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Behavior of Gold Nanoparticles in Physiological Environment and the Role of Agglomeration and Fractal Dimension

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Behavior of Gold Nanoparticles in Physiological Environment and the Role of Agglomeration and Fractal Dimension.

A thesis submitted in partial fulfillment of the
Requirements for the degree of
Master of Science

By

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Abstract

Cooper, Rose, M.S. Pharmacology and Toxicology, Wright State University 2015. Behavior of Gold Nanoparticles in physiological environment and the Role of Agglomeration and Fractal Dimension.

The Present study was designed to examine the role of agglomeration, density and the resulting fractal dimension of nanomaterials in cell culture media. Studies were completed on the kinetics and the process of agglomeration, as well as how to calculate fractal dimensions. The correlation of such complex agglomeration patterns of nanomaterials in culture media, their translocation into cells, and toxic effects were observed. Our results showed that smaller primary particles agglomerated at an accelerated rate when compared to the larger primary particles. They also demonstrated increased cellular uptake, but exhibited lower fractal dimensions. The larger primary particle agglomerates displayed obvious morphology alterations. This could be a result of greater density and sedimentation, which would disrupt cellular structure. These results reveal the biological effects of agglomerates, and how various fractal dimensions may alter cellular interactions with nanoparticles.
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Introduction

The main objective of this project was to characterize the agglomeration of nanomaterials, determine fractal dimension, and correlate these properties to nano-bio effects. The goals were to formerly determine cellular uptake, morphology effects, and correlate them with the structure and fractal dimension of the nanoparticle agglomerates, then the relationship of agglomeration and the fractal dimensions of gold nanoparticles were analyzed along, with the biological responses. The first approach was to synthesize various sizes of gold nanoparticles using the hydrothermal method, next agglomerate nanoparticles via the dispersion method, and lastly, perform characterization of the NP agglomerates to evaluate how well salts in cell culture media agglomerated particles. Furthermore, toxicity of the nanoparticle agglomerates was to be examined in A549 lung macrophage cells. After a 24hr exposure, (a) cellular uptake, and cellular viability were to be measured, (b) morphological effects observed and (c) fractal dimension determined in order to figure if there is any correlation between fractal dimension and biological responses.
Background

Nanoparticles

Nanoparticles were used by artisans in the ninth century in Mesopotamia for generating a glittering effect on the surface of pots (68), but they are now rapidly developing due to their increased economical applications in biomedical, optical and electronic fields (36). Engineered nanomaterials, in the size range of 1-100nm, acquire innovative physical and chemical attributes and are rapidly developing in scientific research (81). The attributes of several common materials are modified when constructed from nanoparticles. Nanomaterials possess increased structural integrity as well as unique mechanical, optical, chemical, electrical and magnetic properties (55). The bio-functionalization of nanometer surfaces can result in aqueous soluble materials which can be further modified with active molecules making them compatible, active, specific capture field agents and useful in biological systems (21). The several unique attributes of nanomaterials resolutely determines their physico-chemical traits, electrical, optical and magnetic characteristics, not shown in their analogous whole assembled complement. The cause of the previous attributes mentioned is typically because nanoparticles have a greater surface area per weight than larger particles which causes them to be more reactive to some other molecules (16). The variation in the attributes of nanoparticles in comparison to their bulk complements has allowed them to become involved in economical applications. Nanoparticle exposure has been steadily incrementing through the manufacturing of engineered nanomaterials for the anteriorly mentioned applications and withal through combustion of fossil
fuels, and utilization of nanomaterials in personal care products, especially cosmetics and sunscreens. Nanoparticle exposure additionally is present in facilities where paints, cement, and other products involving power handling are manufactured, and during processes where nanoparticles are by-products such as baking, welding and polymer processes. Nanoparticle exposure is also widely known to be present in the biomedical field. Nanoparticle utilization in the biomedical field has many accommodations. The advantages of nanoparticles being incorporated in drug delivery systems include: (a) The size and surface characteristics of nanoparticles being easily manipulated for both, passive and active drug targeting, (b) the manipulation of nanoparticles to control and sustain release of the drug during the transportation as well as the location of the release (45). Since dissemination and consecutive release of the drug from the body can be modified, an escalation in drug remedial potency and abatement of side effects can be resolved. Selecting a suitable matrix also helps in expanding the potency and lowering side effects. Anatomical activity of nanomaterials has developed towards substantial concern in recent years owing to the capability for developing sensitive imaging and signaling pathway detection systems as well as possible drug delivery systems. These capabilities expresses nanotechnology where nanoparticles operate as a full system due to its transport and properties. Nanotechnologies are defined as the design, characterization, production and application of structures, devices and systems by controlling shape and size at a nanometer scale (38).
As mentioned throughout this background the unique properties of various types of intentionally produced nanomaterials make these particles novel electrical, catalytic, magnetic, mechanical, thermal, or imaging substances that are highly desirable for applications in commercial, medical, military, and environmental sectors (36). Some of the same special properties that make nanomaterials subsidiary are additionally properties that may cause some nanomaterials to pose hazards to humans and the environment under concrete conditions. Some nanomaterials that enter animal tissues may be able to pass through cell membranes or cross the blood-brain barrier (55).

![Graph showing the number of published papers on nanotoxicology from 1980 to 2013.](image)


Because of these unique physical and chemical properties, that can potentially impact the health of those exposed to them during industrial manufacturing and production, nanoparticle research has been on the rise.
Gold Nanoparticles

Indistinctly gold nanoparticles, considering their exclusive optical-electronic and physical properties, are incrementing in many applications such as photovoltaic, electronic conductors/catalysis, sensory probes, in many therapeutic agents, and drug distribution. These attributes are granted by the interchange of light with electrons on the gold nanoparticle exterior. At a specific wavelength of light, collective oscillation of electrons on the gold nanoparticle surface cause a phenomenon called surface plasmon resonance (figure 2) resulting in strong extinction of light (25).

Colloidal gold nanoparticles, therefore, have dynamic colors owing to their interchange with light. Gold nanoparticles interchange with light or the precise wavelength or oscillation of light solely relies on the environment, size, surface physical dimensions, and agglomeration condition. Gold nanoparticles additionally have the ability to bind amine and thiol groups sanctioning surface modifications and the enhancing utilization of it in biomedical applications. Common oxidation states of gold include +1 (Au (I) or aurous compounds) and +3 (Au (III) or auric compounds). GNP s however exist in a non-oxidized state (AU (0)) (38).
Nanoparticle Synthesis

There are diverse approaches for synthesizing nanoparticles, including attrition, pyrolysis and hydrothermal synthesis. In attrition, macro- or micro-scale particles are pressed in a ball mill, a planetary ball mill or other size-constricting scheme. The proceeding particles are air transferred to yield nanoparticles. In pyrolysis a vaporous precursor (liquid or gas) is compelled through an aperture at high pressure and burned (57). The proceeding solid (an adaptation of soot) is air classified to retrieve oxide particles from by-product gases (46). During standard pyrolysis particles typically convert into aggregates and agglomerates in place of single primary particles. Ultrasonic nozzle spray pyrolysis on the contrary avails in obviating agglomerates from assembling. Thermal plasma can also distribute the energy needed to originate vaporization of small micrometer-size particles (14). The thermal plasma temperatures are at the rate of 10,000 K, causing the solid powder to readily dissipate (87). Nanoparticles are constructed upon cooling.

Figure 2: Basics of localized surface plasmon resonance (LSPR) of gold nanoparticles

Note. Cytodiagnostics. Reprinted from http://www.cytodiagnostics.com/store/pc/Gold-Nanoparticle-Properties-d2.htm Copyright 2015 by the Cytodiagnostics INC. Adapted with permission
while departing the plasma region (52). The central forms of the thermal plasma torches used to yield nanoparticles are DC plasma jet, DC arc plasma, and radio frequency (RF) induction plasmas (41). In the arc plasma reactors, the energy vital for evaporation and reaction is provided by an electric arc located amid the anode and the cathode. Essentially, silica sand can be vaporized with arc plasma at atmospheric pressure, or lean aluminum wires can be vaporized by the exploding wire approach. The derivation coalescence of plasma gas and silica vapor can be promptly cooled by suppressing with oxygen, thence fortifying the merit of the fumed silica engendered.

In RF induction plasma torches, energy coupling to the plasma is cultivated through the electromagnetic field mustered by the induction coil (52). The plasma gas has no association with electrodes, hence excluding available origin of contamination and granting the operation of such plasma torches with a broad range of gases counting inert, reducing, oxidizing, and other corrosive atmospheres (33). The active frequency is typically between 200 kHz and 40 MHz. Laboratory units that function at power levels in the range of 30–50 kW, while the astronomically immense-scale industrial units have been accredited at power ranks up to 1 MW. As the residence time of the instilled feed droplets in the plasma is very brief, it is important that the droplet sizes are sufficiently minute conductive to retrieve an exhaustive evaporation. The RF plasma method has been employed to synthesize distinct nanoparticle materials, for instance synthesis of various ceramic nanoparticles such as oxides, carbohrs/carbides, and nitrides of Ti and Si (79). Inert-gas precipitation is perpetually used to synthesize nanoparticles from metals with neap melting points. The metal is vaporized in a vacuum chamber and then undercooled with an inert gas stream. The undercooled metal vapor compresses into nanometer-size particles, which can be appropriated in the impotent gas stream and installed on a substrate or studied in situ (67).
The hydrothermal synthesis method involves the various techniques of crystallizing substances from high-temperature aqueous solutions at high vapor pressures (75). Hydrothermal is a synthesis approach of individual crystals that confide on the solubility of minerals in sultry water under high pressure (88). Crystal growth is executed in an apparatus subsisting of a steel pressure vessel known as an autoclave in which a nutrient and water are supplied (15). A temperature gradient is cultivated between the adverse ends of the growth chamber. At the hotter end the nutrient solute dissolves, while at the cooler end it is installed on a seed crystal, growing the desired crystal (56). Colloidal particles are sustained by tempering a suspension down after immense heat exposure then amassing after filtration. Colloidal particles are more assessable in biological studies as a result of its conventional dosing, rather than crystal form (43). Nanoparticles can also be formed employing radiation chemistry (73). Radiolysis from gamma rays can establish vigorously active free radicals in solution. This simple technique utilizes a minimum number of chemicals. The chemicals involved include distilled water, a soluble metallic salt, a radical scavenger (often a secondary alcohol), and a surfactant (organic capping agent). In this procedure, reducing radicals will depress metallic ions down to the zero-valence state. Once in the zero-valence state, metal atoms unite to form into particles. A chemical surfactant traps the particle during formation and executes its magnification. In copacetic concentrations, the surfactant molecules stay affixed to the particle. This restrains it from detaching or forming clusters with other particles (14). Formation of nanoparticles using the radiolysis method allows for the altering of particle size and shape by modifying precursor concentrations and gamma dose (9). In our research study, we employed the hydrothermal method considering its many advantages. The hydrothermal method is not only cost-efficient (when synthesizing colloidal particles) and convenient, but it includes: the capability to establish crystalline phases which are not stable at the melting point, the development of materials with
high vapor pressure near their melting points, and it is applicable for the growth of large high quality crystals while preserving exceptional control over their composition (89).

Synthesis of Gold Nanoparticles

Gold nanoparticles can be prepared via various synthesis routes, including chemical, sonochemical, or photochemical paths (53). The most mundane chemical avenue is the hydrothermal process, as previously stated in our research, we employed the hydrothermal synthesis method because of its many advantages, and gold nanoparticles are produced in a liquid by reduction of chloroauric acid (HAuCl4) (59). In the hydrothermal process, the precipitation of GNP is in an aqueous solution from a dissolved gold forerunner. Comparatively, HAuCl4, by a condensing agent such as sodium citrate, ascorbic acid, sodium boron hydride or blocking copolymers. Our research includes the Turkevich method where Sodium citrate is the reducing agent employed. The turkevich method was employed because of its simple preparation procedure and sodium citrate’s ability to prevent agglomeration or further growth of the particles after achieving a desired size (40). Therefore, the sodium citrate acted as a reducing agent as well as a stabilizing agent. Before the addition of the reducing agent, the gold in solution is in the Au3+ form (1). When the reducing agent is integrated, gold atoms are composed in the solution and their concentration elevates hastily until the solution surpasses saturation. Particles then arrange in a process called nucleation. The residual dissolved gold atoms bind to the nucleation sites and growth takes place.
As Gold nanoparticles are synthesized, the color of the particles will also indicate what size the particles have grown to become. For small (~30nm) monodisperse gold nanoparticles the

Figure 3: Addition of Reducing agent to Gold Nanoparticles during synthesis.

surface plasmon resonance phenomena causes an absorption of light in the blue-green portion of the spectrum (~450 NM), while red light (~700 NM) is reflected, yielding a rich red color (74).

As particle magnitude increases, the wavelength of surface plasmon resonance cognate absorption switches to longer, redder wavelengths. Red light is then immersed, and blue light is emulated, creating solutions with a pale blue or purple color. As particle magnitude perpetuates to increment toward the total limit, surface plasmon resonance wavelengths maneuver into the IR segment of the spectrum and most visible wavelengths are imitated, giving the nanoparticles a clear or translucent color. The surface plasmon resonance can be adjusted by fluctuating the size or shape of the nanoparticles, adjusting to particles with custom-made optical traits for various applications (74).

Figure 4: Color change image with gold nanoparticle size.

