Analytical challenges in size and shape determination of drug delivery nanoparticles

by

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12 ECTS
June 2017-July 2017

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Title: “Analytical challenges in size and shape determination of drug delivery nanoparticles”

Literature Master Thesis in Chemistry- Track of Analytical Sciences

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17-Jul-2017

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Nanotechnology is facing great improvements over the last decades, both in the design and synthesis but also in the scope of application. The development of nanoparticles with improved and unique characteristics like size, shape and materials have contributed in major advancements in the field of nano-pharmaceuticals. Their physicochemical properties introduce them as possible alternative treatment in many serious diseases like cancer (Sen Gupta, 2015). As a result, nano-pharmaceuticals offer enormous potential especially in drug delivery as carriers of smaller drugs and diagnostics. There is also another aspect that focuses more on the design of smart materials for tissue engineering. Despite the fact that nanomedicine and drug delivery systems are in the center of the research attention only a few “nano-pharmaceuticals” have been approved by the U.S. Food and Drug Administration (FDA) (Tinkle, 2014). Most nano-pharmaceuticals submitted for evaluation fail to meet the criteria for bioavailability because of their properties and interactions inside the living organisms. It seems that the key for successfully designed nanoparticles appropriate for medical applications lies in the deep understanding of the complexity of these systems and the interactions between the nanoparticles and the organism, which controls their pharmacokinetics, biodistribution and safety.

Trying to understand the role of the analytical chemistry field on this gap, the aim of this literature study is to discuss on the one hand the main characteristics of the nanomedicine with focus on the size and shape and their effect on the biodistribution, clearance and cellular uptake. The main research questions that will be addressed are related to the most effective particle size and its correlation to a specific target, also if an ideal particle size exists how small that should be. In a second section, drug delivery characterization with main focus on size and shape will be discussed. Nanopharmaceuticals should be characterized accurately and for that reason a variety of different methodologies are required in order to achieve reproducible and precise results. The difficulties in obtaining accurate measurements will be highlighted. Not only there is a great variety of techniques used for particle sizing analysis, but their measurement principles are based on many different properties, such as transport, geometry and optical or electrical properties of the nanocarriers. As a result these techniques can measure different size ranges (López-Serrano, 2014)). Additionally, during this literature study it became clear that size determination is sometimes quite poorly reported or even missing. This leads to data misinterpretation and conflicting results from different studies. So the second goal is to describe the different analytical techniques used for the assessment and characterization of nanoparticles (size, shape, morphology) along with their limitations and advances. For the particle size characterization of drug delivery nanoparticles the focus will be on techniques including light scattering techniques (DLS and MALS), microscopy (TEM, SEM, AFM) and chromatography (SEC, FFF). Finally, the aim is to propose a set of standardized techniques that can be used for more universal characterization and also focus on the way that the results should be evaluated and reported.

Figure 1. Accurate size determination of submicron and nanometer size is a major challenge due to the limitations and strengths of each technique, as discussed in (Bell, 2012) study.
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<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AF4</td>
<td>Asymmetric Field Flow Fractionation</td>
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<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
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<tr>
<td>EPR</td>
<td>Passive enhanced permeability</td>
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<tr>
<td>ESEM</td>
<td>Environmental Scanning Electron Microscopy</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FFF</td>
<td>Field Flow Fractionation</td>
</tr>
<tr>
<td>FF-TEM</td>
<td>Freeze-Fracture Transmission Electron Microscopy</td>
</tr>
<tr>
<td>HF5</td>
<td>Hollow Fiber Field Flow Fractionation</td>
</tr>
<tr>
<td>LNP</td>
<td>Lipid Nanoparticles</td>
</tr>
<tr>
<td>MALS</td>
<td>Multi-angle Light Scattering</td>
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<tr>
<td>NIST</td>
<td>Nation Institute of Standards and Technology</td>
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<tr>
<td>NPs</td>
<td>Nanoparticles</td>
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<tr>
<td>NTA</td>
<td>Nanoparticles Tracking Analysis</td>
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<tr>
<td>PDI</td>
<td>Polydispersity Index</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>SEC</td>
<td>Size Exclusion Chromatography</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
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<tr>
<td>SIOS</td>
<td>Scanning Ion Occlusion Sensing</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
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INTRODUCTION

Nanoparticles (NPs) have gained a special role in the biomedical field, as promising drug carriers for targeted drug delivery and imaging. The incredible results and the variety of applications have motivated more and more researchers to focus on the aspects that prevent nanomedicine for being widely applicable and accepted. While the development of new nanomaterials is important, it is equally important to gain a deeper understanding on their physicochemical properties, structure, composition, complexity and toxicity in order to be able to predict and prevent adverse effects.

For a better prediction of the delivery efficiency and prediction of their distribution in the living organisms an investigation of their characteristics is important. Nanoparticles size and shape are believed to have an influence not only on the biodistribution but also on their cellular uptake (Aula, 2015). During this literature it became clear that nowadays research is directed not on the investigation of the biodistribution effect of the physicochemical parameter of the nanoparticles, but on the cellular uptake. However, despite the fact that the effect of size on the NPs (nanoparticles) distribution is extensively reported, the information related to the shape is very limited (Tan, 2013). This will be further discussed in the first sections of this study, as researchers seem to have started focusing on the physicochemical properties of the nanocarriers and especially their size, shape and surface. An overview of the findings related to these two parameters and their importance on the nanomedical field will be highlighted.

Gaining a deeper understanding on the contribution of the most important physicochemical properties of nanoparticles to their functionality will eventually lead to the creation of accurate models in order to be able to predict the optimal particle characteristics depending on the specific goal. The most recent trend for nanomedicine is the development of materials that change their properties size, shape, stiffness according to specific environmental triggers such as pH, temperature etc, and as a result a better understanding on possible hazards and side effects due to these structural changes is of great value. However, despite the fact that physicochemical properties of nanomedicine are of great importance, there has been a great debate over insufficient characterization and documentation regarding their characterization (Khorasani, 2014) (Gaumet, 2008) (Kranz, 2011).

<table>
<thead>
<tr>
<th>Characterization property</th>
<th>Characterization technique</th>
<th>Physicochemical information acquired</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structure</td>
<td>SEM, AFM, SEM-Raman</td>
<td>Nanocarrier’s external structure and texture</td>
</tr>
<tr>
<td>Shape</td>
<td>SEM, TEM, STM, AFM, XRD, SAXS, AUC</td>
<td>FFF, SEC</td>
</tr>
<tr>
<td>Size</td>
<td>DLS, GPC,SAXS, SEM, TEM, AFM, NTA, SIOS, MALDI-TOF-MS, ICP-MS</td>
<td>FFF, SEC, ANUC, CE</td>
</tr>
<tr>
<td>Charge</td>
<td>DLS, ATR-FTIR,CE</td>
<td>Nanocarrier’s averaged charge</td>
</tr>
</tbody>
</table>

This seems to be one of the reasons that nanomedicine research has not fully found application in the clinical settings. Many products are discarded due to poor characterization, leading to revealed unwanted properties at the...
final stages. As a result, the research community agrees (Tinkle, 2014) (Khorasani, 2014) (Wei A. M., 2012) that more standardized universal analytical methodologies are required in order to narrow this gap by producing accurate and reliable results that could be implemented in practical applications for improved efficacy and reduced undesirable effects. Some of the techniques that are currently being used for nanomedicine characterization for some of the more important physical properties are summarized in Table 1. However, not all of these techniques are already approved from FDA but they are considered potential candidates. In this literature not all of these techniques will be discussed, but a selection was made based on the most cited techniques and the newest developed ones suitable for more routine analysis in liquids (or dry powders).

THE FIELD OF NANOMEDICINE

Nanomedicine according to the National Institutes of Health (Singh, 2009) are the technological innovations related to disease diagnosis, treatment and prevention, which have the opportunity to be combined with the recent discoveries related to proteomics and genomics in order to create a new class of drugs beneficial for more patients. The merging of medicine, engineering and biochemistry created the new field ‘nanomedicine’ with applications of nanomaterials focusing on developing new screening, diagnostic and treatment approaches (Hall, 2007). Nanoparticles have the ability to mimic and interact with the organism in order to change and monitor certain biological processes. To be more specific nanotechnology is related with the study of extremely small structures in the magnitude of 0.1 to 100 nm (1nm= 10^-9 m), a schematic representation is presented in Figure 2.

Figure 2. Schematic overview of nanoscale and nanostructures. Inspired by (Nikalje, 2015)

The important difference for the nano scale size is that the properties of the particles, materials can be determined just above the scale of an atom, which means that at least one dimension (height, length, depth) of these particles can be measured between 1 and 100 nm (Nikalje, 2015).
In Figure 3 a schematic representation of the two main areas of nanotechnology related to the medical applications is depicted. Nanomaterials and nano devices can be further sub classified. In this report the focus will be on the drug delivery nano systems, which can be considered as part of the nanomaterials and nanostructure subclass. Clearly with the reduction of size, different new properties have been created (Waters, 2009). The ultimate goal of nanotechnology is to develop methodologies in order to create materials, from the atomic up to supramolecular level, with applications related to their specific function and size. Nanomaterials have a rapid improvement and constantly find new applications due to their advanced and specific physicochemical properties, especially due to their high surface area to volume ratio. These improved physicochemical properties were the main criteria for evaluating them as possible candidate for clinical applications (Boulaiz, 2011).

Another focus of nanomedicine in contrast to most conventional therapies is the eager for smart choices. The goal is to find ways to destroy only specific cells or repair them based on a specific drug release, and not invasively remove the diseased cells by surgery, radiation or chemotherapy, which in most cases can cause severe adverse effect for the patient (Riehemann, 2009). As a result, nowadays the treatment aims to start already from the molecular level with the use of nanoparticles. Equally important is that nanoparticles have already revealed information about biochemical processes in the molecular level of a disease. As a result, also in cases that a treatment had not been found yet, an insight on the biochemical processes behind the diseases is gained, especially related to the biggest enemy of carcinogenesis (Aula, 2015) (Bazile, 2014). Understanding the development and the generation of the diseases enables the design of selective and optimized nanomedicine for their treatment, with ultimate goal the more personalized medicine, which also means the development of multifunctional nanoparticles for more specific targeting, more effective drug delivery or even combinations for optimal delivery and treatment results. So in a nutshell nanotechnology and medicine are combined for the development of new diagnostic methodologies like imaging, drug delivery and regeneration of damaged tissues. Nanomedicine covers and provides advancements on the whole procedure, from offering more accurate and sensitive detection of a disease until developing selective and effective treatment against it with the minimum possible side effects.
APPLICATION OF NANOPARTICLES IN DRUG DELIVERY AND IMAGING.

Nanoparticles and their application in the biomedical field is of great interest, with special focus on the drug delivery and imaging (Bhatia, 2016) (Doane, 2012). Conventional drug treatment is facing some significant limitations related to poor bio-distribution, low effectiveness, severe side effects and very low selectivity. The ultimate goal of drug delivery products is to reduce these limitations by transporting drug molecules accurately and with the higher possible selectivity. This by extent has additional advantages as lower drug doses are required and lower toxic effects. The developed size reduction methodologies led to the creation of nanoparticles, where size and shape can be manipulated in order to produce different particles with unique and enhanced properties that can be used as materials for biomedical applications (Boulaiz, 2011).

If we wanted to describe an ideal drug delivery nanoparticle it should be able to protect the drug/ biopharmaceutical from potential unwanted damages inside the body and also to be able to target only a specific location where it will release the desired amount of drug in a very control manner and in specific time (Sun, 2017). All these enhanced properties have established nano-carriers to be used in many fields of medicine which have opened the horizons for the development of nano-immunology, nano-cardiology nano-ophthalmology, nano-oncology, neurology, dentistry and many more, but most importantly nanomedicine have been used in the targeting against cancer, also brain tumor, and gene delivery (Sen Gupta, 2015). Focusing on the use of nanoparticles as drug delivery carriers against cancer it is important to mention the main root that these molecules have to follow. In most cases the drug delivery product is administered by an intravenous injection. Therefore the nanoparticles are forced to travel through the blood stream and subsequently overcome all the biological barriers before effectively reaching and release the drug. A major limitation of conventional chemotherapy is that it initiates a great resistance mechanism against the drug used, which eventually decreases the levels of successful treatment. Another equally important limitation of chemotherapy is that the drugs used are not specific and as a consequence they damage not only the tumor but also the healthy cells. The use of nanoparticles as vehicles of the drug aim to overcome the limitations of chemotherapy and for that reason specific abilities of these particles have to be manipulated and tuned in order to achieve an effective treatment. Some of the main mechanisms and characteristics that differentiate drug delivery nanoparticles from the conventional treatment are described in this section. Drug delivery nanoparticles can achieve controlled drug release in time and target and simultaneously protect it from enzymatic degradation. They also follow two targeting strategies passive or active. Approaching the target via ‘Passive Enhanced Permeability’ (EPR) meaning that drugs are released in an extracellular environment and then diffused into the target. On the other hand, for active targeting specific ligands are attached to the nanoparticles such as (antibodies, peptides, small molecules etc.) that by ligand-receptor interactions will accumulate on the target (Shi, 2017). All the above mentioned are important and contribute to the final goals of drug delivery which are:

- The decrease of side effects by increasing their selectivity
- Enhancement of pharmacokinetic and dynamic profile
- Assist in increasing drug solubility
- Balance the amount of drug loss and its concentration in the target
- Enhance drug stability, by shielding it from external attacks
- Increase the cellular uptake
- Ensure low toxicity and enhanced biodegradability

Over the years, studies focused on optimizing drug delivery systems have reported the correlation between their physicochemical characteristics and their effect on their final goals. For example, it has been reported that nanoparticles of sizes between 10 and 100 nm, neutral or anionic, are able to increase the local drug concentration in the tumor and escape clearance from the kidneys or uptake from the phagocytes (Danheir, 2010) (Maruyama, 2011).

Another main aspect that nanoparticles are being used for in medicine is for diagnostic imaging. The optical properties of nanoparticles are being used in solar cells, sensors, and in medicine as imaging agents. Fluorescent agents can be used either alone or as drug carriers in order to detect pathogenic areas in the body and in a second stage, by the drug release, to provide treatment. Although the complexity of the targeted system increases the challenges around the imaging with nanoparticles, several inorganic nanoparticles have already been approved for use. The inorganic nanoparticles being used are classified in two categories the semiconductor and metallic nanostructures (Doane, 2012). The quantum confinement of these materials, meaning the change in their electronic and optical properties because of their nanometer scale, equipped them with very useful optical properties. So in this case the important role of nanoparticle size is very worth mentioning. For the inorganic nanoparticles size has an important effect on their properties, which in this case can tune the amount of scattered light with increased particle size (Murphy, 2005) (Jain, 2006).

