Universitat Autònoma de Barcelona

INCREASING THE RESOLUTION AT THE NANOBIOINTERFACE WITH ENGINEERING INORGANIC NANOPARTICLES

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PhD Thesis

INCREASING THE RESOLUTION AT THE NANOBIOINTERFACE WITH ENGINEERING INORGANIC NANOPARTICLES

Memoria presentada por **Sofía Rubio Ponce**
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Prof. Víctor F. Puntes

Sofía Rubio Ponce

Nature, you made us.

Nature, we explore you.

If I could ever learn something from you

from your way to proceed.

At least imitate a pinch of your complexity

a present for the world it would be.

I could better understand myself

could I even help you survive

why not save lifes.

No star in the ceiling I want

a space in the ground will be enough.

Just to take this dream to build

a better place to live.

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PROLOGUE

In your hands, you have a set of pages that collects the knowledge acquired along my PhD academic period. Looking for the exploration at the nanobiointerface, this Thesis dissertation is divided in different sections, in which there will be described the following contents:

Chapter 1: Presents a general overview about the paradigm of nanomaterials and its interaction with biological entities. The contribution of this work in the synthesis and functionalization methods of gold nanoparticles is pointed out.

Chapter 2: A study of gold nanoparticles generation by cytoplasmic reduction in HeLa cells is described along this Chapter. As well as the synthesis of gold nanoparticles mediated by complete cell culture medium and the consequences of mixing gold precursors and their competition in the reduction of gold for nanoparticles formation.

Chapter 3: This Chapter explores the design of functionalized gold nanoparticles. Different methods of gold nanoparticles conjugation were tested and further analyzed by reactive characterization approaches.

Chapter 4: The interaction between nanobioconjugates and cells that have a role in the immune system, such as monocytes, was explored in order to study how the functionalization of gold nanoparticles affects cell responses. The Protein Corona formed onto the surface of conjugated and bared gold nanoparticles while exposing these nanoparticles species to complete cell culture medium was also analyzed.

These studies could be done thanks to the *QualityNano Transnational Access* Grant that I received under the 7th Framework Programme of the European Commission that enabled the collaboration of our group with the Flemish Institute for Technological Research -Vlaamse Instelling voor Technologisch Onderzoek (VITO)- in which experiments could be carried out with the invaluable cooperation of Dr. Sarah Deville and Dr. Inge Nelissen.

Chapter 5: The altered metabolization of proteins by enzymatic digestion with trypsin is tested in this Chapter. Results are part of an excellent work developed by a former Post-Doctoral researcher in the group, Dr. Sonia Goy, in which I was involved.

Chapter 6: Global conclusions of the whole study described along the Thesis are collected in this final Chapter.

Each Chapter is designed to be read individually, presenting its own: Introduction, Experimental Section, Results and Discussion, Conclusions and References.

A list of abbreviations is provided at the end of the Thesis in order to make the reading easier.

Acknowledgments are also included on the page that follows, since I have many to thanks to all those persons that have *walked* with me along these years, making the *walk* more interesting and richer. Without them, many steps had been difficult to take.

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CHAPTER 1:

INTRODUCTION

CHAPTER 1: INTRODUCTION

1.1. An approach to the nanobiointerface

The development of science and technology over the past decade is distinguished by the study of nanomaterials. The focus was placed on the development of new strategies, based on the properties of the novel nano-objects, in order to generate new applications that could give better solutions to non resolved problems. The unique properties of nanomaterials suchs as small size, large surface area and high reactivity, offer new opportunities to meet the needs of modern society such as transference and storage of energy[(1)], decontamination of water[(2)], biofuel production[(3)] or therapeutic treatment[(4)] in nanomedicine could be.

A special interest was born in the field of nanomedicine, where the study of interactions between nanomaterials and biological environment was crucial for precisely understanding mechanisms at the nanobiointerface[(5)] and design proper applications.

When nanoparticles are exposed to biological components, such as molecules, proteins, DNA, vesicles, organelles, cell membrane, cytoplasm or biological fluids, there will be dynamic physicochemical interactions between the colloidal nanomaterial and the biological compound surfaces with kinetics and thermodynamic exchanges.

These interactions lead to the formation of protein coronas, intracellular uptake and biocatalytic processes that could have biocompatible or bioadverse outcomes. By understanding the nanobiointerferences, predictions of structure and activity can be determined by the nanoparticles properties. In this sense, the size, shape, surface charge, chemistry and surface coatings of nanoparticles would compromise their biocompatibility and biological responses and would determine the safety use of nanomaterials.

1.2. Synthesis of gold nanoparticles

One of the most relevant developments of new materials in the nanometer scale has been the generation of nanoparticles. This novel nanomaterial presents advantageous physical, chemical and biological propertied that make it have an important implication in the innovation of technological and biological processes.

A large number of nanoparticles have been synthesized, becoming particularly important those developed from novel metals[6], such as gold. Gold nanoparticles, that can be manufactured into a variety of shapes (including nanospheres, nanorods, nanocages, nanoprisms) present chemical, electromagnetic and optical properties that are strongly influence by their size and shape and determines their wide range of possible applications[7].