Agglomeration of nanoparticles

The terms aggregation and agglomeration are often used conversely, but standards organizations define them distinctly. Where aggregation denotes vigorously bonded or fused particles, agglomeration denotes more impotently bonded particles. Agglomeration is utilized in this study because the method employed doesn’t provide the amount of energy required to tightly bind the particles, as in aggregation. Many Nanoparticles agglomerate when they are placed in biological fluids (1). It has been discovered that when nanoparticles are distributed in
liquids, their hydrodynamic size is often bulkier than the initial particle size. In particular, high ionic strength, neutral pH and the presence of amino acids and other zwitterions in typical culture media alter the surface potential of engineered nanoparticles, often resulting in spontaneous agglomeration (4). Typically it’s not possible to restore agglomerated particles back to a monodisperse suspension consisting only of individual nanoparticles (51). In order to isolate agglomerated nanoparticles, momentums in different directions have to be enforced to each nanoparticle. At diminutively minuscule particle sizes, it is simply not possible to engender sufficient microturbulence that engenders a high enough force gradient to surmount the particle binding forces. In this phenomenon, agglomeration is more ideal than aggregation, because “many types of nanoparticles agglomerate in aqueous biological matrices” (30), and once nanoparticles agglomerate, it is practically impossible to restore them to a monodispersed condition.

Serum proteins found in culture medium also absorb to ENP surfaces, forming a protein corona that can further modulate the dispersion state of some ENPs (72). These physical measures are tremendously dynamic, and discrepancies in the formation of the medium or serum can cause interchangeable ENPs to construct distinctive sizes of agglomerates that comport distinct protein subsets. Understanding the stability and agglomeration behavior of nanoparticles under different solution conditions will facilitate investigations on these matters (39).

Recent in vitro studies have evaluated the cytotoxicity of ENPs whose agglomeration state was altered by (a) dispersion in media containing different concentrations of serum protein (0-20%), (b) different serum proteins (calf serum vs bovine serum), or (c) by varying the polymeric or surfactant dispersants used to prepare the particles (72). In our agglomeration method the stabilization of the agglomeration state by dispersion in media containing a 20% concentration of fetal bovine serum protein was incorporated.
Nanoparticles agglomerate rapidly; therefore, it is essential to understand the fate of these agglomerates upon inhalation, to ascertain whether subsequent toxicological effects are attributable to the nanoparticle physical properties or is a function of their chemical composition (6). Comprehensive experimental mapping of the engineered nanomaterial’s agglomeration for the large number of present and anticipated emerging nanoparticles, over wide ranges of possible environmental water chemistries and nanoparticle properties, is a daunting and possibly impractical task (34). Thus, flourishing attentiveness towards exploring several approaches is in place.

Agglomeration of Ultra-fine Particles in the Human Body

Agglomeration properties of various engineered nanoparticles are unknown, limiting our ability to estimate the size distribution of the airborne nanoparticles and thus their fate in the human body after inhalation (70). It has been reported however that Inhaled particulate matter can be deposited throughout the human respiratory tract, and an important fraction of inhaled nanoparticles deposit in the lung (19). Animal and human studies display that there is a limitation in removal with inhaled nanoparticles than bulkier particles by the macrophage clearance mechanism in the lung, causing lung damage (90). Inhaled nanoparticles can reach the blood and may reach other target sites such as the liver, brain, spleen, heart, or blood cells and possibly the foetus in pregnant women (29). Data on these pathways are inhibited, but the genuine number of particles that shift from one organ to another can be considerable, depending on exposure time. It is not known whether agglomerated particles could become single particles again when introduced in a biological system. While several studies have shown that single particles have the ability to form agglomerates in a biological matrix. (31) In this instance, it can be hypothesized that agglomerates rapidly formed once inhaled may be prone to less adverse biological effects, while single particles that cross barriers then agglomerate
could possibly cause serious health effects. Some nanoparticles dissolve facilely and their effects on living organisms are identically tantamount to the effects of the chemical they are composed of, however; other nanoparticles don’t degrade or dissolve facilely, rather they compile in biological systems and linger for a long time which makes such nanoparticles of circumstantial concern.

Fractal Dimension, Density and Dosimetry

In addition to the agglomeration pattern of nanoparticles, an extensive investigation of the morphological structure, and particularly the fractal dimension of nanoparticle agglomerates, is essential to a better understanding of agglomeration mechanisms, the fate of agglomerates in the aquatic environment, and the link between agglomeration structure and toxicity (48). As a result of particle irregularity, configuration is not meticulously illustrated by Euclidian geometry. Nonetheless, fractal geometry adapts the approach of fractal dimension as a tool to characterize the configuration of particles. The fractal dimension of an object, including nanoparticles, expresses its complexity or level of detail and how much space it possesses between other fractals (71). In this instance, fractal dimension of nanoparticles is an incredibly specific form of characterization that lacks further research. The fundamental guideline for fractal analysis is fractal dimension, which is an authentic, non-integer sum, varying from the more usual Euclidean or topological dimension. The fractal dimension of a line, regarding all shapes; range amidst one and two, and regarding a surface amidst two and three. The fractal dimension cannot acquire just any value, albeit a group of detached points such as a Cantor dust can have a fractal dimension lower than one. For matter to prevail as existence, its fractal dimension must be at least one, considering a line is the only probable approach of linking a set of points.
Additionally, the fractal dimension must be lower than or equivalent to the dimension of the capacity in which the fractal subsists, or else the location cannot accommodate the fractal. Any object we can find in a real physical process must therefore have a mass fractal dimension \( 1 \leq D \leq 3 \) (18). Therefore, it is impossible for any object to have fractal dimensional ranges of \( 1 \geq D \geq 4 \).

Seracettin Arasan found that the sphericity, roundness, and convexity of particles decremented when the fractal dimension incremented, however angularity increases as the fractal dimension increases (71). Hence, there is a differential link amid the particle indicator (sphericity, roundness, angular shape and convexity) and fractal dimension. This is a normal outcome considering higher angularity and lower roundness values produce increased particle surface irregularities. Furthermore, it is notable that escalating particle irregularities increments fractal dimension, given that the fractal dimension is significantly affected by the particle shape in a way that, fractal dimensions increase with increasing angularity or decreasing roundness of the particles (71). Hence an anomalous particle can be characterized by an exponent \( \delta \) (non-Euclidean or fractal dimension) that encloses data about the level of volume filling, surface roughness or ruggedness of the perimeter of the 2D particle outline (projection or section).

Anomalous particles accompanying a rugged exterior or agglomerates can have fractal dimensions 'tween 2 and 3. The fractal dimension of the perimeter of a 2D outline of an irregular particle with a rough surface is between 1 and 2 (81). Lin and coworkers discovered that the low fractal dimension of diffusion limited cluster aggregation (DLCA) aggregates reflects the loose, open nature of aggregates formed (18).

After agglomeration of nanoparticles, the complexity and surface area properties or fractal dimension values reverses, thereby altering the total number of free particles, the total surface area available for biointeractions, and the effective size and density of the particles (24). The physical processes and differences in medium composition that form different sized
agglomerates, which pose significant hurdles for specifying the structural features of ENPS that
determine biocompatibility, (72) may be tackled with the figuring of fractal dimensions. The
modifications in properties additionally reverses the inflammation and toxicity properties. In this
instance, modifications in fractal dimension may change the way in which existing cells respond
to the particles altering their morphology and cellular function.

The efficacious size and density of particles in a cell culture complex are a function of their
agglomerate state, packing density, and shape. In addition, the mechanism and kinetics of
cellular uptake of ENPs are influenced by both particle diameter and shape (72). Yin Yao found
that the surface energy density of nanoparticles increases with the decrease of nanoparticle
diameters (84). Nanoparticle agglomerates are penetrable, encompassing media, captured at
the time of evolution with an effective density which is less than the density of the primary
particles. Notably, in contrast to soluble chemicals as well as their micron-sized counterparts,
nanoparticle agglomerates can settle and diffuse differentially according to their hydrodynamic
diameter and effective density; these are processes that are expected to significantly affect the
delivered cellular dose as a function of exposure time (24). The momentum (and inertia) of
particles and agglomerates of the same size strongly depends on the particle’s density (78).
Randall Troy found that more diminutive particles and rods favored margination, and the
particle density had a more vigorous influence as the lighter particles marignated further than
the denser particles (78). Research has proven that particle size is the most important factor
that determines the amount of material on or in cells and that particle concentration and the
total surface area is of “minor” importance (35).

A number of techniques have appeared in the literature to determine the mass fractal
dimension of aggregates of fine particles (18). The three most prevalent techniques include light
scattering, settling, and image analysis. The most common types of image analysis are box
counting, sand box, and confocal scanning laser microscopy (24). In our study, we employed a settling technique through centrifugation to find the volume of agglomerates delivered to cells, and measured the agglomerates media density. Afterwards particle number, agglomerate density, and fractal dimension was calculated. The methods selected for agglomerate dosimetry are an absolute methodology for in vitro particle dosimetry that includes the particokinetics in an in vitro system and allows precise interpretation and reporting of delivered to cell dose metrics. The currently developed Harvard Volumetric Centrifugation Method (VCM) is a straightforward, convenient approach for experimentally investigating the effective density of agglomerates under the circumstances of study for in vitro systems. The precision of the VCM has been ratified by correlation to entrenched, great precision, but more expensive and time engrossing methods. Straight forward mathematical equations acknowledged as Relevant In-Vitro Dose (RIDf) functions that serve as the rate of distribution of each particle given experimental conditions as a function of time are predicated on a prearranged methodology and are accessible for use by toxicologists. The reported RIDf functions can be used by in vitro nanotoxicologists to accurately calculate the particle mass (RIDm), particle surface area (RIDsa), or particle number (RIDN) delivered to cells as a function of time (24).

Mandelbrot initiated the term fractal dimension and fractal in 1975, a decade after he published his paper; “Self-similarity in the coastline of Britain” (3). The substructure of fractal dimension commence in Mandelbrot’s publications about undifferentiable, illimitable self-homogeneous functions which are consequently in the mathematical definition of fractals, around the time that calculus was discovered in the mid-1600s. A good deal of downtime set in for these functions and regeneration appeared in the late 1800s with the publishing of mathematical functions and sets known as canonical fractals (11). These publications were consorted by probably the most momentous point in the evolution of the perception of a fractal
dimension, through the work of Hausdorff in the early 1900s who defined a fractal dimension that has come to be named after him and is frequently invoked in defining modern fractals (10). Its fame reposes in the promise of a profound cognizance of intricate, tumultuous and deranged systems, which have resisted conventional geometrical endeavors to model them.

The first experiments that explicitly investigated the fractal nature of particle aggregates were reported in 1979 by Forrest and Witten (18). They installed Ž. metallic oxide smoke particles onto transmission electron microscope TEM substrates and the aggregate mass fractal dimension was resolute utilizing image analysis techniques. In a consecutive study, Schaefer evaded the innate circumscriptions of the sample preparation mandatory for microscopy methods.

For Instance in earlier studies transmission electron microscopy (TEM) images of agglomerates and aggregates were used to estimate the aggregate fractal dimension (18). Nonetheless the flat sample groundwork for TEM narrowed the in situ investigation of the fractal dimension for agglomerates/aggregates in the three-dimensional space. Schaefer integrated light and x-ray scattering methods; he imported the utilization of X-Ray and light to figure out the fractal dimension of agglomerates/aggregates in situ. Since then these techniques have been used extensively to measure the fractal dimensions of a wide range of colloidal particles (48).

The significance of A549 Lung cells and Gold Nanoparticles

There is an expanding fixate on the safety of the broad array of engineered nanoparticles utilized in these applications, concretely with apprehensiveness for inhalation as a passage of probable occupational exposure in humans. The large diversity of nanomaterials emerging for a
growing range of applications has led to cellular-based (in vitro) assays play increasingly important roles in evaluating their potential hazards and for prioritization of more costly (in vivo) toxicity testing (72). The in-vitro model used is based on the human permanent lung cell line A549, because the alveolar epithelium is presumably the area of the respiratory tract that is most exposed to fine particles (61). The human lung cell line A549 is frequently used as an in vitro model for studying lung toxicity and genotoxicity of environmental mutagens and carcinogens (76). In particular A549 cells maintain many morphological and biochemical characteristics of pneumocytes type II (80). In this instance, we employed the adenocarcinomic human alveolar basal epithelial cell line (A549 cells), in nature these cells are squamous and responsible for the diffusion of some substances, such as water, electrolytes and possibly even nanoparticles across the alveoli of the lungs (2).

Toxicity of disparate sorts of nano-materials in which humans are exposed to have been progressively reported which has induced nano-toxicology studies. Typical nanoparticles that have been studied are titanium dioxide, alumina, zinc oxide, carbon black, and carbon nanotubes, and "nano-C60 (19).

It is very important to recognize that not all nanoparticles are toxic; toxicity depends on at least chemical composition and shape in addition to simply size and particle ageing (19). Many scientific reports have shown however that gold has non-toxic properties and has therefore been used in several biomedical applications. The question of nanoparticle toxicity, however, is not as simple as examining individual particles only (6). After agglomeration gold’s non-toxicity properties remain unchanged, however due to the change in their properties, difference in morphology, uptake, and inflammation are expected. In this sense, the non-toxic profile of Gold nanomaterial’s has allowed us to use this material as a standard and focus primarily on the pro-inflammatory effects of agglomeration and correlate it to the fractal dimension.
Research Objective

The primary objective of our study is to characterize agglomeration, determine fractal dimension and correlate these properties to nano-bioeffects. The specific aims in our study were to synthesize gold nanoparticles as reference materials, perform characterization and analyze fractal dimensions and agglomeration. After analyzing fractal dimensions and agglomeration expose gold agglomerates to cells to determine bio-effects such as viability and morphology and finally analyze cellular uptake based on agglomeration. Characterization of agglomeration includes determining the agglomeration pattern between different sized nanoparticles, confirming the agglomeration maximum after a certain time point of exposure to biological media and confirming the stabilization properties of fetal bovine serum. To our knowledge this is the first study that correlated fractal dimensional values to agglomeration pattern and furthermore cellular morphology and uptake.