In Table 2 some of the most studied nano-carriers and nanoparticles used for imaging and drug delivery along with their characteristics and applications are mentioned with emphasis on their different size and properties, as this will be a main objective of this literature thesis.
Table 2. Important nanoparticles used in medicine as drug delivery and imaging agents

<table>
<thead>
<tr>
<th>Type of Nanoparticle</th>
<th>Size (nm)</th>
<th>Characteristics</th>
<th>Application</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon nanotubes</td>
<td>0.5-3 nm diameter and 200-1000 length</td>
<td>Allotropic forms of carbon, basic composition of graphite and formed in cylindrical tubes. Mechanical and electronic properties, eg electrical and thermal conductivity. Categories: Single-walled CNT (SWCNTs) and Multi-walled CNT (MWCNTs)</td>
<td>In medicine and pharmacy as therapeutic and diagnostic agents (drugs, genes, vaccines, antibodies, etc.). Also used in tissue regeneration, biosensors and promising as antioxidants</td>
<td>(He, 2013)</td>
</tr>
<tr>
<td>Dendrimers</td>
<td>&lt;10</td>
<td>Hyperbranched macromolecules. Symmetric molecules with an inner and an outer shell. They have self-assembling properties, chemical stability low toxicity and increased solubility.</td>
<td>Dendrimers are being used both for anticancer therapies and imagining. Dendrimers with metal chelates act as contrast agents used in MRI.</td>
<td>(Kobayashi, 2005) (Abbasi, 2016)</td>
</tr>
<tr>
<td>Liposomes</td>
<td>50-100</td>
<td>Phospholipids and polymer based bilayer vehicles. Great biocompatibility, biodegradability and manipulation of size and shape. They increase the solubility of hydrophobic chemotherapeutic drugs, lower the side effects and toxicity.</td>
<td>Drug delivery systems and as nano-therapeutics for cancer treatment. Liposomes can delivery proteins, genes, peptides and other types.</td>
<td>(Allen, 2013)</td>
</tr>
<tr>
<td>Metallic nanoparticles</td>
<td>&lt;100</td>
<td>Usually Au and Fe and Pt colloids. Large surface-to-volume ratios compared to atoms and bulk materials.</td>
<td>Drug and gene delivery, as well as imaging. Specifically studied for diagnosis and treatment of cancer, HIV, tuberculosis and Parkinson disease.</td>
<td>(Rai, 2016)</td>
</tr>
<tr>
<td>Nanocrystals- Quantum dots</td>
<td>2-9.5</td>
<td>NPs with crystalline character and specific nanosize. Quantum dots are nanocrystals made of semi-conductive materials. QDs have unique optical properties. By varying the size of the QDs more desired properties can be achieved.</td>
<td>QDs are being used for in vivo imaging and diagnostics, but they have a few limitations. The core material is Cd metal, which is highly toxic</td>
<td>(Junghanns, 2008)</td>
</tr>
<tr>
<td>Polymeric Nanoparticles</td>
<td>10-1000</td>
<td>Polymers can create a wide variety of formations from macroscale (gels &amp; hydrogels) to nanoscale (polymer-drug conjugates, polymeric micelle etc. Additionally the polymer architectures can vary significantly including linear, branched, hyperbranched and dendritic polymers. Alterations in the polymer composition leads to different properties size, shape, compatibility between core &amp; drug, drug release and stability. New generations of smart polymers responsive to external or internal factors such as temperature, pressure or pH changes has push the limits of polymeric nanoparticles.</td>
<td>Polymeric micelles have been investigated as agents to improve the solubility of pharmaceutical compounds. Thus their applications vary from diagnostic imaging to drug and gene delivery. The most important co-polymers used as DD so far are the PEG-PLGA, PEG-PCL, poly(amino acids).</td>
<td>(Qin, 2013) (Binauld, 2013)</td>
</tr>
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IMPORTANCE OF PHYSICOCHEMICAL PROPERTIES OF NANOPARTICLES

Nanomaterials are the most advanced and developed level of nanotechnology not only in terms of scientific knowledge of the field but also of commercial products and applications. In order to give an insight on the importance of the nanoscale level of particles it is worth mentioning that living organisms constitute of cells that are in the 10 μm range of size, whereas their inner parts are much smaller. Proteins that are equally important have sizes of around 5 nm and these sizes have been achieved also in tailored nanoparticles (Albanese, 2012). The purpose of this comparison is to realize that the particle dimensions (as presented in Table 2) can be manipulated in order to “spy” and understand the biochemistry processes taking place inside the cells at the nanoscale level, but ideally without interfering or damaging the cell.

In order to understand the potential hazards, but also the efficiency of these materials a correlation between their physicochemical properties and the involved interactions for cellular uptake and bioprocessing should be explained. The most crucial physicochemical properties are:

- The surface chemistry of the particles
- Physical properties, such as size, shape, surface area, surface charge, stiffness
- Aggregation or agglomeration
- Stability under physiological conditions

However, it is observed that most published research miss the information which correlate the physicochemical properties and quantitative properties of the nanoparticles with their biological response and fate inside the body (Zhu, 2013). Not having the complete picture between particle’s properties and activity can certainly compromise the safety assessment of the potential pharmaceutical candidates. So in this report the important physical properties of size and shape will be discussed attached with their pharmaceutical impact.

EFFECT OF PARTICLE SIZE- MICRO VS NANO PARTICLES

One of the important characteristics in the nanomedicine systems is the particle size and size distribution, especially for the drug delivery systems. It has been reported into the literature that particle size can determine the organ target of the nanocarrier, the biotoxicity and the fate of the molecule inside the living organism. Particle size is also a very important parameter related to the drug loading, release and stability of the particles. A research question that was thoroughly being investigated was related to the advantages of nano-particles over the micro-particles (Panyam, 2003) (Gaumet, 2008). The size of the drug carrier can vary from 10 nm up to several micrometers in diameter, but the optimal size is an ongoing investigation and debate.

Microparticles are formed from spherical polymeric particles with sizes around 1 to 250 μm polymeric systems. They form microcapsules, which encapsulate the drug into a cavity around a polymeric membrane, or microspheres, in which the drug is distributed throughout the particle. On the other hand nanoparticles are sub micronic (< 1 μm) particles (Couvreur, 1993). Drug delivery microparticles are more often injected locally in the tissue, due to the fact
that they cannot easily pass through the fenestrations, a term that will be in detail explained in the following sections. These particles with a diameter higher than 1 μm, because they are immobilized and stopped from the microscopic blood vessels, they are not suitable for injection (Moon, 2012). This disadvantage can also be used as an advantage. Microparticles can be administrated in a specific location and because of their size they will not be able to escape or move so they will be held in the position of the injection.

An example of such an application is described in the work of (Gu, 2013) where microgels suitable for injection were developed for controlled release of insulin. These microgels consist of a pH responsive polymeric matrix, an enzyme nanocapsule and human recombinant insulin. Under hyperglycemic conditions the enzymes are activated and the conversion of glucose into gluconic acid is triggered. Another research that addresses the differences of micro- and nano-advantages was based on the retention of fluorescent polystyrene particles of sizes 20, 200nm and 2 μm. The particles were tested in rats and monitored for almost two months. It was concluded that nanoparticles of smaller sizes (20 nm) were cleared from the organism already from the first week, whereas almost the entire dose of the microparticles 200 nm and 2 μm were present after the passage of two months, making them possible candidate for drug delivery to the eye retina (Amrite, 2005).

Nanoparticles due to their smaller size have the advantage of higher surface area to volume ratio (s/v), which means that the higher this ratio the more adsorption by drugs or proteins will occur. On the other hand particles of higher volume ration can interact with other particles, which can be at the same time an advantage and a disadvantage in case of aggregation and alterations of the physicochemical properties of the particle (Kohane, 2007).

Another very important size difference between micro and nano particles is related to biological barriers. The most difficult target is the brain, due to its composition of tight and adherent junctions (TJs and AJs). However, under pathological circumstances like brain infection or stroke the blood brain barrier changes and allows drug delivery molecules to enter. It has been found that nanoparticles of sizes between 1nm and not more than 1000 nm can more effectively be used to overcome the brain barriers and delivery anticancer drugs in the brain (Mehmood, 2015) (Saraiva, 2016).

Cellular uptake and phagocytosis is another crucial parameter determining the fate of the particles inside the body and is very important that particles can escape their uptake. The effect of size in the phagocytosis uptake will be further discuss in the following sections. The difference between micro and nano scale is that particles of 2-3 μm are more easily attached to the phagocytes, leading to their clearance from the organism and as a result they cannot reach their target effectively (Champion J. A., 2008). On the other hand, it has been proven that nanoparticles have higher cell uptake and can target a wider variety of organs, exactly because of their smaller size, which means that the smaller the particle size the higher its mobility and can more easily escape from the phagocytosis system. In order to understand the dependence of particle size with the final target it is important to understand the path and the obstacles that the particle has to overcome inside the living organism. However, it is already clear that there is not one specific size or shape for novel application, as there is a significant number of parameters that size and shape can have an influence on.
The path and barriers that nanoparticles have to follow before reaching their targeted cell and how particle size has an influence on this pathway will be discussed in this section. Small nanoparticles before approaching the target and after passing through the epithelial barrier, they undergo changes namely bio-distribution (Panariti, 2012). For example, it has been proven that after administration nanoparticles with sizes in the smaller range of 20-30 nm are discarded via the kidney or liver (Sun T. Z., 2014). However, larger particles follow a different route in order to reach the final target.

It is well reported that specific organs are targets for a specific particle size range as presented also in Table 3. Nanoparticles of the range 150-300 nm are mostly located in the liver or the spleen. Smaller particles of 30-150 nm are accumulated in the bone, heart, kidneys and stomach. The fact that defined nanoparticle sizes can target specific organs is related to the effective escape of these particles from the blood circulation via the fenestrations of the damaged tissues. In the normal healthy tissues there are no fenestration pores. These pores are present in the suffering tissues and in the endothelial cells allowing the exchange of particles between the vessels and the tissue (Gaumet, 2008). In Table 3 the correlation between the fenestration limits and the nanoparticle’s size able to overcome them in order to target a specific organ of a specific animal species is presented.

Table 3. Proved sizes of fenestration limits of the vascular system in different organs or pathological areas, as described in (Gaumet, 2008).

<table>
<thead>
<tr>
<th>Targeted organs/pathological areas</th>
<th>Fenestration Size</th>
<th>Animal Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>20-30 nm</td>
<td>Guinea-pig, rabbit, rat</td>
</tr>
<tr>
<td>Liver</td>
<td>150 nm</td>
<td>Mice</td>
</tr>
<tr>
<td>Spleen</td>
<td>150 nm</td>
<td>Mice</td>
</tr>
<tr>
<td>Lung</td>
<td>1-400 nm</td>
<td>Dog</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>85-150 nm</td>
<td>Guinea-pig, rabbit, rat</td>
</tr>
<tr>
<td>Muscle: cardiac and smooth</td>
<td>&lt;6 nm</td>
<td>Mice</td>
</tr>
<tr>
<td>Skin and mucous membranes</td>
<td>&lt;6 nm</td>
<td>Mice</td>
</tr>
<tr>
<td>Tumor</td>
<td>200-780 nm</td>
<td>Mice</td>
</tr>
<tr>
<td>Brain Tumor</td>
<td>100-380 nm</td>
<td>Rat</td>
</tr>
</tbody>
</table>

The main obstacles that nanoparticles have to face after administration are presented in Figure 4. The first and one of the most important barriers is related to “opsonization”. The definition of opsonization refers to the action of the immune system in order to recognize, target and eliminate unknown particles inside the body by activating the phagocytes (Blanco, 2015). The process of opsonization requires the identification of the unknown particle and its uptake by the phagocyte. The most severe adverse effect of opsonization is the nonspecific distribution of the nanoparticles, which are ending up accumulated in healthy organs such as the spleen and liver.

Additionally, endocytosis mechanisms and drug efflux pumps, which is the mechanism to expel compounds outside the cell, is proved to be highly related to particle size and shape (Kou, 2013). The influence of the size on the uptake from macrophage cells and phagocytes were observed from (Walkey, 2012), who proved that while increasing the particle size up to 100 nm the phagocytosis rate also increased.
Another barrier is related to abnormal blood flow limitations, which influence the penetration through the fenestrations. From Table 3, which presents the size range of the fenestrations of main organs and pathogenic areas, we can observe that in the tumor cases the penetration of bigger nanoparticles is also possible. So depending on the case the “ideal” nanoparticle size for cancer treatment may vary from 70 to 200 nm, which is a quite broad range of sizes (Toy, 2014). A second important obstacle for the nanoparticle efficiency on the tumors is that they enforce a higher inner pressure. So it is clear that especially tumors have abnormal bio-barriers and this is one of the reasons why the nanoparticle size vary significantly in comparison to the optimal size for targeting other organs. The effect of the particle size was one of the first parameters well examined in the case of liposomal doxorubicin in the 80’s and 90’s (Toy, 2014). So the nanoparticle size had already been reported as one of the most critical parameters, which determined the blood circulation, tumor resistance and drug release (Nagayasu, 1999) (Ishida, 1999). Many different studies proved that smaller particles around 20 nm or even smaller can diffuse inside the tumor deeper and more rapidly. For that reason a combination of smaller and bigger carriers are being used in order to increase the EPR effect and the compounds to diffuse deeper into the tumors. So initially nanoparticles of the size of 100 nm reached the tumor by circulation and EPR effect and after due to degradation smaller particles of around 10 nm are released and penetrate the tumor (McKee, 2006) (Popović, 2010) (Wong, 2011). It is very important that the complexity of tumors revealed that nanoparticles can target specific predictable patterns on the tumor but also different regions at the same tumor. There is a strong correlation between the particle size and the tumor environment which determines the appropriate particle size. So the heterogeneity of tumors leads to the conclusion that more complex nanoparticles and not one-size might be a more effective approach. The system complexity becomes even clearer when considering that nanoparticles have first to overcome the natural barriers and then the cellular uptake takes place. Already from the way that molecules enter in the cells there is a difference between the small drug molecules and most nanoparticles. Small drug molecules follow a mechanism of passive diffusion in order to enter the cells. On the other hand bigger in size nanoparticles >750 nm follow processes of endocytosis and particles of ~100 nm enter the cells via pinocytosis or micropinocytosis (Oh, 2014). The endocytosis mechanism will not be in detail explained, but it is presented in the overall Table 4, with brief explanation of the mechanism and examples of nanoparticles following the specific mechanism. From the table it
can be observed that there is not a clear specific size range related to the type of cellular uptake. However, this has also to do with the fact that many parameters play equally and simultaneously a significant role. So the conclusion about the relationship between size and cellular uptake cannot be absolute and it has to be investigated in each specific case.

The understanding of the interactions between nanoparticles and phagocytic cells eg, macrophages is very important as it can provide information on the optimal design of nanoparticles in order to trick the immune system not to respond and as a result to increase their efficiency. Among these interactions as proven the size of the nanoparticles is of great importance. Despite the fact that the experimental results of most studies have proven a size-dependent cellular uptake of nanoparticles it should also be considered that this cannot be implemented in all cases of all cell types. Particles undergo many alterations and aggregation in their travel to reach their target, which sometimes lead to increase of their size. For that reason it is vital for these studies to test if the nanoparticles tend to aggregate in the biological solution before they enter the cell (Zhao, 2012).

