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Synthesis and Characterization Methods of Pure Gold Nanoparticles for Toxicity Assessments

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Abstract

Nanotechnology constantly invades sectors like industry and medicine. Products and devices containing gold nanoparticles and medical treatments based on nanotechnology are interacting with living organisms and the environment with still undefined consequences. The need for investigation of the side effects nanoparticles may cause is pressing. In this work, pure gold nanoparticles were prepared for toxicity tests using two different methods, Spark Discharge technique and Turkevich method. Emphasis was given to the purity and high quality of the samples in order to gain reliable results.

Acknowledgments

I would like to express my gratitude to my supervisor, George Biskos, who gave me the chance to get involved in a really interesting field of research and to explore a whole new world in the nano scale. I would also like to thank Andreas Schmidt-Ott and everyone else who helped me from the Nanolab group of the Chemical Engineering Department, at Delft University of Technology. Thank you all for your help, advice, kindness and patience. I would also like to thank my family for their continuous support and patience.

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1. Introduction

It is generally accepted that the use of the word "nano" is increasing not only in the field of science but also on an everyday level. Products containing nanoparticles are easily accessible to everyone and articles about nanoparticles are appearing quite often in the scientific columns of newspapers. The once imagined use of nanoparticles has now become a reality. This can be assumed by the growing number of publications on ultrafine particles and their sub classification, the nanoparticles, which in the period between 1990 and 2005 show an increase of 97.5% [7]. Nanoparticles are here and they are here to stay.

But what exactly are those popular and much discussed particles? A nanoparticle is a particle with all three dimensions at the size range between 1-100 nanometers (nm) [23]. One nanometer corresponds to one-billionth of a meter (10^{-9}m). The word "nano" comes from the Greek word "nanos" (νάνος) which can be translated as dwarf. The use of this word gives exactly the emphasis needed to the size of the particle. It does not belong just in the micro scale, where micro- (micros/μικρός) in Greek means small, but in an even tinier metric unit of length, the nano scale.

It is the size of the particles that makes them attractive to scientists. Being between the size of an atom and the size of a molecule, one can say that nanoparticles belong at a mesoscopic scale, the scale between the microscopic and the macroscopic worlds [24]. Structures with at least one dimension between 1-100 nm (nanostructures), experience new biological, chemical, electrical, magnetic, mechanical and optical properties, overcoming some of the basic principles of the molecular levels like the fundamental Heisenberg Uncertainty Principle. Very high surface to volume ratio in combination with quantum mechanical properties, like surface plasmon resonance, along with the systematic and dense organization of the nanoscaled structures, make an intriguing variety of new phenomena [49][24]. These novel properties, and many more still unknown but challenging properties, are the subject of research of a constantly increasing number of scientists, introducing the novel field of Nanoscience, with applications like nanotechnology, nanoengineering and nanomedicine.

The term "nanotechnology" can be simply described by the words of Richard P. Feynman at his speech entitled "There's Plenty of Room at the Bottom" on the 29th of December 1959, at the annual meeting of the American Physical Society at the California Institute of Technology, as the art of "*manipulating and controlling things on a small scale"* which eventually leads to *"an enormous number of technical applications"*. It is the field of studies dealing with materials which, according to the United States National Science Foundation [36], have the unique properties of small size, controllable physical and chemical abilities in the molecular scale, and the ability of reorganizing and forming larger structures. It is the promise of the novel, unknown and revolutionary abilities of the new materials that makes the field of nanotechnology so popular.

Nanostructured materials can be classified depending on their dimensionality. As summarized in *Table 1*, there are four subcategories: the zero dimensional (0D)

nanostructured materials with all their dimensions in the nanometer range, the one dimensional (1D) nanostructured materials with one of their dimensions outside the nanometer range, the two dimensional (2D) nanostructured materials with two of their dimensions outside the nanometer range and the three dimensional (3D) nanostructured materials with three of their dimensions outside the nanometer range. The applications shown are indicative examples as the whole number of the possible applications cannot fit in a table. In this study, the focus will be on the zero dimensional nanostructured materials and more specifically on the spherical metallic nanoparticles (nanospheres).

Dimensions	Examples	Examples of Applications
Zero Dimensional 0D	Nanospheres Quantum dots Nanoparticle arrays Core-shell nanoparticles Hollow cubes	Solar cells Lasers Light emitting diodes (LEDs)
One Dimensional 1D	Nanowires Nanotubes Nanorods Nanobelts Nanoribbons Hierarchical nanostructures	Nanoelectronics Nanodevices Alternative energy sources
Two Dimensional 2D	Junctions (continuous islands) Branched structures Nanoprisms Nanoplates Nanosheets Nanowalls Nanodisks	Sensors Photocatalysts Nanocontainers, Nanoreactors
Three Dimensional 3D	Nanoballs (dendritic structures) Nanocoils Nanocones Nanopillers Nanoflowers	Catalysis Magnetic materials Electrode materials for batteries Molecule transport

Table 1. Classification of nanostructured materials depending on their dimensionality [42].

It is believed that the speech of Richard P. Feynman [53] was the introduction to a new era of technological achievements. Nanoparticles indeed began to attract scientists' interest and it was not long until the invention of tools and techniques for the nanoparticle observation and manipulation created enthusiasm among them [36]. The list of nanoparticles' applications like aerospace, agriculture, bioengineering, biology, energy, the environment, materials, manufacturing, medicine, military science and technology is still growing [49].

However, nanoparticles are not considered as a new entry in our environment. Although their observation and fabrication became popular in recent decades, their existence as by-products of cooking, simple combustion and later combustion of well known processes like power generation, is dated back to the millennium. Additionally

nanoparticles are part of plenty of natural phenomena like dust storms, forest fires, volcanoes, ocean and water evaporation that have taken place for thousands of years [7].

A more complete way of nanoparticles classification, is based on their origin of creation. There are two basic categories, the naturally occurring nanoparticles and the anthropogenic nanoparticles. As mentioned above, naturally occurring nanoparticles is a broad category. Natural nanoparticles can be carbon black, iron oxides, fullerenes and they are generated under natural processes. Anthropogenic nanoparticles are engineered nanoparticles manufactured by humans. Some examples can be quantum dots, carbon nanotubes and metallic nanoparticles like gold nanoparticles (AuNPs) [23].Other nanoparticle classifications are based on their morphology [7] or on their chemical composition (organic-inorganic) [12].

Anthropogenic engineered nanoparticles and more specifically metallic nanoparticles (MNPs) have gained an important place in today's industry. The continuously growing field of nanotechnology and its applications lead to the inevitable exposure of humans and environment to nanoparticles. Despite their promising properties, there is still a lot of research that needs to be done when it comes to interactions between living organisms and products containing nanoparticles. The need of further research was the reason that a new branch of toxicology research was created; the field of nanotoxicology. Nanotoxicology is quite new and was introduced only recently, when the first suspicions of possible harmful consequences of nanoparticles came to light [15].

As mentioned above, nanoparticles are used in medicine (nanomedicine) and in commonly used products. A possible toxicity can be observed in uncontained nanoparticles which, released into the environment, can penetrate living organisms [7]. Uncontained nanoparticles are released into the environment through the use of everyday products like cosmetics, food, fabrics etc. Their use can create nanoparticle waste which can either accumulate in living organisms or be released into the environment, putting them at risk.

Nanoparticles may enter the human body through the skin, the respiratory system, the reproductory system and also via ingestion. Other possible not so common entry routes are injection and implants [7]. Once nanoparticles enter the human body they can reach the blood and central nervous system gaining access to other organs like the spleen or liver and affecting the important functioning of the heart and the brain [15][13][25]. *Figure 1* shows the possible entry routes of nanoparticles in the human body, from the circulatory system to the brain, along with some of the most popular diseases that can be caused.

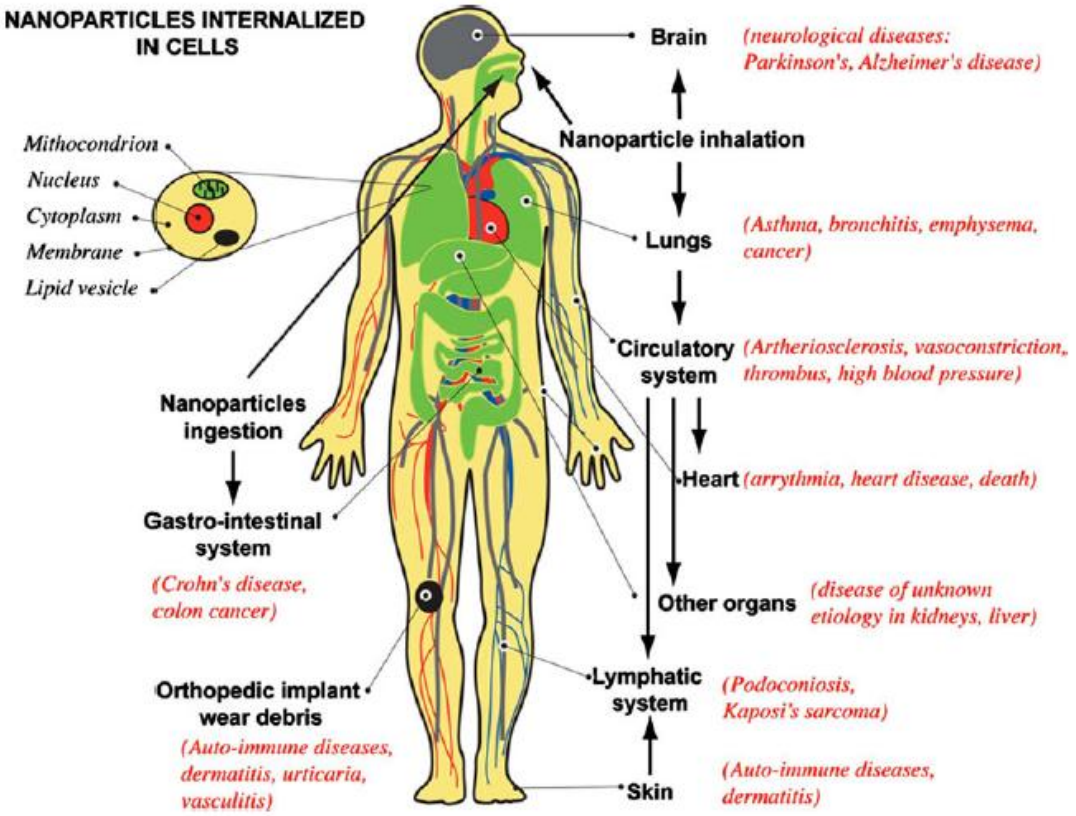


Figure 4. Illustration of the possible entry routes of nanoparticles in the human body [7].

The main focus of this study is on gold nanoparticles and their toxicity. AuNPs were quite popular from ancient times especially for their medical properties. From China in 2500 BC to the medieval Europe, colloidal gold was used as a promising cure of several diseases [18]. Also in China and Egypt in around the fifth or fourth century BC, colloidal gold was part of the decoration of ceramic items and glass, and was also used in cosmetics. The first reported synthesis of colloidal gold was in 1857 by M. Faraday and the systematic research on colloidal gold synthesis was achieved from 1990 and on. The evolution of the research on AuNPs can be confirmed by the increase of around 94% of the publications related to them during the past decade. [33][22].

Right now, gold nanoparticles are involved in numerous applications in the fields of energy (photovoltaic systems), the environment (catalytic behaviour), information technologies (lenses, sensors) and bioapplications [33]. They also have unique properties (optical properties, bioconjugation abilities etc) that can be useful in therapeutic applications and diagnostic medicine [6]. Other biomedical applications of gold nanoparticles are biodiagnostics, drug delivery, imaging and biosensing [45].

However, as mentioned above, their toxic effects are not yet fully understood. The results of several toxicity assessments in the literature were rather contradicting about whether gold nanoparticles make a potential threat or not. Some scientific research groups confirmed their toxicity [2][19][4][29][9][27][45][34], some did not

[38][25][37], while some others insisted that the toxic effects are not on the particle itself but on the chemicals used during the synthesis process [10][43][20]. Since the manipulation of gold nanoparticles is increasing, the need for reliable results is urgent.

The aim of this thesis is to prepare pure gold nanoparticle samples, ideal for toxicity assessments in order to gain reliable and clarifying results. Two methods are presented; one broadly used wet chemical method, the Turkevich method and one gas-phased technique, the Spark Discharge Technique. The methods were chosen regarding their popularity and advantages. Spark Discharge Technique offers significant advantages regarding the purity of the particles while Turkevich method is the most commonly applied method in the toxicity investigation research. Gold nanoparticles were prepared with those two methods and after characterization were ready for toxicity assessments. Additional emphasis was given on the purity of the particles.

2. Literature Review

2.1 Metal nanoparticles synthesis techniques

Metal nanoparticles for applications like toxicity tests can be prepared by several procedures and in a variety of media. Depending on the method used, properties and morphology of the nanoparticles produced differ. It is important to choose a method that serves the demands of the synthesis purpose, so when it comes to toxicity assessments, the method should provide as pure particles as possible. The most popular nanoparticle generation procedures will be briefly introduced in this chapter. They can be organized either based on the phase of the media (gas, liquid and solid) or based on the approach of the strategy (bottom-up and top-down).

2.1.1 Phase of the media

Nanoparticles can be generated embedded in media in the gas phase, in the liquid phase (solution) and in the solid phase (embedded on a substrate) [22]. Since metal nanoparticles are always tiny metallic spheres, their categorization is done according to the phase of the media they are embedded in.

In the gas phase, particles are generated avoiding contact with other substances. The basic synthesis process consists of the generation of metal atoms in the gas phase, their nucleation, condensation and formation of molecular clusters, and finally the formation of nanoparticles due to further condensation of clusters. Generation of metal atoms can be achieved with the submission of the desired metal under resistive heating, laser vaporization and voltage breakdown. Some broadly used synthesis methods of metal nanoparticles in the gas phase are inert gas condensation, ion sputtering, pulsed laser ablation and spark discharge generation. Spark discharge generation method will be presented in the next chapters. Gas phase methods are considered to produce nanoparticles of high purity due to the absence of liquid solvents, which are thought to be the main reason of nanoparticles' toxicity [51].