Approach to Research Objective

Our approach to achieve the research objective is as follows; synthesize various sizes of gold nanoparticles under the hydrothermal method using the turkevich application where sodium citrate is applied for capping and stabilizing the primary particles. Agglomerate nanoparticles by dispersing them in biological media without fetal bovine serum, then in media with fetal bovine serum to stabilize the agglomerates at their reached size and dimensions. Characterize our nanoparticles for confidence in size, shape and structure. Characterize our agglomerates to evaluate how well salts in media agglomerated primary particles, confirm fetal bovine serum stability, and observe size of agglomerates produced from smaller primary particles and larger primary particles. Then ultimately expose gold agglomerates to A549 lung cells to measure
Hypotheses

Numerous studies have postulated nanoscale particles (<100nm diameter) have inherently increased potential for toxicity compared with larger diameter particulates, due to their increased surface to mass ratio and greater proportion of surface available chemically reactive groups (72). In this instance since gold nanoparticles are known to have non-toxic properties it is hypothesized that the smaller gold agglomerates will have a non-significant decreased cell viability.

Particles settle, diffuse and agglomerate at rates that differ in relation to their size, density, and surface physicochemistry (35). According to (figure 6) smaller particles have a higher diffusion rate while larger particles have a higher sedimentation. In this instance we can hypothesize that the smaller particles will diffuse and create larger agglomerates when compared to the larger nanoparticles. Alexandra Noel found that smaller primary TiO2 primary particles produced larger agglomerates than the larger primary particles and suggested that as particle size decreases, the attractive force per unit mass increases, which favors agglomeration (54). In this occurrence the more minute particles are considered to favor a diffusion limited agglomeration while the more bulky nanoparticles will cause a reaction limited agglomeration. An expeditious diffusion limited aggregation (DLA) is betokened by a more diminutive fractal dimension, which yields open, loosely packed agglomerate structures. Since particle repulsion is diminutive, particles cohere instantly upon contact. More gradual aggregation, characterized by reaction-
limited aggregation (RLA), where particle–particle repulsion has a more preponderant effect, is more densely packed. This is because the particles can perforate further into agglomerate structures afore being captured. The RLA mechanism also yields a larger fractal dimension number (54). With these known speculations, it is suggested that the smaller particles will produce smaller fractal dimensions as well as loosely packed agglomerates, while larger particles will produce more densely packed agglomerates with larger fractal dimensions.

It is our suggestion that after exposing primary particles to medium without fetal bovine serum after a longer period of time will cause a possible maximum agglomeration by becoming increasingly associated with the salts in the media which will tend to decrease the mobility of individual nanoparticles (37).

As previously stated it is hypothesized that the smaller primary particles will induce smaller fractal dimensions, an increased agglomeration effect while behaving in diffusion limited agglomeration manner and more loosely packed agglomerates when compared to the larger primary particles. It also has been mentioned that smaller particles has caused greater toxicity effects by the increased uptake in certain barriers and even possible agglomeration after the barriers have been crossed (72). Therefore, it is hypothesized that the gold nanoparticles, which are known to have non-toxic effects will consist that the smaller particles will create larger agglomerates but even then the larger agglomerates from the smaller primary particles are prone to produce decreased non-significant toxicity effects, and greater uptake when compared to larger primary particles. In this instance a decreased fractal dimension produced is hypothesized to be correlated with greater cellular uptake while a greater fractal dimension may have a decreased cellular uptake.
Figure 5: Transportation rate with particle size

Note. Particle and Fibre Toxicology. Reprinted from “ISDD: A computational model by Hinderliter PM, Part Fibre Toxicol 2010” 7:36. Copyright 2010 Hinderliter et al; licensee BioMed Central Ltd. Open Access, Adapted with permission
Brief Findings

Our research confirms our hypothesis that the smaller primary particles induced greater sized agglomerates when compared to the larger primary sized particles. The smaller primary particles induced greater sized agglomerates with lower fractal dimensions, but with a greater cellular uptake. The agglomerates produced from the larger primary particles which increased in sedimentation and fractal dimension caused more seen differences in cellular morphology compared to control and the smaller primary particles.
Materials and Methods

Gold Nanoparticle Synthesis

13nm Gold Nanoparticles

Synthesis of 13nm, 16nm and 36nm gold nanoparticles (GNPs) was performed using an adapted procedure of the methods cited. {60, 44}

Solutions were prepared by dissolving 150.2mg trisodium citrate dihydrate (Na$_3$C$_6$H$_5$O$_7$·2H$_2$O, MW=294.10 g/mol, Fisher Biotech) in 15ml ddH$_2$O (3.4 × 10$^{-2}$ M) and 33.97mg gold (III) chloride trihydrate (HAuCl$_4$·3H$_2$O, MW=393.83 g/mol, Sigma Aldrich) in 100ml ddH$_2$O (8.62 ×10$^{-4}$M). The 8.62 × 10$^{-4}$ M HAuCl$_4$ solution was brought to a boil. The solution was stirred and 7.69 ml of the 3.4 × 10$^{-2}$ M sodium citrate solution was rapidly added, resulting in an immediate color change from pale yellow to purple. The GNP solution was allowed to stir and boil for 10 minutes, where a color change from purple to light red was observed in approximately 2 mins. The solution was removed from the heat and allowed to stir at room temperature for 15 minutes. Subsequently, 5 ml of 3.4 × 10$^{-2}$ M sodium citrate solution was added and the solution was allowed to stir until cooled to room temperature. Following cool down, the GNP solution was filtered through a 0.22 um filter and stored in two 50 ml conical tubes at 4-6.3° C

16nm Gold Nanoparticles
Solutions were prepared by dissolving 40mg trisodium citrate dihydrate (Na$_3$C$_6$H$_5$O$_7$·2H$_2$O, MW=294.10 g/mol, Fisher Biotech) in 4ml ddH$_2$O (0.034M) and 25mg gold (III) chloride trihydrate (HAuCl$_4$·3H$_2$O, MW=393.83 g/mol, Sigma Aldrich) in 5ml ddH$_2$O (0.013M). 1ml of the 0.013M HAuCl$_4$ solution was added to 49mL ddH$_2$O for a final concentration of 0.0013M. The solution was brought to a rapid boil and stirred for 2 minutes. Subsequently 0.871 mL of 0.034M sodium citrate was added resulting in an immediate color change from pale yellow to light red. The solution was allowed to stir and boil for an additional 5 min. The GNP solution was removed from heat and allowed to stir until room temperature was reached. After cool down, the solution was filtered through a 0.22 um filter and stored into a 50ml conical tube for storage at 4-6.3°C.

36nm Gold Nanoparticles

Solutions were prepared by dissolving 40mg trisodium citrate dihydrate (Na$_3$C$_6$H$_5$O$_7$·2H$_2$O, MW=294.10 g/mol, Fisher Biotech) in 4ml ddH$_2$O (0.034M) and 25mg gold (III) chloride trihydrate (HAuCl$_4$·3H$_2$O, MW=393.83 g/mol, Sigma Aldrich) in 5ml ddH$_2$O (0.013M). 1ml of the (0.013M) HAuCl$_4$ solution was added to 49ml ddH$_2$O for a final concentration of 0.0013M. The solution was brought to a rapid boil and stirred for 2 minutes. Subsequently, 0.284 ml of 0.034M sodium citrate was added resulting in an immediate color change from pale yellow to light red. The solution was allowed to stir and boil for an additional 5 min. The GNP solution was removed from heat and allowed to stir until room temperature was reached. After cool down, the solution was filtered through a 0.22 um filter and stored in a 50ml conical tube at 4-6°C.

Concentration of Gold Nanoparticles

The resulting concentration of the gold nanoparticle suspensions was 40 µg/ml. Two milliliters of each size gold nanoparticle suspension were placed in 2ml Eppendorf tubes. The
suspensions were spun down at 4,000 RCF for 8 mins. The supernatant was then decanted in a 2mL Eppendorf tube, leaving the pellet at the bottom. 1mL of ddH₂O was added to pellet. The decanted supernatant was spun down again at 4,000 RCF for 8 min. After a second pellet was retrieved, the supernatant was decanted in another 2mL Eppendorf tube. 1mL of ddH₂O was added to the pellet. Both 1mL GNP pellets in ddH₂O were mixed to create two mL 40 μg/mL GNP suspensions. Concentrations were reconfirmed via Inductively Coupled Mass spectrometry (ICP-MS).

Agglomeration of gold nanoparticles

The methods describe here is an adaptation of the method cited. {85}

Nanoparticle agglomerates were prepared with the following procedure depicted in figure 6a. The NP solution was pipetted into RPMI 1640 medium without fetal Bovine serum (FBS) while vortexing to produce larger agglomerates. In this scenario, the salts in media create a high ionic strength and causes the NPs to agglomerate instantly, and they are allowed to agglomerate for a defined period of time (at 5, 10 and 30 seconds specifically in this work). After the nanoparticles agglomerated at the specific time frames, fetal bovine serum (FBS) is added to coat the NP agglomerates and produce a stable solution of NPs for studying the dependence of cellular uptake and morphology exclusively on agglomeration state. Specifically as described in figure 6b, to obtain the ‘0 s’ agglomerates 0.375ml RPMI and 0.375ml RPMI + 20% FBS were added to a 15ml conical tube. Next 0.25ml NP solution was added with a pipette to this solution while the vortexer was at high speed and it was vortexed for about 30 s after adding the NPs. To obtain the various time points mentioned above 0.375ml RPMI was added to a 15ml conical tube. A timer was set for the needed time point. 0.375ml RPMI +20% FBS was pre-gathered. 0.25ml NP solution was added in a tube while vortexing and the timer was instantly set. After
the timer went off RPMI media with + 20% FBS was immediately added. A control was prepared by mixing 0.375ml RPMI, 0.25ml ddH$_2$O and 0.375ml RPMI + 20% FBS.

**Figure 6b:** A schematic for the preparation of the 0 second agglomerates

**Figure 6a:** A schematic for the preparation of the agglomerates
Characterization

Dynamic Light Scattering

Dynamic light scattering for characterization of primary and agglomerate size were performed on a Malvern Instruments Zetasizer Nano-ZS instrument. As mentioned in the index DLS analyzes the velocity distribution of particle movement by measuring dynamic fluctuations of light scattering intensity caused by the Brownian motion of the particle (50). This technique yields a hydrodynamic radius, or diameter, to be calculated via the Stokes-Einstein equation from the aforementioned measurements. The Malvern Zetasizer Nano-ZS uses the Dispersion Technology Software (V4.20) for data collection and analysis. The mean particle diameter is calculated by the software from the particle distributions measured and the polydispersity index (PDI) given is a measure of the size ranges present in the solution. (Malvern, 2005). The PDI scale ranges from 0 to 1, with 0 being monodisperse and 1 being polydisperse. The software calculates PDI value from the G1 correlation function and from parameters defined in the ISO document 13321:1996 E. All samples were placed in disposable cuvettes and placed in instrument for readings.

UV-Visible Absorption Spectroscopy

The absorption spectra of the NMs were collected on the Cary UV-Vis 5000 Spectrophotometer with WinUV software (Varian, Inc.). As mentioned in the index UV-Vis spectroscopy uses one or multiple light sources that can emit from the ultraviolet, through the visible, and sometimes into the near-infrared spectrums. The light is passed through a sample and the transmitted light is
recorded by a detector. Any absorption of light by the sample is measured allowing identification of any specific wavelengths of absorbance which may be unique to that sample.

Samples were measured at the concentration mentioned in concentration methods. This allowed the solution concentration to fall within the instrument’s calibrated absorption measurement range. For spectrum scans, solutions were placed in quartz cuvettes for measurements, the chamber temperature was set at 25 °C, and the instrument was set to scan wavelengths from 200nm to 800nm.

Transmission Electron Microscopy (TEM)

Transmission Electron Microscopy (TEM) characterization was performed to obtain the primary particle sizes and morphology of NPs using a Hitachi H-7600 tungsten-tip instrument at an accelerating voltage of 120kV in high resolution mode. As mentioned in the experimental instrumentation section a TEM uses a high energy electron beam, produced by a filament and focused onto a sample by electromagnetic lenses, to visualize sample features down to tenths of nanometers. Depending on the beam energy, the electrons can penetrate samples below 100nm in thickness, allowing visualization of the atomic structure for certain samples and crystal structure analysis using diffraction mode settings.

Primary NPs were examined after placing 5 µL onto 200 mesh formvar/carbon coated copper TEM grids which were then allowed to dry. In addition to observing general morphology of the NPs, the mean particle size and standard deviation for each sample was calculated from measuring over 50 NPs in random fields of view using Image J software. The procedure was previously described by Murdock et al. (2008).
Confirmation of Fetal Bovine Serum (UV-Vis, Student’s T Distribution)

Samples were incubated for 24 hours in a Forma series II water jacketed CO2 incubator HEP A class at 37 °C in 5% CO2 afterwards DLS and UV-Vis measurements were obtained. The absorption spectra after 24 hours confirm whether the properties of the particles have altered when compared to initial agglomerates. DLS measurements confirmed the stability of the hydrodynamic radius and confidence levels were calculated using the student’s t-distribution.