Studies have specifically focus on gaining insight on the size dependence endocytosis mechanism of nanoparticles used for cancer cells and fibroblasts. Colloidal gold nanoparticles were investigated as possible treatment for cervical cancer. It has been proven that size has an effect on the uptake mechanism of the particle. The conclusion of this extensive study was that gold nanoparticles of 50 nm size in comparison with other sizes (14-100 nm) showed the

<p>| Table 4. Brief explanation of the main uptake mechanisms. Inspired by the reports of (Zhao, 2012) and (Oh, 2014) and (Sen Gupta, 2015) regarding the cellular uptake of nanoparticles. |
|---------------------------------|-------------------------------------------------|-------------------------------------------------|</p>
<table>
<thead>
<tr>
<th>Endocytosis mechanism</th>
<th>Brief explanation of the mechanism</th>
<th>Proved Nanoparticles following the endocytosis</th>
</tr>
</thead>
</table>
| 1. Clathrin-mediated endocytosis| Particles are bound to plasma membrane vesicles (clathrin coated) which contain proteins and receptors that are specific for the absorbed molecule. | • PVP-coated silver nanoparticles, 80 nm  
• PEGylated NPs, positively charged, 90 nm  
• QDs, 4 nm.  
• Amino-functionalized polystyrene (NPS) nanoparticles, 100 nm,  
• Protein–SWNTs, 50–200 nm |
| 2. Caveolae-dependent endocytosis| Caveolae consists of the cholesterol-binding protein caveolin which forms the caveolar endocytic vesicles that transfer the molecules inside the cells | • Perfluorocarbon nanoparticles: 200 nm,  
• Polysiloxane nanoparticles, 100 nm,  
• Albumin-coated nanoparticles, 20–100 nm |
| 3. Pinocytosis/Macropinocytosis  | Pinocytosis is not selective for the transported compounds. During the pinocytosis small particles enter the cells and then vesicles transport them into the cell. | • PVP-coated silver nanoparticles, 80 nm |
| 4. Phagocytosis                 | During phagocytosis cells are trapping the particles of sizes larger than 0.5 μm | • Nanotube, 20 nm,  
• Multihydroxylated Gd@C82(OH)22 nanoparticles of about 100 nm |
most efficient cellular uptake in all of the three examined types of cells (results presented in the right graphs of Figure 5), (Chithrani, 2007). This study is also one of the examples of poor reported particle characterization.

Despite the fact that this research is aiming to prove the effect of particle size in the cellular uptake the part related to the actual estimation of the particle size is only limited to the report of the used techniques, which were coupled plasma atomic emission spectroscopy (ICP-AES) and UV-visible spectrophotometry.

Another example that proves the size dependence on the cellular uptake used the polymeric nanoparticles in order to study their uptake from the Caco-2 cells. The goal of this investigation (Win, 2005) was to evaluate the cellular uptake of the polymeric nanoparticles from this cell type, which is related to human colon adenocarcinoma. The goal was to evaluate the use of biodegradable polymeric nanoparticles polystyrene as alternative drug delivery systems for oral chemotherapy. The examined particles were in the size range of 50-1000 nm and it has been proven that the smaller nanoparticles exhibit greater cellular uptake from the Caco-2 cells. This study is also an example of the existent problem related to the poorly documented analytical methods and experimental models that are being used in the investigation of size-dependence cellular uptake was discussed, due to some contradictory results with other studies. Smaller particles showed higher cellular uptake, but the smallest 50 nm particles deviating from this behavior. The researcher’s explanation was first of all that there might be a lower limit that below it the size does not play such a crucial role anymore and also that contradictory results and opinions in the literature around this subject may be driven from the wide variety of analytical methods and procedures used for the nanoparticle characterization.

These two examples of studies first of all show that particle uptake is inversely proportional to the particle size and that also the complexity of the systems requires very specific studies related to more than just the size in order to make a conclusion. Other parameters such as cancer cell type and composition of the nanoparticle also contribute significantly to their fate. The second equally important conclusion from these studies is that in order to have
accurate conclusions, especially for potential biopharmaceutical products the reported properties should be in detail explained. From the current literature it is clear that the particle size characterization can bring a lot of conflicts, due to the complexity of the systems and the high polydispersity caused from the imperfection of preparative methodologies (Wicki, 2015) (Morachis, 2012), but also due to the limits of each analytical technique used for size determination.

It is also clear that not only the size of the nanocarriers can provide them with unique characteristics but also their shape, high surface-to-volume ratio and other physicochemical properties that can manipulate the pharmacokinetic and dynamic of the drugs, in order to enhance their action.

**EFFECT OF PARTICLE SHAPE**

From the literature research related to the effects of particle size and shape, it was observed that more recent studies tend to focus on explaining the motion and movement of anisotropic nanoparticle inside the blood stream and on a second stage mostly focus on the uptake mechanisms and applications for tumor and vaccination. The fate of nanocarriers inside the living organism regardless the administration way (Champion J. A., 2008) is affected of the particle shape, as well as size. In this section the driven forces of transportation inside the blood vessels in accordance to shape will be explained, and the influence of shape in the cellular uptake will follow.

One of the main goals in nanomedicine is to create particles with improved selectivity and adhesion efficiency and in order to approach this goal the design of nanoparticles of controlled sizes, shapes and composition is necessary, but also it is equally important to create computational models that provide information in order to be able to create improved nanocarrier designs (Shah, 2011).

Because of the improvements on the synthetical procedures of the nanoparticles new aspects for the creation of a wide variety of new shapes is opened (Jones, 2011) (Kochkar, 2011). The detailed explanation of these methodologies is beyond the scope of this literature thesis. However, the most important shapes created should be mentioned. Nanorods, nanoshells, nanoplates, nanocubes, nanostars are some of the key structures that are being tested or already used in the nanomedicine field (Sen Gupta, 2015).

According to the literature the predominantly reported particles are spherical because of the novel approaches for their synthesis. The basic mechanism behind the formation of spherical particles is due to thermodynamic and entropic rules. The existent forces make the particles to form spherical molecular self-assemblies in order to reduce their energy. But there are also proven advantages of the anisotropic shapes inside the blood flow environment such as better margination (meaning the tendency of particles to drift laterally towards the vessel walls (Gentile, 2008)), or control of the interactions with the blood vessel walls and attachment via ligand-receptor binding. Ideally, in terms of margination particles should be close to the walls, in order to increase the interactions with the targeted organ or tumor (Toy, 2014).

In order a nanoparticle to travel and finally bind with its target it has first to escape and survive phagocytosis. In the previous section the correlation between nanoparticle size and macrophage uptake was described and in this section the effect of shape will be discussed. Having escaped the attack of phagocytes and during travelling in the blood nanocarriers should be able to marginate towards the blood vessel walls.
IMPACT OF NANO SHAPE ON CELLULAR UPTAKE AND TRANSPORTATION INSIDE THE LIVING ORGANISM.

One of the most important research investigating the role of particle shape in the phagocytosis was conducted from Mitragotri and Champion from University of California in 2006 (Champion J. A., 2006). They examined as targets of alveolar macrophages a variety of anisotropic polysterene (PS) particles. They aimed to observe how the shape will influence the cells in phagocytosis. They examined six shapes, with sizes of the high nano and macro-scale. Despite the fact that these results give an indication on what is might happening, they cannot be directly projected to natural targets. The PS particles were tuned to a specific shape, which might not fully represent natural phagocytic targets but served the purpose of gaining an insight on the procedure. The results of this research showed that the phagocytes attack was much more complicated than expected. Phagocytes were attacking differently nanoparticles of different shapes but they selected also areas of the same nanoparticle that differentiate in shape. So for example elliptical disk particles interacted and internalized the phagocytes very fast and effectively along their major axis, whereas when the particles were attached to the phagocytes from their flat side they did not engulf the particles not even after 2h, as shown in Figure 6.

![Figure 6. Proof of shape and orientation dependence of polysterene particles on the cellular uptake from macrophages by a time-lapse video microscopy. The two particles presented are identical in terms of composition and shape but they interact with the cells from two different orientations a) major axis which leads in complete uptake of the particle and b) cell is attached in the flat side of the particle and effective uptake is not observed. (Champion J. A., 2008)](image)

Despite the fact that this research was quite extensive and seemed to give a nice insight on the shape effect on the phagocytosis once again the problem of not having reported important information remains. In this research both the shape and the size are discussed and conclusions about both are reported. However, there is not a clear discussion about the method of size determination.

In Table 5 examples of recent studies related with the effect of nanoparticle shape on the cellular uptake are listed in addition to the biological conclusion and method of fabrication. Additional comments about the validity of these studies, based on the size and shape determination are also presented in the size determination column of Table 5.
Table 5. Different non-spherical particles and their effect on cellular uptake, along with the size and shape determination as presented in the publication.

<table>
<thead>
<tr>
<th>NanoParticle</th>
<th>Method of fabrication</th>
<th>Shape</th>
<th>Investigated Cell type</th>
<th>Size determination-Comments</th>
<th>Conclude biological effect</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silica particles</td>
<td>Stober method</td>
<td>Worm-like, cylindrical, and spherical</td>
<td>Primary human alveolar and tissue macrophages, primary epithelial cells, immortalized RAW 264.7 mouse macrophages, and A549 human lung tumor epithelial cells.</td>
<td>The particles had similar dimension of approximately 200 nm, and average equivalent positive charge density. <strong>However:</strong> Particles were characterized only by one analytical technique TEM.</td>
<td>Spherical particles follow a clathrin-mediated endocytosis mechanism. Worm particles appear to be internalized primarily through macropinocytosis or phagocytic mechanisms. All particles regardless of shape share a degree of both uptake mechanisms.</td>
<td>(Herd, 2013)</td>
</tr>
<tr>
<td>Polystyrene nano- and microparticles</td>
<td>Film-stretching procedure and trastuzumab as the targeting antibody</td>
<td>Spherical, rod-, and disk-shaped</td>
<td>Breast cancer cell lines: BT-474, SK-BR-3, and MDA-MB-231</td>
<td>The shape and size are presented as (length ± width) and (diameter ± thickness) not as aspect ratio. So comparison between the size of these particles is difficult. <strong>Also:</strong> The sizing analysis is not reported at all.</td>
<td>Rod shaped showed higher specific uptake in all cells compared with spheres. RoIs coated with an antibody-coating exhibited greater inhibition of BT-474 breast cancer cell growth.</td>
<td>(Barua, 2013)</td>
</tr>
<tr>
<td>PEGDA-based nanoparticles</td>
<td>Jet and Flash Imprint Lithography (J-FILTM)</td>
<td>Discoidal, cuboidal (rod-shaped)</td>
<td>HeLa, HEK293, HUVEC and mouse lung microvasculature endothelial cells</td>
<td>The particles used had similar volumes and surface area, but they differ significantly in their largest dimensions (height). For the discoidal it is reported (diameter * height) and for the rod shaped is reported (length<em>width</em>height). <strong>Also:</strong> Only one analytical technique is used for the size and shape determination SEM.</td>
<td>Conclusion based both on size and shape. For all the tested cell types, nanodsics of larger or intermediate sizes were internalized more efficiently in comparison to nanorods or the smallest-size discs.</td>
<td>(Agarwal, 2013)</td>
</tr>
</tbody>
</table>
After having escaped from the phagocytes, nanocarriers should travel inside the blood stream in order to reach the target. The motion of nanoparticles in the blood flow show differences between the anisotropic particles and the conventional spherical, which are related to the dynamics present in the blood flow. Computational models have been developed in order to examine the motion inside the blood vessels along with the influence of shape and aim in the design of particles with optimal shape properties.

The transport of particles is based on drag forces such as fluid flow (shear flow), adhesion and Brownian motion (Tan, 2013). Particles tend to rotate inside the circulation system mostly because of differences in velocity caused by non-uniform forces surrounding them (Decuzzi P. &., 2006). These motions are important as they significantly affect the adhesive and margination process and by extend the interactions of the particles with its targeted cells, as described from Figure 7.

The study of (Shah, 2011) provided a more clear understanding on the effects of shape on the transportation and targeting mechanism of nanodrug delivery systems. In this study the forces and trajectories of rod-shaped and spheroid shaped nanoparticles of the same composition were compared. The results showed different trajectory behavior and different adhesion. The nanorods due to more effective binding and tumbling motion lead to better adhesion in comparison to the spherical shaped particles, as shown in Figure 8 A and B. It was also found that nanorods with the same volume as the nanospheres are more likely, up to three times, to bind. The most influential parameters for the binding seemed to be the flow shear rate, and the height of the vessels, the increase of which has an adverse effect on the binding.
It is important to mention that computational models are developed in order to describe these motions and reveal differences. Some of the most successful examples of such models are the (Decuzzi P. &., 2006) and (Gentile, 2008) models, which focus mainly on the van der Walls, hemodynamic, electrostatic and steric interactions. The computational results of these models have also been proven from experimental results (Geng, 2007). From the comparison of the margination of the disc-shaped and spherical particles, it was observed that discoidal particles are not accumulated in the liver, most likely due the rotational and surface properties (Decuzzi P. G., 2010). The importance of the coherence between modelling and experimental results is that the models can provide a practical guide for improvement in the design of nanoscale drug carriers according to the target and goal.

To sum up, shape seems to play an equally important role in the nanoparticle drug delivery efficiency. Although, the current fabrication techniques are not able to control size and shape in order to investigate the effect of each property separately, the controlled synthesis of various shapes in combination with theoretical prediction models seems to start pointing in the correct direction for the manipulation of the shape effect. However, despite the great progress in these two fields, the shape has not yet proven its true potentials in the nanomedicine.

**SIZE AND SHAPE DETERMINATION - ANALYTICAL CHALLENGES**

In the previous section a discussion about the importance of size and shape of nanoparticles for their function and fate inside the living organisms was discussed. It has been proven that size and shape influence not only the clearance, but also the bio-distribution of nanoparticles and they play a significant role in the cellular uptake of the nanomedicine. In parallel the issue of existing gaps not only in the analytical techniques used but also in the documentation and presentation of the results related to these parameters was highlighted. Another issue is that data from different studies are not easily comparable especially when different methods have been used. This new section is aiming to more critically report the strengths and limitations of the analytical techniques used for size and shape determination of the nanomedicine and also to investigate the reasons that size determination is not a straightforward analysis and answer.

![Figure 8. A] Adhesion from ligand-receptor binding depends on the nanocarrier shape and rotation due to the forces inside the fluid. Comparison of the binding effectiveness between nanorods and spherical particles. The laying position of nanorods indicates maximum binding interactions. B) Shear flow and shape influence on the orbit of non-spherical nanoparticles inside a capillary channel. C) Probability for adhesion regarding different shapes and particle volume. In this case γ is the aspect ratio, meaning the ratio of length to width of a nanoparticle. D) Percentage of number of Si particles accumulated in each organ according to different particles and shapes (Sen Gupta, 2015) (Liu, 2012) (Tan, The
The main purpose of particle size analysis is the quantitative calculation of the mean size, particle size distribution (PSD) and the shape of the particles that are meant to be used as biopharmaceuticals. Another aspect of the particle size analysis is that it aims to assure the quality of the final product before and ideally after administration. However, the inconsistency of the data not only between different instruments but also between similar instruments of different brands and depending on the data processing used make the validation of these methods very difficult. It is already discussed that this is one of the reasons why there is a big gap between research and actual clinical applications in the nanomedicine field (Shekunov, 2007). The difficulty to find more novel acceptable levels for quality control is also due to the intrinsic polydispersity of nanoparticles, which is very difficult to manipulate after synthesis.