The precisely control in the generation of nanoparticles has been challenging. The control over shape and size is achieved through careful experimental conditions where the specific reducing agent, reaction time or temperatures of reaction have a critical role in the synthetic procedure. As well as the possible use of a capping agent, which would bind to select nanoparticle facets and block the growth beyond a certain nanometer range. Kinetically controlled processes were needed in order to generate nanoparticles of the desired properties that could enable their use for the expected applications.

Traditionally, in the field of monodispersed spherical gold nanoparticles which is of interest in the development of this Thesis, the most common synthesis procedures have been done based on the method pioneered by Turkevich et *al*. in 1951[8] and later refined by Frens et *al*. in 1973[9] with some modifications[10]. This method is based on the electrochemical reduction of gold (III) precursors, such as hydrogen tetrachloroaurate ($HAuCl_4$), using citrate as the reducing and capping agent. The method generates monodisperse spherical gold nanoparticles between 10-20nm in diameter.

Nowadays, the control of spherical gold nanoparticles sizes is possible by other approaches as the seeding growth of gold nanoparticles where different sizes up to

200nm can be obtained[11]. In the same way, the synthesis of size controlled gold nanoparticles under 10nm of size is also possible by the combination of two competitor reducing agents, such as sodium citrate and traces of tannic acid[12].

The degree of monodispersity and stability of the final colloidal solution is critical in the optical behavior and the biological effect of the nanoparticles.

Gold nanoparticles have been widely used for labeling[13, 14]. They present interesting optical properties because of the plasmon resonance inherent to the metallic particles and because they generate enough contrast for the detection in Transmission Electron Microscopy (TEM) images. Gold nanoparticles are also used as sensing systems[15] due to changes in the optical properties enables the detection of molecule-analyte interactions.

Furthermore, gold nanoparticles are used in delivering processes as they can be used as carriers for drug delivery and gene therapy[16]. Moreover, because of the dissipation of absorbed light, gold nanoparticles can be used in heating process where the temperature increases in the vicinity of the gold nanoparticles enabling the hyperthermal therapy[17, 18].

Because of the wide utility of gold nanoparticles it is of huge interest to study synthesis of gold nanoparticles, not only the chemical procedure but also the use of biological templates for the generation of gold nanoparticles inside the cells[19].

For this reason, the study of gold precursors in cell culture has been also carried out along this Thesis in order to analyze if cytoplasmic conditions could leads to the generation of gold nanoparticles in situ, for diagnosis of the cell status via SERS or hyperthermia.

The focus is put into looking for the biological machinery to generate a biogenic synthesis inside the cytoplasm where gold precursors can be transformed to gold nanoparticles by reducing agents inside the cells. Consequently, gold nanoparticles would intimately remain inside the cells gaining the possibility to become more bioactive and opening new approaches in the interaction of nanoparticles with cells and the appropriate control of cell responses.

1.3. The role of the functionalization

Due to their high surface energy, nanoparticles have a high affinity that makes them interact with organic molecules, peptides or proteins that would cover the nanoparticle surface. These compounds can be absorbed, chemically or electrostatically attached, onto the nanoparticle surface modifying their properties and generating a new object due to the conjugation procedure.

Colloidal stability is sometimes compromised by the surface charge alteraion of nanoparticles. For this reason, it is extremely important to control the functionalization process in order to generate stable conjugates. In this case, the colloidal stability of nanoparticles will not be interfered by the conjugated compounds and, at the same time, the conformation of molecules onto the nanoparticles surface will be controlled. The organization of molecules onto nanoparticle will have direct consequences in the way in which conjugated nanoparticles will interact with their environment.

The functionalization of nanoparticles involves the use of bifunctional ligands in which a moiety is used for anchorage to the nanoparticle surface while the other is directed to the outer-surface for specific interaction with biomolecules (the specific functionality). In the case of gold nanoparticles, among all the possible mechanisms, the conjugation through thiol grups (SH-Au bond) is the most widely used due to its pseudo-covalent character.

The high bond strength of the thiolated molecules allows the exchange with other ligands which bind more weakly to the gold nanoparticle surface (such as citrate, amines or phosphates). Moreover, the thiol mediated bond maintains stable over time making this strategy a very common functionalization method for gold nanoparticles conjugation.

For example, gold nanoparticles can be conjugated via thiol bond with thiol-modified oligonucleotides for specific detection of nucleic acid sequences in biological samples. Proteins or peptides containing a superficial cysteine residue, and polymers with a terminal SH group can be also used for the functionalization of gold nanoparticles through this strategy[20].

Other largely used ligands for functionalization of gold nanoparticles are the alkanethiols, such as 11-mercaptoundecanoic acid (MUA)[21]. These ligands will also bond via thiol groups and, due to a hydrophobic chain, will be able to generate a very compact layer obtaining the characteristic of self-assembled monolayers (SAMs)[20] onto the gold nanoparticle surfaces.

In this case, the SAM layer will have a potential role in the biological properties of the conjugated gold nanoparticles. Since the terminal groups of the layer will determine the final charge that the conjugate will present. This will affect the functionality of the conjugate as well as their interaction with the environment[22]. Additionally, further conjugation can be enhanced by the presence of these terminal groups that could favor the coupling of other molecules of interest.