Cellular Viability by Alamar Blue Assay

Cells were seeded in a 96-well plate and incubated for 24 hours. After 24 hours and essential characterization of agglomerates with DLS and UV-Vis the cells were washed with 1x PBS and dosed with 0, 5, 10 and 30 s agglomerates that were prepared the same day and 0, 5, 10 and 30 s agglomerates that were incubated after 24 hours. Cells were also dosed with a control, with RPMI media, RPMI media + 20% FBS and ddH2O. Cells were then incubated for 24 hours. Cell viability was assessed using the Alamar Blue Assay. For positive controls the medium was replaced and 20ul of 0.1% triton x was added to positive control wells and sat for approximately 2 minutes. Briefly the medium with agglomerate solutions and negative control wells was replaced with 100ul RPMI cell culture medium containing 10ul reagent and was placed in positive control wells also and was allowed to incubate for 1 hr. Following incubation a microplate reader (BioTec Synergy HT Spectrophotometer plate reader) was used to determine the absorbance of the dye. Absorbance was monitored at 570nm, using 600nm as a reference wavelength. The absorbance intensity from agglomerate and triton x treated cells was compared with those from untreated control cells to determine percent viability.
Cellular Morphology and Nanoparticle Distinction

Hyperspectral Imaging (HSI) was performed on certain samples as a potential methodology for detecting agglomeration state in a biological system and the morphology of the A549 cells after dosing. HSI collects a spectra similar to that of UV-Vis, except that the spectra obtained is for reflected light from the sample instead of the absorbed spectra as in UV-Vis and can provide visual change in distinction after particles and other substance are introduced to a biological system. Cells were seeded on 2 chambered slides at 20,000 cells/ml for 24 hours. After 24 hours the cells were dosed with 2.6 mls of 40 ug/ml agglomerates and control, and were incubated for 24 hours. The following day the cells were washed with pre-warmed PBS, fixed with 4% paraformaldehyde for 10 minutes and the slides was allowed to air dry. A few drops of thawed Prolong Gold reagent with DAPI counterstain were applied and the slide was covered with a coverslip and sealed with clear nail polish around the edges. The slides were permitted to sit overnight in the dark at 2-6°C to be scanned properly on the CytoViva Hyperspectral Imaging System (HSI). Scan information was collected and processed through ENVI software.

Quantitative Association of Cellular Uptake (ICP-MS)

5ml of A549 cells were seeded in 6-well plates at approximately 20,000 cells/ml in growth media for 24 hours. Afterwards the growth media was aspirated and dosed with 2-3mls of 40ug/ml agglomerates and control. The following day samples were washed with pre-warmed DPBS modified 1x Phosphate buffered saline (PBS), trypsinized with 0.25% trypsin, neutralized with growth media and counted with cellometer vision (Nexcelcom Bioscience). The samples were then centrifuged at 900g for 5 min and the supernatant was removed and resuspended in ddH₂O. The cells were frozen at -20°C overnight. The following day the samples were thawed and 500µl of 1% Triton-X 100 (Fisher Scientific, Pittsburg, PA) was added and the samples were
vortexed. 144µl of aqua regia (1:5.5 v/v ratio of 69% Nitric acid [HNO3, Sigma-Aldrich, St.Louis, MO] to 810µl 37% Hydrochloric acid [HCL, Fisher Scientific, Pttsburg,PA] was added to the samples. After adding 20µl of internal standard mix (Perkin Elmer, Waltham,MA), the volume was brought up to 10 mL using deionized water creating final concentrations of 0.05% Triton X-100, 3% HCL, 1% HNO3, 20 µg/L of internal standard mix. ICP-MS (Perkin Elmer Nexion 300D, Waltham, MA) was used to determine the contents of Au agglomerates in the samples prepared above. Calibration plots of standards of Au were obtained by injecting a series of standard solutions (2, 50,100,300 ppb, in 1% HNO3 and 3% HCl) with flow rate of 1.0ml/min. Three samples of each agglomerate were measured with three trial runs respectively.

Qualitative Measurement of Cellular Uptake (TEM)

Transmission Electron Microscopy (TEM) characterization was performed to obtain the, morphology of agglomerates and cellular morphology using the same previous instrument mentioned in characterization section. To further view agglomerates in cellular environment the A549 were fixed. After seeding cells in a 6-well plate and incubated for 24 hours, the cells were dosed with Au agglomerates and incubated for additional 24 hours. The following day the cells were washed with pre-warmed 1x PBS and trypsinized. Growth media was added and transferred into a 15ml conical tube. The samples were then spun down for 10 mins at 900g or until a pellet was seen. The supernatant was removed and the cells were fixed in 2% paraformaldehyde for 2hrs at room temperature. The paraformaldehyde was removed and the cells were washed with 1x PBS 3x for 30 min respectively. The cells were then stained in 1% osmium tetroxide (OsO4) /99% PBS for an hour. After the removal of OsO4 the cells were neutralized in corn oil and washed with 1x PBS. Following neutralization and washing the cells were dehydrated with ethanol (EtOH) in the following manner: 300µl of 50% EtOH/50% H2O for 10 min, 300 µl of 70% EtOH/30% H2O 3x for 10 min, 300µl of 80% EtOH/20% H2O for 10min,
300µl of 90% EtOH/10% H2O for 10min and finally 300µl of 100% EtOH 3X for 10 mins. The samples were then switched to a beam capsule and 300µl of 1:1 dilution of 100% EtOH and resin was placed and sat in samples for an hour. The capsule was then filled with LR white resin (Electron Microscopy Sciences, Hatfield, PA) for curing and placed in a vacuum oven on at 60ºc and 15psi. The resin embedded pellet was then sectioned using a microtome and imaged by TEM.

Calculation of Fractal Dimension, Densities and particle number

A549 cells were seeded in a 96 well plate at approximately 20,000 cells/ml in growth media for 24 hours. Agglomerated gold ultrafine particles at the indicated time points and media density ($P_{\text{media}}$) was calculated from the mass of a 1 ml sample by subtracting the weight of a 50 ml volumetric flask and 15ml conical tube from, the weight of the same flask and conical tube containing 1 ml nanoparticle suspension. The agglomerates were then exposed to cells for 24 hours. After exposure time the one ml agglomerates were dispensed into TPP packed cell volume (PCV) tubes (Techno Plastic Products, Trasadingen, Switzerland) and centrifuged at 2,000×g for one hour. Agglomerate pellet volumes, $V_{\text{pellet}}$, were measured with the measuring scales on tubes. Effective agglomerate densities were calculated from $V_{\text{pellet}}$ values of triplicate samples of each Au agglomerate. Delivered to cell dose metrics were then calculated based off the order of calculations followed in “An Integrated Approach for the in Vitro Dosimetry of Engineered Nanomaterials,” Joel M Cohen. The order of calculations is as follows: Mass concentration $M = \gamma \times V$ where $\gamma$ is the known mass concentration and $V$ is the volume of the exposure media (ml) applied directly to the cells in culture. Effective agglomerate densities $pEV = p_{\text{media}} + \left( \frac{M_{\text{ENM}}}{V_{\text{pellet}}SF} \right) \left( 1 - \frac{p_{\text{media}}}{p_{\text{ENM}}} \right) p_{\text{media}}$ or media density was measured as mentioned previously, M ENM is the standard atomic weight of the AU material and converted
into grams using the atomic mass unit of $1.66 \times 10^{-24}$ g, $V_{\text{pellet}}$ is the volume of the pellet after centrifugation, SF or stacking factor is a theoretical constant of 0.634 and $\rho ENM$ stands as the standard density of the material at room temperature. The total particle number dose

$$N = \frac{M}{(\frac{4}{3}\pi r^3 H) \times p_{\text{EV}}}$$

where M is the mass concentration, Hydrodynamic radius, $r_H$ (obtained from DLS and converted into cm), and pEV effective agglomeration densities calculated. The estimated $f(t)$ function was then fitted as a Gompertz sigmoidal function as described by the authors {23}

$$f_D(t) = 1 - e^{-\alpha t}$$

where $\alpha$ (hrs$^{-1}$) is the material media specific deposition constant, and t (hr) is exposure duration. Using the estimated $f(t)$ function, particle number delivered to cells can be figured

$$R_{\text{IDn}} = (1 - e^{-\alpha t}) \times N$$

with N as the total particle number dose. The hypothetical stacking factor (SF) of 0.634 is approximated on stacking of sporadic spheres. The figure of the SF confide in the competence of agglomerates stacking. In the event of homogenous spheres, viable units for SF may measure from 0.634 for irregular tight stacking, to the hypothetical maximum of 0.74 for arranged stacking. We foresee SF units to proximate the hypothetical unit for unarranged tight stacking (0.634), while for non-agglomerating spherical ENMs we foresee SF to aim towards the hypothetical unit for arranged stacking (0.74). The theoretical SF values can be verified from the sedimentation coefficients measured (27).

The number of individual particle in each agglomerate, the hydrodynamic radius via DLS, and the size of the primary particle via TEM were gathered and placed in the theoretical equation

$$R_{\text{IDn}} = \left(\frac{R_{h,N}}{r_p}\right)^{DF}$$

with the tenth root of $R_{\text{IDn}}$ as the number of primary particles in each agglomerate, Rh,N as the hydrodynamic radius, $r_p$ as the primary particle size and DF as the unknown fractal dimension. The 10th root of The $R_{\text{IDn}}$ was then figured for a real number amount of particles in agglomerate. The theoretical equation

$$N = \left(\frac{R_{h,N}}{r_p}\right)^{DF}$$

was assessed from the following research article: “Validity Range of Centrifuges for the Regulation of
Nanomaterials from Classification to AS-Tested Coronas” “Wendel Wohlleben” J Nanopart Res 2012. This particular theoretical equation was chosen as it best fits the data that was extracted with our particular methodology and is based on the theory that primary particles agglomerate to form fractal structure where the number of primary particles is directly related to the diameter of the conglomerate (33).
Results:

Characterization of Primary Particles

Ultra-Violet Visible Spectroscopy:

Figure 7: UV-VIS peaks of primary Au particles. (a) 13nm (b) 16nm and 36nm
The UV-VIS peaks show that the 13nm particles had a sharp peak and a high absorbance of light. The 36nm particles had a slight broader peak when compared to the smaller particles and had an absorbance of 1.3. The 16nm had a sharp peak and the lowest absorbance of light at approximately 0.95. The larger primary particles demonstrated a slight right shift in wavelength when compared to the smaller particles.

**Dynamic Light Scattering**

The Hydrodynamic radius given from DLS of each individual primary particle are indicated in the table below. The hydrodynamic radius provided in the DLS data is 1.3-2.3 times larger than the diameter. The polydispersity index which is a measurement of the distribution of molecular mass, shape or size in a sample of nanoparticles is less than 1 indicating that the primary particles are uniform or monodispersed.

<table>
<thead>
<tr>
<th>Au Particle Size</th>
<th>Z Ave (NM)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>13nm</td>
<td>22.86 ± 0.57</td>
<td>0.26±0.003</td>
</tr>
<tr>
<td>16nm</td>
<td>38.29± 0.68</td>
<td>0.40±0.008</td>
</tr>
<tr>
<td>36nm</td>
<td>46.64± 0.64</td>
<td>0.40±0.024</td>
</tr>
</tbody>
</table>
Transmission Electron Microscopy:

The transmission electron microscopy Images, show that the different size particles were all uniformed throughout, and mostly spherical in shape. All particles remained single primary particles which confirmed the stabilization of the sodium citrate coating. The morphological properties of the primary particles are indicated in the TEM pictures below. The sizes of the primary particles were measured with image J application and are indicated in the table below.

**Figure 8:** Representative Transmission Electron Microscopy Images of synthesized Gold Nanoparticles. All Particles were spherical throughout

<table>
<thead>
<tr>
<th>Size (nm)</th>
<th>TEM Image</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td><img src="16nm.png" alt="Image" /></td>
</tr>
<tr>
<td>36</td>
<td><img src="36nm.png" alt="Image" /></td>
</tr>
<tr>
<td>13</td>
<td><img src="13nm.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Table 2:** Measurement of single primary particles with image J application via TEM
<table>
<thead>
<tr>
<th>Au Particle Size</th>
<th>Size measured</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>13nm</td>
<td>11.95nm</td>
<td>±2.86</td>
</tr>
<tr>
<td>16nm</td>
<td>23.91nm</td>
<td>±4.32</td>
</tr>
<tr>
<td>36nm</td>
<td>38.89nm</td>
<td>±6.46</td>
</tr>
</tbody>
</table>

Characterization of agglomerates

I. **Ultra-Violet Visible Spectroscopy**

After agglomeration the particles displayed a significant decrease in the absorbance of light and left shift in wavelength. The peaks of the particles are much broader which displays diversity in the size range of the agglomerates. There is no seen differences in the absorbance of light amongst the agglomerates with different starting primary sizes as most of the peaks below are significantly close in range.
Dynamic Light Scattering

From the DLS data it shows that the particles agglomerated immediately with an increase in the Z average at 0 seconds. The 13nm particles increased in Z average slightly at 0 sec and then showed a significant increase in radius as the time of exposure increased. The 16nm particles displayed a rapid increase in the hydrodynamic radius (HDR) approximately doubling in measurement at 0 sec and significantly increased in HDR as time increased, but displays a maximum agglomeration potential at 30 seconds. The larger 36nm particles didn’t immediately agglomerate at 0 seconds. After 5 sec the larger particles displayed a slight increase in HDR and then an onward increase with the time of exposure to RPMI media without fetal bovine serum. From this data it shows that the smaller particles exhibited a DLCA agglomeration behavior while the larger particles exhibited an RLCA behavior. The 13nm and 36nm displayed a similar

**Figure 9:** Ultra-violet visible spectroscopy peaks of agglomerates
HDR at 5 and 10 sec. Since fractal dimension is based on complexity in relation to density and size comparing the 13nm and 36nm at these time points were selected for further experimentation with fractal dimension and it’s relation to cellular uptake and morphology.

### Transmission Electron Microscopy

The representative transmission electron microscopy images below displays that the different agglomerates qualitatively represented the DLS data. The smaller particles produced agglomerates with a greater population of individual particles which exemplifies the faster agglomeration rate when compared to the larger particles. The larger particles produced agglomerates with a decreased population of individual particles but had a similar size in agglomeration when compared to the smaller particles. The greater dimension of the larger particles could account for the production of similarity in size when compared to the smaller particles.