According to the Nation Institute of Standards and Technology (NIST), a PSD can be considered as monodisperse when not less than 90% of the distribution is within 5% of the median size, which means that a relative standard deviation below 2% for Gaussian distributions is required (Xu, 2005). The problem behind this definition of monodisperse nanoparticles is that it is way too far from reality, as most available techniques cannot achieve such a high resolution. So the accuracy of the polydispersity and particle size estimation strongly depends on the technique used, the particle composition itself and the conditions used for the analysis. Many parameters and their effect should be taken into consideration during the interpretation of the results. Temperature, concentration, pH, viscosity are only a few examples that may lead to aggregation and changes in the particle size. The same and many more parameters take place also after administration in the living organism. Alterations in the particle size under these conditions seems to be more important to be evaluated as they can significantly influence the particle’s final functionality.

To initiate the discussion for the need of complementary techniques used for size and shape determination, an example will be presented. The difficulty of measuring size distribution and structure of nanodrugs was already in proved from the analysis of the FDA approved Doxil (Caelyx), which consists of PEGylated liposomes. From cryo-TEM analysis an ellipsoidal shape of crystalline nanorods was revealed when loaded with doxorubicin sulfate salts. However, cryo-TEM analysis was not able to show the PEG layer and therefore the size measurement was misleading. Additionally, when DLS was included, the diffusion coefficients and the hydrodynamic radius of the liposomes including the PEG layer was estimated. However, as it will be discussed in the following sections, DLS assumes a spherical shape for the liposomes, which according to cryo-TEM is not true in this case. The final FDA approval came after 25 years of preclinical and clinical studies when also the X-ray diffraction was used for the determination of the PEG-layer. This is only one example of the need for a broad spectrum of techniques based in different approaches and methods needed in order a nano-drug to be approved (Barenholz, 2012) (Tinkle, 2014).

All the above mentioned aspects will be further discussed in comparison also with specific techniques. The techniques used in particle size characterization that will be evaluated in this literature thesis can be separated to light scattering techniques, microscopy techniques, single particle analysis and separation techniques. The main principles of each technique as well as strengths and limitations will be discussed along with new developments.
Light scattering techniques are used in nanoparticle size characterization as reference techniques because of their advantages. However, their disadvantages indicated that they cannot be used as stand-alone techniques, but complementary results from high resolution techniques are in most cases necessary.

To begin with, light scattering is the consequence of the interaction between a light beam and the electric field of a small particle or molecule. It can be considered as the redirection of light that happens when an electromagnetic wave (in this case light) has to overcome an obstacle for example a heterogeneous solution particles. When the electromagnetic wave interacts with a particle, the particle’s electron orbit oscillate with the same frequency as the coming wave. This oscillation forms the dipole moment of the particle when the charges separate in the molecule. This interaction finally leads to the electromagnetic radiation or scattered light. Because light scattered is emitted at the same frequency as the incident light the process is often called elastic scattering (Hahn, 2006).  

Explanation of the light scattering theory follows two theoretical frameworks, Rayleigh scattering and Mie scattering (Li X. X., 2012). Rayleigh scattering theory is preferred because of its simplicity in comparison to Mie scattering and it can be used to calculate the scattering of particles, which is much smaller than the wavelength of incident EM wave.

\[
I = I_0 \frac{8\pi \alpha^2 (1 + \cos^2 \theta) v}{\lambda \pi r^2} \tag{Equation 1}
\]

In the presented equation for the calculation of the intensity of the scattered light \(I\), \(I_0\) the intensity of incident light, \(\alpha\) polarizability of particles, \(\theta\) angle used to measure the scattered light, \(r\) distance between sample and detector, \(\lambda\) wavelength of incident light and \(v\) particles per unit of volume are used.

Rayleigh scattering occurs when the dimensions of the spherical particle radius is much smaller than the wavelength of the incident electromagnetic radiation and exhibits a strong wavelength dependence. The scattering produced by such small particles is equal in all directions, as shown in Figure 9. The produced intensity of the light is directly proportional to the particle diameter.

Mie scattering occurs when the dimensions of the scattered particles is much larger than the wavelength of the incident electromagnetic radiation. An example is when light is scattered by small water droplets in clouds (Instruments, 2012).
It is important to mention these two theories in order to explain one of the main disadvantages of DLS. In the most commonly used Rayleigh approximation the intensity of scattered light is proportional to the $d^6$, where $d$ is the particle diameter and inversely proportional to $\lambda^4$, where $\lambda$ is the laser wavelength. This fact can explain why DLS results are biased over the larger particles, which can hide the smaller particles present and subsequently make accurate estimation more difficult and also that at lower wavelengths a higher scattering intensity is observed. On the other hand, Mie theory can provide accurate estimations over all wavelengths, sizes and angles, but because of the more difficult calculations not all software are using it.

**DYNAMIC LIGHT SCATTERING (DLS)**

Dynamic light scattering is undoubtedly the most commonly used technique for particle size analysis, mainly because of the limitations that govern the microscopy techniques, but also due to some of the greatest advantages that it offers. DLS is a non-invasive technique of relatively low resolution that also provides concentration information from the size distributions. The difference between static light scattering and dynamic is that static light scattering is related to the measured property. In static light scattering the time averaged scattering intensity is measured whereas dynamic light scattering is related to the time fluctuations in the intensity of the scatter light caused by a Brownian motion which induce Rayleigh scattering (Brar, 2011). Analysis of the fluctuations in intensity in time allows the determination of the diffusion coefficient and leads to the estimation of the hydrodynamic diameter ($R_h$), radius (also known as Stokes radius) via the Stokes-Einstein equation.

$$R_h = \frac{kT}{6\pi\eta D}$$

Equation 2

The hydrodynamic diameter is estimated based on Boltzmann’s constant $k$, absolute temperature $T$, viscosity $\eta$ and diffusion coefficient-velocity of the Brownian motion, as shown in the above mentioned equation. Hydrodynamic diameter is considered as the diameter of a hard sphere, which is a theory that will be explained in the next sections, that diffuses at the same speed as the measured particle and depends not only on the size of the particle “core”, but also on the surface structure and the ionic strength of the solution. As a result, there are many parameters that can influence the outcome of DLS.

In a common DLS experiment, a laser passes through a polarizer and then this light goes through the sample (incident beam). If the size of the analyzed particles is smaller compared to the wavelength of the incident light, then the light will scatter in all directions as described from the Rayleigh theory. The scattered light goes then through an analyzer, in order a specific polarization to be selected, which will then enter the detector. The position of the detector defines the scattering angle $\theta$. In most cases the detector is placed at 90° to the laser beam, in order to collect the scattered light intensity. The autocorrelator basically compares signals, in order to find
the degree of similarity between two signals or even between one signal that changes in time. Autocorrelator can also give a lot of information about the sample. If the sample particles are large then the signal will not face rapid changes and the correlation will be similar for a longer time frame. In case of smaller particles that can move faster, then the correlation will drop faster and as a result already qualitative information about the particle’s size can be extracted.

The effectively measured size range in DLS for nanoparticles is from 1 nm up to 500 nm and working concentration range is of $10^8-10^{12}$ particles/mL (Sapsford, 2011). However, there are systems that a broader range up to micrometer level is claimed. This level of resolution and sensitivity can be achieved with the new technology of the non-invasive backscattering optics (NIBS), which allows a size determination from 0.3 nm up to 10 μm (Zetasizer Nano ZS; Malvern Instruments, Malvern, UK, measurement range of 0.3 nm–10.0 μm) (Lee, 2016).

As every technique, DLS has its limitations and in this case that means that despite the fact that it is a relatively easy technique to use and can easily be used for routine analysis, on the other hand it cannot handle multimodal particle size distributions. The fact that the intensity of the scattered light is proportional to the sixth power of the particle diameter makes this technique biased to the presence of larger particles as already discussed in the Rayleigh explanation. Additionally, DLS assumes spherical nature of the particles and its use in non-spherical nanomaterials provide inaccurate results with underestimated size values (Brar, 2011). For non-spherical particles certain assumptions are required. The most challenging part in DLS is the understanding of the final outcome as there are various algorithms in order to estimate the size from the correlation function. The two most commonly used approaches are based on different statistical procedures. In the one case the fit of a single exponential to the correlation function is used in order to obtain the mean size or z-average diameter and the polydispersity index. In the other case the fit is based on a multiple exponential function, such as non-negative least squares or the most commonly used CONTIN, and the outcome is the distribution of the particle sizes (Instruments, 2012).

DLS is considered a quite standardized and suitable technique for quality control, which is very important for the characterization of pharmaceutical products. Examples of the obtained results from DLS also in comparison to the other techniques are given in the separate sub-sections of each technique since DLS is the most commonly used technique for complementary evaluation of the results obtained from microscopy (TEM, SEM, AFM) or single particle analysis (NTA, SIOS).
MISCOSCOPY TECHNIQUES

TRANSMISSION ELECTRON MICROSCOPY (TEM)

Microscopy plays a very important role in particle size analysis, as it is often used for validation of the results obtained with more routine techniques such as laser diffraction or dynamic light scattering. The use of electron microscopy opened a window in the nano and micro world with the more detailed images ever obtained. The most cited electron microscopy techniques for drug delivery and nanomedicine are the transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

Transmission Electron Microscope (TEM) uses electrons in order to provide information about the structure and composition of the samples. It provides black and white images because of the interaction between the sample and the electrons inside the vacuum chamber. Based on the contrast image, TEM is mostly suitable for particles that contain heavier atoms, such as gold and silver nanoparticles. TEM has an atomic or a sub-nanometer spatial resolution which means that it can magnify particles of approximately 1 nm, providing high resolution images (Malatesta, 2016).

During TEM analysis the system has to be under vacuum so the electrons can move. The air is pumped out of the chamber and vacuum is created. The beam of electrons is crossing a very thin layer of sample, where the electrons interact and the sample properties can be estimated. The electrons follow a path, in which they have to go through many electromagnetic lenses and are covered with coil as shown in Figure 12. In the final step of this path the electron beam is focused on a screen, where the electromagnetic signal is converted into light and the final image is formed. The resolution and quality of the image can be adjusted in two ways, either by tuning the voltage of the electron source in order to adjust the speed of the electrons or by adjusting the electromagnetic wavelength by the lenses. The faster the electrons and shorter wavelength the better the quality (Microscopemaster, n.d.). Because TEM has been proven to cause alterations in the samples, due to the vacuuming processes that lead to very harsh dehydration conditions, developments in TEM focused on improvements during this process.

Cryo-transmission electron microscopy (cryo-TEM) and freeze-fracture TEM (FF-TEM) are two of the newest developments in TEM. These new types of TEM aim to keep the sample under very low (cryogenic) temperatures without any dye to be used (López-Serrano, 2014). They are not too harsh techniques as the rapid freezing of the sample with the use of liquid nitrogen offers advantages. The ice crystals are limited, and it enables the proteins and other biological compounds to be preserved. Although both techniques can provide valuable information related to size, aggregation and shape with minimum structural changes, the instrumentation is quite expensive and not easily accessible and as a result not widely used. Additionally, the sample preparation is laborious and time-consuming (Wei A. M., 2012).
An example of the information and the data that can be obtained are presented in Figure 10. Cryo-TEM was used for two purposes. First of all to monitor the evolution of a micelle solution over 21-day period and secondly to monitor the size evolution depending on the concentration of micelle. The sizes of the micelles were estimated from the cryo-TEM pictures and they were visualized as frequency histograms (Figure 11 a,b). It was observed that at the beginning of the experiment there was a quite monodisperse distribution, which after only one day started to convert into two distinct distributions. The second distribution was identified as aggregation products of large and small micelles. The same trend was followed for days until the 16th day, in which the distribution started again to reveal a monodisperse population. It is important to mention that the following trend of these results was consistent with the results obtained from DLS. On the other hand, the size evolution depending on the concentration of micelles was examined with three different techniques DLS, cryo-TEM and SANS (small angle neutron scattering). The used techniques provided complementary but different results, as DLS showed a monomodal or close to bimodal distribution, which means that it was not possible to relate the observed changes as a result of the different concentrations. However, cryo-TEM and SANS provided the necessary sensitivity to monitor these changes. As shown from Figure 11c cryo-TEM showed that the 10th or 11th day the 2 mg/ml and 5 mg/ml solutions contained a more bimodal population of micelles, whereas in the 10 mg/ml solution larger micelles had formulated. So in conclusion three techniques were needed in order to cross validate the results and have a more accurate conclusion.

**In situ TEM (Wet TEM)**

Transmission electron microscopy on the one hand has achieved the most significant resolution for nanoparticle characterization. On the other hand the fact that pretreatment can affect significantly the size and structural
properties makes it very risky, when these properties are important to be accurately measured, to rely only on this technique for characterization. In-situ or wet TEM was developed, in order to address this problem and be able to study dynamic processes in a more liquid environment. The term in-situ in this case means that the properties of the particles are being measured, while they are changing (Carlton, 2012). Although, in a way every TEM experiment can be considered in situ because of the effect of the electron beam to the particles, in the actual in situ experiments the aim is to cause a change on purpose and monitor its effect in order to learn something from this alteration (De Jonge, 2011). This method has the advantage that the observed result can be directly correlated with the resulting change in the structure and after for verification complementary techniques can be used. It is also important that because both the implemented change and its outcome can be simultaneously monitored and record also quantitatively results can be obtained, leading to a better understanding of fundamental processes and aggregation.

Despite the important advantages that in-situ TEM can offer the experimental complexity is still of an issue. The effects of both sample preparation, with the need of creating a thin layer of the sample and the electron beam should be understood and taken into account.

In-situ TEM is reported to be used for liposome characterization in liquid environment. Usually, the characterization of liposome size and structure involves a combination of dynamic light scattering (DLS) and TEM. In order to achieve a resolution of nanometer scale that will give the details of lipid structures usually one of three TEM techniques is used: freeze–fracture, cryo-TEM, or staining is required. Despite the fact that these techniques can quite successfully image the liposomes in the radiation and high-vacuum environment of TEM, liposomes are removed from their natural liquid environment which makes the correlation of structure and size with liposome’s functionality quite inaccurate. For these reasons (Hoppe, 2013) used an in-situ microfluidic TEM to image the lipid bilayer structure in real time inside deionized water. The aim of this study was not directly to measure the liposome’s size but their stability under different effects.