As a consequence of their easy functionalization, gold nanoparticles are appropriated for in vivo approaches, biological imaging[15], diagnostics[23] and therapeutic treatment[24]. They could be used as contrast agent or drug delivery devices presenting an enormous potential for developing new tools.

However, the stability of conjugates can be compromised by their exposure to biological media. The control of conformation of molecules onto the gold nanoparticle surfaces is critical for their maintenance in the dispersed phase in physiological conditions. The stability will enable the designed conjugated nanoparticles to be functional in biological environments and also inside the body.

For this reason, a study of the mechanisms of functionalization is carried out along this Thesis in order to elucidate how the conjugation method will influence the design of the conjugates and the methodologies to analyze the stability of different conjugates states.

1.4. Exposure to biological media

Biological fluids are a complex mixture of salts, sugars, peptides and proteins which may cause different phenomena such as the aggregation and corrosion of nanoparticles. The former usually occurs as a consequence of the compaction of the stabilization layer around nanoparticles induced by a high ionic strength of the media[25].

The interaction between nanoparticles and biological media will be determined by the control of the stability of the sample. Aggregation should be avoided[26] in order to let the nanoparticles and conjugates be functional for *in vitro* and *in vivo* applications.

Apart from the functionalization of nanoparticles, one of the strategies to maintain stability of nanoparticles and conjugates in physiological media is the Protein Corona formation[27]. This a mechanism that will be generated as a consequence of the adsorption of proteins through electrostatic attraction onto the gold nanoparticles or conjugates surfaces when they are exposed to biological media, containing proteins. This mechanism will avoid aggregation of samples while exposing them to biological media increasing their biocompatibility[28].

In the process of bioconjugation with proteins, the nanoparticle or conjugate sizes and surfaces states should be taken into account as well as the compositions and concentration of both nanoparticles and biological media[29]. These parameters will influence the temporal evolution of the protein adsorption[30] onto colloidal inorganic nanoparticles and conjugates, stabilized electrostatically.

The Protein Corona formation follows a two-step process, clearly explained by Casals *et al.*[31], in which the nanoparticles exposed to media that contains proteins will be coated, at early incubation states, by the protein adsorption onto their surfaces. This protein coating, also known as 'Soft' Protein Corona, will be in equilibrium with the medium and it will be reversible. However, it can change to an irreversible protein coating, or 'Hard' Protein Corona, when the nanoparticles or conjugates are subjected to longer incubation times forming a permanent protein corona (due to crowding

effects) onto the nanoparticles or conjugates surfaces that will maintains even in protein-free media.

So, in this way, the final surface state of bioconjugates can be controlled. It is important to establish methodologies that would enable to evaluate the stabilization of nanoparticles in biological media before *in vitro* and *in vivo* assays. With this at hand, interactions and behavior of nanoparticles and biological systems can be explored.

Regarding cell internalization, several pathways have been considered to mediate the process of nanoparticles uptake. It can be divided in two main possibilities: via receptor-ligand internalization or non-specific ways (passive internalization). The functionalization of the nanoparticles surfaces can favor the penetration into cells, since it can generate a specific recognition and trigger receptor-mediated endocytosis[32-34]. There are other non-specific attractive forces that promote cellular contact and particle uptake that come from the intrinsic nanoparticles characteristics such as surface charge (cationic), hydrophobicity and roughness. They generally lead to non-specific binding forces that promote surface binding and cellular uptake[35].

Once nanoparticles are internalized into cells, the physical properties of nanoparticles will determine their in vivo biocompatibility, regarding their therapeutic use. The main parameters that would influence nanoparticle biocompatibily have been described again to be related to size, surface charge and hydrophobicity[36].

Realted to hydrophobic nanoparticles, which present high dispersibility, their *in vivo* half-life is generally short (seconds to minutes) since they are rapidly removed from circulation by the reticuloendothelial system, particularly in the liver and spleen. Due to this lack of bioavailability of the hydrophobic nanoparticles, their applications rely on the fast macrophages uptake that will carry nanoparticles to lymph nodes or inflammation sites.

In relation with the nanoparticle size, it has an important interest when nanoparticles are injected into the bloodstream, being the size critical in the routes of clearance from the body. Nanoparticles smaller than 6 nm[37], can be excreted by the kidney.

Meanwhile, the liver and spleen can trap nanoparticles larger than 200 nm. In this case, the clearance from the bloodstream does not necessarily imply excretion (since the bile duct is around 30 nm in diameter). Kupffer cells can hold onto the nanoparticles until the liver degrades them. Finally, nanoparticles that present sizes between 30–40 nm to a few hundred nanometres can undergo passive accumulation at tumour sites through the enhanced permeation and retention (EPR) effect. These mid-range sizes and relatively neutral surface charges nanoparticles are therefore optimal for chemotherapeutic drug delivery to cancers.

To conclude with the nanoparticles properties that would determine biocompatibility, the surface charge is intimately related to the cytotoxicity of nanoparticles. Being the cationic nanoparticles the one that present a higher surface reactivity and being, therefore, more likely to be toxic inducing more cytotoxic effects than neutral or anionic nanoparticles[*(38)*]; as well as than the relatively hydrophobic or poorly dispersed particles, which were rapidly and safely removed by the reticuloendothelial system (RES).