Nanoparticles generated in the liquid phase are products of wet chemical methods. Those methods are considered the oldest for colloidal synthesis [16]. Colloidal metals can be synthesized using a broad range of methods with a significant part of them based on the reduction of metal salts. Depending on the desired metal nanoparticles, the chosen metal salt is reduced by a reducing agent to metallic ions. Nucleation takes place, nanoparticles are formed and with the addition of stabilizing agents, they become stable. Variation of the reducing and stabilizing agents provides the ability of choosing between a wide range of nanoparticle sizes, and results to the development of several different synthesis methods. Some popular wet chemistry methods are the Turkevich method, the Brust-Schiffrin method and the sol-gel method [46]. Reduction of the salts can be also achieved electrochemically, radiolytically, sonochemically and also biosynthetically [22]. Furthermore, generation of MNps via biosynthetic methods also provides a promising alternative to the classic wet chemistry methods because of the absence of reducing agents and stabilizers. The nanoparticles are generated by the reduction of chloroauric acid with the help of microorganisms, enzymes and plants or plant extracts [35][14][40]. In general, liquid based methods usually have the disadvantage of a great amount of impurities dissolved in the nanoparticle solution. Those impurities are by-products of the chemical reactions and can lead to misleading results during a toxicity assessment test [3].

Nanoparticles in the solid state can be generated with methods of decomposition of metal complexes. Both thermal and photolytic decomposition can lead to formation of particles with the desired dimensions [22]. Some popular methods are the electron-beam lithographic method and the laser-based ablation method, which both provide an adequate level of control over the particle size [46]. A significant disadvantage of this category of methods is the lack of control on the particles size and surface characteristics. Additionally, the chances of impurities are higher than other methods [48].

2.1.2 Top-down and Bottom-up

Synthesis of metal nanoparticles has two basic approaches: the bottom-up and the top-down. Top-down strategies start from larger sizes while bottom-up strategies start from the molecule size range. They both result to the formation of particles with size range within the nano scale. Illustrated in *Figure 2* are possible methods for metallic nanoparticle generation, separated into top-down and bottom-up approaches.

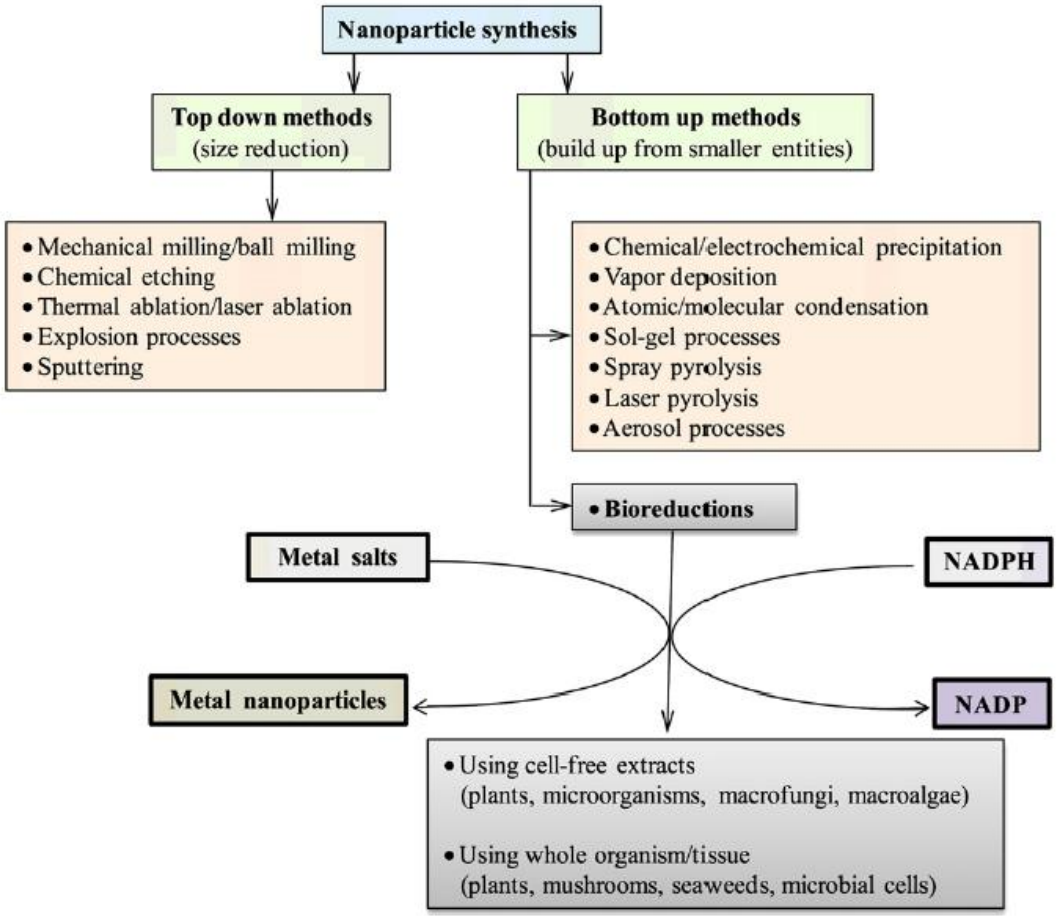


Figure 5. Nanoparticle synthesis methods classified as top-down and bottom-up [28].

Top-down strategies are based on the decomposition of bulk state materials until the generation of particles of the desired dimensions [23]. The generation of MNps is achieved with several physical and chemical ways of decomposition illustrated in figure 2. Electron-beam lithographic method and laser-based ablation method are some of the most popular top-down methods [46]. Moreover, a significant part of the top-down methods are the metal vapor techniques which provide the ability to synthesize a wide range of nanostructured metal colloids [47]. However, the imperfections in the surface structures of the nanoparticles make a significant disadvantage of this category of methods along with the high chances of impurities in the final solution [48][28].

Although top-down strategies have more in common with physical procedures, bottom-up strategies are utilized more frequently. According to these strategies, individual molecules are formed by biological or chemical reduction and under certain circumstances they form nanoparticles of various sizes. The most popular bottom-up strategies are the wet chemical [46]. Popular bottom-up strategies are citrate reduction methods and several biosynthetic methods.

2.2 Toxicity of nanoparticles

2.2.1 Toxicity mechanisms of gold nanoparticles

Nanoparticles have the ability to affect the membrane of a cell and cause physical damage or cell death. Depending on the nanomaterial, different kind of damage can be done to the membrane, organelles and DNA of the cell. The damage can be provoked either by chemical or by physical mechanisms which eventually lead to a "cellular response", the desperate try of the cell to survive under the new circumstances. Usually the response turns out to be lethal for the cell. Some chemical mechanisms are the production of reactive oxygen species (ROS), dissolution and release of toxic ions (like Ag^{+}), disturbance of the electron/ion cell membrane transport activity or mechanisms based on the surfactant properties. Physical mechanisms are mostly connected to the morphology of the particle and can lead to disruption of membranes and their activity, change of protein conformation and obstruction of transport processes [15]. Illustrated in *Figure 3* are the basic categories of damage nanoparticles can cause to a typical cell.

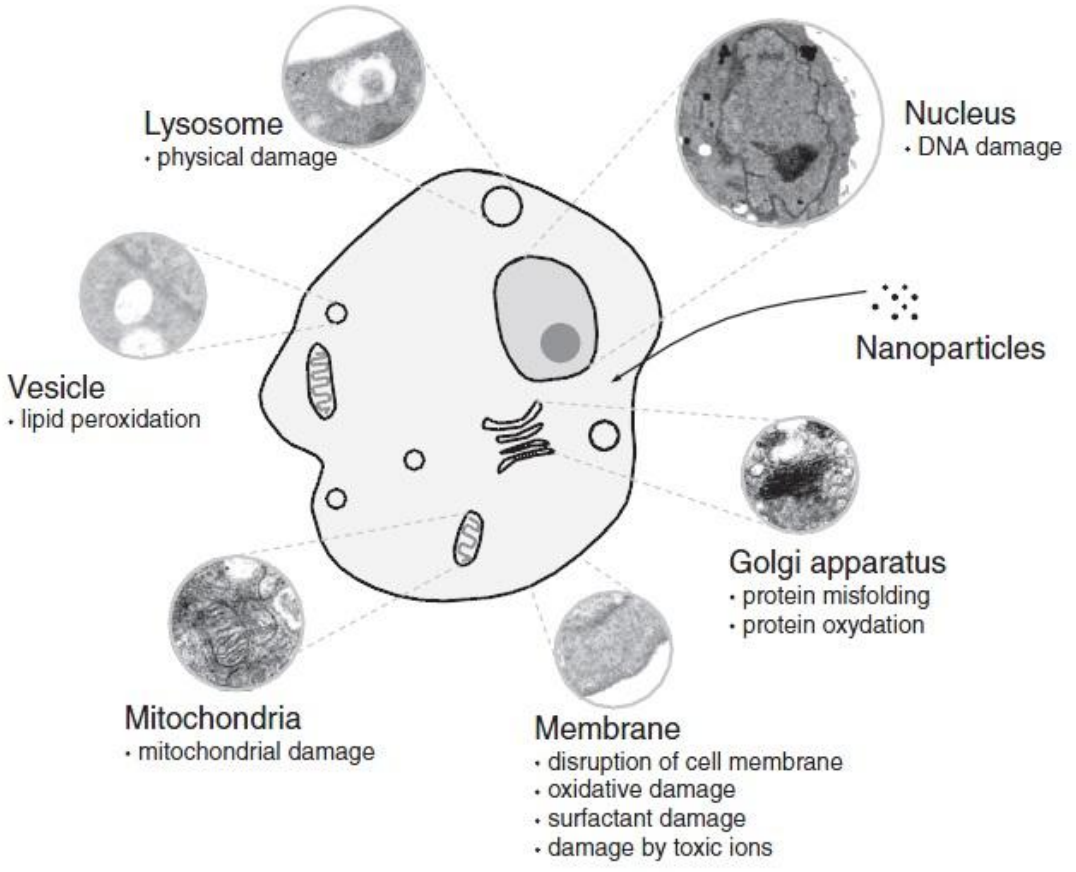


Figure 6. Possible nanoparticle damage to a typical cell [15].

Gold nanoparticles have easy access to cells because of their small size [26]. In most cases, toxicity of gold nanoparticles is related to their interactions with the cell membrane. Those interactions are directly connected to the nanoparticles' surface

coating and charge density. Due to electrostatic attraction, the nanoparticles' cationic surfaces can penetrate the negative charged cell membrane. By altering the AuNPs' coating, one can theoretically have control on AuNPs toxicity levels and therefore manipulate their unique abilities [39][26][20].

Furthermore, small gold nanoparticles (4-5nm) can penetrate the nuclear membrane and bind to the DNA, resulting to alternations of the original genome. On the contrary, larger gold nanoparticles (<5.5 nm) tend to accumulate in tissues and organs, residing within the cells for a long time [39].

Cui et al., (2012), investigated the antibacterial action of gold nanoparticles on bacteria and more specifically on *E. coli*. They concluded in two possible mechanisms that gold nanoparticles can affect bacteria: the inhibition of the ATP synthesis process, which affects the cells' metabolism leaving the cell without energy, and the inhibition of the ribosomal functions, without which no proteins are synthesized and the cell is left without structural material. Either one of those mechanisms is activated or both, cellular death is certain.

2.2.2. Toxicity assessment of gold nanoparticles on human cells, organisms and bacteria

Although gold nanoparticles have promising properties making them suitable for medical and biological applications, it is necessary to assess their potential toxicity before any in vivo applications take place [24]. Several groups have enrolled in the investigation of the gold nanoparticles toxicity during the past decades concluding to contradicting and confusing results. The literature available refers to experiments on bacteria and on human or mammalian cells.

As the noble metal gold was known for its therapeutic and medical properties, gold nanoparticles were not expected to be toxic [39]. Some research groups indeed confirmed the non-toxic nature of gold nanoparticles, like Simon-Deckers et al., (2008), who tested 37.5nm particles with concentration of 6 mg/mL on *E.coli*, NRK-52E cell line (rat kidney cells) and Hep-G2 hepatocarcinoma cell line. AuNPs synthesized by the Turkevich method (citrate reduction) and purified with centrifugation. AuNPs did not induce any toxic effects.

Lasagna-Reeves et al., (2010) tested *in vivo* the bioaccumulation and toxic effects of gold nanoparticles on mice. Different doses of 40, 200 and 400 μg/kg gold nanoparticles were administrated everyday in mice for 8 days. The 12.5 nm sized nanoparticles were synthesized by citrate reduction of HAuCl4 and were filtered and purified. Despite the accumulation of gold nanoparticles in mice organs, no toxicity was observed, as well.

Shenoy et al., (2006), tested the toxicity of unfunctionalized gold nanoparticles and surface-functionalized gold nanoparticles (methoxy-PEG-thiol AuNPs and coumarin-PEG-thiol AuNPs) on MDA-MB-231 human breast carcinoma xenograft cells. Gold nanoparticles were synthesized by the citrate reduction of HauCl4 method and the

concentration applied to the cell line was 20μl. No toxicity effect was observed for the three types of AuNPs.

Some studies showed selective toxicity activity of gold nanoparticles. That depended on the concentration like the study of Hernandez-Sierra et al., (2008) that showed that the bactericidal effect of gold nanoparticles was weak compared to the equivalent effect of silver and zinc oxide nanoparticles. Gold 80nm particles were prepared by a wet chemistry technique while no purification processes are mentioned. The concentration of AuNPs applied was from 0.192 to 197μg/mL and only in the 197μg/mL they presented bactericidal effect.

Also the group of Connor et al., (2005), that investigated the potential toxicity of gold nanoparticles with different modifiers and stabilizers on human leukemia cells (leukemia cell line K562), blamed the toxicity effect on a capping agent. The tests employed cysteine and citrate-capped particles of 4nm, glucose-reduced particles of 12nm and cetyltrimethylammonium bromide (CTAB) –capped particles of 18 nm in a variety of concentrations (0, 50, 100, 200 and 250 μM). The results showed that despite the penetration of all the tested particles in the cells, only the CTAB-capped ones were found toxic and lead to cell death. However, it is worth mentioning that when washed, the CTAB-capped particles didn't show any toxicity while CTAB alone showed similar toxicity to the initial experiment. The conclusion of the work was that toxicity of gold nanoparticles is mostly a result of the precursors and not of nanoparticles themselves.