For this study the Poseidon in-situ microfluidic TEM stage, from Protochips, Inc. instrumentation, shown in Figure 12, was used and prior to the TEM investigation DLS experiments were performed. Poseidon in situ TEM contains a liquid flow holder equipped with a microfluidic cell (Figure 12 b) that nanoparticles pass through an electron transparent viewing window (Klein, 2011). The in-situ microfluidic TEM was successfully image liposomes of 100 nm diameter in water, and most importantly no staining or sample preparation was necessary in order to increase the contrast, providing less harsh conditions for the liposomes. From the TEM analysis the measured diameters for the liposomes were ranging from 69 nm to 1.46 μm. Although, these results were not aligned with the expected size obtained from the synthesis technique, they were aligned with the results from DLS. The explanation given is that liposomes have changed significantly after their preparation. Additionally, the fact that DLS gave a broad peak, proved that it is unable to resolve the smaller particles. Since the results of both techniques were in consistence it
was concluded that the particle’s structure was not affected by the microfluidic TEM (cell size, pressure, surface chemistry etc.) but indeed the particle properties changed after synthesis.

![Figure 94. a) Representation of the inside of a microfluidic cell during a liposome experiment. Two SiN membranes separate the solution from the TEM vacuum while allowing electrons to pass through. Fluid flow is from front to back of the cell, between the Au spacers. A population of liposomes of different particle sizes are depicted as opaque spheres for clarity. (b) Microfluidic tip showing a small Si chip and a large O-ring that keeps the liquid chamber sealed.](image)

Lastly, except the size, also morphological information about the liposomes could be extracted as shown in Figure 14 b, c. In-situ TEM has successfully been applied not only for the imaging of liposomes (Hoppe, 2013) and gold nanoparticles (Klein, 2011) but also for the understanding and monitoring of biological pathways, such as in the case of carbon nanotubes and their degradation due to macrophagocytosis (Elgrabli, 2015).

![Figure 105. a) POPC liposomes imaged in deionized water using plasma cleaned Si chips. (b) Denatured liposome structures resulting from surface interactions. (c) Incomplete and non spherical structures resulting from DSIDA/POPC liposomes imaged in water.](image)
The main difference between SEM and TEM is the way that electrons interact with the sample. In scanning electron microscopy electrons are produced from the sample surface, whereas in TEM electrons penetrate the sample. Some of the main compartments of SEM, such as the electron source and electromagnetic lenses that produces and focus the electron beam, are similar with TEM. However, because in SEM only the lenses above the specimen are needed for focusing of the electrons the column is significantly shorter than the one used in TEM, as presented in Figure 15. Additionally, with SEM in comparison to TEM, the sample size is only limited due to the chamber size. Despite the fact that TEM principles are quite different than SEM, they both provide same type of data (López-Serrano, 2014). The particle size estimation is in both cases based on image analysis software, which uses the information of the image to calculate the size distributions and shape and morphological parameters (Gaumet, 2008). The differences in the principles is due to the fact that in SEM the produced electron beam is focused into a specific spot, around 1 nm on the surface of the specimen/sample. This beam is then scanned in a rectangular pattern. This way the interactions between the beam electrons and the specimen surface produce signals that are measured and depicted on an image. The image is formed by the difference in brightness from the signals produced in the different scanned areas.

The main advantage of SEM over TEM is related to the sample preparation. Since the specimen thickness is not important anymore, as the electrons do not penetrate it. This simplifies significantly the sample preparation and its effects on the sample’s properties. However, also in this case sample preparation is not harmless as it can cause changes in the size of the particles. The sample has to dry and be coated with a conductive material. An important advantage of SEM is that a larger amount of sample can be measured at once, which can significantly improve the statistical reliability of nanoparticle size and shape distribution measurements (Sapsford, 2011).

Environmental SEM (ESEM)

Given the abovementioned disadvantages of TEM and SEM sample preparation such as staining or freezing a recent development of scanning electron microscopy is the environmental SEM (ESEM), in which the samples are analyzed at low pressure gaseous environment and at high relative humidity (up to 100%). The conditions of ESEM, in combination with its resolution make it a quite appropriate technique for analysis of biologically unstable samples.

In the research of (Ma, 2010) ESEM is used as a complementary confirmation of the results obtained with AF4-MALS ans DLS for the size and size distribution determination of DNA/chitosan-rhodamine nanoparticles. From the DLS results it was concluded that the nanoparticle sample of DNA/Ch-rho was polydisperse with an $R_h$ (hydrodynamic radius) of 20-160 nm. The $R_h$ was calculated for each fraction from the autocorrelated function of DLS, which leads to the diffusion coefficient and by extent to the $R_h$. The validity of the obtained results from AF4-DLS was confirmed
by ESEM, after the collection of fractions in different elution times, as presented in Figure 16. The estimated sizes of the first and second fractions were quite similar with both ESEM and DLS. However, in this experiment the limitations of both techniques caused problems. DLS as already discussed, it assumes hydrated spherical particles, whereas ESEM analyses dried particles. As a result, in the last AF4 fraction, with larger particles, there was a discrepancy between the estimated radii of the two techniques. A reason for that might be that particles tend to shrink under the vacuum conditions of ESEM. For ESEM the average particle size and standard deviation were determined by measuring the diameter of more than 150 particles from at least 6 different fields for each fraction using the microscope software in order to gain statistical significance.

![Figure 17. AF4-MALS-DLS fractograms of nanoparticles chitosan-rhodamine and complexes DNA/chitosan-rhodamine. The lines and dots represent the normalized Rayleigh ratio at $90^\circ$ and $\text{Rh}$ of DLS in time. On the right side the ESEM micrographs of the three fractions are presented.](image)

The minimum sample preparation of ESEM is one of the potential advantages over SEM. However, from this experiment it has been proven that also ESEM can cause up to a certain extent alteration of the particle size, which should always be considered and investigated either with a complementary technique of by using statistical tools to verify the validity of the outcome.
**ATOMIC FORCE MICROSCOPY (AFM)**

The two most commonly used techniques for particle size characterization are Electron Microscopy (EM) and Dynamic Light Scattering (DLS). Another technique that can be used as complementary technique is Atomic Force Microscopy (AFM), which falls in the category of scanning microscopes and has been named as “nanoscopy”, because of the level of detail that it provides (Khorasani, 2014). AFM provides 3D images that contain information about size, shape and surface with a nanometer resolution. The basic working principles of AFM, as can also be seen from Figure 17, are relatively simple and include the use of a cantilever with a sharp tip. The cantilever measures the force between the probe and the sample and the final image is a result of the measurements of the vertical and lateral deflections of the cantilever as measured by an optical lever. A photoelectric detector is altering the signal in order to create an image at the software, in which the critical dimensions of the nanoparticle can be measured. More specifically the size calculation is possible due to the three dimensional structure presented in this image (Guo, 2013).

The fact that the conductivity of the sample is not significant makes it a very useful tool for characterization of a variety of samples and this is especially important as it can be used for the imaging of “softer materials”, such as biomolecules, whereas TEM and SEM are not able to analyze not electron dense materials. Important is also to mention that for AFM analysis coating, staining or freezing are not required and most importantly analysis can be performed in air (Garg, Characterizing particulate drug-delivery carriers with atomic force microscopy, 2005), so sample preparation is significantly faster and easier, which makes AFM a technique suitable for routine analysis. As a result AFM in contrast to electron microscopy is able to perform analysis without causing severe damage to the sample and has the capability to analyze also in liquid environment, meaning that more native conditions can be used. The same liquid as the one used for storage and preparation can be used in order to have more accurate size determination (Parot, 2007).

Many comparative studies (Kanno, 2002), where AFM and TEM or SEM were used, it has been proven that AFM is able to produce similar results with TEM, which is supposed to be the highest resolution technique. In Kanno’s research the size of liposomes was estimated with AFM and TEM and the obtained results were quite comparable (Figure 18). However, due to the liquid environment of the analysis of AFM the obtained size distribution is considered more accurate and closer to the reality after administration of the liposomes.
On the contrary, in the research of (Anabousi, 2005) a combination of AFM, TEM and DLS was used. From AFM a clear increase in the size could be observed with parallel formation of small globular structures. In this case AFM was used according to the authors in order to visualize and confirm the particle size and morphology of the formed liposomes, with the obtained results from TEM. The method used for size determination included the size evaluation of all visible particles present in a specific scan area. In the same study DLS was used for size determination, and a deviation between the obtained mean size was observed, as presented in table 6. The explanation lies most likely in the interactions between the liposomes with the surface of the silicon wafer used for AFM analysis.

As a result, AFM is a non-destructive technique that enables the direct observation and estimation of the nanocarrier’s size and shape with a resolution equivalent to TEM’s and with little sample preparation. Despite the several advantages that make AFM a useful tool for the characterization of drug delivery nanoparticles, it has been proven that it cannot be used as a stand-alone technique and again complementary techniques based on other principles should be used in order to gain a better understanding on the parameters that affect the final result of size determination.

<table>
<thead>
<tr>
<th>Liposome type</th>
<th>AFM Before transferrin addition</th>
<th>AFM After transferrin addition</th>
<th>DLS Before transferrin addition</th>
<th>DLS After transferrin addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-glutaryl-PE</td>
<td>165±16 nm (n=45)</td>
<td>192±22 nm</td>
<td>121.40 ± 5.57</td>
<td>129.00 ±1.66</td>
</tr>
<tr>
<td>DSPE-PEG2000-COOH</td>
<td>157±12 nm (n=57)</td>
<td>168±17 nm</td>
<td>148.43 ± 2.9</td>
<td>154.80 ± 0.85</td>
</tr>
<tr>
<td>DSPE-PEG2000-MAL</td>
<td>172±9 nm (n=29)</td>
<td>180±12 nm</td>
<td>166.00 ± 10.41</td>
<td>169.33 ± 8.76</td>
</tr>
</tbody>
</table>

Figure 19. AFM results of liposomes with smooth surface morphology of round or spherical shape. The spherical structures’ size was estimated as 10.53 ± 1.45 nm in diameter (Anabousi, 2005).
NANOPARTICLE TRACKING ANALYSIS (NTA)

Nanoparticle tracking analysis is a quite recent technique as it was commercialized in 2006 and because of shared principles is quite comparable to DLS (Shekunov, 2007). The technique combines visualization of the nanoparticles and then from estimation of the Brownian motion of the particles the size and size distribution is calculated. Brownian motion of each individual particle is followed in real-time via video. Scattering or fluorescence properties of particles are measured with a laser light scattering microscope coupled to a CCD camera that can record many individual particles in solution. The main principles of NTA are presented in Figure 19.

![Figure 19. Schematic representation of the main principles of NTA as presented from Malvern NanoSight’s technology.](image)

From the software and based on the Stokes-Einstein equation the particle size is estimated. Particles of about 30 nm up to 1 μm can be detected. The lower detection limit of this technique is strongly related to the refractive index of the nanoparticles. An important advantage of NTA is that it can also provide quantification information as the estimation of the concentration is possible because in this case the volume is known (Carr, 2008).

One of the doubts concerning the accuracy of NTA is driven from the fact that many influential parameters are chosen from the user. During NTA analysis the user has to focus on specific areas and capture with the camera, which can clearly be both an advantage a disadvantage. The operator can select the settings focusing on a certain population of particles, while missing other particles. So the results in most cases are biased from individual judgment and experience of the user (Filipe, 2010) (Carr, 2008). The solution in order to increase accuracy of the results is the aim for a higher statistical significance, which means that the user should thoroughly investigate many different areas under the microscope trying to identify the presence of different particle size classes. Method development in the case of NTA means to optimize the video settings in order to capture all the present moving nanoparticle sizes. A few examples of tunable software parameters along with their effect are presented in Table 7. However, it is concluded that because of these limitations NTA cannot be used as a standardized method.
Table 7. Example of critical parameters and possible outcome based on setting values (Filipe, 2010).

<table>
<thead>
<tr>
<th>Critical parameter</th>
<th>Description</th>
<th>Incorrect settings</th>
<th>Desired result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection Threshold</td>
<td>This is one of the most critical parameters that can influence the final result. Is the minimum gray area of a particles image that is used for considering a signal as traceable particle or not</td>
<td>- If set at an extreme high value then there is the risk that smaller particles will be missed - if set at an extreme low threshold loss of larger particles is possible or additional estimation of the noise</td>
<td>Ideally the threshold should be set in a value that all the particles can be detected and the noise is excluded.</td>
</tr>
<tr>
<td>Blur</td>
<td>It works more as a smoothing effect, as it eliminates visual noise from areas around and within the particles.</td>
<td>- If a too harsh smoothing effect is applied, there is always the risk to “erase” small particles - If insufficient additional light scattering will be consider as signal</td>
<td>Sufficient smoothing can help the software in the detection of only the real signal and particles.</td>
</tr>
<tr>
<td>Capture duration</td>
<td>Determines the length of the recording</td>
<td>If set too short then the statistical accuracy is doubtable and results in poorly determined size distributions</td>
<td>Set accordingly in order to have statistical accuracy robustness and reproducibility.</td>
</tr>
</tbody>
</table>

From the comparative study between DLS and NTA of (Filipe, 2010) it has been proven that these two techniques are comparable and can provide complementary results. In this study three classes of drug delivery nanoparticles were investigated, more specifically PLGA particles, TMC (N-trimethyl chitosan) particles and liposomes. The problematic fact that different techniques report different values and the importance of deeper understanding of these values or even the determination of a more novel terminology is clear from the outcome of this study. The comparison of the two techniques is based on values that are not entirely comparable. The obtained results from NTA analysis for the three classes of drug delivery nanoparticles are presented in Figure 20. For simplicity reasons only the results related to TMC particles will be discussed. From DLS the Z-average and PDI was calculated and the results indicated a quite monodisperse sample. The results from NTA are expressed as relative low standard deviation and mean size. Although the results from both techniques were in accordance, there was a difference of about 90 nm between the mean size from NTA and the Z-average from DLS, which may

![Figure 21. Results of three drug delivery nanoparticles obtained with NTA and DLS. In the middle graphs the size distribution of the samples obtained with both techniques is presented and on the left side the 3D graphs of size vs intensity vs concentration obtained with NTA are depicted (Filipe, 2010).](image-url)
indicate the presence of more populations and a polydisperse sample. The shift of size distribution towards larger sizes by DLS might be due to the fact that DLS measurements are intensity distributions, whereas NTA measurements are number distributions. From theory is known that intensity distributions provide a larger shift in case of higher polydispersity samples (Filipe, 2010).

As a conclusion, despite the fact that NTA and DLS provide similar results they are based on different statistical values and thus they cannot be directly comparable. Additionally, NTA provides more quantitative results, but the statistical sampling of DLS is superior, so considerably consistent results can be obtained (Filipe, 2010) (Carr, 2008). This supports again the statement that DLS can be used as a standardize technique, whereas for NTA maybe after the development of suitable standards it can be considered to be used as a more standardized technique.