The connection between nanoparticles and biological systems compromises dynamic interactions that depends on the intrinsic properties of both entities and would determine whether the nanoparticles are bioavailable and may participate in biocompatible or bioadverse effects.

For this reason, a deep understanding of the mechanisms involved at the nanobiointerface is crucial for a potentially useful safe design of functionalized nanoparticles features for applications in nanomedicine.

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CHAPTER 2: ENGINEERING FOR NANOBIO

The suitability of inorganic nanoparticles to monitor and manipulate biological states

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ENGINEERING FOR NANOBIO

The suitability of inorganic nanoparticles to monitor and manipulate biological states

2.1. Introduction

Autometallography, an old process to identify cellular targets with gold and silver nanoparticles grown in physiological conditions[1-3], and other biomineralization processes have been employed and observed to study the behavior of prokaryote and eukaryote cells.

Indeed, the redox activity of cell metabolism is able to reduce and oxidize soluble ions into mineralized insoluble species which are deposited in the cell surroundings or inside the cells. This biological phenomena is used by many microorganisms to detoxify ionic bioavailable species into more inert and poorly available ones.

This is also performed intendedly to synthesize magnetosomes, exquisite iron oxide or iron sulfide magnetic nanoparticles used for navigation[4]; or clearly, endo and exo skeletons[5]. Despite tremendous efforts, nanobiointeractions are still a mystery. Consequently, this should provide some information and point of view about the interactions of nanoparticles with cells[6-9].

Besides, the reduction processes of photosynthesis and chemosynthesis and the oxidation process of combustion ultimately determines the equilibrium between CO_2 and hydrocarbons in the biosphere and has determined chemical potentials, and redox alteration may have significant effects on biology[10], as for example, the universality of generation of ROS (Reactive Oxygen Species) by cells exposed to corroding nanoparticles [11-13] probably the first paradigm of nanotoxicity.

Recently, there have been studies of the formation of nanoparticles of gold and silver in HeLa and other cells, either alive or lysed (of course, the cell lysiate can be further

oxidized as organic matter can be, therefore being it a soup that can reduce noble metal cations as those studied)[14, 15]. And also inside living plants[16] and microorganisms[17] (this is because biological matter is not the most oxidized state of carbon, of course, and therefore it can be further oxidized reducing metallic species of their environment).

Despite the redox potential of cells depends on the cell status, cell type and cell compartment, in all cases, noble metal cations have a higher redox potential and the consequent tendency to be reduced oxidizing their environment. A simple and paradigmatic example is the environmental synthesis of silver nanoparticles by humic acid[18, 19], and the many examples of gold spontaneous mineralization[20]. In fact we postulate that gold synthesis in cells could tell us about cell status. Since gold reduction would be determined by the status (sick or healthy) of the cells. Observing the kinetics of synthesis and the designed posology of the gold salt one can infer that the anti-inflammatory effect was produced by the synthesis of gold salts, probably altering the ROS generation in the inflamed area.

In order to advance in our integration of nanometric tools within the biological machinery one would imagine the ability to design orthobiological synthesis of inorganic nanoparticles in order to generate in situ the nanoparticles, for diagnosis (as imaging) [21] or therapy (as hyperthermia)[22] inside the body at the region of interest. It is difficult to imagine a process truly orthobiologic since here is the whole biological system the reducer of the cationic salt. However, it is possible to control the mineralization processes of gold in biological experiments, as it is subsequently described along this Chapter with little effect on cell viability.

This is useful for two main reasons, on one side, it allows us to synthesize nanoparticles inside the cytosol where they will remain for a certain extension of time before being exocitosed, something which is extremely challenging since nanoparticles are normally endocytosed and kept in vesicles[23-26] which, despite being inside the cells, from the metabolic point of view, it is outside the cell, as what is inside our stomach is still outside of our body. This localization of nanoparticles inside the cytoplasm should be extremely efficient for hyperthermia[27, 28] compared to the use of

gold nanoparticles which are outside the cell and outside the cytosol as is commonly the case. Also, having the nanoparticles intimately in the cytosol will open the way to unprecedented cell metabolism analysis via Surface-enhanced Raman Spectroscopy (SERS) as was previously shown[29, 30], but only achieved via intracellular injection.

Moreover, pure bio-gold nanoparticles, synthesized inside cytoplasm by a biological synthesis[17, 31, 32] without any type of surfactant (contrary to what occur in the case of the pre-synthesized gold nanoparticles), would generate a biogenic gold which is the most biocompatible.

Besides, in the control of the synthesis, using different gold precursors[33], we observe how the use of aurothiomalate, a drug for the treatment of rheumatoid arthritis[34-37], leads to the generation of nanoparticles that will finally explain why the treated persons had purple urine not due to hemorrhage. It could also cast some light in the process of gold based drugs. Drugs as aurothiomalate already approved by FDA&EMA generated nanoparticles. So, indirectly, it could be argued that FDA&EMA have already approved the use of nanoparticles for biomedical treatments.

The effect of the therapeutic process is the synthesis of gold nanoparticles inside the body where the small fraction (<6nm) is expulsed via the urinary system[38] and the large ones and aggregates are supposed to be accumulated in the injected area[39-41].