![Table 3: Dynamic Light Scattering of agglomerated Au nanoparticles in fresh media at various serum free time points. All particles displayed a gradual increase in agglomeration. The 16nm particles however displayed a max agglomeration at 30 seconds. The standard deviations show there is a maintained difference in agglomerate size rather than a non-maintained difference.](image)
primary particles, even though there wasn’t as much particles agglomerating. There is an increase in the polydispersity of the particles when compared to the primary particles which confirms the DLS data of the agglomerates above in Table 3.

A) 13nm 5 seconds, DLS: $69.69 \pm 5.03$

B) 13nm 10 seconds, DLS: $89.93 \pm 6.23$

C) 36nm 5 seconds DLS: $61.46 \pm 0.42$

D) 36nm 10 seconds, DLS: $85.73 \pm 5.12$
Confirmation of Fetal Bovine Stabilization

There are many research articles that observed the stabilization of FBS on agglomerating nanoparticles. Our stabilization testing of the Fetal Bovine Serum was to confirm these existing observations. After 24 hours UV-Vis measurements were obtained to confirm the agglomerates wavelength of light in relation to its absorbance. The agglomerates wavelength was stabilized in the ranges of approximately 560-575nm. The absorbance of light increased slightly in the ranges of 0.2-0.35abs compared to 0.15-0.28, but not significantly.

Figure 10: TEM images of agglomerates exposed to cells, A: 13nm 5 sec B: 13nm 10 sec C: 36nm 5 sec D: 36nm 10 sec

Figure 11: UV-Vis peaks of agglomerates after 24 hours

Agglomeration after 24 hours with RPMI 1640
DLS measurements were obtained after 24 hours to confirm the stability of the HDR and afterwards student t distribution calculations were performed to measure the confidence levels of the FBS stabilization within 15nm. In the Table it appears that the overall agglomeration pattern remains stable, however the 16nm didn’t show a maximum agglomeration as it had initially. Using the Students T-Distribution test it was proved that the fetal bovine serum stabilized the 13nm and 16nm gold particles at a 90% confidence level and stabilized the 36nm gold nanoparticles at a 97.5% confidence level.

<table>
<thead>
<tr>
<th>Time</th>
<th>Z Ave (NM)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 seconds</td>
<td>45.44 ± 1.25</td>
<td>0.57 ± 0.04</td>
</tr>
<tr>
<td>5 seconds</td>
<td>65.47 ± 0.33</td>
<td>0.47 ± 0.003</td>
</tr>
<tr>
<td>10 seconds</td>
<td>84.67 ± 0.46</td>
<td>0.47 ± 0.007</td>
</tr>
<tr>
<td>30 seconds</td>
<td>122.6 ± 1.05</td>
<td>0.52 ± 0.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time</th>
<th>Z Ave (NM)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 seconds</td>
<td>71.55 ± 1.82</td>
<td>0.51 ± 0.03</td>
</tr>
<tr>
<td>5 seconds</td>
<td>82.50 ± 0.70</td>
<td>0.56 ± 0.002</td>
</tr>
<tr>
<td>10 seconds</td>
<td>91.85 ± 1.14</td>
<td>0.32 ± 0.002</td>
</tr>
<tr>
<td>30 seconds</td>
<td>104.3 ± 1.35</td>
<td>0.33 ± 0.006</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time</th>
<th>Z Ave (NM)</th>
<th>PD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 seconds</td>
<td>36.56 ± 0.41</td>
<td>0.51 ± 0.002</td>
</tr>
<tr>
<td>5 seconds</td>
<td>49.70 ± 0.38</td>
<td>0.50 ± 0.002</td>
</tr>
<tr>
<td>10 seconds</td>
<td>54.59 ± 1.06</td>
<td>0.56 ± 0.007</td>
</tr>
<tr>
<td>30 seconds</td>
<td>108.67 ± 3.06</td>
<td>0.60 ± 0.04</td>
</tr>
</tbody>
</table>

Table 4: Dynamic light scattering measurements of agglomerates after 24 hours and confidence intervals
Cellular Viability

Cellular Viability measured with alamar blue, overall the results display that the Au agglomerates didn’t significantly affect the viability. This presents that even after agglomeration Au nanoparticles still contains non-toxic properties. The 13nm 0 sec agglomerate however after being incubated for 24 hours then exposed to the cells for an additional 24 hours has a slight decrease in viability but not significant enough to account for toxicity concerns. This however supports the statement that smaller ultrafine particles are more adhesive and usually causes more damage as they are able to cross barriers larger particles are unable to.
Figure 12: Cellular viability graph
Cellular Uptake Association with Inductively Coupled Mass Spectrometry

The smaller nanoparticles when agglomerated have a higher PG/cell when compared to the larger primary particles. The 13nm particles when agglomerated after 30 seconds has a surprisingly increase in uptake and also has the greatest HDR in relation to the rest of the agglomerates. The Larger primary particles when agglomerated has a significantly lower uptake rate and has an overall slightly smaller agglomerate size in relation to the smaller primary particles. The smaller particles capability of greater uptake even though their agglomerate size is larger could account towards their loser packing and minor complexity.

Figure 13: Nanoparticle Deposition graph
Nanoparticle Distinction with Hyperspectral Imaging

The agglomerates of the 13nm primary particles exposed to cells has a short ranged minor peak whereas the agglomerates of the 13nm primary particles that aren’t exposed to cells has only a major peak indicating that when the particles were exposed to the cells the polydispersity index increased, meaning there was a change in mass, shape and size of particles. The agglomerates of the 13nm primary particles exposed to cells displayed a 10 degree right shift compared to agglomerates that weren’t exposed to cells. This possibly indicates that there was a change of the morphology of the agglomerates which possibly could result in differences of fractal dimension between agglomerates outside of the cellular environment and those that are inside the cellular matrices. The larger primary particles consist of a greater polydispersity index which

<table>
<thead>
<tr>
<th>Au Particle and Agglomeration time</th>
<th>Cellular uptake (PG/cell)</th>
<th>Z Ave (NM)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>13nm 0 second</td>
<td>1.96 ± 0.18</td>
<td>29.22 ± 8.90</td>
<td>0.59 ± 0.16</td>
</tr>
<tr>
<td>13nm 5 second</td>
<td>1.93 ± 0.12</td>
<td>69.69 ± 5.03</td>
<td>0.46 ± 0.01</td>
</tr>
<tr>
<td>13nm 10 second</td>
<td>1.84 ± 0.14</td>
<td>89.93 ± 6.23</td>
<td>0.41 ± 0.15</td>
</tr>
<tr>
<td>13nm 30 second</td>
<td>2.59 ± 0.02</td>
<td>124.48 ± 26.23</td>
<td>0.53 ± 0.004</td>
</tr>
<tr>
<td>36nm 0 second</td>
<td>1.52 ± 0.02</td>
<td>40.09 ± 11.02</td>
<td>0.54 ± 0.04</td>
</tr>
<tr>
<td>36nm 5 second</td>
<td>1.43 ± 0.03</td>
<td>61.46 ± 0.42</td>
<td>0.50 ± 0.04</td>
</tr>
<tr>
<td>36nm 10 second</td>
<td>1.33 ± 0.02</td>
<td>85.73 ± 5.12</td>
<td>0.54 ± 0.01</td>
</tr>
<tr>
<td>36nm 30 second</td>
<td>1.22 ± 0.01</td>
<td>106.83 ± 7.92</td>
<td>0.58 ± 0.01</td>
</tr>
</tbody>
</table>
also exists after agglomeration. The agglomerates display a slight right shift and sharp round peak indicating agglomeration whereas the non-agglomerates display a round narrow peak indicating stability. In the scenario where the agglomerates weren’t exposed to the A549 cell line the agglomerates of the larger primary particles resulted in a left shift when being referenced to the agglomerates of the smaller nanoparticles, this explains that the larger particles resulted in a reaction limited agglomeration pattern. The A549 cell line displays a significantly broad and round peak which represents a background biological environment.

**Figure 14:** The larger particles have a minor and major peak. The minor peak of the larger particles displays a left shift and the major peak has a slight right shift. The particles with lower wavelengths for each size has round narrow peaks while the major peak for the larger particles display a round broad peak. The A549 cells without particles have two major peaks that are significantly broad.
Figure 15: The 13nm 10 sec displays a right shift in wavelength with higher intensity compared to 13nm 5sec. The 36nm agglomerated particles continue to a major and minor peak when referenced to non-agglomerated particles. The 36nm 10 sec minor peak had a lower range in wavelength and a lower intensity and broader peaks compared to 36nm 5 sec. The 36nm agglomerates displays a left shift when compared to 13nm agglomerates.
Cellular morphology and Qualitative Cellular Uptake Association with Hyperspectral Imaging and Transmission Electron Microscopy.

Changes in cellular morphology of the Au agglomerate exposed cells were observed as compared to unexposed cells (86). In the absence of Nps the cells displayed a typical polygonal shape and intact morphology (Fig 18A). Whereas; in presence of NPs, changes in the cell morphology were observed (FIG 18: B, C, D, and E). The changes in the cellular morphology were more pronounced with increase from agglomerates produced from a greater primary particle size. There were slight morphological distinctions between the 13nm 5 sec agglomerates and the 13nm 10 sec agglomerates. It was seen that with the agglomerates produced from the larger primary particles the cellular wall became less intact, the proplastids increased in size and number and that the proportion of the cells with stress granules, and lysosomal vacuoles increased. The TEM images confirms the ICP-MS data that more particles of the agglomerates from the smaller particles were uptaken and the particle number calculations that quantified more particles in the agglomerates of the smaller primary particles. The TEM qualitatively confirms the HSI data that described an increase in polydispersity of the particles once introduced in the cellular environment. From the representative HSI images it appears that there were an increased number of cells in the presence of agglomerates created from smaller primary particles when compared to the agglomerates created from the larger primary particles.

**Figure 16:** The 13nm 10 sec agglomerates have a slight right shift in the minor wavelengths and also higher intensity when compared to 13nm 5 sec agglomerates. The 36nm 5 sec agglomerates display a higher intensity whereas the 36nm 10 sec agglomerates has a slight right shift in the major peak and a left shift in the minor peak. The 13nm agglomerates has a very slight right shift in the minor peaks and the 36nm agglomerates have an overall higher intensity.
18A: Control Cells without nanoparticles

18 B: Cells with 13nm 5 second agglomerates, DLS: 69.69nm
18 C: Cells with 13nm 10 second agglomerates, DLS: 89.93nm

18 D: Cells with 36nm 10 second agglomerates, DLS: 61.46nm
**18 E:** Cells with 36nm 5 second agglomerates, DLS: 85.73nm

**Figure 17 (A, B, C, D, and E):** TEM images of cellular morphology exposed to various agglomerates

**Figure 18:** The HSI representative images above appears to show that there are more cells with agglomerates from the smaller primary particles when compared to the agglomerates created from the larger primary particles.
Calculation of Fractal Dimension, Density and Dosimetry

The agglomerates from the smaller primary particles have an overall smaller fractal dimension which correlated with a higher cellular uptake and a decreased agglomerate density.

Agglomerates from the larger primary particles have an overall increase in fractal dimension and density which causes a decrease in cellular uptake. As mentioned above agglomerates from the larger primary particles with an increased fractal dimension and density caused more noticeable morphological changes. (Figures 18 D and E) In this instance a higher fractal dimension may indicate a RLCA agglomeration pattern with increased morphological disturbances, whereas a smaller fractal dimension may indicate a DLCA agglomeration pattern with less morphological changes but with increased cellular uptake.
<table>
<thead>
<tr>
<th>Au Particles</th>
<th>Fractal Dimension</th>
<th>Total Uptake (pg/cell)</th>
<th>Concentration uptake of Au particles (ug/ml)</th>
<th>Amount Au particles dosed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>_______</td>
<td>0.01 ± 0.15</td>
<td>0.00188</td>
<td>2ml</td>
</tr>
<tr>
<td>13nm 5 sec</td>
<td>2.16</td>
<td>1.93 ± 0.12</td>
<td>0.6045 ± 0.6193</td>
<td>2ml</td>
</tr>
<tr>
<td>13nm 10 sec</td>
<td>1.84</td>
<td>1.84 ± 0.14</td>
<td>0.3438 ± 0.4306</td>
<td>2ml</td>
</tr>
<tr>
<td>36nm 5 sec</td>
<td>2.82</td>
<td>1.43 ± 0.03</td>
<td>0.1836 ± 0.2539</td>
<td>2ml</td>
</tr>
<tr>
<td>36nm 10 sec</td>
<td>2.38</td>
<td>1.33 ± 0.02</td>
<td>0.0593 ± 0.0188</td>
<td>2ml</td>
</tr>
</tbody>
</table>
Table 7: Fractal Dimensions of Agglomerates with Correlating Mass Concentration, Densities and Particle Number

<table>
<thead>
<tr>
<th>Au particle</th>
<th>DF</th>
<th>Mass {}</th>
<th>Density</th>
<th>Particle number asoc</th>
<th>RIDN</th>
<th># of particles</th>
</tr>
</thead>
<tbody>
<tr>
<td>13nm-5 sec</td>
<td>2.16</td>
<td>40 ug/ml</td>
<td>.9621 ul/cm^3</td>
<td>3.31*10^16 ug/ul</td>
<td>2.764*10^16 ug/ul</td>
<td>44</td>
</tr>
<tr>
<td>13nm-10 sec</td>
<td>1.84</td>
<td>40 ug/ml</td>
<td>.9465 ul/cm^3</td>
<td>1.39*10^16 ug/ul</td>
<td>1.16*10^22 ug/ul</td>
<td>40</td>
</tr>
<tr>
<td>36nm-5 sec</td>
<td>2.82</td>
<td>40 ug/ml</td>
<td>1.0075 ul/cm^3</td>
<td>4.08*10^16 ug/ul</td>
<td>3.40*10^16 ug/ul</td>
<td>≈ 3-4</td>
</tr>
<tr>
<td>36nm-10 sec</td>
<td>2.38</td>
<td>40 ug/ml</td>
<td>1.0021 ul/cm^3</td>
<td>1.51*10^16 ug/ul</td>
<td>1.26*10^16 ug/ul</td>
<td>≈6-7</td>
</tr>
</tbody>
</table>
Discussion

Synthesizing the gold nanoparticles with sodium citrate allowed the particles to be stabilized as primary particles so that observation of the agglomeration effects of the salts in the RPMI media 1640 media was at its best potential. Once the primary particles were placed in RPMI without fetal bovine serum they instantly agglomerated and produced larger units’. The UV-Vis of the primary particles displayed sharp peaks and higher absorbance of light compared to the agglomerates. Agglomeration in UV-Vis was noticed in lower absorbance, broader peaks and shifts in wavelength. In contrast to UV-Vis, HSI indicated agglomeration with right shifts and sharper peaks. From the Dynamic light scattering measurements of the primary particles the hydrodynamic radius was slightly larger than the particle size measured in transmission electron microscopy. After agglomeration the DLS data displayed an increase in polydispersity from the larger particles. The agglomeration pattern of the smaller primary particles from the dynamic light scattering data displays that the smaller primary particles agglomerate at a faster rate than the larger primary particles, which is further confirmed with preliminary data with sp-icpms (single particle inductively coupled mass spectrometry noted in appendix) data. In this instance the smaller primary particles agglomerated in a DLCA agglomeration pattern. In sp-icpms the increase in agglomerate size with the larger primary is practically stabilized. This is most likely due to the interaction forces and the energy needed to agglomerate smaller particles is much lower therefore causing an increase in agglomeration and an overall DLCA agglomeration pattern.