Another study that accused CTAB as the substance that causes the toxicity effect was performed by Wang et al., (2008). The cytotoxicity of gold nanoparticles and nanorods was tested on the human skin cell line, HaCat keratinocytes. Gold nanoparticles of 5, 12, 20, 30, 50 and 70 nm were synthesized by citrate reduction of HauCl4. Gold nanorods were generated using a seed-mediated growth method with the help of CTAB as surfactant and were purified in order to remove the excess surfactant. Surprisingly, gold nanoparticles didn't show any toxicity while gold nanorods proved to be highly toxic. To clarify whether the toxicity of nanorods was due to the presence of CTAB or not, Wang et al., tested the toxicity of PSS (poly-styrenesulfonate) coated nanorods. The PSS-coated nanorods were not toxic and the toxicity of gold nanorods was blamed on the presence of CTAB.

Several groups concluded that gold nanoparticles are partly toxic depending on the concentration, surface characteristics and size. Patra et al., (2007), tested the response of human carcinoma lung cell line (A549), baby hamster kidney cell line (BHK21) and human hepatocellular liver carcinoma cell line (HepG2) to gold nanoparticles. The 33nm samples were prepared by the citrate reduction of HauCl4 and were centrifuged to eliminate non-AuNPs components. The concentrations (0, 10, 30, 60 and 120 nM) were tested with several methods. The results showed no effect of gold nanoparticles on cell lines BHK21 and HepG2 but a significant death response in cell line A549 dependent on the concentration added. The results are considered positive,

as a new cancer-fighting possibility is implied. Nevertheless, the cell response to gold nanoparticles needs to be further investigated before gold will be actively introduced to nanomedicine.

Goodman et al., (2004), tested the effect of gold nanoparticles on cell viability. Cationic (ammonium-functionalized) and anionic (carboxylate-functionalized) gold nanoparticles with concentrations from 0.38 to 3 mm were tested on Cos-1 cell line, red blood cells and *E. coli* cultures. Cationic nanoparticles proved to be toxic for the cells while the anionic ones didn't show any particular toxicity, a fact explained by the electrostatic attraction between the negatively charged cell membrane and the cationic AuNPs.

Pan et al., (2007), tested the cytotoxicity of TPPMS/TPPTS-modified AuNPs of 0.8-15nm, prepared with a wet chemistry method and centrifuged in order to be pure. The toxicity assessment was performed against HeLa cervix carcinoma epithelial cells, SK-Mel-28 melanoma cells, L929 mouse fibroblasts and mouse monocytic/macrophage cells. The results showed that the smaller particles (1-2 nm) were toxic while the larger particles did not induce any toxicity.

There are also groups that confirmed the hypothesis that gold nanoparticles present bactericidal activity and cytotoxicity against human cells. Arshi et al., (2011), tested antibacterial activity of several gold nanoparticles on bacteria *E. coli*. Gold nanoparticles of average size 4 and 1 nm were synthesized with a microwave irradiation method and capped with CTAB as binding agent. No purification method was mentioned. *E. coli* was used to test the antibacterial activity of gold nanoparticles of both sizes at the concentrations of 0 and 20μL. The results showed growth delay of *E. coli* for both nanoparticle sizes at 20μL with a slightly better bactericidal activity for the smallest particles.

Antimicrobial activity of gold nanoparticles was tested also on eight different bacteria *S. aureus*, *S. faecalis*, *E. faecalis*, *E. coli*, *P. aeruginosa*, *P. vulgaris*, *B. subtilis*, *Y. enterocolitica*, and the fungus *C. albicans.* AuNPs were prepared by a green synthesis technique, the reduction of HauCl4 with the help of *T. decandra.* Their size was 33-65nm and they were washed and purified with centrifugation. The concentration applied to bacteria was 10 mg/mL and the results showed excellent bactericidal activity against all of the samples tested [19].

Another study on the toxicity of greenly synthesized gold nanoparticles was performed by Bindhu et al., (2014). Gold nanoparticles of an average of 16nm were prepared using *Ananas comosus* as reducing agent of chloroauric acid. The antibacterial tests were done on the bacteria *S. aureus* and *P. aeruginosa* with 50μg/ml concentration of AuNPs. Antibacterial activity of AuNPs was confirmed.

Gold nanoparticles of average size 50nm were synthesized by Muthuvel et al., (2014) with a green method using the leaf extract *S. nigrum* in water as the reduction agent of

chloroauric acid. Their antibacterial potential was tested against the gram-positive bacteria *Staphylococcus saprophyticus*, *Bacilus subtilis* and against the gram-negative bacteria *E. coli* and *Pseudomonas aeruginosa*, with concentrations of 50 and 100μL. The antibacterial activity against gram-negative bacteria was better than on the gram-positive ones. However, in both cases the cell membranes were penetrated by gold nanoparticles, confirming their toxic effect.

Chueh et al., (2014), assessed the cytotoxicity of AuNPs on the mammalian cell lines: PK-15 (porcine kidney), Vero (African green monkey kidney), NIH3T3 (mouse embryonic fibroblast) and MRC5 (human normal lung fibroblast). Nanoparticles in the form of nanorods and with average length of 10-40nm were purchased in an aqueous solution. The cytotoxicity effect was confirmed for all the cell lines and several cell responses were observed.

Toxicity of gold nanoparticles was tested on MRC-5 human fetal lung fibroblast cells by Li et al., (2010). Coated with fetal bovine serum nanoparticles of 20nm were prepared by a citrate reduction of HauCl4 method and were washed in a phosphate buffer saline (PBS) solution. The cell culture was treated with 1nM concentration of gold nanoparticles and toxicity caused by oxidative stress was observed along with the activation of defense cell mechanisms like formation of stress response proteins and autophagosomes.

In the research work by Yen et al., (2009), cytotoxic effect of a broad size range of gold nanoparticles was investigated in blood cells J774 A1 macrophages. The particles were prepared by a physical method without any surface modifiers and stabilizers and were separated in three groups according to their size: small AuNPs with size range 2-4nm, medium AuNPs of 5-7nm and large AuNPs of 20-40nm. The concentrations applied to the cells were 1ppm and 10ppm. For the concentration of 1ppm no toxic effects were observed along with no morphological changes. However, for the concentration of 10 ppm, all three sizes showed a significant cytotoxicity response of the macrophage with greater cytotoxic effect observed at the smaller AuNPs.

Pernodet et al., (2006), investigated the human dermal fibroblast cell processes affected by gold nanoparticles. Concentrations of 0.1 and 0.6 mg/mL of 14nm sized gold nanoparticles were incubated with the fibroblast cells. The results showed the accumulation of AuNPs in the cells and the decrease of cell proliferation, adhesion and motility.

Reference	Bacteria – Cells	Preparation method	Purification method	Size	Concentration	Results
Simon-Deckers et al., 2008	*E.coli*, NRK-52E and Hep-G2	Turkevich method	Centrifugation	37.5nm	6 mg/mL	No toxic effects
Lasagna-Reeves et al., 2010	Male C57/BL6 mice of 12 weeks	Citrate reduction	0.45 µm *Millipore* syringe filters	12.5nm	40, 200 and 400 µg/kg	No toxic effects, only accumulation in organs
Shenoy et al., 2006	MDA-MB-231 human breast carcinoma xenograft cells	Citrate reduction and conjugation with methoxy- and coumarin-PEG-thiol		10-15nm	20µl	No toxic effects
Hernandez-Sierra et al., 2008	*Streptococcus mutans*	NaBH4 reduction method		80nm	0.192 to 197µg/mL	Weak bactericidal effect at 197µg/mL
Connor et al., 2005	K562 leukemia cells			4, 12 and 18nm	0, 50, 100, 200 and 250 µM	Only the CTAB-capped NPs were found toxic
Wang et al., 2008	HaCat keratinocytes cells	Citrate reduction method	Centrifugation	5, 12, 20, 30, 50 and 70 nm	10µL	Only the CTAB-capped nanorods were found toxic
Patra et al., 2007	A549 human carcinoma lung cells, BHK21 baby hamster kidney cells and HepG2 human hepatocellular liver carcinoma cells	Citrate reduction method	Centrifugation	33nm	0, 10, 30, 60 and 120 nM	Concentration dependent death response only in A549
Goodman et al., 2004	Cos-1 cell line, Red blood cells and *E. coli*	ammonium- and carboxylate-functionalization		2nm	0.38 to 3 µM	Toxicity induced only the cationic NPs
Pan et al. (2007)	HeLa cervix carcinoma epithelial cells, SK-Mel-28 melanoma cells, L929 mouse fibroblasts and mouse monocytic/macrophage cells	Wet chemistry method and modification	Centrifugation	0.8-15nm	30µM to 10mM	Only the smaller particles were toxic

Arshi et al., 2011	*E. coli*	Microwave irradiation method – CTAB as binding agent		1 and 4nm	20µL	Growth delay of *E. coli* for both nanoparticle sizes
Geethalakshmi et al., 2012	*S. aureus, S. faecalis, E. faecalis, E. coli, P. aeruginosa, P. vulgaris, B. subtilis, Y. enterocoliticaa*n d *C. albicans*	Green synthesis method	Centrifugation	33-65nm	10 mg/mL	Excellent bactericidal activity against all the samples
Bindhu et al., 2014	*S. aureus* and *P. aeruginosa*	Green synthesis method		16nm	50µg/ml	Confirmed antibacterial activity
Muthuvel et al., 2014	*Staphylococcus saprophyticus, Bacilus subtilis, E. coli* and *Pseudomonas aeruginosa*	Green synthesis method		50nm	50 and 100µL	Antibacterial activity was better against gram-negative bacteria than against gram-positive ones
Chueh et al., 2014	Mammalian cells PK-15, Vero, NIH3T3 and MRC-5	Purchased in aqueous solution		10-40nm		Cytotoxicity and cell response were observed
Li et al., (2010)	MRC-5 human fetal lung fibroblast cells	Citrate reduction method – coating of PBS		20nm	1nM	Toxicity caused by oxidative stress and activation of defense responses
Yen et al., 2009	J774 A1 macrophages	Physical method without stabilizers		2-4nm, 5-7nm and 20-40nm	1ppm and 10ppm	Toxic effect only for the concentration of 10ppm for all sizes
Pernodet et al., 2006	Human dermal fibroblast cells	Turkevich method		14nm	0.1 and 0.6 mg/mL	Accumulation in the cells and decrease of cell proliferation, adhesion and motility

Table 2. Summary of the literature review for toxicity experiments with gold nanoparticles.

3. Materials and methods

In this chapter two different nanoparticle synthesis methods will be presented; the gas-phased Spark Discharge Generation method (SDG) and the Turkevich method, a wet-chemistry method. Additionally, three characterization methods will be described; the Scanning Mobility Particle Sizer (SMPS), UV Spectroscopy and Transmission Electron Microscopy.

3.1 Nanoparticle Synthesis

The nanoparticle synthesis methods described were employed to prepare gold nanoparticle samples for toxicity assessments at the Department of Chemical Engineering of the Delft University of Technology in The Netherlands. The selection of those two methods was premeditated as the SDG method is known for its samples of high purity and the Turkevich method (one of the citrate reduction methods) is the most popular synthesis method for nanotoxicity assessments.

3.1.1. Spark discharge Technique

The spark discharge technique is aerosol based. Particles generated with this technique are embedded in a gas flow avoiding contact with other materials and impurities. Thus, high purity is a significant advantage of this technique especially when it comes to applications like nanoparticle toxicity (nanotoxicity) research. Nanoparticles of all the conducting and semiconducting materials can be formed with this technique and can be embedded in gases like nitrogen, helium and argon. Furthermore, control on the morphology and size of the particles can be easily achieved as well as transferring the particles to a liquid suspension. Consequently, the spark discharge is a convenient and suitable technique for the production of nanoparticle samples for toxicity tests [51].

The synthesis process employs the spark discharge particle generator (SDG) *(Figure 4)* which consists of the spark discharge generator chamber, a current source, a capacitor and two opposing metallic electrodes of the desired material (e.g. Gold) placed a few millimeters apart from each other (adjustable). A constant flow of gas flows across the chamber. A high voltage coming from the power source is applied to the electrodes and an electrical breakdown occurs between them, generating a spark within microseconds. Hundreds or thousands of sparks can be generated per second. For every spark that occurs, the energy consumed corresponds to *Equation 1*, where C is the capacitance of the spark and V the discharge voltage between the two electrodes.

$$E = \frac{1}{2} C * V^2 \qquad (1)$$

Due to this phenomenon, plasma, ionized gas consisting of gold molecules, atoms, ions, electrons and photons, is created. This is called the vapour cloud. Embedded in the gas flow, the vapour cloud cools rapidly and, because of its small size comparing to other evaporation-condensation processes, a high concentration of tiny particles

forms. Those tiny particles condensate into nanoparticles and as agglomeration takes place, larger nanoparticles of different sizes are forming and are being carried away of the spark chamber by the gas flow through the aerosol outlet. At the end of the SDG process, an aerosol of gold nanoparticles has been created.