The observed adverse effects produced by the use of aurothioamalate affecting the immune response, as it is immediate rather than delayed, suggest that it's the intended interference with the immune system of the redox process the responsible for this immune disregulation rather than the produced and remaining gold nanoparticles. Therapeutic effects cannot be attributed to gold pre-synthesized nanoparticles since they have no shown significant immunomodulatory effects[42].

The control of the synthesis of nanoparticles remains basically the same as in the most common recipes[43, 44]. The gold precursor has to be reduced and then the insoluble reduced atoms aggregate into nanoparticles in a process of nucleation and growth determined by the solubility of the ions and the nanoparticles, the reduction rate and

relative concentrations and the energy needed for nucleation and growth of the nanoparticles.

As the biological matrix is well defined, we play with the dose, the stability and reactivity of the precursor to control the synthesis of large nanoparticles outside the cells or small ones inside the cytoplasm, once the gold salt is introduced in the cell media, presumably by the copper metalloprotein[45, 46] responsible of internalization of a similar compound as the antitumoral drug cisplatin[47, 48]. In this later case, the stability of the platinum ion and its lower redox potential avoids the spontaneous synthesis of platinum nanoparticles.

Gold precursos (aurothimalate and $HAuCl_4$) have been exposed to HeLa cell type as well as to complete cell culture medium (c-CCM) in order to explore the generation of gold nanoparticles by the intrinsic reductive biology of the cell and the media. Morphology of nanoparticles and their location in cells could be determined by high-resolution microscopy. Moreover, explored nanoparticles could be unequivocally assigned to gold by crystallographic experimental techniques such as Selected Area Electron Diffraction (SAED) patterns.

Characterization of the gold nanoparticles inside the biological matrix is challenging, specially the smaller ones. In this work we use crystal contrast imaging techniques, as standard (TEM, SEM, STEM) and High-Resolution Transmission Electron Microscopy TEM (HRTEM), High Angle Annular Dark Field (HAADF)-STEM and Energy-dispersive X-ray spectroscopy (EDX) Elemental mapping.

The toxicity of this process (gold reduction) has been also assessed and compared to the exposure of such nanoparticles to cells when they have been previously synthesized. Finally, as expected, white light irradiated cells when having the nanoparticles intimately dispersed in the cytoplasm suffer from hyperthermia more significantly than when similar previously formed nanoparticles are incubated with the cells. In both cases, either due to the synthesis process in the presence of the biological species, or due to the differential location of the nanoparticles, those produced by in situ synthesized nanoparticles are more biological active than in the case of pre-synthesized nanoparticles.

2.2. In situ synthesis of gold nanoparticles by cytoplasmic reduction

2.2.1. Study of gold precursors in HeLa cells

EXPERIMENTAL SECTION

Incubation of gold precursors in HeLa cells

Two different types of gold precursors were incubated in HeLa (human cervical adenocarcinoma) epithelial cells. Gold precursors used were: Gold(III) chloride trihydrate ($HAuCl_4$) and Sodium Aurothiomalate Hydrate (named as ThioAu), which corresponds to the active principle of the antirheumatic drug auranofin[(49)]. Cells were grown for 24h to ~90% confluency with a density of $8 \cdot 10^5$ cel/mL in 100mm culture plates in Minimum Essential Medium Eagle (MEM) alpha with 2mM of glutamate and 10% of Fetal Bovine Serum (FBS). Gold precursors (at final concentrations of 100µM, 250µM, 500µM and 1000µM) were added to culture plates after cell growth and incubated for 24h. FBS was previously incubated at 56°C for 30 minutes to deactivate the proteins of the complement system in serum.

Chemical fixation of HeLa cells for TEM

HeLa cells, previously incubated with gold precursors, were prepared by chemical fixation for Transmission Electron Microscope (TEM). For the fixation procedure, glutaraldehide was used as fixative agent in Phosphate Buffer solution (PB) with a final concentration of 0.1M of PB and 2.5% of glutaraldehide. Culture plates were washed in order to remove the cell culture media and to add the fixative compound to cells. This process was repeated twice with a fixation period of 30 minutes and 1 hour each time.

After that, the scraping of culture plates with PB 0.1M was carried out in order to collect the cells. The scraped material was brought under three cycles of centrifugation (at 2000rpm for 10min). Then, dehydration of samples by the replacement of water with organic solvent (acetone) was done followed by infiltration with embedding epoxy resin. Generation and polymerization of blocks of Epon 12 resin were done and ultrafine cuts (~50nm) were carried out in order to prepare the samples for TEM. Samples were stained by uranyl acetate 2% for 20 minutes.

Synthesis of control gold nanoparticles

A solution of 2.2mM sodium citrate in milli-Q water (150 mL) was heated with a heating mantle in a 250mL three-necked round-bottomed flask for 15 min under vigorous stirring. A condenser was utilized to prevent the evaporation of the solvent. After boiling had commenced, 1 mL of $HAuCl_4$ (25 mM) was injected. The color of the solution changed from yellow to bluish gray and then to red in 10 min. The resulting particles (~10 nm, ~$3 \cdot 10^{12}$ NPs/mL) are coated with negatively charged citrate ions and hence are well suspended in H_2O.