This phenomenon experimentally relates to the point where smaller particles create larger agglomerates to their high diffusion rate and larger particles tend to produce smaller
agglomerates because of sedimentation. (Figure 6). Therefore with the smaller primary particles faster agglomeration rate the difficulty of the fetal bovine serum to stabilize the smaller primary particles was increased, however the FBS was still able to confirm its optimal stabilization properties and stabilized the agglomerates produced from the smaller primary particles at a 90% confidence level. The TEM images displays that the morphological properties of the nanoparticles before agglomerated and exposed to biological matrices were spherical and uniformed throughout. After agglomeration and exposure to a cellular environment the polydispersity of the nanoparticles increases and the overall structure of the particles doesn’t seem to be maintained, this data was also confirmed with hyperspectral imaging.

Gold nanoparticles have shown to have non-toxic properties and these are displayed in the alamar blue cellular viability tests, however with other known toxic ultra-fine materials it has been proven that smaller particles produce greater toxicity effects. Here it is shown that with gold as a potential nontoxic particle the smaller particles still display a non-significant decrease in cellular viability after 24 hours proving that smaller nanoparticles in general produced increased toxicity concerns possibly due to crossed biological barriers. The smaller primary particles resulted in an increased cellular uptake even after producing larger agglomerates. Increased uptake with smaller nanoparticles even after larger agglomerates were produced is possibly due to the looser density and smaller fractal dimension of the agglomerates created. Although the agglomerates from smaller primary particles indicated an increase in cellular uptake and non-significant decrease in cellular viability from a known non-toxic ultra-fine particle, the agglomerates from the larger primary particles induced the greater morphological changes. The possible explanation for this phenomenon is that the increased density of the agglomerates from the larger primary particles causes a biological environment to become more manipulated and an overall maintained structure is depleted. In this case a higher fractal
dimension may present a RLCA agglomeration pattern with increased morphological disruption, whereas a smaller fractal dimension may present a DLCA agglomeration pattern with less morphological changes but with increased cellular uptake. In the case of a toxic ultra-fine material a smaller fractal dimension may indicate increased toxicity results.
Conclusion

Smaller primary particles contain a faster agglomeration pattern due to the low energy and interaction forces while larger primary particles because of the increased mass inducing sedimentation have a slower agglomeration pattern. The smaller primary particles may have a potential to cross biological barriers and potentially cause an increased toxicity concern. Larger primary particles caused increased morphological disruptions the potential for toxicological effects correlating with their ability to disrupt the cellular environment is still an ongoing area of study. Lower fractal dimensional values are potentially correlated with increased cellular uptake and a faster agglomeration pattern, while higher fractal dimension values correlates with a slower RLCA agglomeration pattern and increased morphology changes in a biological environment. It has been proven from previous literature that smaller nanoparticles are able to increase toxicity concerns in our project even though gold displayed non-significant decreases in viability the smaller gold particles had an increased cellular uptake and a very slight decrease in viability after 24 hours. Therefore a smaller fractal dimension may indicate increase in toxicity however more investigation is needed. In this instance fractal dimension could possibly exists as a unique characterization that will lead toxicologists to predetermine biological effects before carrying out toxicity, pro-inflammatory and morphology studies.
Appendix

I. Students T-Distribution for FBS stabilization

13nm

1. Ho: μ= 15nm HA: μ < 15nm
2. T = 1.215nm-15nm/10.10 /√4= -13.785/5.05= -2.73
3. T=2.73 degrees of freedom 4-2=2 t.90< 2.73 < t.95 Therefore .05 < p< .10
4. Although the exact value of p is unknown, it is known that p < 0.10, therefore we can conclude at the 0.10 level of significance or we can be 90% confident that the fetal bovine serum is stabilizing the 13nm gold nanoparticles lower than an agglomeration factor of 15nm.

16nm

1. Ho: μ= 15nm HA: μ < 15nm
3. T= 2.44 degrees of freedom 4-2=2, t.90<2.44<t.95 Therefore .05<p<.10
4. Although the exact value of p is unknown, it is known that p<.10, therefore we can conclude at the 0.10 level of significance or we can be 90% confident that the fetal bovine serum is stabilizing the 25nm gold nanoparticles lower than an agglomeration factor of 15nm.

36nm

1. Ho: μ= 15nm HA: μ < 15nm
2. T = -8.65nm-15nm/10.03 /√4= -23.65/5.015= -4.72
3. T= 4.72 degrees of freedom 4-2=2, t.975< 4.72<t.99 therefore .01<p<.025
4. Although the exact value of p is unknown, it is known that p<.025 therefore we can conclude at the 0.025 level of significance or we can be 97.5% confident that the fetal bovine serum is stabilizing the 50nm gold nanoparticles lower than an agglomeration factor of 15nm.
II. Fractal Dimension Calculations

These mathematical junctions including the Relevant In vitro Dose Functions and the theoretical fractal dimension equation is combined and displayed here in its entirety with permission from Wendel Wohlleben and corresponding author; Philip Demokritou. Theoretical fractal dimension calculations has been carried out in numerous publications. The methods describe here is an adaptation of the methods cited, “Validity Range of Centrifuges For The Regulation of Nanomaterial’s: From Classification to as-tested Coronas.” “Wendel Wohlleben,” J Nanopart Res (2012) 14:1300. “An Integrated Approach for the In Vitro Dosimetry of Engineered Nanomaterials.” “Joel M Cohen,” Particle and Fibre Toxicology (2014) 11:20.

13nm- 5 second exposure to RPMI media without Fetal Bovine Serum

1. Density Estimation using VCM Calculations

“For an ENM suspension of known mass concentration, $\gamma$ (µg/ml), the total dose, $M$ (µg), can be calculated as: $M = \gamma \times V$. Where $V$ is the volume of the exposure media (m) applied directly to the cells in culture.

$$M = 40 \, \mu g/ml \times 1 \, ml = 40 \, \mu g$$

After centrifuging agglomerates and measuring the volume of the pellet and media density. The effective density of the agglomerates, PEV, can then be calculated as a volumes weighted average of ENM density, PENM, and media density, Pmedia.

$$p_{EV} = p_{media} + \left[ \left( \frac{M_{ENM}}{V_{pellet \, SF}} \right) \left( 1 - \frac{p_{media}}{p_{ENM}} \right) \right]$$

“The value of SF depends on the efficiency of agglomerate stacking. In the case of uniform spheres, possible values for SF may range from 0.634 for random close stacking, to the theoretical maximum of 0.74 for ordered stacking. We expects values to approximates to the theoretical value for random close stacking (0.634) whereas for non-agglomerating spherical ENMs we expect SF values to approach the theoretical value for ordered stacking (0.74).

$$p_{EV} = 0.9621 \, g/cm^3 + \left[ \left( \frac{3.27 \times 10^{-22}}{0.25 \mu l \, (0.634)} \right) \left( 1 - \frac{0.9621 \, g/cm^3}{19.3 \, g/cm^3} \right) \right]$$

$$p_{EV} = 0.9621 \, g/cm^3 + 1.957 \times 10^{-21} \, g/\mu l$$

$$p_{EV} = 0.9621 \, \mu l/cm^3$$
The total particle number dose, \( N \) (#), can be calculated from the total mass, \( M \) hydrodynamic radius, \( r_H \) (cm, determined by DLS for ENMs in suspension), and agglomerate effective density, \( p_E \) (g/cm\(^3\)). Assuming spherical agglomerates as:

\[
N = \frac{M}{\left(\frac{4}{3}\pi r_H^3\right) \times p_E}
\]

\[
N = \frac{40 \, \mu g}{\left(\frac{4}{3}\pi 6.96 \times 10^{-6} \text{cm}^3\right) \times .9621 \mu l/cm^3}
\]

\[N = 3.31 \times 10^{16} \mu g/\mu l\]

For particle number delivered to cells \( RID_n = (1 - e^{\alpha t}) \times N \). Where \( \alpha \) (hrs\(^{-1}\)) is the material media specific deposition fraction constant, and \( t \) (hr) is exposure duration.

\[
RID_n = \left(1 - e^{-0.0747h^{-1} \times 24hrs}\right) \times 3.316 \times 10^{16} \mu g/\mu l
\]

\[
RID_n = 2.764 \times 10^{16} \mu g/\mu l
\]

Fractal dimension was calculated as, \( RIDn = (\frac{R_hN}{r_p})^{DF} \) with \( N \) as the number of primary particles in each agglomerate, \( R_h, N \) as the hydrodynamic radius, \( r_p \) as the primary particle size and \( DF \) as the unknown fractal dimension.

\[
2.764 \times 10^{16} \mu g/\mu l = \left(\frac{6.969}{11.95}\right)^{DF}
\]

\[
FD = \log_{5.8} (2.764 \times 10^{16} \mu g/\mu l)
\]

\[
FD = \ln \left(\frac{2.764 \times 10^{16} \mu g/\mu l}{10}\right) / (5.8)
\]

\[
FD = \frac{3.78}{1.75} = 2.16
\]

\[
\ln(44) = 3.78 \text{ therefore 44 particles are in this agglomerate}
\]

13nm- 10 second exposure to RPMI media without Fetal Bovine Serum

\[M = 40 \, \mu g/ml \times 1 \, ml = 40 \, \mu g\]

\[
pEV = p\text{ media} + \left[\left(\frac{\text{MENM}}{V\text{ pellet SF}}\right)\left(1 - \frac{p\text{ media}}{p\text{ENM}}\right)\right]
\]

\[
pEV = .9465g/cm^3 + \left[\left(3.27 \times 10^{-22} g / (51\mu l) (0.634)\right)\left(1 - .9465g/cm^3\right)\right]
\]

\[
pEV = 9.62 \times 10^{-22} g/\mu l
\]

\[
pEV = .9465 \, \mu l/cm^3
\]
\[ N = \frac{M}{\left(\frac{4}{3} \pi r^3 H \right) \times pE} \]

\[ N = \frac{40 \, \mu g}{\left(\frac{4}{3} \pi 8.99 \times 10^{-6} \, \text{cm}^3 \right) \times 0.9465 \, \mu l/cm^3} \]

\[ N = 1.389 \times 10^{16} \, \mu g/ul \]

\[ RIDn = \left(1 - e^{-0.0747h^{-1} \times 24 \text{hrs}}\right) \times 1.389 \times 10^{16} \, \mu g/\mu l \]

\[ RIDn = 1.158 \times 10^{16} \, \mu g/\mu l \]

\[ RIDn = \left(\frac{Rh, N}{rp}\right)^\Delta DF \]

\[ 1.158 \times 10^{16} \, \mu g/\mu l = \left(\frac{8.993}{11.95}\right)^\Delta DF \]

\[ FD = \log_{7.5} \left(1.158 \times 10^{16} \, \mu g/\mu l\right) \]

\[ FD = \ln \frac{(1.158 \times 10^{16} \, \mu g/\mu l)}{10} \frac{1}{\left(7.5\right)} \]

\[ FD = \frac{3.69}{2.01} = 1.84 \]

\[ \ln(40) = 3.68 \, therefore \approx 40 \, particles \, are \, in \, this \, agglomerate \]

36nm- 5 second exposure to RPMI media without Fetal Bovine Serum

\[ M = 40 \, \mu g/ml \times 1 \, \text{ml} = 40 \, \mu g \]

\[ pEV = p \, \text{media} + \left[\left(\frac{MENM}{V \, \text{pellet} \, SF}\right) \left(1 - \frac{p \, \text{media}}{pENM}\right)\right] \]

\[ pEV = 1.0075 \, g/cm^3 + \left[\left(\frac{3.27 \times 10^{-22} \, g}{0.27 \, \mu l \times (0.634)}\right) \left(1 - \frac{1.0075 \, g/cm^3}{19.3 \, g/cm^3}\right)\right] \]

\[ pEV = 1.0075 \, g/cm^3 + 1.811 \times 10^{-21} \, g/\mu l \]

\[ pEV = 1.0075 \, \mu l/cm^3 \]

\[ N = \frac{M}{\left(\frac{4}{3} \pi r^3 H \right) \times pE} \]
\[
N = \frac{40 \mu g}{\left(\frac{4}{3} \pi \times 6.146 \times 10^{-6} \text{cm}^3\right) \times 1.0075 \mu l/cm^3}
\]

\[
N = 4.084 \times 10^{16} \mu g/ul
\]

\[
RIDn = \left(1 - e^{-0.0747h^{-1} \times 24 \text{hrs}}\right) \times 4.084 \times 10^{16} \mu g/ul
\]

\[
RIDn = 3.404 \times 10^{16} \mu g/ul
\]

\[
RIDn = \left(\frac{Rh \cdot N}{r_p}\right)^\text{DF}
\]

\[
3.404 \times 10^{16} \mu g/ul = \left(\frac{61.46}{38.89}\right)^\text{DF}
\]

\[
FD = \log_{1.58} \left(3.404 \times 10^{16} \mu g/ul\right)
\]

\[
FD = \ln \left(\frac{3.404 \times 10^{16} \mu g/ul}{10}\right)
\]

\[
FD = 8.406
\]