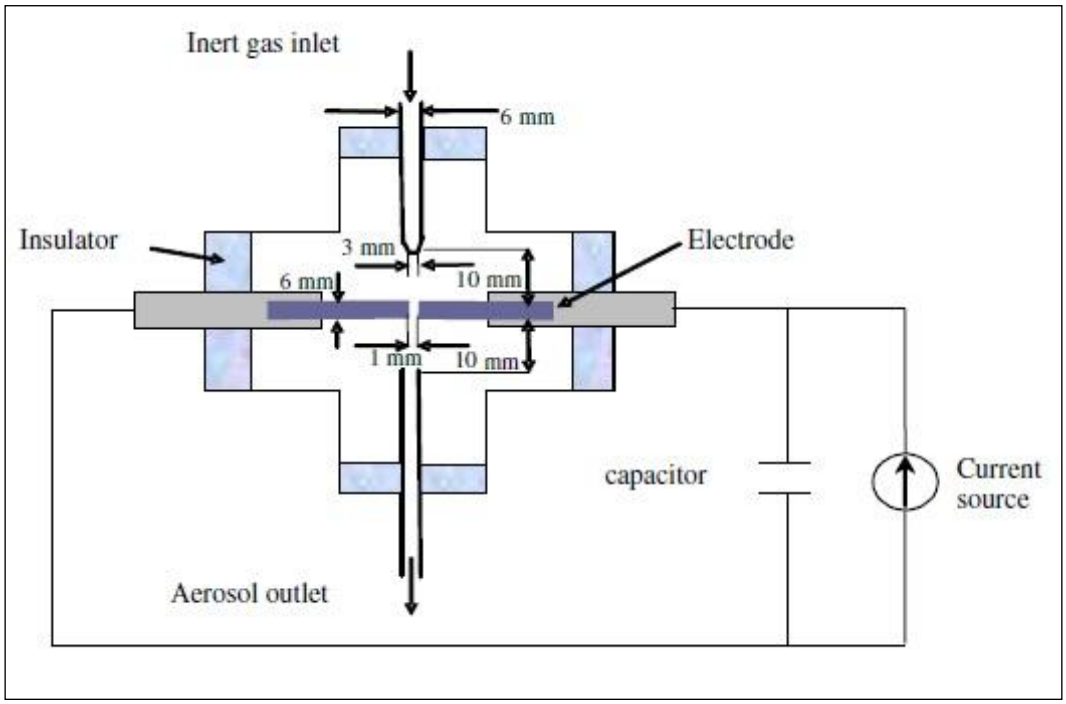


Figure 4. Electrical circuit of a typical spark discharge generator [41].

The synthesis process can be influenced by the parameters of the SDG. First of all, the mean particle diameter is directly affected by the energy of the spark and the gas pressure. Also, the mean particle size increases as the gap distance between the electrodes grows and more energy is needed to generate a spark between them. Moreover, by increasing the capacitance, the sparks produced are stronger (higher energy) which results to the evaporation of more material. More material is equal to higher metal vapour loading in particle growth region and leads to higher particle concentration and mean diameter. The key to control the concentration is to combine high frequency with high gas flow. In General, by altering all these parameters and by combining them properly, total control over particle diameter, shape, concentration can be achieved [51].

By taking into account those characteristics, one can conclude that the aerosol-based spark discharge method has plenty of advantages and meets the need for reliable toxicity assessments. High purity is achieved with the absence of liquid solvents. The synthesis process takes place in a, relatively to other methods, small area while it only acquires electrical power to operate. The particles generated are crystalline, a characteristic demanded for many applications. Furthermore, the method enables the control on the characteristics of the nanoparticles generated, providing a wide range of materials, sizes and media.

3.1.1.1 Experimental Set Up

The experimental set up described in this subchapter was used for the synthesis of gold nanoparticles in aerosol form and their transfer into a liquid solvent. *Figure 5* illustrates the gold nanoparticles aerosol synthesis, the control on their shape and size and the deposition into liquid solvent.

Right after the aerosol outlet of the spark discharge chamber, the particles are practically agglomerates of different shapes and sizes without being spherical. In order to calculate their size, the particles must be spherical because all the calculations are based on spherical shapes. Therefore, a tube oven is set at the melting point of gold, approximately at 910°C. As the particles pass through the oven, they sinter, shrink and, embedded in the gas flow become spherical. This way, control on the particle morphology can be easily achieved.

The tube after the oven carries the nanospheres to the next step of the process, the size selection. The particles are exposed to high temperatures during the sintering process and they need to cool before they proceed to the rest of the setup. Therefore, the tube passes through a cooling system where water reduces the high temperatures.

The next stop for the particles is the neutralizer. The aerosol particles coming from the oven are not considered homogeneous when it comes to their charge. The role of the neutralizer is to bring all the aerosol nanoparticles in to charge equilibrium [41]. The constant production of ions by a radioactive source provides a saturated environment for the charging of the particles [50]. As a result, ions transfer their charge to the particles, which coming out of the neutralizer, all carry the same charge. This process is a necessary preparation for the next component of the setup, the Differential Mobility Analyzer.

The DMA, further described in the next chapter, is an instrument that separates the particles according to their electrical mobility [41]. It creates an electric field and particles with a certain force are attracted. By applying a specific voltage value, particles of a certain size can be attracted while the ones of different sizes are discarded. Size selection is important and useful as it gives us the ability to control the size and thus the properties of the particles produced.

After the size selection, the nanoparticles can be deposited into a liquid suspension. This is necessary for the characterization of the samples and for the toxicity assessments. We chose to deposit the particles in demineralized water (demi water). With the nanoparticles embedded in, the gas flow is bubbled through a column, shown in *Figure 6*, containing a predetermined amount of demi water. The nanoparticles are diffused into the liquid and a colloid suspension is created. The nanoparticle capture of the bubble column is around 50%, so the final concentration of the suspension is half of the initial concentration. The bubble column is connected directly to the exhaust so that the gas and some of nanoparticles that did not success to diffuse could be discarded safely. At the end of the process, the gold nanoparticle

suspension can be collected in a round bottom glass container and stored in dark place at around 4°C.

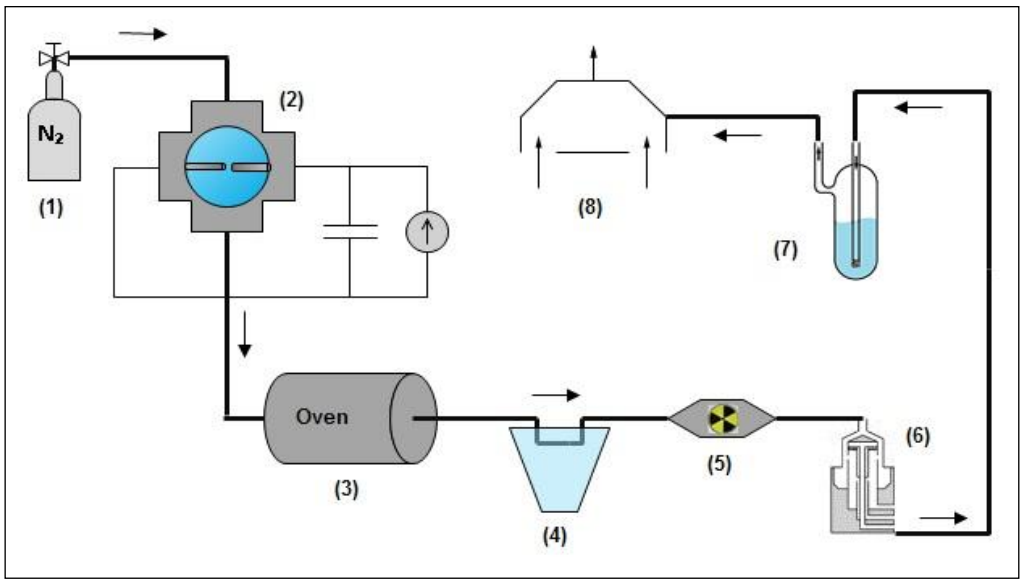


Figure 5. Schematic of the set-up with the DMA; **(1)** Nitrogen tank, **(2)** Spark discharge generator, **(3)** Oven, **(4)** Container filled with water for tubes' cooling, **(5)** Neutralizer, **(6)** DMA, **(7)** Bubble Column, **(8)** Exhaust.

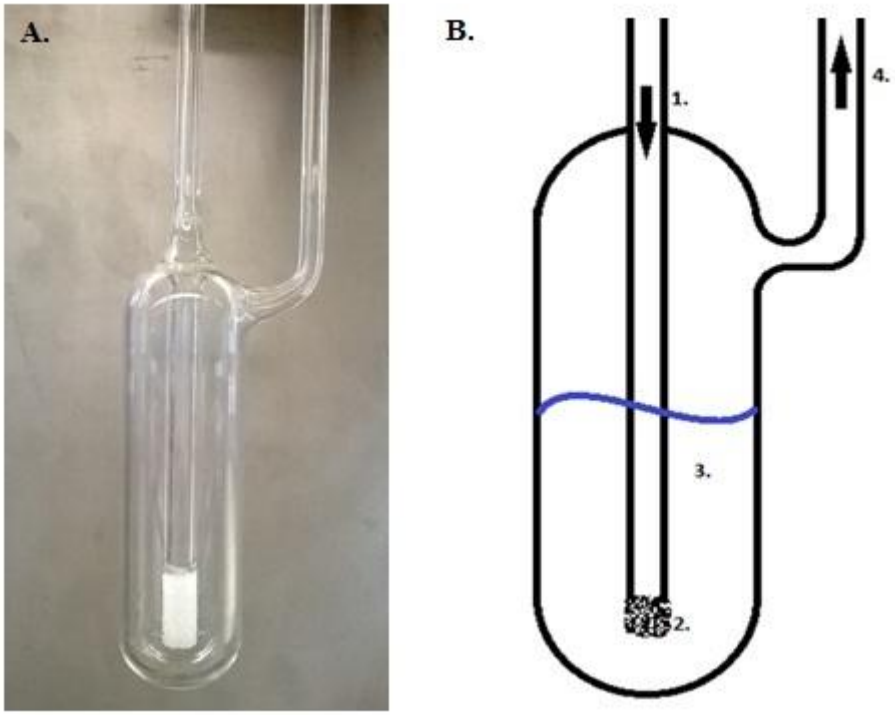


Figure 6. A. The bubble column, **B.** Schematic of the bubble column: **(1)** Aerosol inlet, **(2)** Porous frit, **(3)** Liquid, **(4)** Gas outlet.

3.1.1.2 Experimental settings

The process described in the previous subchapter took place in order to generate spherical gold nanoparticles of an average size of 20nm. The gas used was nitrogen. In the table below the settings of the spark and the DMA are presented.

Settings for the spark discharge generator and the DMA	
Spark voltage (kV)	0.8±0.10
Current (mA)	30.00
Sample volume (mL)	40
Total N_2 Flow rate (L/min)	3.0±0.005
Temperature Oven (°C)	910
Run Time (min)	60
DMA voltage (kV)	0.25
DMA selected size (nm)	20

Table 3. Experimental settings for the Spark Discharge Generator and the DMA.

During the experiment the settings were almost held constant with minor fluctuations. In order to deposit only 20nm particles a certain voltage was applied to the DMA. The value of the voltage that corresponds to the particular particle diameter can be determined through the particle size distribution, which will be further discussed in one of the following subchapters. At the end of the deposition a 34 ml solution of 20 nm gold particles was obtained with concentration $2*10^5$ p/cm³.

3.1.2 Turkevich Method

Colloidal AuNPs can be also synthesized with liquid methods based on chemical reduction. The most common and convenient methods involve the reduction of tetrachloroauric acid ($HAuCl_4$) by various reducing agents. The method chosen for this work was originally introduced by Turkevich in 1951 [52] and further developed by Frens in 1973 [17]. As observed in the literature review, most of the toxicity studies tested nanoparticles synthesized by the Turkevich method or other similar citrate reduction methods. It can be assumed that this wet chemistry method is the most popular gold nanoparticles synthesis method in the nanotoxicity field.

According to this method, spherical gold nanoparticles are synthesized by reduction of tetrachloroauric acid ($HAuCl_4$) with trisodium citrate dehydrate ($Na_3C_6H_5O_7.2H_2O$). When the colorless citrate solution is added to the yellow chloroauric acid solution, the reduction process occurs and due to nucleation the solution turns dark blue. After a few seconds nanoparticles start to form and the solution changes to bright red or dark purple depending on the size of the particles. This method has the advantage of using the trisodium citrate dehydrate both as reducing and stabilizing agent [17], which leads to effortless control of the nanoparticle size, depending on the amount of the agent added [30]. Although the range of the particle size that can be obtained is quite wide (15 to 150 nm), particles over 20 nm are always polydispersed [46].

The synthesis reaction is described by the equation suggested by Balasubramanian et al., 2010 (*Equation 2*). By the reaction of $HAuCl_4$ with $Na_3C_6H_5O_7$ in water, gold nanoparticles are gained along with many byproducts like sodium ketoglutarate, chloride and acetone.

$$\underset{\text{tetrachloro auric acid}}{2HAuCl_4} + \underset{\text{trisodium citrate}}{3Na_3C_6H_5O_7} + 1.5H_2O \rightarrow \underset{\text{nanogold}}{2Au^0} + \underset{\text{sodium ketoglutarate}}{Na_2C_5H_6O_5} + \underset{\text{sodium acetate}}{3CH_3COONa} + \underset{\text{sodium chloride}}{4NaCl} + \underset{\text{chloride}}{4Cl^-} + \underset{\text{hydrogen}}{2H^+} + \underset{\text{acetone}}{0.5C_3H_6O} + \underset{\text{carbondioxide}}{5.5CO_2} \quad (2)$$

Those byproducts can affect the results of a nanoparticle toxicity test. Therefore, purification of the suspension is considered necessary. The easiest and most commonly applied method of purification is centrifugation. As an easy, costless and effective method, centrifugation can remove most of the unwanted by-products [3].

3.1.2.1 Materials

The nanoparticle synthesis process followed was also successfully applied by Nguyen Ngoc Long et al., 2009. Gold chloride/chloroauric acid ($HAuCl_4.3H_2O$) and trisodium citrate dehydrate ($Na_3C_6H_5O_7.2H_2O$) were purchased from Sigma Aldrich. The equipment used was a heating plate equipped with a magnetic stirrer and a thermocouple, magnets, Millipore water (milliQ water), 100ml beakers, pipettes and glass containers for the storage of the nanoparticle solutions. The whole process took place in the fume hood and a lab coat, safety glasses, protective gloves and a respiratory mask were used. The solutions were prepared as follows.

- Trisodium citrate dehydrate solution $Na_3C_6H_5O_7.2H_2O$:

For the preparation of the trisodium citrate dehydrate solution, 0.5080g of $Na_3C_6H_5O_7.2H_2O$ were weighed and added to 5ml of pure milliQ water. The solution was stirred for approximately 10 minutes on the magnetic stirrer and stored in a glass container.