MTT and Light exposure assay

For this purpose, 96-well plates with a HeLa cellular density of 3500cells/well were used. After 24h of cell growth, different concentrations (100µM, 500µM and 1000µM) of gold precursors and pre-synthesized gold nanoparticles were added to HeLa cells for three different replicas measurements. These gold drugs were incubated in HeLa cells at 37°C for 24h, 48h and 72h.

Afterwards, each 96-well plate was treated with tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide through the EZ4U cell proliferation and toxicity assay. This method will enable to study the cytotoxicity effect of gold drugs due to the color change (derived from the reduction of the yellow-colored tetrazolium salt to insoluble purple-colored formazan by NAD(P)H-dependent cellular oxidoreductase enzymes which are largely in the cytosolic compartment of the cell).

The absorbance of this colored formazan solution can be quantified by measuring at certain wavelengths (450 and 620 nm) by a spectrophotometer and reflects the number of viable cells present. Therefore, cytotoxicity (loss of viable cells) due to gold drugs can be measured by this tetrazolium dye assay.

The cytotoxic effects of $HAuCl_4$ gold precursor were studied in HeLa cells and compared with the cytotoxicity generated by pre-synthesized citrate-stabilized gold nanoparticles after their exposure to HeLa cells. Control measurements of all

concentrations of gold nanoparticles tested were done, in order to correct the final signal without optical interference for the own gold nanoparticles absorbance.

At each incubation time, cells with the considered gold drugs were exposed to white light lamp for one hour at room temperature in laminar flow hood. A control of gold-treated cells without light exposure was carried out at each incubation time.

RESULTS AND DISCUSSION

In the presence of cells, gold could be transported into the cytoplasm and cytoplasmic conditions –with its relative high reducing potential- could lead to the formation of gold nanoparticles through the reduction of gold precursors due to their higher redox potential against cell environment.

Both types of gold precursors ($HAuCl_4$ and ThioAu) were incubated at concentrations of 100µM, 500µM and 1000µM in HeLa epithelial cells for 24h (detailed explanation in *Experimental Section*). These concentrations include doses close to the standard Turkevich (166µM) and auranofin ($2.5x10^3$ µM) doses.

Samples of HeLa cells, previously incubated with gold precursors, were chemically fixed by glutaraldehide followed by dehydration of samples with acetone and infiltration in resin. Ultrafin cuts of around ~50 nm were done after polymerization (see *Experimental Section for further details*). After this process, samples could be subsequently characterized by Jeol 1010 TEM (Transmission Electron Microscope) and FEI Magellan 400L XHR SEM (Scanning Electron Microscope) used as STEM (Scanning Transmission Electron Microscope) for the study of cells and the detection of nanoparticles inside them.

Gold nanoparticles generated in HeLa cells by the addition of different concentrations of both types of gold precursors, were characterized by these microscopy techniques. Images are shown in Figure 2.1. Detected nanoparticles inside cells are of a small size, around 18.12nm (Figure 2.2.). However, higher sizes were also found related to the agglomeration of the small nanoparticles.

Interestingly, gold nanoparticles seem to be localized outer cell membrane as well as inside cells (where the nanoparticles appear both in vesicles and completely inside the cytoplasm). This cytoplasmic localization would confirm the generation of gold nanoparticles by reduction of gold precursors by the own biological environment within cells, indicating an endogenous gold nanoparticles synthesis.