SCANNING ION OCCLUSION SENSING (SIOS)

Scanning ion occlusion sensing (SIOS) is a recently developed approach for particle analysis, in which each particle is analyzed individually as it travels through a nanopore (Yang, 2012). In Figure 21 a schematic representation of the basic principles of SIOS is presented. This technology has the effect that the method can be tuned selectively only for the particles of interest. This method is suitable for particle analysis of sizes from 60 nm up to several micron. There are two Ag/AgCl electrodes above and below the nanopore, which are applying a potential difference across the pore. When an electrolytic solution is passing through the pore then a formed current pulse can be measured, which is called blockage event. Consequently, the resulted motion of charged nanoparticles is due to electrophoresis or electro-osmosis. Particles can also travel through the pore due to pressure difference when using a VPM (variable pressure module), in order to create either vacuum or pressure. This mode of the instrument is particularly useful for weakly or even neutral nanoparticles and the detection limits are quite low (Yang, 2012).

The calibration of the pores so far is based on synthetic carboxylated polystyrene nanoparticles (CPS100, CPS217 and CPS355). The fact that calibration products exist is important to be mentioned because in general reference components are difficult to synthesize and characterize and there are not standards for all the techniques used for size determination (NTA, microscopy), however they are absolutely important for a technique to be approved as a standardized acceptable technique (Tinkle, 2014).

The study of (Yang, 2012) is a comparative study between SIOS and DLS using both liposomes and polystyrene nanoparticles in order to find the advantages and disadvantages of each technique. Here only

Figure 22. Schematic representation of SIOS. In a. the pressure module is presented and in b. the monitored blockage event, moment when a particle passes through the pore (Yang, 2012).
the results regarding the polysterene nanoparticles will be discussed but the accuracy and sensitivity of SIOS could also be proved by the size determination of the bimodal liposome samples. In the case of polysterene nanoparticles bimodal samples were prepared in different volume ratios and also one trimodal sample of 1:1:1 ratio and the samples were analyzed both by SIOS and DLS. The obtained results are presented in Figure 22, where also the comparative table is included. Two comments can be concluded from this study. First of all, it seems that SIOS can clearly be used for the size and size distribution determination of bimodal sample, with quite good precision and accuracy. Secondly, the ongoing discussion about the way of reported results seems to stand, as different research groups report the results using different, although somehow equivalent, values, but also the results obtained from different methods may differ, as can be seen from the table where for DLS the Z-average and PDI (polydispersity) are used whereas for SIOS the results are reported as mean, medium and mode size.

![Figure 22](image_url)

**Figure 23.** a. The resulted size distribution of the different ratios of the examined polysterene nanoparticles is presented as obtained from SIOS and in b. the resolution between the trimodal sample mixture is presented. The table is an overview of the results obtained from both DLS and SIOS for the polysterene nanoparticles.

### SEPARATION PRIOR TO SIZE DETERMINATION

As it was discussed in the previous section of light scattering, also in case of dynamic light scattering the technique is not per se suitable for polydisperse samples. For example during the synthesis of micelles or vesicles using self-assembly methods, because of the complexity of the interactions quite heterogeneous mixtures are produced (Robertson, 2016). In this case sample preparation or purification techniques are absolutely necessary prior to detection and size determination.
Many properties of the nanoparticles are strongly related to their size characteristics as already proved in the first sections and as a result homogeneity and purification of the nanoparticles has been pointed also from FDA and the European commission as in many cases strongly required. The more widely, high resolution techniques used, TEM and SEM are discussed in the previous section, but they are not without their limitations. Because of the harsh sample preparation conditions required, they can significantly influence and alter the properties of the nanocarriers giving misleading results. So analysis in a more native-like environment is strongly required in order to be able to correlate their properties with the expected or unexpected functionality.

Dynamic light scattering accuracy is compromised when polydisperse samples are analyzed. The same stands also for MALLS, where the accuracy is strongly affected by the presence of large nanoparticle aggregates. So hyphenation of these detection techniques with size-based chromatographic separation methods can significantly enhance the accuracy and simultaneously provide solution conditions that are not denaturing. Some of the separation techniques that are being used are size exclusion chromatography, field flow fractionation, analytical ultracentrifugation and capillary electrophoresis (Sapsford, 2011). Because this literature study is not aiming to provide a full report of all the principles and limitations of every analytical technique used, emphasis will be given mainly in FFF and SEC, because these separation techniques are compatible and being used in combination with the previous light scattering and microscopy techniques, either online or offline.

**SIZE EXCLUSION CHROMATOGRAPHY (SEC)**

Size exclusion chromatography separates molecules according to their hydrodynamic volume. SEC is being used for molar mass determination and due to its hyphenation with light scattering techniques such as DLS, LALS, RALS, MALS, the radius of gyration, hydration and by extent shape can be estimated. Most SEC columns are available for protein separation however, there are a few available and suitable for nanoparticle separation, of around 100 nm (Zhang, 2012).

The sensitivity and accuracy of the method after hyphenation is also determined from the detection system. For example in case of MALS and DLS there are some limitations for size determination. Accurate size determination for particles below 10 nm, such as Quantum dots is not possible, basically because the light scatters equally at all angles (Wagner, 2014).

The mechanism of SEC, which is also its main difference from other chromatographic techniques is that it is not an enthalpically driven separation but it is an entropically driven process. Larger particles cannot enter the pores, thus eluting first, whereas smaller particles enter the pores and elute later. In an ideal SEC separation the analyte should not have any interaction with the stationary phase (García, 2005). However, this is not always possible and thus is one of the main reasons for the low selectivity of the method. Interactions with the stationary phase can significantly alter the morphology and size of the nanoparticles. Sometimes manipulation of the non-ideal SEC interactions is necessary, so from the combination of size exclusion mechanism and adsorption better separation can be achieved. Due to SEC mechanism is able to separate both hard and softer nanoparticles, from proteins to carbon nanotubes, only driven by their size characteristics (Pitkänen, 2016). Another advantage of SEC is that a wide selection of mobile
phases can be used, both aqueous and organic depending on the nanoparticle, and this is important in order to make sure that the chromatographic conditions do not impose any additional denaturation or aggregation.

One of the main challenges in SEC is the selection of a mobile phase appropriate for the final cause of the analysis. For example in the case of metal nanoparticles and quantum dots adsorption of the analytes into the column is taking place and can cause several problems. First of all, due to loss of recovery not accurate quantitative results will be obtained. Secondly, because of the interaction of the analyte with the column packing material the hydrodynamic diameter of the pores will be biased and as a result shifting in the retention volumes will encounter. Nowadays, the main focus of research for improvement of SEC is mostly related to the mobile phase and the manipulation of the adsorption on specific column resins. For that reason, surfactants or coatings and stabilizing agents are sometimes being used (Wei G. T., 1999).

In the research of (Zhang, 2012) lipid nanoparticles (LNP) based on small interfering RNA (siRNA) are being analyzed using multiple detection after the separation with SEC. In this report the limitations and advantages of SEC are also being discussed, forming the final goal to be the high-resolution analysis of LNP for the estimation of their size distribution under native conditions. From the online coupling of SEC with UV-Vis, MALS and RI the size distribution, molecular weight and amount of siRNA loading were estimated. The obtain size values showed that not only the separation was efficient but the results were also reproducible and because six LNPS of different sizes were analyzed, SEC has been proven to be able to separate a relatively broad range of LNPS. Additionally, in the case where the particles showed similar properties, cryo-TEM and DLS were used for further validation of the results. The complementary information of the multiple detection techniques revealed differences even between these particles. In Figure 23 the resulted size values of LNP A are presented and from their comparison with the respective results from LNP B (not shown here), differences were revealed.

Although in this study DLS and cryo-TEM were also used for the estimation of particle size, these methods were not able to provide a high resolution and also accurate quantitative results. On the other hand, a comparative study between FFF and SEC showed that the two techniques in this case provided similar results and that SEC can be used

Figure 24. Results of the separation of LNP A population with G6000PWxl-CP column. a) presents an overlay of UV 260, LS 90°, and RI signals; b) shown the UV spectra of the SEC fractions of 3 min elution; c) shows the root mean square (RMS) radius; e) the molar mass of LNP during the 3 minute of fractions is presented and f) the cumulative and differential distribution of the molar mass.
to estimate the size distribution of the LNPS under native conditions. So in conclusion, although the SEC optimization and method development might be time consuming, once optimization is achieved the implementation of the method is easy and the conditions used make it a quite efficient method for quality control of intermediate size nanoparticles (Wei G. T., 1999) (Zhang, 2012).

FLOW FIELD FLOW FRACTIONATION (F4)

Flow field flow fractionation (F4) is another technique used as a separation method for nanoparticles characterization under native-like conditions (Contado, 2017). The hyphenation of F4 with multiple detector systems can increase the accuracy and provide information of more complex and polydisperse systems. F4 is not only used for the particle size estimation but it provides also information about the stability, drug release effects and interactions of the nanoparticles.

F4 is part of a bigger family that is called field-flow fractionation (FFF), and they are flow-based separation techniques able to separate particles from the nano up to micro scale. The main principles of field flow fractionation (FFF) is that there is an empty capillary channel and an external field, with an ideal parabolic flow, which is applied perpendicularly to the mobile phase flow (Contado, 2017). The nature of the external field is what differentiate and leads to the types of FFF, which have specific areas of size and applications range. Specifically the separation mechanism of F4 is based on the particle diffusion coefficient In F4 this external field is a flow from a second mobile phase, which is applied across the laminar flow (cross-flow) and as a result F4 separates the particles based on their diffusion coefficient (Müller, 2015). So the main mechanism is a hydrodynamically driven mechanism, which forces the components towards the wall of the channel. Simultaneously, because smaller particles have higher diffusion coefficient move in to the center of the channel where the flow is faster. The whole mechanism and separation are described in Figure 24 by (Müller, 2015). So contrary to SEC, the smaller particles elute earlier and the larger later. In this case retention time is inversely proportional to the hydrodynamic diffusion coefficient and directly proportional to the hydrodynamic size of the particle.

The biggest advantage of F4 is that the separation takes place inside an empty capillary, so the absence of stationary phase implies more “native” conditions, in which extra interactions and stress are reduced. For that reason F4 is also consider as a more selective separation technique in comparison to SEC.

![Figure 1125. Basic separation principle of AF4. Smaller particles have higher diffusion coefficients, so they are stabilized more on the center of the cartridge and the elute faster than the larger particles. (Müller, 2015)](image-url)
Previously it was mentioned that the type of external field can separate the type of field flow fractionation. Additionally, based on the separation channel design three main FFF categories are reported: symmetrical F4 (SF4), asymmetrical F4 (AF4) and hollow fiber F4 (HF5). AF4 will be further discussed as it has been proven to be the most successful version of F4. Different types of drug delivery nanoparticles including liposomes and organic nanoparticles eg micelles are reported (Zattoni, 2014). The separation device of AF4 is presented in the Figure 24. The main difference in this case is that an ultrafiltration membrane is being used. The purpose of this membrane is to let the buffer components pass through and prevent the analytes to exit the channel (Müller, 2015).

However, despite the great success of AF4 it still has a few limitations related to the separation channel, the analysis time and the solvent consumption. For these reasons the HF5 was designed, which basically is a miniaturization of AF4, but that seems to provide equally good results. In HF5 the channel has a cylindrical geometry with walls made of polymeric or ceramic material (Zattoni, 2014). The membrane responsible for letting the buffer components to pass through is a hollow-fiber membrane. The advantages of HF5 is that it has a very low channel volume, which leads to reduced sample dilution and it can separate under really low flow rate, which helps in the compatibility with other methods such as mass spectrometry. Despite the fact that HF5 has very successful protein applications in the field of nanotechnology there is still space for research in the application side.

![Figure 26](image.png)

**Figure 26.** Analysis of drug delivery nanoparticles with multidimensional analysis of AF4-UV-RI-FL-LS. After the separation either online or offline determination of the size distribution can be performed. (Zattoni, 2014)

The hyphenation of AF4 or HF5 with MALS provides unique size separation and determination. From AF4 and subsequently from HF5 information related for the $R_g$ can be derived and they can be correlated with MALS and DLS with the $R_g$ values, in order to obtain size but also shape information. In Figure 25 a schematic representation of the multidimensional analysis of AF4 with a variety of detectors is depicted and the potential use of the fractions for further analysis with bioassays or high resolution techniques as TEM or SEM.

Two other types of FFF have been reported for their use in the drug delivery nanoparticle field, sedimentation (SdFFF) and magnetic FFF (MgFFF). The sedimentational field in SdFFF is either gravitational or centrifugal. SdFFF was used for the parallel study of size and charge in the uptake of polystyrene drug delivery particles (Andersson, 2005). In a more recent study of (Esposito, 2012) they did a comparative study between SdFFF and SEC in order to detect the free drug bromocriptine in a lipid dispersion. It has been proven that SEC was not able to detect such small differences due to the interaction with the stationary phase, whereas SdFFF could effectively separate the two populations. On the other hand, magnetic FFF (MgFFF) is not a novel method, but it has been successfully used for the characterization of magnetic particles, and for toxicity determination, as described from (Carpino, 2007).
So in conclusion, field flow fractionation and especially AF4 has been successfully used for drug delivery nanoparticle characterization, as it can be coupled to a variety of detectors and be performed under native-like conditions. With AF4-MALS accurate size distribution results can be obtained and also of the smaller particle range, which is an important difference and advantage over DLS, which has a limited resolution over the smaller particles, when larger particles are also present. Another advantage of AF4 over other separation membrane techniques or ultracentrifugation is that it is faster and gives equal or even better resolution. The information that can be derived are not only about size and distribution but also about the shape and morphology and the drug release and stability of the particles.

SHAPE DETERMINATION AND TERMINOLOGY

In the previous sections both the impacts related to the nanocarriers geometry (shape, aspect ratio and ratio of particle dimension to vessel diameter) on the bio-distribution and cellular uptake of the particles as well as the techniques used for their characterization were discussed. For shape the determination of the terminology describing the shape is necessary, in order to make the measurement and data analysis comparable and feasible. Particle shape is determined in most cases with imaging techniques, but there are also techniques that can give indications and not the exact shape of the particles (AF4-MALS-DLS). Particle shape parameters can be estimated from a 2D projection using simple geometrical calculations.

The first parameter that is being used is the aspect ratio (Agarwal, 2013). Aspect ratio is defined as the width to length ratio of a particle and it can be used to distinguish between symmetrical particles, spheres or cubes and more irregular particles such as tubes. Commonly, spherical nanocarriers are characterized by their diameter, whereas rod-shaped particles are reported based on their rod dimensions (w and L) and aspect ratio (γ) as presented in Figure 26 (Tan, 2013). In the example of table 8 the volume of the nanorods was kept the same, whereas two different aspect ratios of 3 (γ = 3) and 5 (γ = 5) were considered in order to ensure the same drug loading capacity.

