heath GR, Scheuring S. Advances in high-speed atomic force microscopy (HS-AFM) reveal  
573 dynamics of transmembrane channels and transporters. *Curr Opin Struct Biol.* 2019;57:93-  
574 102. DOI:10.1016/j.sbi.2019.02.008
- 575 [45] Humphris ADL, Antognozzi M, McMaster TJ, Miles MJ. Transverse dynamic force  
576 spectroscopy: A novel approach to determining the complex stiffness of a single molecule.  
577 2002;18(5):1729-1733. DOI:10.1021/la015537k
- 578 [46] Anderson W, Kozak D, Coleman VA, Jämting ÅK, Trau M. A comparative study of submicron  
579 particle sizing platforms: Accuracy, precision and resolution analysis of polydisperse particle  
580 size distributions. *J Colloid Interface Sci.* 2013;405:322-330. DOI:10.1016/j.jcis.2013.02.030
- 581 [47] Finsy R. Particle sizing by quasi-elastic light scattering. *Adv Colloid Interface Sci.*  
582 1994;52(C):79-143. DOI:10.1016/0001-8686(94)80041-3
- 583 [48] Gross J, Sayle S, Karow AR, Bakowsky U, Garidel P. Nanoparticle tracking analysis of particle  
584 size and concentration detection in suspensions of polymer and protein samples: Influence of  
585 experimental and data evaluation parameters. *Eur J Pharm Biopharm.* 2016;104:30-41.  
586 DOI:10.1016/j.ejpb.2016.04.013

- 587 [49] *Introduction to Nanoparticle Tracking Analysis (NTA) Measurement Principle of ZetaView®*.  
588 [www.particle-metrix.de](http://www.particle-metrix.de).
- 589 [50] NanoSight NS300 | Characterize Nanoparticles | Malvern Panalytical.  
590 [https://www.malvernpanalytical.com/en/products/product-range/nanosight-](https://www.malvernpanalytical.com/en/products/product-range/nanosight-range/nanosight-ns300)  
591 [range/nanosight-ns300](https://www.malvernpanalytical.com/en/products/product-range/nanosight-range/nanosight-ns300).
- 592 [51] Wright M. Nanoparticle tracking analysis for the multiparameter characterization and  
593 counting of nanoparticle suspensions. *Methods Mol Biol.* 2012;906:511-524.  
594 DOI:10.1007/978-1-61779-953-2\_41
- 595 [52] Steen HB. Flow cytometer for measurement of the light scattering of viral and other  
596 submicroscopic particles. *Cytom A.* 2004;57(2):94-99. DOI:10.1002/cyto.a.10115
- 597 [53] Saywell A, Bakker A, Mielke J, et al. Light-Induced Translation of Motorized Molecules on a  
598 Surface. *ACS Nano.* 2016;10(12):10945-10952. DOI:10.1021/acsnano.6b05650
- 599 [54] Welsh JA, Holloway JA, Wilkinson JS, Englyst NA. Extracellular Vesicle Flow Cytometry Analysis  
600 and Standardization. *Front Cell Dev Biol.* 2017;5:78. DOI:10.3389/fcell.2017.00078
- 601 [55] Nolan JP, Duggan E. Analysis of individual extracellular vesicles by flow cytometry. In:  
602 *Methods in Molecular Biology.* ; 2018. DOI:10.1007/978-1-4939-7346-0\_5
- 603 [56] Groot Kormelink T, Arkesteijn GJ, Nauwelaers FA, van den Engh G, Nolte-'t Hoen EN, Wauben  
604 MH. Prerequisites for the analysis and sorting of extracellular vesicle subpopulations by high-  
605 resolution flow cytometry. *Cytom A.* 2016;89(2):135-147. DOI:10.1002/cyto.a.22644
- 606 [57] van der Pol E, van Gemert MJ, Sturk A, Nieuwland R, van Leeuwen TG. Single vs. swarm  
607 detection of microparticles and exosomes by flow cytometry. *J Thromb Haemost.*  
608 2012;10(5):919-930. DOI:10.1111/j.1538-7836.2012.04683.x
- 609 [58] Roberts GS, Yu S, Zeng Q, et al. Tunable pores for measuring concentrations of synthetic and  
610 biological nanoparticle dispersions. 2012;31(1):17-25. DOI:10.1016/j.bios.2011.09.040
- 611 [59] Maas SL, Broekman ML, de Vrij J. Tunable Resistive Pulse Sensing for the Characterization of  
612 Extracellular Vesicles. *Methods Mol Biol.* 2017;1545:21-33. DOI:10.1007/978-1-4939-6728-  
613 5\_2
- 614 [60] Vogel R, Willmott G, Kozak D, et al. Quantitative sizing of nano/microparticles with a tunable  
615 elastomeric pore sensor. *Anal Chem.* 2011;83(9):3499-3506. DOI:10.1021/ac200195n
- 616 [61] Maas SLN, Vrij J De, Broekman MLD. Quantification and size-profiling of extracellular vesicles  
617 using tunable resistive pulse sensing. *J Vis Exp.* 2014. DOI:10.3791/51623
- 618 [62] van der Pol E, Hoekstra AG, Sturk A, Otto C, van Leeuwen TG, Nieuwland R. Optical and non-  
619 optical methods for detection and characterization of microparticles and exosomes. *J Thromb*  
620 *Haemost.* 2010;8(12):2596-2607. DOI:10.1111/j.1538-7836.2010.04074.x
- 621 [63] Coulter WH. Means for counting particles suspended in a fluid. October 1953.
- 622 [64] Fraikin J-L, Teesalu T, Mckenney CM, Ruoslahti E, Cleland AN. A high-throughput label-free  
623 nanoparticle analyser. 2011. DOI:10.1038/NNANO.2011.24
- 624 [65] Rhyner MN. The coulter principle for analysis of subvisible particles in protein formulations.  
625 *AAPS J.* 2011;13(1):54-58. DOI:10.1208/s12248-010-9245-6
- 626 [66] Graham MD. The Coulter Principle: Foundation of an Industry. 2003. DOI:10.1016/S1535-  
627 5535(03)00023-6