- Chloroauric acid solution $HAuCl_4.3H_2O$:

For the preparation of the chloroauric acid solution, 0.0077g of $HAuCl_4.3H_2O$ were weighed carefully using a plastic container. By the time the chloroauric acid was placed on the plastic container, its form changed from crystalline to liquid. Quickly, the chloroauric acid was added to a 100ml beaker containing 50ml of milliQ water by rinsing carefully the plastic container with a small amount of the milliQ water so that the final solution was in total 50 ml.

3.1.2.2 Synthesis Process

The chloroauric acid solution was heated to boiling on the heating plate while stirring, inside the fume hood. At the time the solution started boiling, 200μl of the freshly made trisodium citrate dehydrate solution were quickly added. During the reduction, gold atoms were released from the $HAuCl_4$ molecules and after a few seconds, the solution changed colour from yellow to black. The gold atoms formed nanoparticles

and the solution became bright red. Change of colour lasted a few seconds. The solution was heated for 10 more minutes and then it was removed from the heating plate to a magnetic stirrer without heating where it kept stirring until it reached room temperature. Because of the heating, the solution was evaporated resulting to a final 33 ml volume. The nanoparticle solution was stored in a glass container, in a dark place at 4°C. The whole process duration was approximately 45 minutes.

3.1.2.3 Purification Process

As explained above, due to a great amount of byproducts in the nanoparticle suspension, purification was necessary. These unwanted components would probably affect the results of the toxicity tests. Centrifugation is an easy and effective way to remove most of the unwanted components. Balasubramanian et al., 2010, proposed a purification process based on centrifugation as shown in the figure below.

Between 5 different centrifugation forces and five different durations the authors chose to use the 7000g force for 20 minutes due to 79% recovery of the particles combined with a satisfactory removal of non-AuNP components. The process contains two "rounds" of centrifugation, each one containing two centrifugations as shown in *Figure 9.*

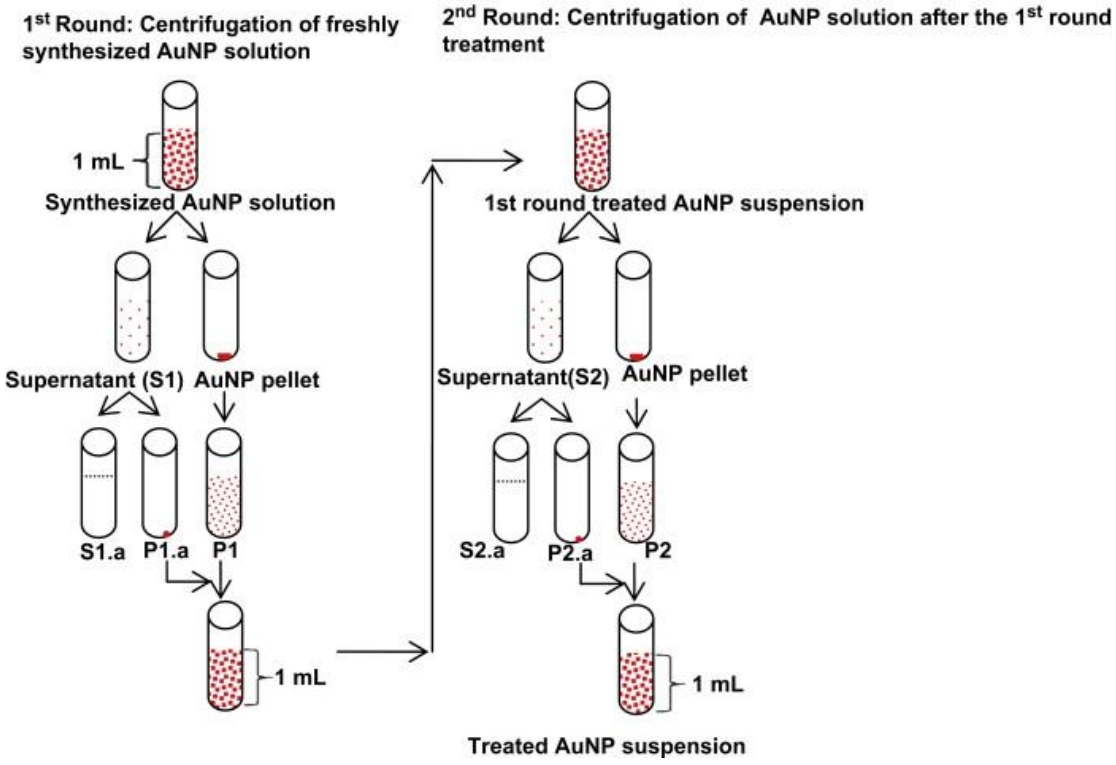


Figure 7. Purification process of nanoparticles with centrifugation [3].

In the first round, the synthesized nanoparticle solution was centrifuged under 7000g force for 20 minutes. It was then separated in two solutions, one containing the precipitated particles and one containing the rest of the solution with the expected losses of nanoparticles. The last one was centrifuged one more time under the same

conditions and then separated again in two solutions, one with the precipitated particles and one with the solution containing the non-AuNP components and a small amount of nanoparticles. The two solutions with the precipitated particles were combined in one and dispersed in ultrapure water until the original volume of the first solution. With this process a significant amount of non-AuNP components was removed but for better results and more pure nanoparticle solution, a second round was considered necessary.

During the second round the same process as in the first round took place. At the end of the process, a significantly pure nanoparticle solution was obtained without great loss of particles. The solution was again stored in a glass container at 4°C.

3.2 Characterization Methods

The next step after the nanoparticle synthesis is their characterization. Characterization is considered necessary as it provides information about the content of the samples prepared. Knowledge of the basic characteristics of the nanoparticles is useful when it comes to applications like toxicity assessments. The methods presented below are applied either to nanoparticle aerosols (SMPS) or to nanoparticle solutions (UV Spectroscopy and Transmission Electron Microscopy).

3.2.1 Scanning Mobility Particle Sizer (SMPS)

The characterization of the spherical nanoparticles directly in the gas phase can be done with the addition of the Scanning Mobility Particle Sizer (SMPS) in the previously described experimental setup *(Figure 8)*. This way, the online particle size distribution can be made before the deposition process.

The SMPS system consists of three components: the neutralizer, the Differential Mobility Analyzer (DMA) and the Condensation Particle Counter (CPC). The first two components are already used in the basic setup, so the only addition is the CPC right after the DMA. Since the measurement is about the gas phased particles, the bubble column is removed from the setup.

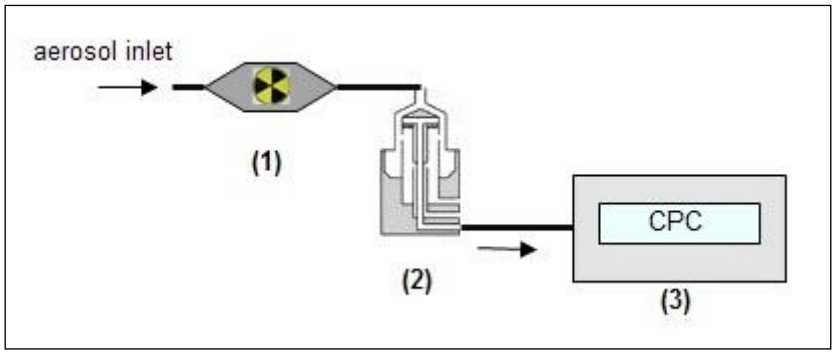


Figure 8. Schematic of the Scanning Mobility Particle Sizer (SMPS); **(1)** Neutralizer, **(2)** DMA, **(3)** CPC.

The first component of the SMPS set up is the neutralizer which was described at the subchapter *3.1.1.1.* The neutralizer is responsible for the homogeneous charging of the aerosol particles.

The DMA is a particle classifying and analyzing cylindrical instrument with two flow inlets on the top and two outlets at the bottom *(Figure 9).* Its function is based on the separation and selection of particles according to their electrical mobility [51]. A polydisperse aerosol flow enters the DMA (Qa) and surrounds the rod placed at the center of the instrument. A sheath flow of purified air also enters the DMA from the top inlet (Qsh) and exits from the bottom outlet (Qex). The sheath flow stream keeps the flow stream inside the DMA laminar and does not conflict with the aerosol flow stream. A controlled voltage is applied to the inner rod creating an electric field. The electric field attracts only charged particles with high electrical mobility (Z). Those particles are being deposited on the upper part of the rod and exit the DMA as a monodisperse aerosol flow (Qs). Particles with lower electrical mobility are attracted to the outer wall and the sheath flow is carrying them out of the instrument. By altering the voltage applied to the electrode, particles of different size are being selected.

The electrical mobility of a particle is defined by the *Equation 3*, where n is the number of elementary charges on particle and is considered to be equal to 1 (single charged particles), e is the elementary unit of charge, Cc is the slip correction factor, m is the gas viscosity and dp is the particle diameter.

$$Z = \frac{n*e*Cc}{3*\pi*m*dp} \qquad (3)$$

By applying a specific voltage value, particles of a certain electrical mobility are attracted while the others are discarded. The voltage applied corresponds to a specific particle diameter and is given by *equation 4*, where V is the voltage applied on the rod, L is the effective axial distance between aerosol inlet and aerosol outlet, R_1 and are the outer and inner radius of the electrode respectively.

$$V = \frac{Qsh*\ln\left(\frac{R2}{R1}\right)}{2*\pi*L*Z} \qquad (4)$$

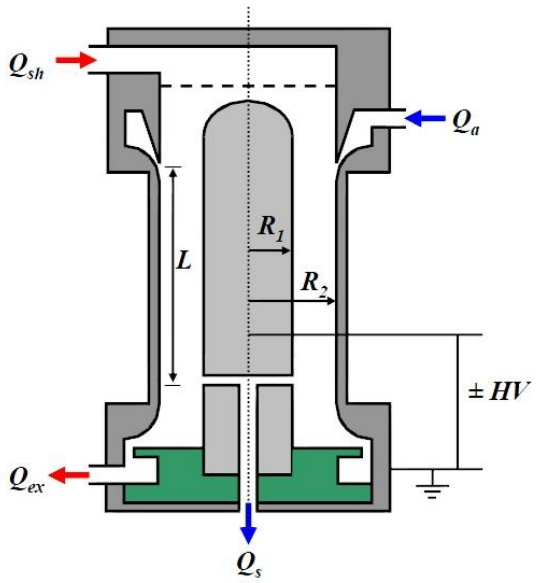


Figure 9. Schematic cross-section of a Differential Mobility Analyzer (DMA) [50].

As can be assumed by the equations, the DMA function can be determined by geometry parameters (L, R1, R2) and operating conditions (Qsh, Qa, Qe, Qs) [50]. For correct calculations, it is important to use the right DMA dimensions and also to measure the flows carefully. Any declinations can lead to false calculations and therefore selection of the wrong particle size.

The monodisperse aerosol exiting the DMA heads to the Condensation Particle Counter (CPC). The CPC is an instrument that detects the size selected particles coming out of the DMA and calculates their concentration. It lacks the ability to detect the very small particles so it makes them larger and detectable using a supersaturated vapour environment [44]. With the CPC the concentration of each particle size is measured and the particle size distribution (PSD) can be created with further calculations. The PSD gives information about the variety of the sizes of particles contained in the aerosol. Detailed calculations are explained in the next chapter.

The SMPS system provides direct information about the nanoparticle aerosol produced. The ability to create the particle size distribution online and to provide information about the particles at the moment of their synthesis is precious. By altering the settings of the spark one can directly observe the effects on the particles produced and judge the results, saving time and resources simultaneously.

3.2.2 Transmission Electron Microscopy (TEM)

Electron microscopy techniques are the most popular and accurate techniques for optical size and shape characterization of nanoparticles in the liquid phase. They are considered ideal for characterizing individual particles but they can also observe the existence of clusters [8], small nanoparticles containing fewer than 10^{-4} molecules or atoms. Particle characterization by transmission electron microscopy requires the transmission of a beam of high energy electrons through the sample [5]. Imaging of particles down to 0.1 nm can be achieved by visualizing the absorbance of the beam.

The images are of high resolution and they provide information about the size, the shape and the crystalline structure of individual particles [12]. A particle size histogram can be obtained by analyzing such images with the help of image processing software like imageJ.

3.2.3 UV Spectroscopy

UV Spectroscopy is an optical method of particle characterization. It is a quick and easy method that does not affect the samples tested. The data of a UV spectrum provide information about the formation, the size and the stability of the nanoparticles in the suspension. More specifically, size, concentration and aggregation level are properties that can be estimated by a method combining Mie theory and Gans model [1]. As the size of the particles is directly related to the wavelength of the absorption peak, it can be also calculated with a specific software program based on Mie's theory about light absorption by particles.

The measurement is done with a spectrometer. First a blank measurement takes place. The blank liquid suspension is placed in two cuvettes and the spectrometer measures the absorption of the particles contained. For the second measurement one of the two cuvettes is filled with the particle solution while the other one stays filled with the blank solution. The spectrometer measures the absorption of the particles and saves the data which are plotted and the UV absorption spectrum curve is created.

The optical absorption spectrum is obtained by plotting data of the absorbance of the particles on each wavelength. The existence of a significant peak confirms the presence of nanoparticles in the solution. The shape and position of the peak indicates the size and shape of the particles. More specifically, the broadness of the peak is clear indicator of the size of the nanoparticles. It is observed that the optical absorption spectrum shifts to longer wavelengths when the particle size is increasing [2]. In general, this measurement is considered quite useful when it comes to the primary characterization of a nanoparticle solution.

4. Results and Discussion

Gold nanoparticle samples prepared with the two aforementioned techniques were characterized and the results are presented in this chapter. The characterization techniques are the previously described Scanning Mobility Particle Sizer, Transmission Electron Microscopy and UV Spectroscopy.

4.1 Characterization of gold nanoparticles prepared by Spark discharge Generation

The samples synthesized with the Spark Discharge Generation technique were characterized first in the gas phase using the SMPS system and after the deposition in the liquid phase employing the other two methods.