![Figure 27. Schematic representation of rod particle of specific aspect ratio γ (Tan, 2013).](image)

<table>
<thead>
<tr>
<th>Table 8. Dimensions for nanospheres and nanorods (Tan, 2013)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphere</td>
</tr>
<tr>
<td>d (nm)</td>
</tr>
<tr>
<td>100</td>
</tr>
<tr>
<td>200</td>
</tr>
</tbody>
</table>

Equation 3

\[ \gamma = \frac{\text{width}}{\text{length}} \]
SIZE DETERMINATION AND TERMINOLOGY

It is clear that there is not one method that can be used for universal analysis of nanomedicine and this is a result of both the complexity of the system and the strengths and limitations of each technique. Because of the fact that the techniques used for size determination are using different physical principles, this makes any possible comparison of data obtained from different techniques quite uncertain and challenging (Gaumet, 2008). In this section the different values that particle size can be determined and reported will be discussed along with the differences of these values.

Particles are 3D objects and very rarely are perfect spheres (Figueiredo, 2013). The complexity of their size determination starts from the fact that they cannot be described the same way as spherical particles just by a single dimension using only their radius or diameter. A theory of equivalent spheres has been developed in order to simplify the measurement and create a comparable terminology (Horiba Instruments, 2016). According to this theory the particle size is translated by the diameter of an equivalent spherical particle based on the same property which is either volume or mass. So the main concept behind this theory, is to try to describe a particle as if it was a sphere, without actually being a sphere. An extra obstacle in size determination is that different techniques base the size calculation on different equivalent sphere models and therefore they will not necessarily provide the exactly same result for the particle diameter (Pabst, 2007).

Another important limitation of the equivalent spheres theory is that it works quite accurately for regular shaped particles, but for shapes such as rods or cubes, that at least one dimension can differ significantly (eg. the length significantly different from height) it is not always applicable and for more precise measurements, techniques that take into consideration multiple dimensions are needed.

Another source of difficulty in the size determination derives from the complexity of the sample size distribution terminology. A nanomedicine product does not consist only of one nanoscale particle but a mixture of particles, as

Figure 28. On the left: Equivalent spherical diameters (ESD) estimated based on specific nanoparticle properties and/or behavior (Figueiredo, 2013). On the right is a schematic representation of the equivalent spheres theory retrieved from (Malvern Instruments, 2012)
a result when referring to nanoparticles size the estimation of size distribution is equally important. The size distribution can be monomodal, if there is one distinct population, or plurimodal, when several populations coexist (Gaumet, 2008). It can also be monodisperse, presented as a narrow distribution, or polydisperse, as a broader distribution. When a sample with a mean size of e.g 200 nm is described in the publication as “a monodisperse sample distribution”, this means that the particle size distribution is quite symmetric and the center exists around 200 nm, but this doesn’t directly mean that the particle size is 200 nm. The term of polydispersity will be more in detail discussed.

In most cases when the particle size is reported additional information about the particle size distribution is also present, unless the sample is perfectly monodisperse. However, there are different statistical distributions measured from the different techniques.

Number weighted distributions

In most image analysis the result is expressed as number weighted distribution, as for example in Nanoparticle Tracking Analysis (NTA), SEM and TEM (Shekunov, 2007). In this case an equal statistical weight is given to each particle, which is independent of the particle size. This distribution is being used for counting techniques where high resolution and absolute number of particles is important.

Volume weighted distributions

This is the resulted measurement of a static light scattering technique. In this case the weighting given to each particle is related to its volume or mass. This terminology is often being used in order to express the composition of a sample as volume to mass ratio. Despite the fact that DLS provides intensity distribution, the results can be converted, using the rather complex Mie theory, into volume distribution. However, for this transformation some assumptions are necessary, which can up to an extent compromise the result. All particles are considered as spherical and homogeneous, the refractive index should be known and finally and riskier is that the intensity distribution is assumed to include no error (Malvern Instruments, 2012).

Considering these assumptions it can be concluded that the volume and number distributions derive from the intensity distribution can be successfully used for comparative purposes but should not be considered as absolute. So it is proposed that a good practice would be to report the most accurate intensity distribution and the relative percentages from the volume distribution and not the size values.

Intensity weighted distributions

In comparison to the volume weighted distributions that are being used in static light scattering, intensity weighted distributions are being used to express results of DLS. As a result there is already a difference between NTA and DLS as they measure different distributions. The distribution obtained from NTA is number-weighted and not intensity-weighted as in DLS, which is weighted towards the larger particles (Filipe, 2010). In the case of intensity weighted distribution the weight given to each particle is related to the intensity of the light scattered by the particle. Particle scattering intensity is directly proportional to the sixth power of particle diameter (as discussed in the Rayleigh theory and DLS), which means that in the presence of aggregation or agglomeration where bigger particles can be
formed the intensity distribution can be misleading. However, the use of this distribution serves the purpose of expressing the presence of larger particles in a sample.

Having explained these differences along with the fact that different techniques are based on a different concept of equivalent spheres is important to realize the differences in the reported results. This can be clearly understood from Figure 28, where an example of a sample that consists of an equal number of particles with diameters of 5 nm and 50 nm is presented. It is obvious that the different expressions of statistical distributions provide different results. From the number weighted distribution an equal weight is given in both particle types. On the other hand, the intensity and volume weighted distribution gives a higher signal for the bigger particles.

![Figure 29](image1.png)  
*Figure 29. The differences between the number, volume and intensity weighted distributions of the same sample are presented, obtained from (Instruments M., 2012).*

The conversion from one statistical distribution type to another is possible, as mentioned also in the case of DLS but under certain assumptions based on the physical properties of the particles.

In the application example given for the comparison of SIOS and DLS (in the subsection of SIOS) the results are reported based on the mean, media, mode values for SIOS and Z-average and polydispersity (PI) for DLS. These values are being used in order to simplify the interpretation of particle size distribution data. The choice of the most appropriate statistical parameter is related not only to the sample and the data but also to the aim of the comparison and what is actually being compared. The mean, media and mode values are being used in order to report the most common particle size in the sample (Horiba Instruments, 2016). The differences between the values that can be obtained from the statistical distribution are presented also in Figure 29.

**Mean:** is the expression for the average size population

**Media:** includes the size where 50% of the population falls above or below that specific value

**Mode:** represents the size of highest frequency

![Figure 30](image2.png)  
*Figure 30. Schematic representation of the statistical parameters that can be obtained from a statistical distribution. Scheme retrieved from Horiba, Ltd.*
To address the fact that all these statistical parameters can be calculated and reported, some values have been reported from ISO, in order to create a universal way of reporting the size of nanoparticles especially for quality control purposes. Two widely used parameters is the Z-average and polydispersity (PDI).

Z-average size is mostly used in DLS and is the most trustworthy value, as it is the primary and most mathematically stable resulted parameter for the expression of the average diameter of particles (Malvern., 2012). The Z-average size is calculated based on a standard process of comparing the particles with a sphere. However, also this value is only comparable with the size measured from other techniques under certain criteria. The sample has to be monomodal, of a spherical or spherical-like shape and monodisperse and prepared in a suitable and similar solution because the Z-average can detect even small changes in the sample, as for example the presence of small amounts of aggregated particles. Another important obstacle is that the Z-average is considered as a hydrodynamic parameter and as a result it can only be used for particles in a dispersion medium or solution (Panchal, 2014).

Another value that should be reported is the term of polydispersity (IUPAC) or polydispersity index. Polydispersity has two different possible definitions depending on the underlying property of interest. The term polydispersity is used in SEC and DLS (Nobbmann, 2014). In SEC the aim is to calculate the molecular weight of a sample and the obtained distribution is typically a molecular weight distribution that describes the amount of material present in the molecular weight “slices”. The distribution is calculated from the ratio:

\[ PDI = \frac{M_w}{M_n} \]  

Equation 4

MW is the mass weighted molecular weight and it describes the average molecular weight by mass and Mn is the number weighted molecular weight, which is an average molecular weight by number. In this case a sample is considered as “monodisperse”, when both Mw and Mn are equal (PDI=1). For polydisperse samples this ratio indicates how far away the calculated size distribution is from the uniform distribution. On the other hand, in DLS the polydispersity and size distribution of the particles are the properties of interest, which means that the molecular weight is not of interest but the different sizes (Panchal, 2014). The distribution in DLS represents the amount of light that was scattered from the various sizes. From the comparison of the width and the mean of the distribution the relative polydispersity is obtained.

\[ \text{relative PDI} = \frac{\text{Absolute width of the distribution}}{\text{mean}} \]  

Equation 5

However, not even the relative polydispersity is used but an overall polydispersity, which is normalized. For an ideal Gaussian distribution an overall polydispersity would be equal to the relative polydispersity of the distribution. The reported value is the polydispersity index PDI, which is calculated from the square light scattering polydispersity and for a monodisperse sample PDI is 0.0.
Table 9. Summary of approximate values for polydispersity parameter (Nobbmann, 2014)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Equation</th>
<th>Type of Distribution</th>
<th>monodisperse</th>
<th>polydisperse</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDI from SEC</td>
<td>( \frac{M_w}{M_n} )</td>
<td>uniform</td>
<td>1.0</td>
<td>1.0-1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>narrow</td>
<td>1.1-2.0</td>
<td>&gt;2.0</td>
</tr>
<tr>
<td>PDI from DLS</td>
<td>((\frac{\text{width}}{\text{mean}})^2)</td>
<td>Moderate*</td>
<td>0.1</td>
<td>0.1-0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>broad</td>
<td>&gt;0.4</td>
<td></td>
</tr>
</tbody>
</table>

*A moderate polydispersity is an intermediate situation when the sample cannot be characterized as polydisperse or broad, but neither narrow.

So a polydispersity index of 0.1 and lower is considered as a highly homogeneous particle population, whereas higher values indicate the presence of polydisperse samples or even more than one populations. As a result an accurate linear correlation between the PDI value and the monodispersity of a sample is not possible. An example that can verify this claim is presented in Figure 30.

![Figure 30](image1.png)

This is a comparison experiment between DLS and SEM (Gaumet, 2008). The PDI of PLGA (a.) and latex (b.) nanoparticles were obtained with light scattering and the size distribution of the same samples was estimated with SEM. The results obtained for the PLGA with the SEM microscope indicated that this was a quite polydisperse sample with a size range from 100 nm to 1 μm, which is a quite broad range. Contradictory results were obtained when measured the same sample with DLS. The measurement showed a mean size of about 318 nm with a PDI of 0.093, which also according to table 9 shows a monomodal distribution. On the other hand, on the right picture of Figure 30(b.) a quite monodisperse latex sample is presented as estimated with SEM. Despite the fact that from this picture a highly homogeneous population is expected the DLS measurement showed a PDI of 0.12, which suggests a moderately polydisperse sample. This example aims to point the difficult of size determination even between exactly the same sample, which derives both from the instrumentation but also from the data processing and the defined values used.
CRITICAL VIEW OVER REPORTED SIZE DETERMINATION

Having discussed in the second section the difficulties for size determination and the difficulties in the reported size values a few questionable research examples will be reported. The main focus will be on the fact that in many cases the term of mean size is misused and the determined values from the particle size distribution, as well as the conditions of analysis are not reported.

To begin with the very first source of confusion, except the different terminology used, is the data processing of the measurements, which also leads to wrong conclusions. Usually, in most publications, the size is calculated only from a single sample and the statistical significance of the measurement is presented, meaning the standard deviation (SD). The meaning of standard deviation is often wrongly used. SD represents the distribution of the size around the calculated mean. However, when a result is presented, as for example $156 \pm 3$ nm, the direct translation of this result is not that the particle size is between these values. The real meaning of this reported result is based on the fact that the size measurements of a sample are performed several times (eg. for DLS in most cases triplicates), so the standard deviation at this point represents the error that is made on these measurements and not in the distribution of the particles.

An important factor for size determination is the conditions used, which can strongly influence the final result. For example, it is already mentioned that the sample preparation of TEM and SEM, regardless the high resolution power of these techniques can strongly influence the particle size as the particle can shrink and aggregate during drying and staining. The determination of the particle size with dynamic light scattering is also strongly influenced from parameters such as viscosity, pH, temperature, and particle core and surface structure (Sapsford, 2011).

An example of the influence of the solution matrix was described from (Wu, 2005). In this research a number of different parameters was investigated for the preparation of nanoparticles. The characterization of the chitosan nanoparticles was based on TEM and DLS measurements. The measured size from the light scattering experiments was estimated at 182 nm with a PDI of 0.17. Contrary from the TEM experiments the suggested size was estimated between 20 and 80 nm. Such a significant deviation cannot be only attributed to sample preparation and instrumental limitations. So in one hand the sample preparation of TEM is quite harsh and the drying can lead to size alterations, but on the other hand physicochemical properties of the sample itself may play equal or even more significant role. In this case the properties of the nanocarrier and the solution environment is the explanation of such a deviation. Chitosan is a cationic polysaccharide and as an extent its properties is highly influenced by pH and ionic strength. Measurements were performed in acetic aqueous solution, so not a buffer solution. Chitosan chains might be denatured thus giving a larger hydrodynamic diameter in DLS.
In the research of (Beletsi, 2005) the particle size effect on the pharmacokinetic and photothrombic activity of PLA nanocarriers with meso-tetra(carboxyphenyl)porphyrin (TCPP) was investigated. The mean particle size of four batches of PLA nanoparticles of varying mean sizes (from 121-343 nm) was estimated only with DLS. The results of these experiments are summarized in Table 10. The obtained PDI for the four different PLA batches are quite high, in the range that we could say that the sample shows a moderate or even broad polydispersity (according to table 9), which means that the sizes of the different batches are quite close and there is a high chance of overlapping populations. However, the reported conclusion is that the smaller nanoparticles may be consider as the optimal size, due to the fact that this batch exhibited higher levels of vascular thrombosis and lower extravasation. (Gaumet, 2008) is opposed to this conclusion, claiming that the same effect can be independent of the size but due to surfactant residuals that affect the surface properties and by extend the interactions between particles and target cells. This opinion seems to be valid as in the paper it is reported that different concentrations of poly(vinyl) alcohol were used as stabilizing agents.

In other studies a high homogeneity is claimed, but neither PDI nor size distribution is reported, but only the standard deviation of the three measurements. A relevant example is the study of (Tammam S. N., 2015), in which despite the fact that for the characterization both DLS and AFM were used, and it is claimed that the results are in agreement, the mean particle size is only reported based on the standard deviation, for the larger nanoparticles as 158 ± 4 and for the smaller as 25 ± 1 nm. No comment about the homogeneity or the size distribution is mentioned. In this study the aim is to describe the effect of nanoparticle size and nuclear localization sequence (NLS) density on the cellular uptake and targeting ability, so particle size is of importance for the final conclusion. Additionally, the next study of the same group based its research on the same particle characterization and formulation (Tammam S. N., 2017) to prove again the effect of particle size for cancer targeting, which is one of the most difficult targets that it has been proven that particle size play an important role.

These are only a few examples of wrongly reported nanoparticle characterization, but according to the literature it is an existing problem that has an important impact on the future of nanomedicine.