Fractal dimension values can’t be over 3, the possible explanation for this is that the agglomerates were so dense that their sedimentation speed increased and bonded to agglomerates outside of the defined cubical space. According to TEM images there were only a few particles agglomerating together and the density calculations were a little higher than the smaller primary particles. In this instance it is theoretically plausible to divide by the fraction dimensional value of 3 which sections off the different agglomerates and produces a higher FD value that correlates with the increased density.

\[
FD = \frac{8.406}{3} = 2.819
\]

Particle number association with the given fraction dimensional value

\[
\frac{\ln(X)}{0.45} = 2.819
\]

\[
X = 1.26, \ln(3.5) = 1.26
\]

Therefore there are ≈ 3-4 particles per agglomerate

36nm- 10 second exposure to RPMI media without Fetal Bovine Serum

\[
M = 40 \mu g/ml \times 1 \text{ ml} = 40 \mu g
\]

\[
pEV = p \text{ media} + \left[\left(\frac{\text{MENM}}{V\text{pellet SF}}\right)\left(1 - \frac{p\text{media}}{p\text{ENM}}\right)\right]
\]
\[ pEV = 1.002 \, g/cm^3 + \left[ \left( \frac{3.27 \times 10^{-22} \, g}{(0.251 \, \mu l) (0.634)} \right) \left( 1 - \frac{1.002 \, g/cm^3}{19.3 \, g/cm^3} \right) \right] \]

\[ pEV = 1.002 \, g/cm^3 + 1.949 \times 10^{-21} \, g/\mu l \]

\[ pEV = 1.002 \, \mu l/cm^3 \]

\[ N = \frac{M}{\left( \frac{4}{3} \pi r^3 H \right) \times pE} \]

\[ N = \frac{40 \, \mu g}{\left( \frac{4}{3} \pi (8.57 \times 10^{-6} \, cm)^3 \right) \times 1.002 \, \mu l/cm^3} \]

\[ N = 1.51 \times 10^{16} \, \mu g/\mu l \]

\[ RIDn = (1 - e^{-0.0747 h^{-1} \times 24 \, hrs}) \times 1.51 \times 10^{16} \, \mu g/\mu l \]

\[ RIDn = 1.26 \times 10^{16} \, \mu g/\mu l \]

\[ RIDn = \left( \frac{Rh \times N}{rp} \right)^{DF} \]

\[ 1.26 \times 10^{16} \, \mu g/\mu l = \left( \frac{85.73}{38.89} \right)^{DF} \]

\[ FD = \log_{2.20} (1.26 \times 10^{16} \, \mu g/\mu l) \]

\[ (1.26 \times 10^{16} \, \mu g/\mu l) \]

\[ FD = \ln \frac{10}{(2.20)} \]

\[ FD = \frac{3.70}{0.78} = 4.75 \]

Fractal dimension values can’t be over 3, the possible explanation for this is that the agglomerates were so dense that their sedimentation speed increased and bonded to agglomerates outside of the defined cubical space. According to TEM images there were only a few particles agglomerating together and the density calculations were a little higher than the smaller primary particles. In this instance it is theoretically plausible to divide by the fraction dimensional value of 2 which sections off the different agglomerates and produces a higher FD value that correlates with the increased density

\[ FD = \frac{4.75}{2} = 2.375 \]

\[ \frac{\ln(X)}{(0.45)} = 2.819 \]

\[ X = 1.852, \ln(6) = 1.79 \]

Therefore there are \( \approx 6-7 \) particles per agglomerate
### III. Table 8: Preliminary Single Particle Inductively Coupled Mass Spectrometry

<table>
<thead>
<tr>
<th>Sample</th>
<th>Analyte</th>
<th>Most Freq. Size (nm)</th>
<th>Mean Size (nm)</th>
<th>No. of Peaks</th>
<th>Mean Inten. (counts)</th>
<th>Part. Conc. (parts/mL)</th>
<th>Diss. Inten. (counts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I 13-0</td>
<td>Au 196.96</td>
<td>16.01563</td>
<td>20.16027</td>
<td>1373</td>
<td>2.261471</td>
<td>13569.24</td>
<td>0.016942</td>
</tr>
<tr>
<td>I 13-5</td>
<td>Au 196.96</td>
<td>18.58642</td>
<td>29.22101</td>
<td>153</td>
<td>9.633987</td>
<td>1512.085</td>
<td>0.013476</td>
</tr>
<tr>
<td>I 13-10</td>
<td>Au 196.96</td>
<td>24.99008</td>
<td>38.13629</td>
<td>166</td>
<td>23.72892</td>
<td>1640.563</td>
<td>0.018908</td>
</tr>
<tr>
<td>I 13-30</td>
<td>Au 196.96</td>
<td>34.33319</td>
<td>51.56045</td>
<td>47</td>
<td>60.91489</td>
<td>464.4968</td>
<td>0.013842</td>
</tr>
<tr>
<td>I 36-0</td>
<td>Au 196.96</td>
<td>41.03282</td>
<td>41.95175</td>
<td>507</td>
<td>25.11834</td>
<td>5010.636</td>
<td>0.022676</td>
</tr>
<tr>
<td>I 36-5</td>
<td>Au 196.96</td>
<td>15.18733</td>
<td>46.52125</td>
<td>316</td>
<td>44.84177</td>
<td>3123</td>
<td>0.011227</td>
</tr>
<tr>
<td>I 36-10</td>
<td>Au 196.96</td>
<td>15.28816</td>
<td>45.53651</td>
<td>149</td>
<td>49.38926</td>
<td>1472.554</td>
<td>0.010707</td>
</tr>
<tr>
<td>I 36-30</td>
<td>Au 196.96</td>
<td>15.57886</td>
<td>46.85339</td>
<td>92</td>
<td>69.79348</td>
<td>909.2278</td>
<td>0.010448</td>
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<tr>
<td>24hr 13-0</td>
<td>Au 196.96</td>
<td>15.92919</td>
<td>19.96917</td>
<td>1048</td>
<td>2.242366</td>
<td>10357.29</td>
<td>0.013329</td>
</tr>
<tr>
<td>24hr 13-5</td>
<td>Au 196.96</td>
<td>21.84607</td>
<td>30.46933</td>
<td>314</td>
<td>11.07006</td>
<td>3103.234</td>
<td>0.013895</td>
</tr>
<tr>
<td>24hr 13-10</td>
<td>Au 196.96</td>
<td>19.07069</td>
<td>31.0289</td>
<td>184</td>
<td>12.41304</td>
<td>1818.456</td>
<td>0.01253</td>
</tr>
<tr>
<td>24hr 13-30</td>
<td>Au 196.96</td>
<td>15.52147</td>
<td>22.2655</td>
<td>518</td>
<td>10.0695</td>
<td>5119.348</td>
<td>0.010108</td>
</tr>
<tr>
<td>24hr 36-0</td>
<td>Au 196.96</td>
<td>38.52135</td>
<td>39.94398</td>
<td>156</td>
<td>21.75641</td>
<td>1541.734</td>
<td>0.013118</td>
</tr>
<tr>
<td>24hr 36-5</td>
<td>Au 196.96</td>
<td>15.30531</td>
<td>29.95921</td>
<td>633</td>
<td>15.93049</td>
<td>6255.883</td>
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</tr>
<tr>
<td>24hr 36-10</td>
<td>Au 196.96</td>
<td>15.54192</td>
<td>42.816</td>
<td>229</td>
<td>40.9345</td>
<td>2263.187</td>
<td>0.009669</td>
</tr>
<tr>
<td>24hr 36-30</td>
<td>Au 196.96</td>
<td>32.18351</td>
<td>43.54372</td>
<td>150</td>
<td>34.02</td>
<td>1482.437</td>
<td>0.014682</td>
</tr>
</tbody>
</table>

**Table 8**: The data shows that the mean size of the smaller primary particles agglomerate gradually while the larger primary particles remain stable and have a reaction limited agglomeration pattern. After 24 hours the smaller particles agglomeration tends to slow down and the larger particles agglomerates break up and after 10 seconds re-agglomerate but still remain stable. Compared to DLS the particles agglomerated at larger sizes shown but this may be due to the significantly lower concentration needed.
Background on experimental instrumentation

Dynamic Light Scattering

According to the semi-classical light scattering theory (12), when light imposes on matter, the electric field of the light incites an emission of electrons in the molecules that wavers back and forth from matter to the source of light. Hence the molecules administer a secondary source of light and consecutively scatter light. The recurrent shifts, the angular circulation, the polarization, and the excitement of the scattered light are resolved by the size, shape and molecular synergy in the scattering material. Dynamic Light Scattering is also known as Photon Correlation Spectroscopy. This technique is one of the most prominent methods used to determine the size of particles (47). Shining a monochromatic light beam, such as a laser, onto a solution with spherical particles in Brownian motion causes a Doppler Shift, when the light hits the moving particle it changes the wavelength of the incoming light (18).

This adjustment is linked to the size of the particle. It is practical to cipher the sphere size dispersion and deliver a description of the particle’s movement in the medium, by checking the diffusion coefficient of the particle and using the autocorrelation function. The experiment’s theory is established vitally on two conditions. The first condition is that the particles are in Brownian motion (also called ‘random walk’); in this situation we know the probability density function, given by the formula: \( P(r, t | 0, 0) = (4\pi D t)^{3/2} \exp \left(-\frac{r^2}{4Dt}\right) \), where \( D \) is the diffusion constant. The second condition is that the beads used in the experiment, are spherical particles with a small diameter compared to the molecular dimensions (47).
In this case, it is feasible to implement the Stoke-Einstein relation and hence have a formula that readily remits the diffusion constant: \( D = \frac{k_B T}{6 \pi \eta a} \); where \( a \) is the radius of the beads, \( k_B \) is the Boltzmann constant, \( T \) is the temperature in Kelvin degrees and \( \eta \) is the viscosity of the solvent (47). From the image it can be visually perceived that the laser gorges through a collimator lens and then strikes the cell with the solution. The light is dispersed and identified by a photomultiplier that modifies a variation of intensity into a variation of voltage. There is an additional collimating lens afore the photomultiplier. The utilization of the collimating lenses is fundamental: the first lens allows us to center the beam into the cell, so that the area we will strike is distant enough from the side of the cell. The second lens is employed to gain a measure of scattered light that is neither extravagant nor a scarce amount. The photomultiplier is situated at a scattering angle of 90 degrees; undeniably for this scattering angle it is probable to disregard the nonlinearity of the linewidth with the scattering angle. This is because at this angle \( \Delta \Gamma/\Gamma < 0.1 \), after the photomultiplier, the signal is instantly pre-amplified and then emitted to the computer where the voltage is embellished through a program in Lab-view (18).

The photomultiplier adjusts an intensity variation into a voltage variation that is complementary to it. Since the particles move separately one from the other, the frequency spectrum of the intensity of the scattered light will have the form of a Lorentzian shaped line whose width depends on \( D \) and the scattering angle: \( S_I(\omega) = \frac{\Gamma(\Theta)}{[\omega^2 + \Gamma^2(\Theta)]} \). Where \( \omega = 2 \pi f \) with \( f \) the roll-off frequency and \( \Gamma(\Theta) = 2D \left(4\pi \left(\frac{\lambda}{n}\right) \sin \frac{\Theta}{2}\right)^2 \) Where \( \lambda \) is the wavelength of the incident light, \( n \) is the index of refraction of the solution and \( \Theta \) is the scattering angle (18). Since the process is arbitrary, implementing the WIENER KHINTCHINE theorem gets the spectrum through the correlation function.
In this way spatial correlations are translated into phase correlations according to: \( S_l(\omega) = e^{i\omega t} R_l(t) \, dt \). The half-width at half-height of the Lorentian curve is the correlation time, \( \tau \), and represents the time for a particle to move out of phase, \( \tau = \pi / DK^2, K = 4\pi / (\lambda / n) \sin (\theta / 2) \) (18).

From these formulas it is practical to figure D and thus the radius from \( D = k b T / 6\pi \eta a \).

The amplification and the filter-characteristics of the measurement apparatus are determined by four variables: Amplifier gain \( (10^4 - 10^{10}) \), amplifier suppression \( (10^{-10} - 10^{-3}) \) A, amplifier rise time \( (0.01; 0, 03; 0.1; 0.3 \ldots 300) \) ms, PMT voltage supply \([0-2000]\) V (18).

Ultra-Violet visible spectroscopy

Ultraviolet–visible spectroscopy (UV-Vis) implies to absorption spectroscopy or reflectance spectroscopy in the ultraviolet-visible spectral sector. Therefore (UV-Vis) employs light in the visible and adjacent ranges (near-UV and near infrared [NIR]). The absorption or reflectance in the visible range specifically revises the visually perceived color of the correspondent chemicals. In this vicinity of the electromagnetic spectrum, molecules undergo electronic transitions. In this instance absorption spectroscopy measures transitions from the ground state to the excited state (28).