4.1.1 SMPS

As mentioned above, the SMPS system can measure the particles size online creating directly their size distribution. During the measurements different voltages were applied to the DMA and concentrations of particles with different diameters were counted every time in the CPC. These concentrations were converted to particle diameters using MATLAB. The particle diameter calculation was done using the constants and properties shown in table 2. Those constants and properties are about nitrogen gas and they are based on the particular experiment.

Constants		Gas and DMA Properties	
Gas Pressure (P)	1.013×10^{5} Pa	DMA length (L)	0.114 m
Temperature (T)	298.15 K	Inner Radius DMA (R_1)	0.935×10^{-2} m
Boltzmann Constant (k)	1.38065×10^{-23} J/K	Outer Radius DMA (R_2)	1.96×10^{-2} m
Elementary Charge (e)	$1.6021765 \times 10^{-19}$ C	Viscosity of the gas (m)	1.75×10^{-5} Pa s
		Mean Free Path (La)	65.3×10^{-8} m

Table 4. Constants, Gas properties and DMA properties [50],[51].

Assuming that the particle diameter (dp) is 50nm, the Cunningham slip correction factor was calculated using *Equation 5*. The slip correction coefficients A=2.492, B=0.84, C=0.43 and the mean free path (La=65.3nm), are considered known for standard conditions (p=1013 mbar, T=25°C) [50].

$$Cc = 1 + \left(A * \left(\frac{La}{d}\right)\right) + \left(B * \left(\frac{La}{d}\right) * e^{-C*\left(\frac{d}{La}\right)}\right) \qquad (5)$$

The electrical mobility of a particle is defined by the *Equation 3* and the voltage that corresponds to the assumed particle diameter was calculated by *Equation 4*.

These calculations were reversed with a specific MATLAB code making it possible to calculate all the particle diameters that corresponded to each voltage value measured. The particle size distribution was created by plotting the diameter and the concentration values *(Figure 10).* From the PSD we can assume that the higher concentration is achieved by particles of around 30 nm.

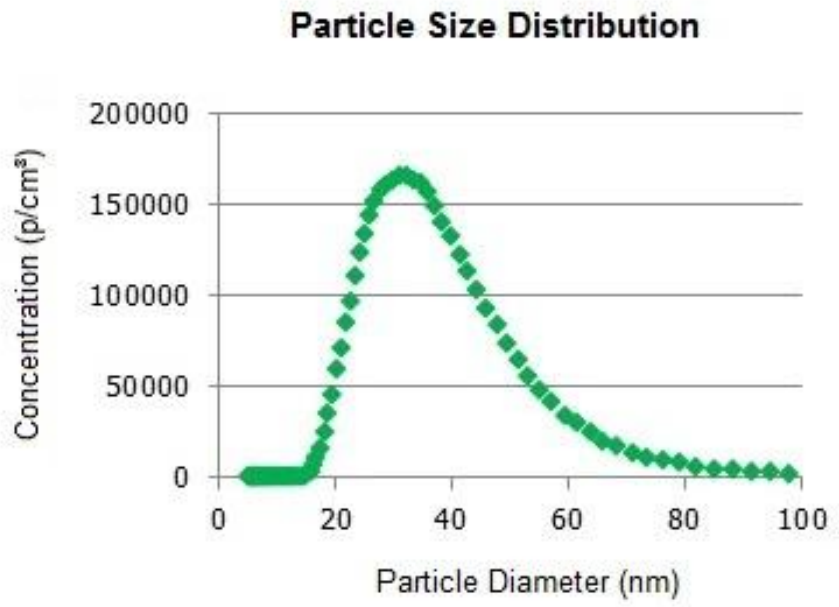
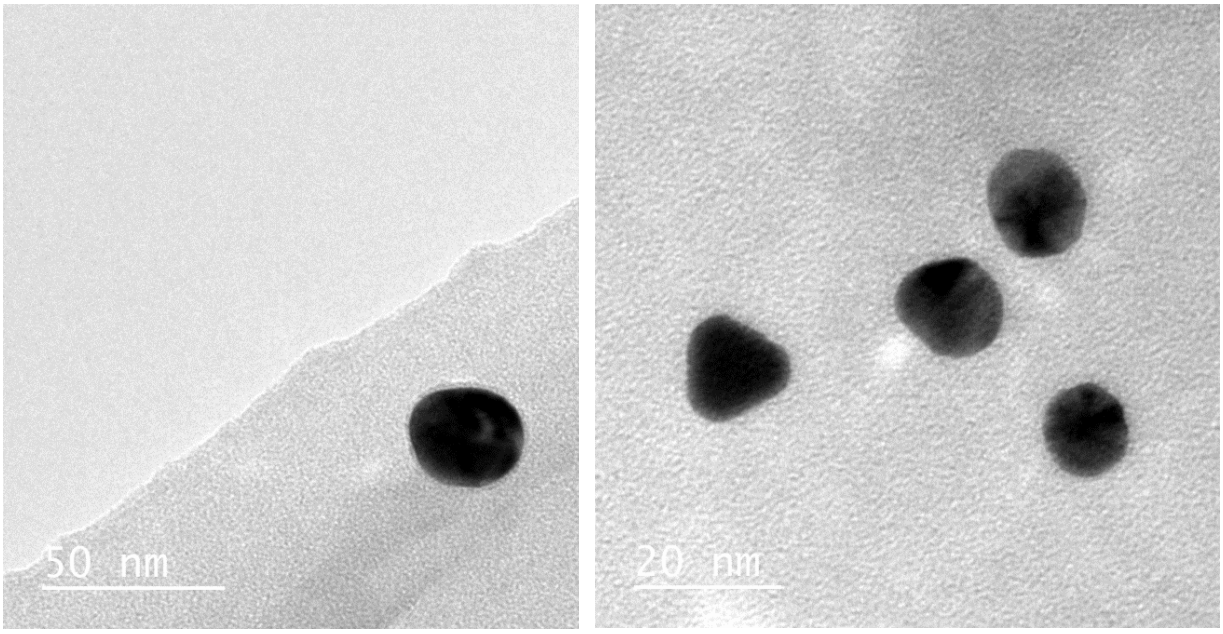


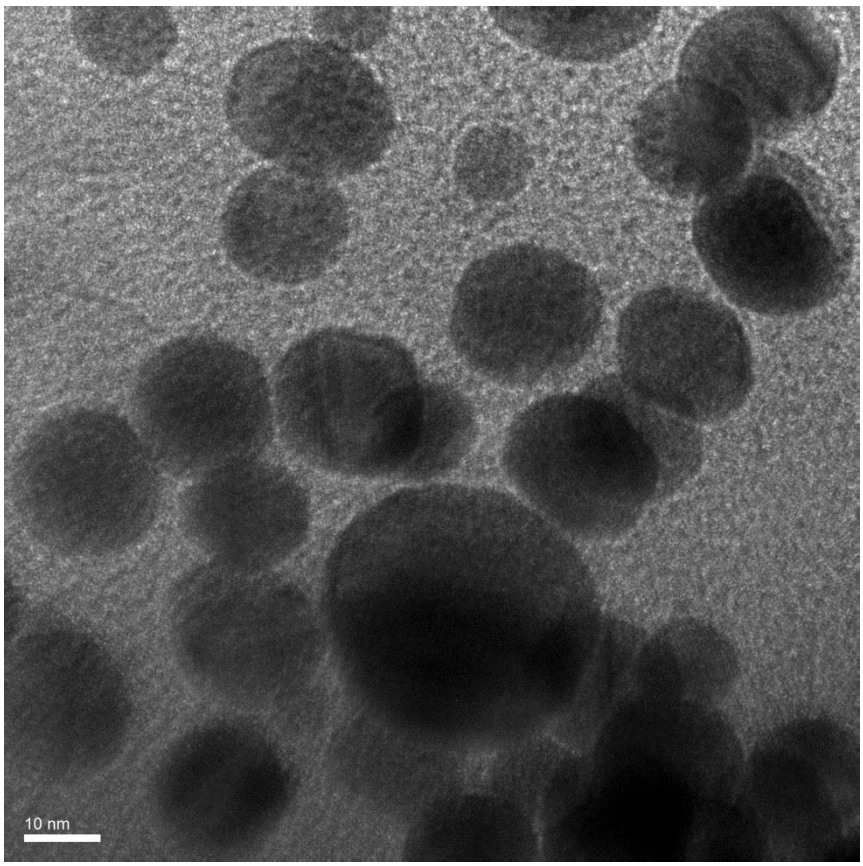
Figure 10. Size distribution of gold nanoparticles generated with the SDG.

4.1.2 TEM

A transmission electron microscopy instrument was employed to examine the synthesized gold nanoparticles. The measurements were done by a specialized technician. The pictures gained can confirm: the existence of nanoparticles in the suspension, their crystalline form, their almost spherical shape (with the exception of some triangle particles) and their average size. The particle in *picture 1* is about 30nm large while the particles in *picture 2* are smaller, around 15nm. By analyzing picture 3, a more complete view of the suspension was obtained and the size histogram of the particles was created *(Figure 11),* which appeared to be quite monodispersed and confirmed 20nm average size.



Picture 1 & 2. TEM pictures of large and small gold nanoparticles generated with the Spark Discharge technique.



Picture 3. TEM picture of gold nanoparticle suspension generated with the Spark Discharge technique.

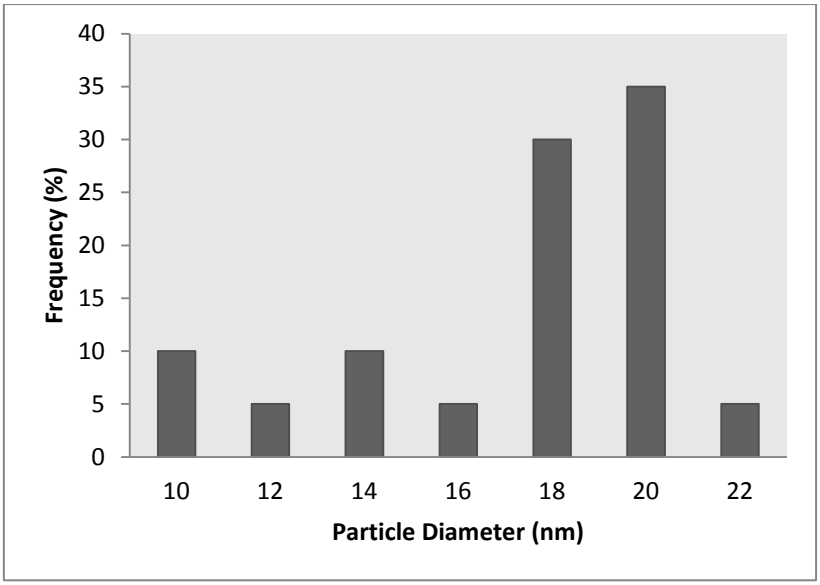


Figure 11. Size histogram of gold nanoparticles generated with the SDG.

4.1.3 UV spectroscopy

A spectrometer was employed in order to obtain data about the absorbance of the nanoparticles in the suspension generated. For the blank measurement demi water was used. In *figure 11* the absorption spectrum of the particles is illustrated. The peak of the wavelength is shown to be at 523 nm, a value that according to the theory corresponds to an average size of 20 nm particles.

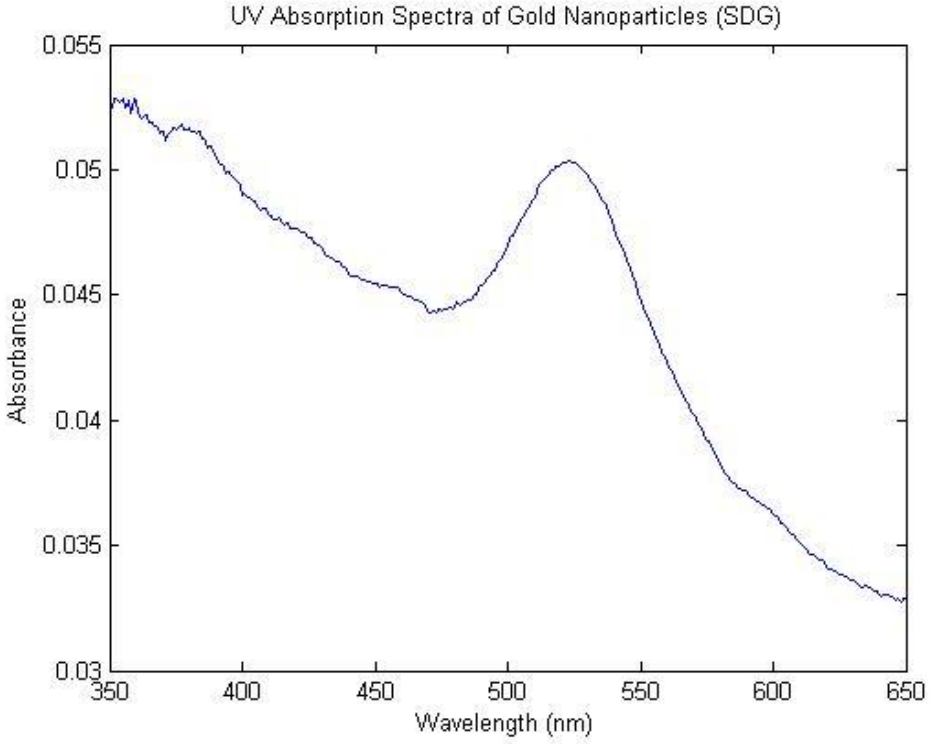


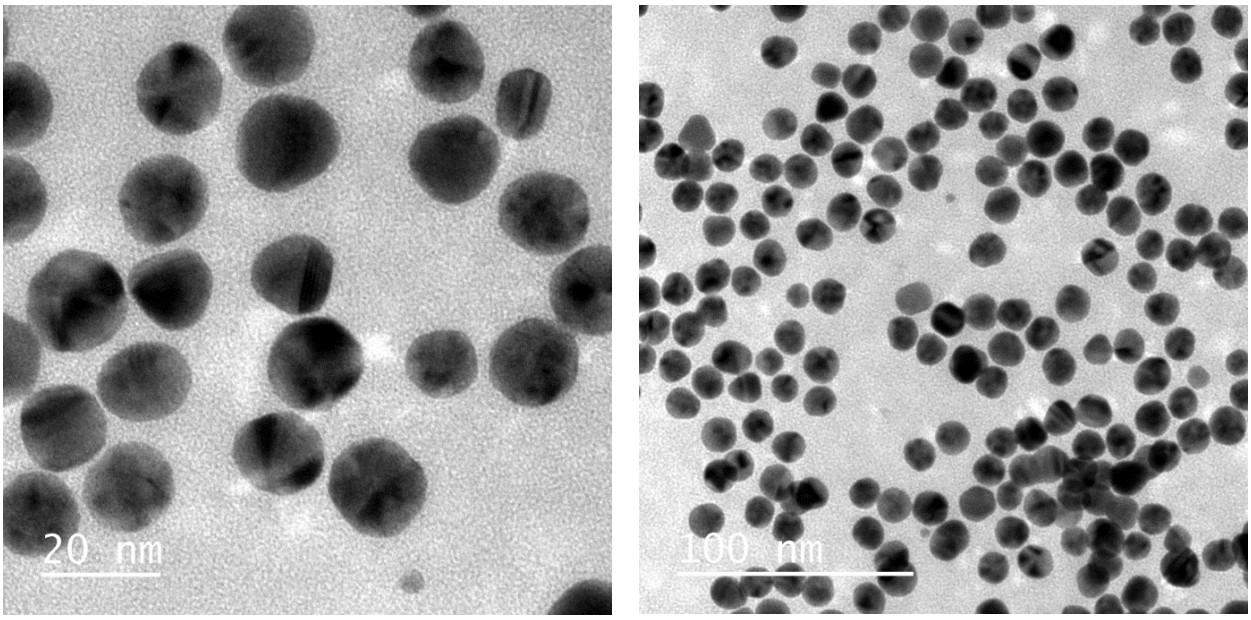
Figure 12. UV absorption spectrum of gold nanoparticles obtained by the spark discharge technique.