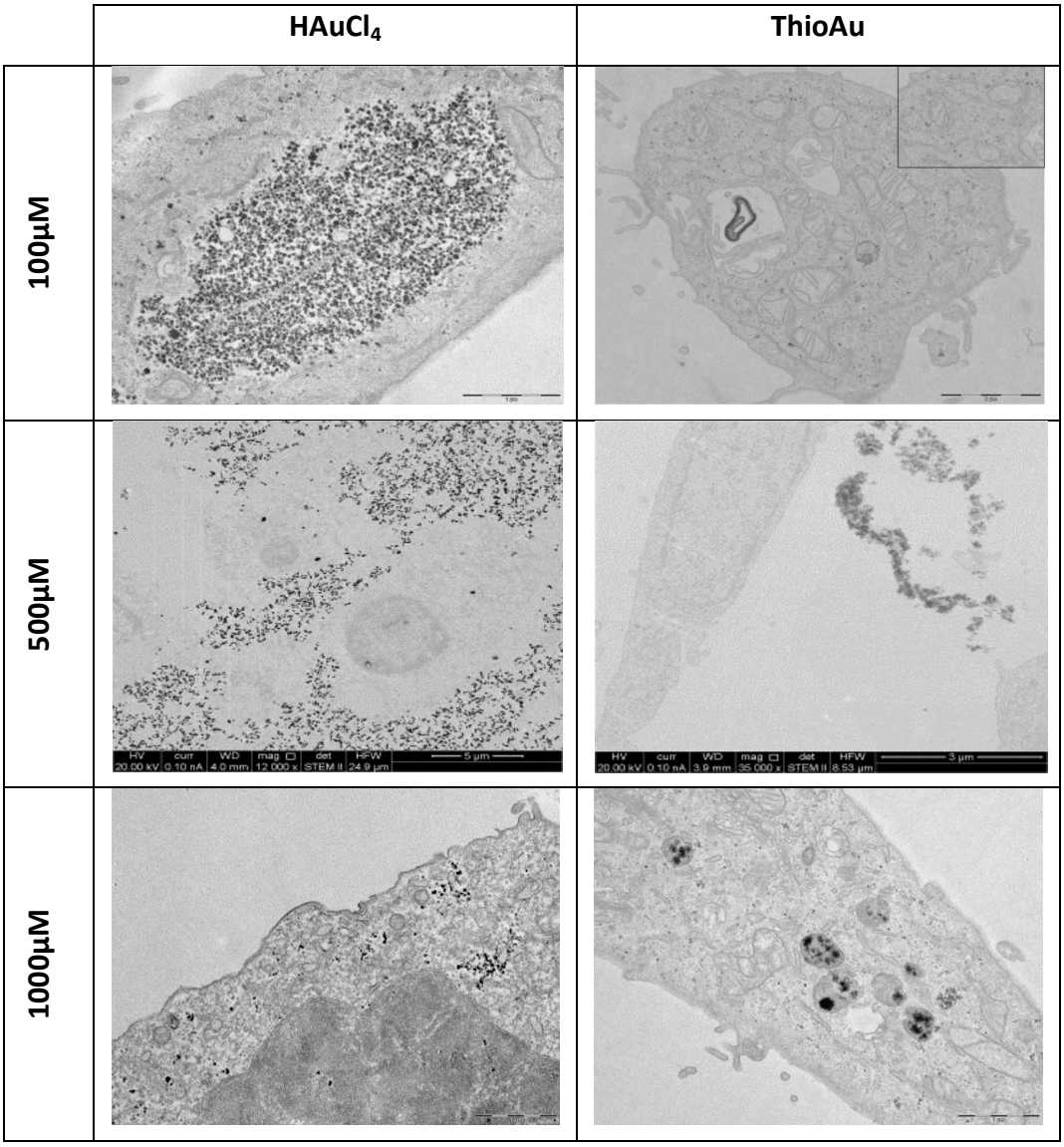


Figure 2.1.: **TEM Characterization** of the formation of gold nanoparticles with both types of gold precursors ($HAuCl_4$ and ThioAu) at different concentrations (100µM, 500µM and 1000µM) incubated for 24h in HeLa cells.

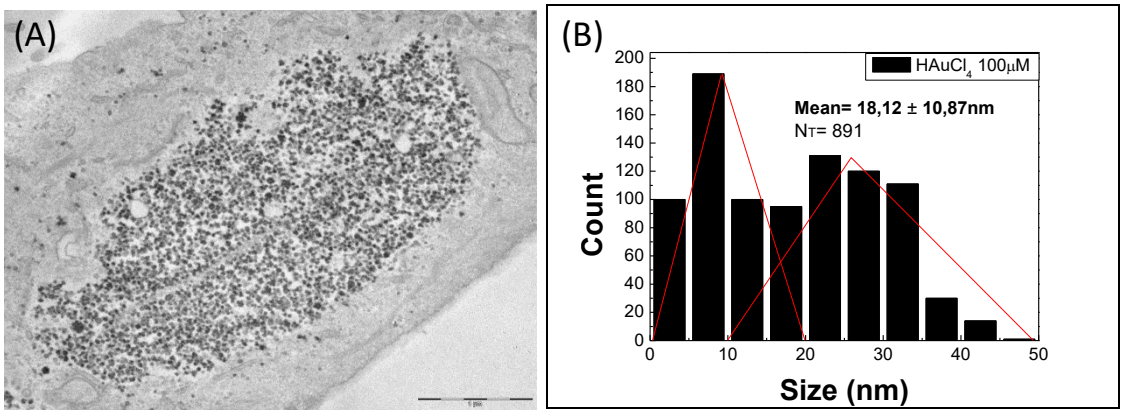


Figure 2.2.: (A) TEM Characterization of the formation of gold nanoparticles inside HeLa cells by the addition of $HAuCl_4$ gold precursor (100μM). **(B)** Respective size-distribution diagram is represented, showing that gold nanoparticles generated inside cytoplasm of HeLa cells are around ~18.12nm.

To detect small nanoparticles inside cells where highly dense nano objects as ribosomes are present is challenging.

Additionally, FEI Magellan 400L XHR SEM Microscope was also used for imaging of the structure of samples using STEM mode with a high-angle detector (Figure 2.3.). The High Angle Annular Dark Field (HAADF) Scanning Transmission Electron Microscopy (STEM) can generate Z-contrast imaging. By using a STEM detector with a large inner radius, a HAADF detector, electrons are collected which are not Bragg scattered. As such HAADF images show little or no diffraction effects, and their intensity is approximately proportional to Z^2.

Through this method, it is possible to perform atomic resolution images, highly sensitive to variations in the atomic number of atoms in the sample. The strong contrast generated is directly related to the atomic number in the sample (Z-contrast image).

As a consequence, an intense contrast resolution –that corresponds to nanoparticles- can be seen in Figure 2.3. Due to the high contrast, it can be confirm that the nanoparticles seen outside the cells and inside them (both in vesicles and cytoplasm) correspond to a compound which presents higher atomic weight than organic compounds of cells and which could might be gold.