<table>
<thead>
<tr>
<th>Mean particle size (nm) of each batch</th>
<th>Polydispersity index (PI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>121 ± 10</td>
<td>0.42</td>
</tr>
<tr>
<td>194 ± 12</td>
<td>0.21</td>
</tr>
<tr>
<td>250 ± 5</td>
<td>0.30</td>
</tr>
<tr>
<td>343 ± 10</td>
<td>0.23</td>
</tr>
</tbody>
</table>
DISCUSSION AND FUTURE RECOMMENDATIONS

Nanomedicine are becoming more and more sophisticated. New techniques to create multifunctional and smart nanocarriers are being developed. While researchers are developing this next generation of nanomedicine, the gaps on both the regulations and characterization methods become an important issue. It has been proven that the existing gap between the academic research and the final products reaching the market, which according to (Tinkle, 2014) is a “valley of death”, for the nanomedicine in order to be tackled, the cooperation of experts from the academia, industry and federal regulatory bodies is required (Tinkle, 2014). The reasons behind this issue are quite complex, but undoubtedly it is also related to the characterization and understanding of the nanomedicine properties, as discussed in this literature thesis. Consequently, the development of analytical techniques and methods is aiming to gain more resolution. However, from a literature search using as keywords the term “nanomedicine” and “analytical chemistry or techniques”, it becomes clear that the current published literature on this emerging field is quite limited and the focus is mostly on the challenges, but also on the potential opportunities of analytical chemistry to solve some of the current limitations of nanomedicine (Kranz, 2011). As a result because of the importance of the analytical field on the faith of nanomedicine, and its current limitations, there is a significant support and funding in order to narrow the existing gap. Additionally, among the reasons for delays or rejections of possible nanomedicine and drug delivery nanoparticles is the lack of universal standards and standardized techniques for their characterization. So the proposal of techniques that can be used for more universal nanoparticle characterization is of great essence.

In the first section the effect of nanoparticle size and shape both on the clearance and the bio-distribution was discussed in order to prove the importance of these physicochemical parameters. The size and shape limits to target a specific organ is a quite complicated study as there are many parameters that might play a more significant role and are still poorly understood. An overview of the complexity of the nanomedicines was provided by (Shi, 2017), as presented in Figure 3. Focused on size and shape it was concluded that in most studies related to the nanocarriers shape effect, both the size and the shape of the examined nanoparticles varied, so an accurate conclusion is not possible and the effect of this physicochemical property is still poorly understood. For a deeper understanding of the nanoscale geometry effect on cellular uptake highly monodispersed, of specific shape and size nanoparticles having equivalent volumes and identical surface properties and material compositions are required (Agarwal, 2013). Despite the inaccuracy of some experiments it seems that indeed in many cases and especially in the escape or uptake of the phagocytosis system, the shape and size do play a role. Thus the importance of developing computer-based predictive models in order to be able to predict the optimal size and shape properties of the nanocarrier, depending a specific target is also discussed, since these tools will help bridge the gap and give a better insight on the potential benefits and risks of the engineered nanomedical products.
Finally, it seems that most studies related both for the effect of particle size and shape are recently more directed in the importance of these properties in the cellular uptake and not in the bio-distribution.

In the second section some of the current analytical techniques used for nanomedicine size characterization along with their principles and limitations are discussed. Examples of the existing problem of poorly characterized and reported size and shape determination are presented in order to highlight the significance of this matter. The comparison of the discussed techniques are presented in the overall Table 11. It is clear that techniques are based on different physical principles and thus data obtained from different methods is difficult to be directly and absolutely comparable. So as a conclusion there are some important points for consideration that have been highlighted in respect to the particle sizing analysis:

- Each particle sizing technique has its own limitations and advantages and as a result there is not one universal technique to be used in all cases. The selection of the appropriate technique should be based on the purpose of the analysis and the analyses nanocarriers.

- There is an important need for development of standards that can correlate the bio-distribution of various nanomedicine with their activity, safety and efficacy based on size, surface charge, stability and other important parameters.

- Most of these techniques report particle size in based on the equivalent spheres theory. Non-sphericity of nanoparticles should be taken into account during the interpretation of the results. Only electron microscopy technique are recommended in order to provide accurate shape information of the samples, most of the other techniques give only an indication of the particle shape.
- Each technique requires a different sample preparation and optimization. As a result detailed protocols and measuring conditions should be available for consistent and reproducible results. Additionally, the development of guidance on what type of data should be reported is needed. The fact that different research groups use different ways of interpretation and reporting of the results may cause difficulties in the comparison of the outcome with results obtained from other groups.

- Data interpretation is equally important and should be properly and accurately reported, since there are differences in the reported values. Not only different techniques are based on different model and properties, but also in case of eg. tailing in the size distribution then there is a significant deviation from its Gaussian counterpart.

- For the single-particle sizing techniques (NTA, SIOS) in order to increase the accuracy a certain number of particles should be counted. It is recommended that during the optimization this number should be evaluated before the actual measurements, so the uncertainty of the measurements would be in acceptable limits.

An overall conclusion is that there is the need for a greater variety of techniques and a more universal guidance/protocol on the final reported size values and experimental conditions, in order to avoid inaccurate results that lead to different conclusions between different studies of the same nanocarriers. It seems that the most appropriate method is often selected based on the specific case, which might have advantages as the method can be very accurate for the specific application, but it might also lead to problems if the results cannot be comparable to other techniques. As a result in order to have a more accurate conclusion at least two techniques should be combined, one of which should be a microscopic technique and if possible prior separation with AF4/HF5 is recommended. Dynamic light scattering is a quite appropriate technique especially for quality control, as it is the most user friendly, fast and relatively cheap technique. However, due to its existing limitation a stand-alone analysis of DLS is not sufficient. Additionally, the design of better characterized nanoparticles available for calibration purposes seem to be of great importance. The final step is the careful discussion of the results, meaning that particle size along with standard deviation and polydispersity, and especially polydispersity should be carefully discussed as overlapping of populations might be present. As a result, all the above mentioned parameters in both the measurement, the used technique and the data interpretation add to the inaccuracy of the particle characterization.

The suggested techniques are only a small part of the characterization techniques that can be used for nanomedicine size characterization and as the field evolves the list of possible techniques available will continue to grow with new developments and applications. A close collaboration between academia, industry and FDA will definitely accelerate the development of the field.
<table>
<thead>
<tr>
<th>Technique</th>
<th>Proved Applications</th>
<th>Measurement principle</th>
<th>Size and concentration range</th>
<th>Result</th>
<th>Advantages</th>
<th>Limitations</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM</td>
<td>In general: It is used for structural determination of NPs; Interactions of NPs with biological components can be observed (eg. cellular uptake)</td>
<td>Absorption and diffraction of electrons</td>
<td>High resolution technique 0.2–1 nm</td>
<td>Concentration range $10^{10}$, $10^{12}$ particles/mL</td>
<td>Phase-contrast TEM images (pixel * pixel) - size - size distribution - aggregation/agglomeration - aspect ratio can be determined - Number weighted distribution</td>
<td>- Fast imaging - Provides both compositional and structural information - Cryo-TEM and wet (in situ) TEM provide less harsh and liquid analysis</td>
<td>Sample preparation: - Increases the possibility of structural alterations (eg. drying and staining) - Less than 50 nm thickness required - Time consuming process Analysis: - Sample can be damaged due to the high voltage electron beam - Limited contrast between the nanoparticles and background from the film increase the inaccuracy in the particle size estimation - Expensive and not user friendly</td>
</tr>
<tr>
<td>SEM</td>
<td>In general: It is used for determination of surface properties and size of NPs.</td>
<td>Scattered electrons and X-rays</td>
<td>Lower resolution than TEM 1–10 nm</td>
<td>Concentration range $10^{10}$, $10^{12}$ particles/mL</td>
<td>- size - size distribution - shape - aggregation and dispersion - Number weighted distribution</td>
<td>- Fast imaging, with a large field of view (up to 20 μm) - Simpler sample preparation than TEM - Larger amount of sample can be screened improving the statistical significance - ESEM can be used to image biomolecules</td>
<td>Sample preparation: - Process of drying and addition of contrasting agents may cause shrinkage of the NPs and alter their characteristics - Drying of particles means non-physiological environment (unless ESEM is used) Analysis: - Expensive equipment and experts are needed in order to obtain accurate data.</td>
</tr>
</tbody>
</table>
| **AFM** | **In general:** It is used for determination of surface and size of NPs. Interactions between particles and biomolecules can also be observed.  
**Application examples:**  
- AuNPs  
- carbon nanotubes and quantum dots functionalized with proteins  
- Pegylated gelatin NPs | **The information is gathered from the deflection angle of the cantilever probe.**  
**High resolution 0.5 nm**  
**Appropriate for particle sizes of 0.5 nm up to 50 nm** | **Similar with TEM and SEM**  
- size  
- size distribution  
- shape  
- aggregation and dispersion | **- Able to analyze nonconductive, wet soft samples**  
- NPs under physiological-like liquid conditions can be analyzed  
- Non destructive for the sample  
- Simple and cheap sample preparation  
- Measurements can be performed both in gaseous and liquid environments  
- Able to monitor in real time interactions of nanoparticles with supported lipid bilayer (not possible with SEM & TEM) | **- Non automated technique, as both the set up and the interpretation is quite complex**  
- Size of cantilever is larger than the dimension of the NPs, leading to overestimation  
- 3D mapping of the surface  
- It is considered a subjective technique and requires extensive optimization | (Li K. D., 2014)  
(Garg, 2005)  
(Figueiredo, 2013)  
(Khorasani, 2014) |
| **NTA** | **In general:** It is used for determination of surface and size of NPs.  
**Application examples:**  
- Liposomes and other drug delivery vehicles  
- Quantum dots  
- Multi-walled carbon nanotubes | **Single particle analysis by video tracking. It measures Brownian motion.**  
**Limited concentration range (10^7 – 10^9)**  
**Minimum size limit is 10-40 nm and maximum is 1-2 μm** | **- Individual particle sizing**  
- number distribution  
- Diffusion Coefficient  
- Scattering/fluorescence properties  
- Particle concentration/number  
- Intensity weighted distribution | **- Relatively fast (between 5 min to 1h)**  
- Multimodal, polydisperse samples and heterogenous/mixed sample types can be well resolved  
- Provides also particle concentration information, whereas DLS and EM techniques don’t.  
- There is no need for knowledge of the refractive index of the solvent, as the scattered intensity of the light is not measured | **- Sufficient dilution of the sample is required, so that the observation fields are not over-crowded**  
- Optimization by a skilled operator is required mainly due to the video capturing settings and analysis  
- For accurate results time consuming | (Filipe, 2010)  
(Shang, 2014)  
(Montes-Burgos, 2010) |
<table>
<thead>
<tr>
<th><strong>DLS/ PCS/ QLS</strong></th>
<th><strong>In general:</strong></th>
<th><strong>Application examples:</strong></th>
<th><strong>Measures the rate that intensity of the scattered light fluctuates</strong></th>
<th><strong>Size range</strong></th>
<th><strong>Hydrodynamic radius</strong></th>
<th><strong>- Liquid analysis</strong></th>
<th><strong>- Well-known pitfall of low peak resolution, meaning that heterogeneous size distributions can only be resolved if they differ in a size at least by a factor of 3</strong></th>
<th><strong>- Non spherical measurements are biased because spherical particles is a priori assumed</strong></th>
</tr>
</thead>
</table>
| **In general:**  | It is used for size, shape, aggregation determination of particles and can also investigate binding interactions | - Au nanoparticles  
- Liposomes  
- Trimethyl chitosan (TMC) nanocarriers  
- poly(lactic-co-glycolic acid) (PLGA) nanocarriers | Size range 1nm up to 10 μm  
Broader concentration range from 10^8-10^{12} particles/mL | - Approximate size distribution  
- Z-average  
- Polydispersity  
- Diffusion coefficient  
- No concentration information  
- Intensity weighted distribution  
- Volume weighted distribution | - Liquid analysis  
- Non invasive  
- Fast experiment (minutes)  
- Relatively cheap and user friendly instrumentation  
- High reproducability  
- High accuracy in hydrodynamic determination of monodisperse samples  
- Able to analyze a wide range of concentrations  
- Requires knowledge of refractive index and viscosity | (Malvern Instruments, 2012)  
(Figueiredo, 2013)  
(Hall, 2007)  
(Lin, 2014) |

<table>
<thead>
<tr>
<th><strong>SIOS</strong></th>
<th><strong>In general:</strong></th>
<th><strong>Application examples:</strong></th>
<th><strong>Particle size from 60 nm up to a few microns</strong></th>
<th><strong>Concentration range from 10^{-7}-10^{9} particles/mL</strong></th>
<th><strong>- size</strong></th>
<th><strong>- size distribution</strong></th>
<th><strong>- High resolution due to the tunable pore</strong></th>
<th><strong>- Equally important and challenging to adjust the pore diameter in order to ensure detection of high polydispersity samples</strong></th>
<th><strong>- Several particle sizes might be detected simultaneously</strong></th>
</tr>
</thead>
</table>
| **In general:** | It is used for size, aggregation determination of particles and can also investigate binding interactions | - Liposomes and interaction between liposomes and plasma proteins  
- DNA coated nanoparticles  
- adenovirus | | | | - mean, mode)  
- Number weighted mean | | (Yang, 2012)  
(Bell, 2012)  
(Yang, 2012)  
(Wei A. M., 2012) |
| **SEC** | **In general:**  
It is used for size separation and purification of complex NP samples | **Chromatography, Interactions with the stationary phase** | **Particle size <100 nm** | **- Molar mass polydispersity (Mw/Mn)** | **- can be coupled to a wide variety of detectors (eg. SEC-UV-MALS-RI)** | **- Optimization makes the method development time consuming** | **(Pitkänen, 2016)** |
| - neutral liposome particles  
- siRNA containing lipid nanoparticle | **Retention is governed by the diffusivities of the particles and the interaction with an external physical field** | **Particle size 20 nm- 1 μm** | **- size** | **- detector dependent size distribution** | **- native conditions can be used** | **- Not high through put** | **(López-Serrano, 2014)**  
**(Zhang, 2012)**  
**(Sapsford, 2011)** |

| **FFF** | **In general:**  
It is a separation and purification method able to size-sort and isolate NPs | **Retention is governed by the diffusivities of the particles and the interaction with an external physical field** | **Particle size 20 nm- 1 μm** | **- size** | **- detector dependent size distribution** | **- can be coupled to a wide variety of detectors (eg. FFF-MALS-DLS)** | **- Difficult to handle** | **(Figueiredo, 2013)**  
**(Ma, 2010)**  
**(López-Serrano, 2014)**  
**(Wagner, 2014)**  
**(Andersson, 2005)** |
| - QD-DNA  
- polymer NP-peptides  
- polymer NP-drug  
- Liposomes  
- solid lipid NPs | **Retention is governed by the diffusivities of the particles and the interaction with an external physical field** | **Particle size 20 nm- 1 μm** | **- size** | **- detector dependent size distribution** | **- native conditions can be used** | **- Slow analysis** | **- High solvent consumption** | **(Figueiredo, 2013)**  
**(Ma, 2010)**  
**(López-Serrano, 2014)**  
**(Wagner, 2014)**  
**(Andersson, 2005)** |
REFERENCES