4.2 Characterization of gold nanoparticles synthesized with the Turkevich method

The samples prepared with the Turkevich method were characterized employing the Transmission Electron Microscopy method and the UV Spectroscopy method.

4.2.1 TEM

A transmission electron microscopy instrument was again employed to examine the synthesized gold nanoparticles. The pictures gained were clear and helped to obtain information about the morphology and size of the nanoparticles. As shown in *picture 4* and *picture 5*, the suspension contained some nearly spherical and some triangle nanoparticles of around 14nm. By analyzing the TEM pictures the size histogram of the particles created *(Figure 13)* appeared to be monodispersed and confirmed average 14nm size.



Picture 4 & 5. TEM pictures of colloidal gold nanoparticles.

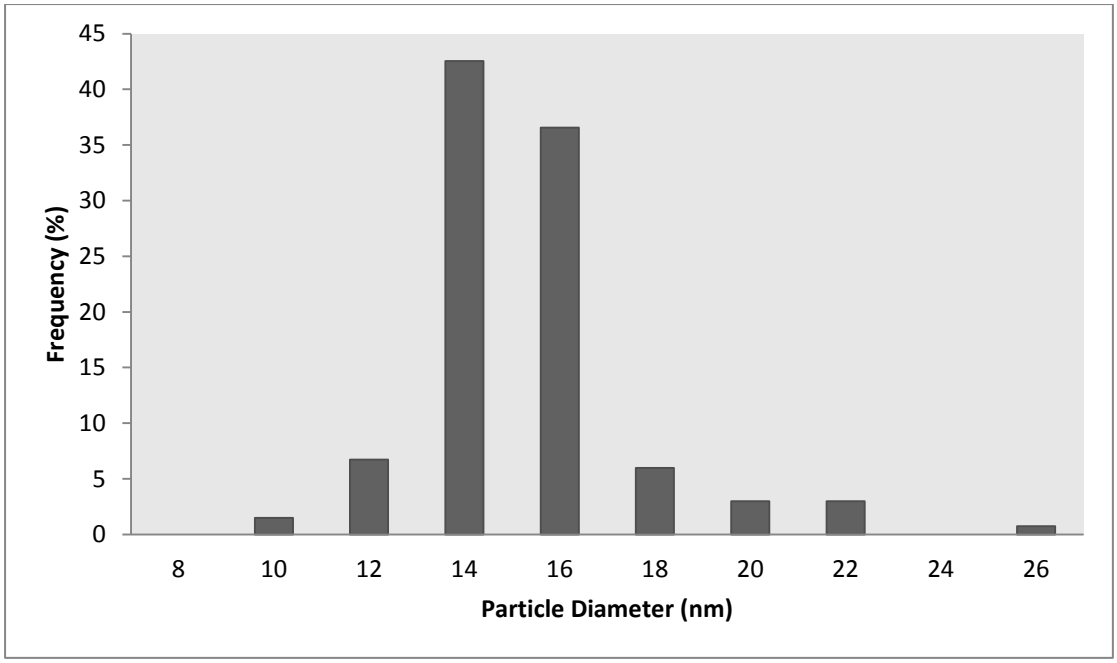
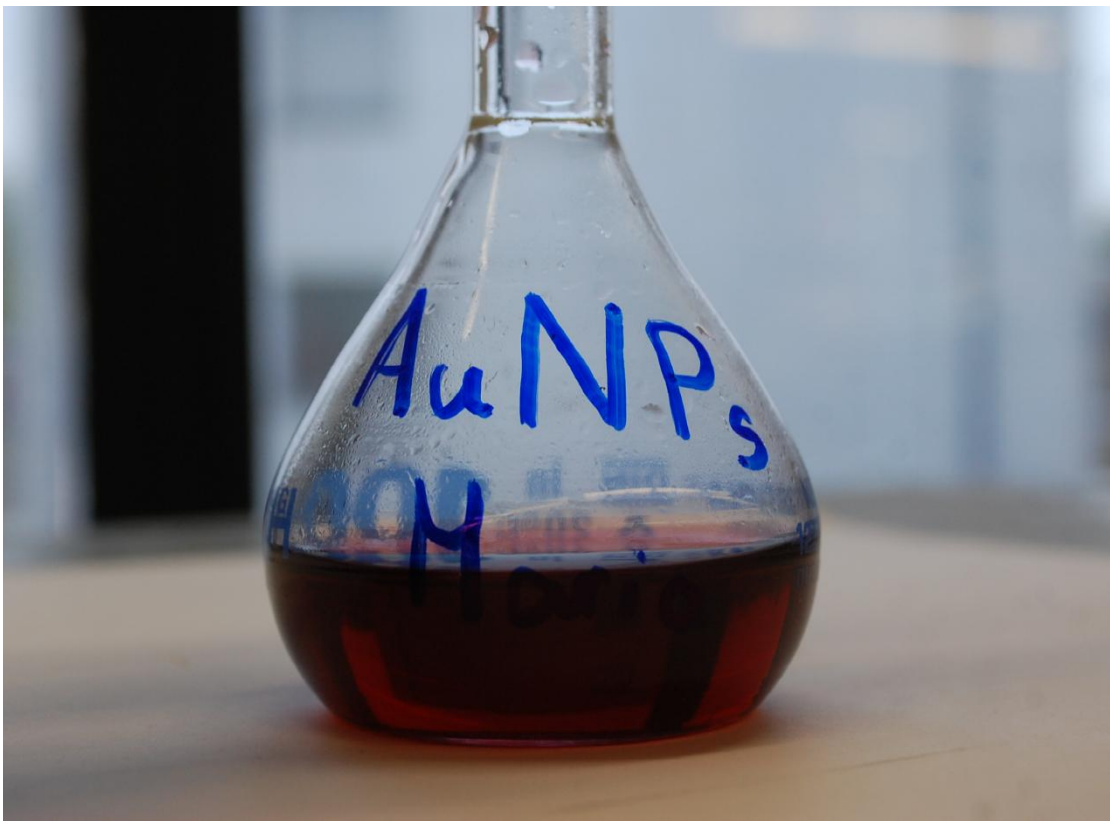


Figure 13. Size histogram of colloidal gold nanoparticles measured from TEM images.



Picture 6. Gold nanoparticles synthesized with the Turkevich method.

4.2.2 UV spectroscopy

The spectrometer was again employed for the measurement of the suspension. For the blank measurement pure Millipore water (milliQ water) was used. *Figure 14* shows the data of the measurement. The peak of the wavelength is at 518 nm.

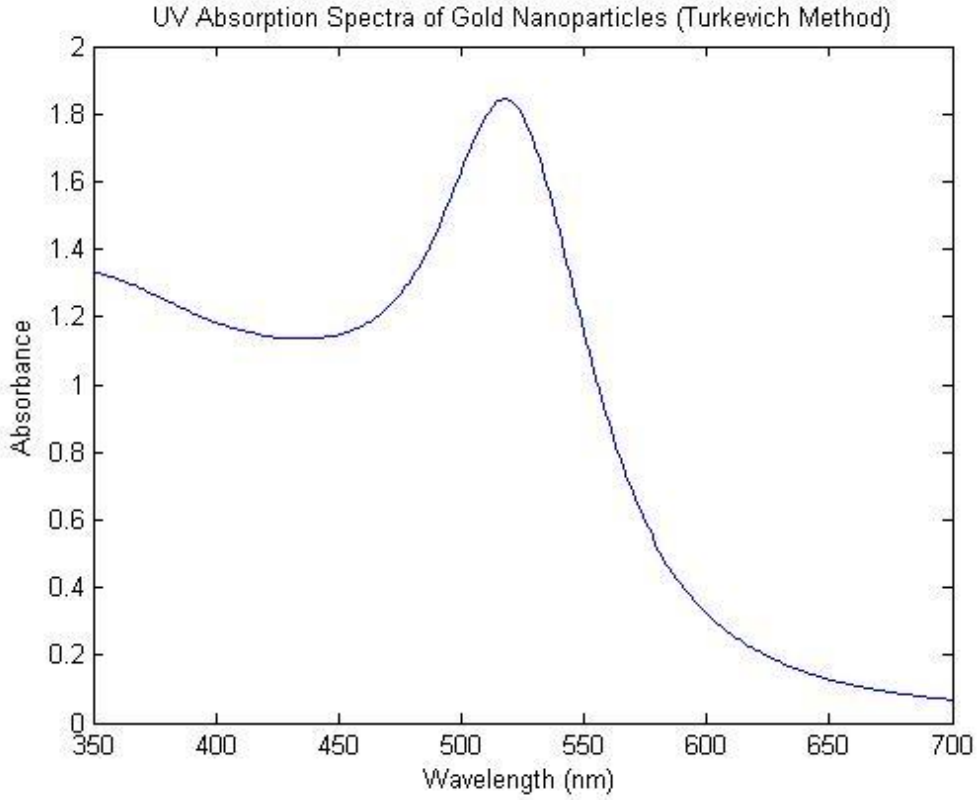


Figure 14. UV absorption spectrum of colloidal nanoparticles.

4.3 Discussion

The comparison of the two methods is inevitable. As can be observed by the results, the UV absorption spectra of the two samples differ a lot. Although they both show a peak in the wavelength region between 500 and 550 nm, there is a huge difference in the height of the peak. As illustrated in *Figure 15*, the curve of the Turkevich particles is very well formed while the SDG is barely showing and it seems more like a straight line. Despite its well-defined curve in *Figure 12*, in the comparison figure the SDG absorption spectrum is outshined by the Turkevich absorption spectrum. This can be explained with the concentration of the nanoparticles in the solution. The Turkevich method solution contains much more material than the SDG one resulting to this huge difference between the two curves. This can be also confirmed by the color of the solutions. While the Turkevich method solution has a bright red color, the SDG method solution is light pink.

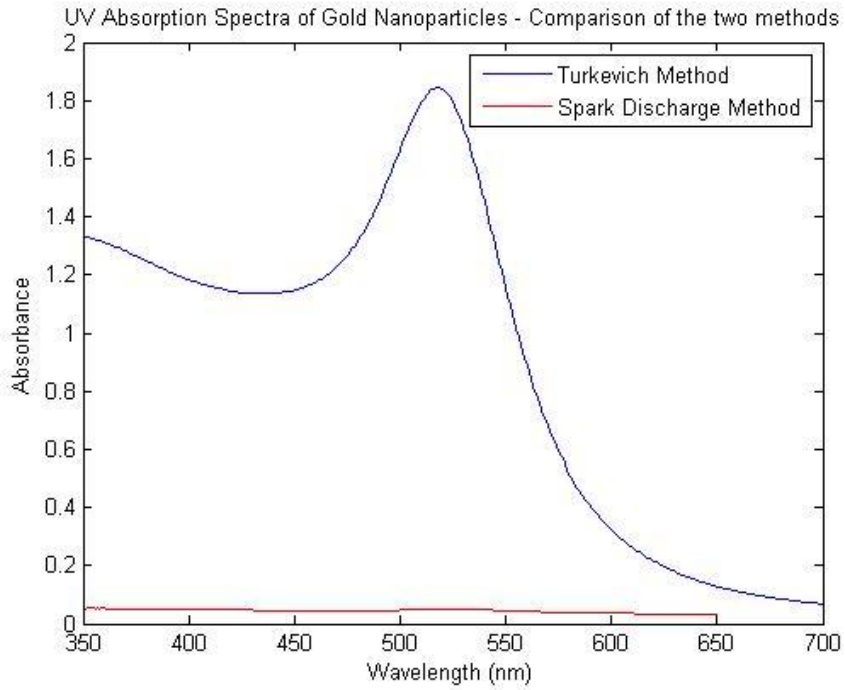


Figure 15. Comparison of the UV absorption spectra of the two different gold nanoparticle synthesis methods.