UV-Vis functions on the precept that molecules involving \( \pi \)-electrons or non-bonding electrons (n-electrons) can consume the energy in the arrangement of ultraviolet or visible light to excite the designated electrons to increased anti-bonding molecular orbitals, the greatest efficiently excited electrons, absorbs the longest wavelength of light (20).

A UV-Vis spectrophotometer measures the intensity of light passing through a sample and compares it to the intensity before it passes through the sample (28). The scale among the two analyses is regarded as the transmittance and is commonly conveyed as a percentage, the absorbance is dependent on the transmittance.
A spectrophotometer includes a light source, sample holder, a diffraction grating in a monochromator or a prism to disperse the distinct wavelengths of light and a detector. The radiation sources are usually a tungsten filament (300-2500 nm), a deuterium arc lamp (190-400nm), xenon arc lamp (160-2,00nm) and the recent light emitting diodes (LED) for the visible wavelengths (28).

The detector is typically a photomultiplier tube, a photodiode, a photodiode array or a charge-coupled device (CCD). Single photodiode detectors and photomultiplier tubes are used with scanning monochromators, which filter the light so that only light of a single wavelength reaches the detector at one time (28). The scanning monochromator moves the diffraction grating to "pass-through" each wavelength so that its intensity may be quantified as a conduct of wavelength (91). Fine-tuned monochromators are utilized with CCDs and photodiode arrays. As both of these gadgets subsist of various detectors massed into one or two spatial arrays, they are able to collect light of various wavelengths on disparate pixels or groups of pixels concurrently.

Spectrophotometers can exist as a single beam instrument or a double beam. Single beam instrument permits the entire light to cross the sample cell. The intensity of light afore it crossed the sample ($I_o$) is computed by abstracting the sample. The double beam instruments which are the preferably contemporary versions separate the light into two beams afore approaching the sample. One beam is enforced as the reference; the other beam crosses the sample (92). The reference beam intensity is 100% Transmission (or 0 Absorbance), and the measurement displayed is the ratio of the two beam intensities (28). Double-beam instruments have two detectors (photodiodes), the sample and reference beam are measured simultaneously (28). In other instruments, both beams cross a beam
chopper, which closes off one beam at a time. The detector intersperses amid calibrating the sample beam and the reference beam in adherence with the chopper. There may additionally be one or more dark intervals in the chopper cycle. In this incident, the calibrated beam intensities may be amended by deducting the intensity evaluated in the dark interval afore the ratio is taken (92).

Samples are generally positioned in a clear container noted as a cuvette. Cuvettes are usually rectangular in shape, with a width of 1 cm, (this width turns into the path length, L, in the Beer-Lambert law). Test tubes can additionally be utilized as cuvettes in certain instruments. The type of sample container used must admit radiation to pass over the spectral region of interest (28). The universally suitable cuvettes are composed of supremacy fused silica or quartz glass because these are translucent throughout the UV, visible and near infrared regions. Glass and plastic cuvettes are widespread, however glass and most plastics absorb in the UV, which inhibits their profitability to visible wavelengths.

Hyper-spectral Imaging

Hyperspectral microscopy is a leading visualization approach that coalesces hyper-spectral imaging (HSI) with progressive optics and computer software to facilitate the accelerated identification of materials at the micro- and nanoscales. HSI is an approach that was first plenarily recognized and enforced in NASA’s airborne visible/infrared imaging spectrometer. The principle is to combine spectrophotometry and imaging: instead of taking a single photograph with a single dominant wavelength per pixel, advanced optics and algorithms allow the capture of an entire spectrum per pixel (65). Since this technique integrates another dimension to a contrarily two-dimensional image, a hyperspectral image is occasionally mentioned as a data cube. Hyperspectral imaging, like most spectral imaging,
accumulates and develops information from across the electromagnetic spectrum (93). The goal of hyperspectral imaging is to obtain the spectrum for each pixel in the image of a scene, with the purpose of finding objects, identifying materials, or detecting processes (22). Hyperspectral imaging correlates spatial and spectral data and has been utilized in many various fields such as pharmaceutical, forensics and medical. With the development of incipient and more expeditious imaging instruments (cameras), computers and software; the hyperspectral imaging approach have been broadened to retrieve qualitative and quantitative data. When observed via image analysis software, each pixel of a hyperspectral image provides the complete spectral response of that pixel’s spatial area within the VNIR or SWIR spectral range (63). At 100x magnification, a hyperspectral illustration will consist of 700,000 pixels as diminutive as 128nm each. This spectral data is recorded at approximately 2nm of spectral resolution in the VNIR range, enabling minute spectral differences to be measured from pixel to pixel within the image (26). This approach has been mostly utilized for molecular imaging with plasmonic NPs as contrast agents and/or sensors. Here we find this approach valuable to characterize the agglomeration and stability of plasmonic NPs in biological media and their interactions with cells (32).

Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Inductively coupled plasma mass spectrometry (ICP-MS) has been established as the most dynamic technique for the determination of trace elements in distinct matrices due to its attractive features: high sensitivity, wide linearity, multi-element capability and good precision (42). Notwithstanding, ICP-MS established analysis of metal species frequently needs the usage of a separation procedure prior to detection, because the element-specific techniques as well as ICP-MS cannot separate the species of a given element.
ICP-MS is a mass spectrometric multi-element method based on counting the number of atoms in a sample (49). It provides straightforward sample development, multi-elemental discovery linked with high sensitivity (ng L\(^{-1}\) range), a massive dynamic range (up to nine orders of magnitude), and can consistently be calibrated by effortless standards, hence donating the advantage of administering quantitative data. This instrument joins a high-temperature ICP (Inductively Coupled Plasma) source with a mass spectrometer. The ICP source adjusts the atoms of the elements in the sample to ions. These ions are then isolated and identified by the mass spectrometer. Argon gas surges inside the concentric channels of the ICP torch. The RF load coil is associated to a radio-frequency (RF) generator. As power is supplied to the load coil from the generator, fluctuating electric and magnetic fields are fixed at the terminus of the torch. When a spark is executed to the argon streaming through the ICP torch, electrons are broken off of the argon atoms, assembling argon ions. These ions are apprehended in the fluctuating fields and strike other argon atoms, constructing an argon discharge or plasma (66).

The sample is commonly imported into the ICP plasma as an aerosol, either by aspirating a liquid or dissolved solid sample into a nebulizer or using a laser to directly convert solid samples into an aerosol (66). Once the sample aerosol is transported into the ICP torch, it is exclusively desolvated and the elements in the aerosol are converted first into gaseous atoms and then ionized towards the edge of the plasma. Once the elements in the sample are reconstituted into ions, they are then sent into the mass spectrometer via the interface cones. The interface sector in the ICP-MS sends the ions maneuvering in the argon sample stream at atmospheric pressure (1-2 torr) into the depressed pressure sector of the mass spectrometer (<1 x 10\(^{-5}\) torr). The ions from the ICP source are then focused by the electrostatic lenses in the system. The ions originating from the system are positively
charged, so the electrostatic lens, which also has a positive charge, works to assemble the ion beam and fixate it into the entrance aperture or slit of the mass spectrometer. Once the ions permeate the mass spectrometer, they are split by their mass-to-charge ratio (94).

The quadrupole mass filter is the type of instrument that was used in our experiment. In this type, 4 rods (approximately 1 cm in diameter and 15-20 cm long) are arranged as in (ICP-MS figure 21 in appendix,). In a quadrupole filter, shifted AC and DC voltages are connected to adverse pairs of the rods (95). These voltages are then swiftly modified along with an RF-field. The outcome is that an electrostatic filter is entrenched that only admits ions of a single mass-to-charge ratio (m/e) to pass through the rods to the detector at a given present in time (66). Therefore the quadrupole mass filter is a subsequent filter, with the settings being transposed for each distinct m/e at a time. The voltages on the rods can be replaced at a very punctual rate. This permits the quadrupole mass filter to isolate up to 2400 amu (atomic mass units) per second! Universal quadrupole mass spectrometers utilized in ICP-MS have resolutions between 0.7 - 1.0 amu (96). Once the ions have been split by their mass-to-charge ratio, they are identified and computed by a detector. The function of the detector is to reverse the multiple ions striking the detector into an electrical signal that can be measured and associated to the amount of atoms of that element in the sample through the function of calibration standards. Once the ion collides into the effective surface of the detector, a measure of electrons is released which then collide into the next surface of the detector, intensifying the signal (94).
Transmission Electron Microscopy (TEM)

A transmission electron microscope (TEM) grants visualization and investigations of specimens in the range of micro-space (1 micron/1μm = $10^{-6}$m) to nano-space (1 nanometer/nm = $10^{-9}$m). Theoretically, the maximum resolution (d) that one can obtain with a light microscope has been limited by the wavelength of the photons that are being used to probe the sample, $\lambda$ and the numerical aperture of the system, NA (17). TEM exposes grades of detail and complexity unattainable by light microscopy because it uses a focused beam of high energy electrons which exhibit a small de Broglie wave-like behavior (5). Equivalent to all matter, electrons have both wave and particle properties (as theorized by Louis-Victor de Broglie), and their wave-like properties mean that a beam of electrons can be contrived to operate to resemble a beam of electromagnetic radiation (58). The wavelength of electrons is proportionate to their kinetic energy exemplified in the de Broglie equation. Transmission electron microscopy lightens the sample with electrons (i.e. the electron beam) within a high vacuum, and selects the electrons that are vacillated through the sample. The electrons are composited from underneath the sample and come in contact with a phosphorescent screen or through a camera (98).

Transmission electron microscopy (TEM) is universally used to access nanometer scale information about intracellular components (7). TEM concedes detailed micro-structural examination through high-resolution and high magnification imaging (5). For balanced activity of image stability and brightness, the microscope is frequently conducted to transfer an eventual magnification of 1,000–250,000× and a resolution below 1 nm is collected on
the screen. If a greater ultimate magnification is compulsory, it may be accessed by photographic or digital enlargement. The magnification of the TEM corresponds to the ratio of the distances between the specimen and the objective lens' image plane (62).

TEM incorporates three fundamental systems: (1) an electron gun, which produces the electron beam, and the condenser system, which centers the beam onto the object, (2) the image carry out system, consisting of the objective lens, movable specimen stage, and intermediate and projector lenses, which centers the electrons passing through the specimen to form a real, highly magnified image, and (3) the image-recording system, which amends the electron image into some scheme distinguishable to the human eye (69). The image-recording scheme commonly incorporates a fluorescent screen for observing and fixating the image and a digital camera for perpetual records. In inclusion, a vacuum scheme, containing pumps and their correlated gauges and valves, and power supplies are critical. The threshold of electrons; the cathode, is a heated V-shaped tungsten filament. The filament is enclosed by a control grid, periodically suggested as a Wehnelt cylinder, with a midway aperture arranged on the axis of the column; the apex of the cathode is located to lie at, just above or below this aperture (97). Electrons are propagated in an electron microscope by a procedure considered thermionic emission from a filament, usually tungsten, in the same manner as a light bulb, or alternatively by field electron emission (4). The cathode and control grid are at a negative potential that is equivalent to the anticipated accelerating voltage and is sequestered from the remnant of the instrument. The terminal electrode of the electron gun is the anode, which takes the composition of a disk with an axial hole. Electrons leave the cathode and shield, advance towards the anode, and, (if the stabilization of the high voltage is competent), pass through the central aperture with a perpetual energy (69). The intensity and angular aperture of the beam are governed by the
condenser lens scheme amid the gun and the specimen. A single lens can be exploited to assemble the beam onto the object, but, more frequently, a double condenser is utilized. The latest instruments resort to two projector lenses (one called the intermediate lens) to transfer an immense range of magnification and to execute an increased broad magnification without a coextensive increase in the physical length in the column of the microscope (5). In this the initial lens is forceful and fulfills an attenuated image of the source, which is then imaged by the second lens onto the object. This adjustment secures the space amid the electron gun and the object stage and is more multifarious because the reduction in size of the image from the source (and hence the eventual size of the illuminated area on the specimen) may be assorted greatly by governing the first lens. The application of a meager spot area reduces interruption in the specimen owed to heating and irradiation (98). The specimen grid is toted in a minuscule holder in a peripatetic specimen stage. The objective lens is ordinarily of minute focal length (1–5 mm [0.04–0.2 inch]) and regulates an entire intermediate image that is furthermore magnified by the projector lens or lenses. A proper projector lens can execute a range in magnification of 5:1, and by the enactment of conversable pole pieces in the projector a vast breadth of magnifications may be collected (99).

The improvement of the final image in the electron microscope confides merely upon the precision of the diverse mechanical and electrical alterations with which the single lenses are aligned to one another and to the illuminating system. The lenses require power supplies of a high degree of stability; for the highest standard of resolution, electronic stabilization to better than one part in a million is necessary (69).
Accompanying TEM, nano-sized structures can be identified in the cell environment and within cellular organelles (8). Therefore TEM is consistently handled by many researchers to resolve the uptake and localization of NPs inside cells, in conjunction with contributing proof to the uptake process whether it is endocytic or not. In TEM, an electron beam is translated through samples that are ordinarily < 100 nm thick to develop a bright-field (BF) image accommodating data about the internal framework of the sample. TEM contains the convenience of displaying the distinguished particle shapes and sizes as well as their position due to its high resolution. TEM requires extensive sample preparation (fixation, drying, staining, and slicing) and allows the investigation of only a tiny fraction of a single cell in an individual experiment (64).
Figure 19: ICP-MS schematic

**Figure 20**: UV-Vis schematic,

Note. Schematic of UV-Vis spectrophotometer. Reprinted “Characterization of group 12-16 semiconductor nanoparticles by UV-visible spectroscopy,” by Andrew R Barron, OpenStaX College, Copyright 1999-2015 Rice University, Creative Commons Attribution License May 16, 2014. Adapted with Permission
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