Another remark that can be drawn from the results is that while the conversion of the peak wavelength of the SDG method to particle size showed reasonable results, the conversion for the Turkevich method particles was confusing. For a peak at 523nm the corresponding size according to the calculations of the program is 20nm, a value that can be confirmed by the TEM analysis. Nevertheless, the size that corresponds to 518nm according to the same program is much smaller than 1 nm which is impossible according to the TEM analysis. The philosophy of the program is based on nanoparticles synthesized in the gas phase, without any impurities. On the other hand, the solution prepared with the Turkevich method contains a high concentration of by-products, even after the purification. Those by-products probably affected the UV spectroscopy measurement, absorbing light and altering the results.

5. Conclusions

Considering the increasing penetration of gold nanoparticles in fields like industry and medicine, the need to assess their toxicity is pressing. The results of the previous research works on nanotoxicity are contradicting. This can be explained by several reasons like the wrong testing conditions or the low quality of the nanoparticle samples tested. In order to achieve an accurate assessment, it is necessary to use pure quality nanoparticles.

The goal of this work was achieved as pure spherical gold nanoparticles with crystalline form were prepared with two completely different methods. Complete

control over the characteristics of the particles was also accomplished for both synthesis methods. The characterization of the nanoparticle samples shows the flexibility of the two methods which can fulfill the demands of a typical toxicity assessment. The samples prepared are ready for toxicity testing and can be diluted to the required concentration.

The Spark Discharge Method has the advantage of producing undoubtedly pure nanoparticles in an aerosol form. The size and shape characteristics are controllable. The disadvantage of low concentration samples can be fixed by longer depositions. Furthermore the particles can be tested either to the gas or to the liquid phase.

The Turkevich method is more commonly used because of its short-time duration and the preparation of high concentrated nanoparticle samples with controllable size. However, the purity of the samples is questionable.

A suggestion for further research on nanoparticles' purity is to use the samples prepared in this work in a toxicity assessments. This way, an objective comparison between the samples prepared with the two methods can be achieved as the quality and purity of the two samples will then be tested and either confirmed or denied. Furthermore, with the same toxicity tests a comparison between the two methods can also occur.

6. References

1. Amendola V. and Meneghetti M., (2009), 'Size Evaluation of Gold Nanoparticles by UV-vis Spectroscopy', *J. Phys. Chem. C*, vol. 113, pp. 4277–4285.

2. Arshi N., Ahmed F., Kumar S., Anwar M.S., Lu J., Koo B. H., Lee C. G., (2011), 'Microwave assisted synthesis of gold nanoparticles and their antibacterial activity against *Escherichia coli* (*E. coli*)', *Current Applied Physics* vol. 11, pp. S360-S363.

3. Balasubramanian S. K. et al., (2010), 'Characterization, purification, and stability of gold nanoparticles', *Biomaterials*, vol. 31, pp. 9023-9030.

4. Bindhu M.R.and Umadevi M., (2014), 'Antibacterial activities of green synthesized gold nanoparticles', *Materials Letters,* vol. 120 pp. 122–125

5. Boyd R. D., Pichaimuthu S. K. and Cuenat A., (2011), 'New approach to inter-technique comparisons for nanoparticle size measurements; using atomic force microscopy, nanoparticle tracking analysis and dynamic light scattering', *Colloids and Surfaces A: Physicochem. Eng. Aspects*, vol. 387, pp. 35– 42.

6. Brown C. L., Whitehouse M. W., Tiekink E. R. T. and. Bushell G. R, (2008), 'Colloidal metallic gold is not bio-inert', *Inflammopharmacology*, vol. 16, pp. 133–137.

7. Buzea, C., Pacheco Blandino, I.I., Robbie, K., (2007), 'Nanomaterials and nanoparticles: Sources and toxicity', *Biointerphases*, vol. 2, no.4, pp.MR17-MR172.

8. Capannelli1 G., Castello E., Comite A., Costa C., and Mamolini G., (2011), 'Electron microscopy characterization of airborne micro- and nanoparticulate matter', *Journal of Electron Microscopy* , vol. 60, no.2, pp. 117–131.

9. Chueh P. J., Lianga R.Y., Leea Y. H., Zenga Z. M. and Chuanga S. M., (2014), 'Differential cytotoxic effects of gold nanoparticles in different mammalian cell lines', *Journal of Hazardous Materials* vol. 264, pp.303– 312.

10. Connor E. E., Mwamuka J., Gole A., Murphy C. J. and Wyatt M. D., (2005), 'Gold Nanoparticles Are Taken Up by Human Cells but Do Not Cause Acute Cytotoxicity', *Small*, vol. 1, no. 3, pp. 325 –327.

11. Cui Y., Zhao Y., Tian Y., Zhang W., Lü X. and Jiang X., (2012), 'The molecular mechanism of action of bactericidal gold nanoparticles on *Escherichia coli*', *Biomaterials*, vol. 33, pp. 2327-2333.

12. Da Silva B.F., Perez S., Gardinalli P., Singhal R.K., Mozeto A. A., Barcelo D., (2011), 'Analytical chemistry of metallic nanoparticles in natural environments', *Trends in Analytical Chemistry*, vol. 30, no. 3, pp. 528-540.

13. Donaldson K., Stone V., Tran C. L., Kreyling W. and Borm P. J. A., (2004), 'Nanotoxicology', *Occup Environ Med*, vol.61, pp. 727–728.

14. Dubeya S. P., Lahtinenb M. and Sillanpaa M., (2010), 'Green synthesis and characterizations of silver and gold nanoparticles using leaf extract of *Rosa rugosa*', *Colloids and Surfaces A: Physicochem. Eng. Aspects*, vol. 364, pp. 34–41.

15. Elsaesser A. and Howard C.V, (2012), 'Toxicology of nanoparticles' *Advanced Drug Delivery Reviews*, vol.64, pp. 129–137.

16. Faraday M., (1857), 'Experimental relations of gold (and others metals) to light', *Phil Trans Royal Soc (Lond), vol.* 147, pp.145–81.

17. Frens, G., (1973), 'Controlled Nucleation for the Regulation of the Particle Size in Monodisperse Gold Suspensions', *Nature Physical Science*, vol. 241, no. 1, pp. 20-22.

18. Fricker, S.P. (1996), 'Medical Uses of Gold Compounds: Past, Present and Future', *Gold Bulletin*, vol. 29, no.2, pp. 53-60.

19. Geethalakshmi, R. and Sarada, D.V.L., (2012), 'Gold and silver nanoparticles from *Trianthema decandra*: synthesis, characterization, and antimicrobial properties', *International Journal of Nanomedicine*, vol. 7, pp.5375-5384.

20. Goodman C. M, McCusker C. D., Yilmaz T. and Rotello V.M., (2004), 'Toxicity of Gold Nanoparticles Functionalized with Cationic andAnionic Side Chains', *Bioconjugate Chem.*, vol. 15, pp. 897-900.

21. Hernández-Sierra J. F., Ruiz F., Corina D., Pena C., Martínez-Gutiérrez F., Martínez A. E., De Jesús Pozos Guillén A., Tapia-Pérez H., Martínez Castañón, (2008), 'The antimicrobial sensitivity of Streptococcus mutans to nanoparticlesof silver, zinc oxide, and gold', *Nanomedicine: Nanotechnology, Biology, and Medicine* vol. 4, pp. 237-240.

22. Johnston R. L., (2012), 'Metal Nanoparticles and Nanoalloys', *Frontiers of Nanoscience*, vol. 3.

23. Ju-Nam, Y. Lead J. R., (2008), 'Manufactured nanoparticles: An overview of their chemistry, interactions and potential environmental implications', *Science of the total environment*, vol.400, pp. 396-414.

24. Khlebtsov N. G. and Dykman L. A., (2010), 'Optical properties and biomedical applications of plasmonic nanoparticles', *Journal of Quantitative Spectroscopy & Radiative Transfer*, vol. 111, pp. 1–35.

25. Lasagna-Reeves C., Gonzalez-Romero D., Barria M.A., Olmedo I., Clos A., Sadagopa Ramanujam V.M., Urayama A., Vergara L., Kogan M. J. and Soto C., (2010), 'Bioaccumulation and toxicity of gold nanoparticles after repeated administration in mice', *Biochemical and Biophysical Research Communications* vol. 393, pp. 649-655.

26. Lewinski N., Colvin V. and Drezek R., (2008), 'Cytotoxicity of Nanoparticles', vol. 4, no. 1, pp. 26 – 49.

27. Li J. J., Hartono D., Ong C. N., Bay B. H., Yung L.Y.L., (2010), 'Autophagy and oxidative stress associated with gold nanoparticles', *Biomaterials* vol. 31, pp.5996-6003.

28. Mittal A. K., Chisti Y., Banerjee U. C., (2013), 'Synthesis of metallic nanoparticles using plant extracts', *Biotechnology Advances*, vol. 31, pp. 346–356.

29. Muthuvel A., Adavallan K., Balamurugan K., Krishnakumar N., (2014), 'Biosynthesis of gold nanoparticles using Solanum nigrum leaf extract and screening their free radical scavenging and antibacterial properties', *Biomedicine & Preventive Nutrition* vol. 4, pp.325–332.

30. Nguyen Ngoc Long et al., (2009), 'Synthesis and optical properties of colloidal gold nanoparticles', *Journal of Physics: Conference Series*, vol.187, pp. 012-0126.

31. Pan, Y., et al., (2007), 'Size-Dependent Cytotoxicity of Gold Nanoparticles', *Small*, vol. 3, no.11, pp. 1941-1949.

32. Patra H. K., Banerjee S., Chaudhuri U., Lahiri P., Dasgupta A. K, (2007), 'Cell selective response to gold nanoparticles', *Nanomedicine: Nanotechnology, Biology, and Medicine* vol. 3, pp. 111-119.

33. Pelaz B. and Del Pino P., (2012), 'Synthesis Applications of Gold Nanoparticles', *Frontiers of Nanoscience*, vol. 4.

34. Pernodet N., Fang X., Sun Y., Bakhtina A., Ramakrishnan A., Sokolov J., Ulman A., and Rafailovich M., (2006), 'Adverse Effects of Citrate/Gold Nanoparticles on Human Dermal Fibroblasts', *Small*, vol. 2, no. 6, pp. 766 – 773.

35. Philip D., (2010), 'Green synthesis of gold and silver nanoparticles using *Hibiscus rosa sinensis*', *Physica E*, vol. 42, pp. 1417–1424.

36. Roco M. C., Williams S. and Alivisatos P. (Editors) 'Nanotechnology Research Directions: IWGN Workshop Report - Vision for Nanotechnology R&D in the Next Decade' WTEC, Loyola College in Maryland, September (1999).

37. Shenoy D., Fu W., Li J., Crasto C., Jones G., Di Marzio C., Sridhar S. and Amiji M., (2006), 'Surface functionalization of gold nanoparticles using hetero-bifunctional poly (ethylene glycol) spacer for intracellular tracking and delivery', *International Journal of Nanomedicine* vol. 1, no.1, pp. 51-57.

38. Simon-Deckers A., Brun E., Gouget B., Carriere M. and Sicard-Roselli C., (2008), 'Impact of gold nanoparticles combined to X-Ray irradiation on bacteria', *Gold Bulletin*, vol. 41, no. 2, pp. 187-194.

39. Soenen S. J., Rivera-Gil P., Montenegro J. M., Parak W. J., De Smedt S. C. and Braeckmans K., (2011), 'Cellular toxicity of inorganic nanoparticles: Common aspects and guidelines for improved nanotoxicity evaluation', *Nano Today*, vol. 6, pp. 446-465.

40. Suman T.Y., Radhika Rajasree S.R., Ramkumar R., Rajthilak C. and Perumal P., (2014), 'The Green synthesis of gold nanoparticles using an aqueous root extract of *Morinda citrifolia* L', *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, vol. 118, pp. 11–16.

41. Tabrizi, N.S., et al., (2009), 'Generation of nanoparticles by spark discharge', *Journal of Nanoparticle Research*, 11, pp. 315-332.

42. Tiwari J. N., Tiwari R.N. and Kim K. S., (2012), 'Zero-dimensional, one-dimensional, two-dimensional and three-dimensional nanostructured materials for advanced electrochemical energy devices', *Progress in Materials Science*, vol.57, pp. 724–803

43. Wang S., Lu W., Tovmachenko O., Shanker Rai U., Yu H., Ray P. C., (2008), 'Challenge in understanding size and shape dependent toxicity of gold nanomaterials in human skin keratinocytes', *Chemical Physics Letters* vol. 463, pp. 145-149.

44. Winkler et al. (2008), **'**Heterogeneous nucleation experiments bridging the scale from molecular ion clusters to nanoparticles', *Science*, vol. 319, no.5868, pp. 1374-1377.

45. Yen H. J., Hsu S. and Tsai C. L., (2009), 'Cytotoxicity and Immunological Response of Gold and Silver Nanoparticles of Different Sizes', *Small*, vol. 5, no. 13, pp. 1553–1561.

46. Zhao, P., Na Li, Astruc, D., (2013), 'State of the art in gold nanoparticle synthesis', *Coordination Chemistry Reviews*, vol. 257, pp. 638– 665.

47. Zhou J., Ralston J., Sedev R. and Beattie D. A., (2009), 'Functionalized gold nanoparticles: Synthesis, structure and colloid stability', *Journal of Colloid and Interface Science*, vol. 331, pp. 251–262.

Books

48. Gogotsi Yury, (2006), *Nanomaterial Handbook*, Taylor& Francis group, LLC.

49. Mansoori, A. (2005), *'Principles of Nanotechnology: Molecular-based Study of Condensed Matter in Small Systems'*, USA: World Scientific.

Theses

50. Steiner, M.G., (2011). *High resolution mobility spectrometry of molecular ions and their effect on the charging probabilities of airborne particles under bipolar diffusion charging conditions*. Ph.D. University of Vienna.

51. Tabrizi N. S., 2009. *Generation of Nanoparticles by Spark Discharge*. Master's Thesis. Delft University of Technology.

Websites

52. Turkevich J., Cooper Stevenson P. and Hillier J., (1951), *'A study of the nucleation and growth processes in the synthesis of colloidal gold',* http://dx.doi.org/10.1039/df9511100055 (last visit 15th May 2014).

53. Zyvex, Ralph Merkle, (n.d.), '*Feynman's Talk*', http://www.zyvex.com/nanotech/feynman.html (last visit 18th May 2014).