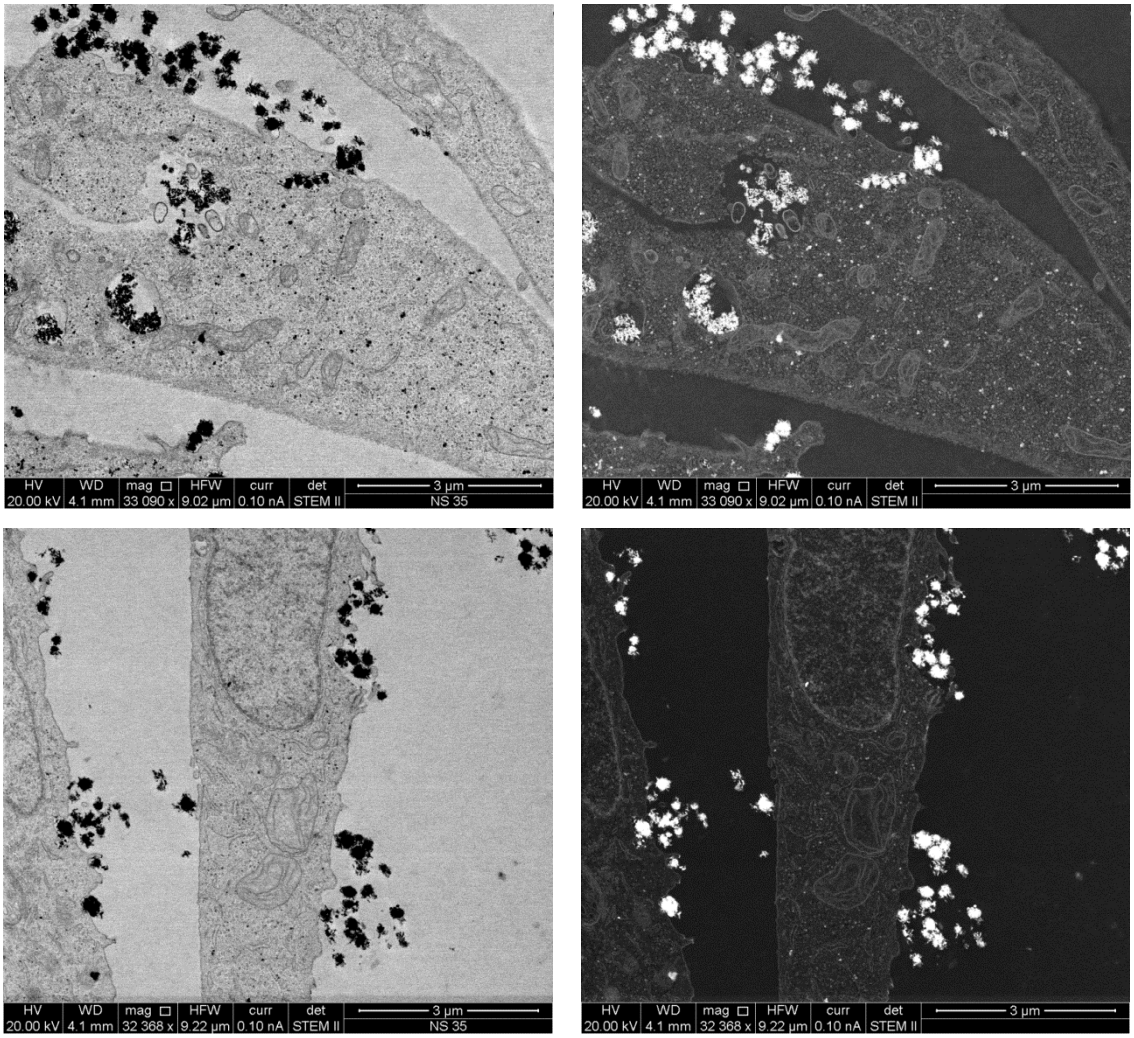


Figure 2.3.: STEM characterization of HeLa cells incubated with $HAuCl_4$ precursor (250µM) for 24h. High-angular microscopy was used in order to obtain Z-contrast images in *dark field* (images in the right side).

Moreover, for the determination of gold nanoparticles in HeLa cells, samples can also be analyzed by Energy-dispersive X-ray spectroscopy (EDX). This analytical technique is used for the elemental analysis of a sample that relies on an interaction of some source of X-ray excitation and the sample. Each element can be characterized since it has a unique atomic structure allowing unique set of peaks on its X-ray spectrum.

A Jeol 7100F type Field Emission Scanning Electron Microscopy (FE-SEM), with an Energy Dispersive X-ray Spectroscopy (EDX) detector was used to analyze the composition of nanoparticles and to stimate that the nanoparticles previously characterized by TEM and STEM analyses could correspond to gold element.

Samples characterized by FE-SEM belong to both types of gold precursors ($HAuCl_4$ and ThioAu) at an intermediate concentration of 250µM, which were incubated for 24h in HeLa cells.

Analyzing the spectra obtained through this microscopy technique (Figure 2.4. and 2.5.), it can be said that the nanoparticles present in both types of samples could correspond to gold nanoparticles. It is consistent with the peaks corresponding to gold atoms that appear in the spectra of HeLa cells samples treated with each type of gold precursors.

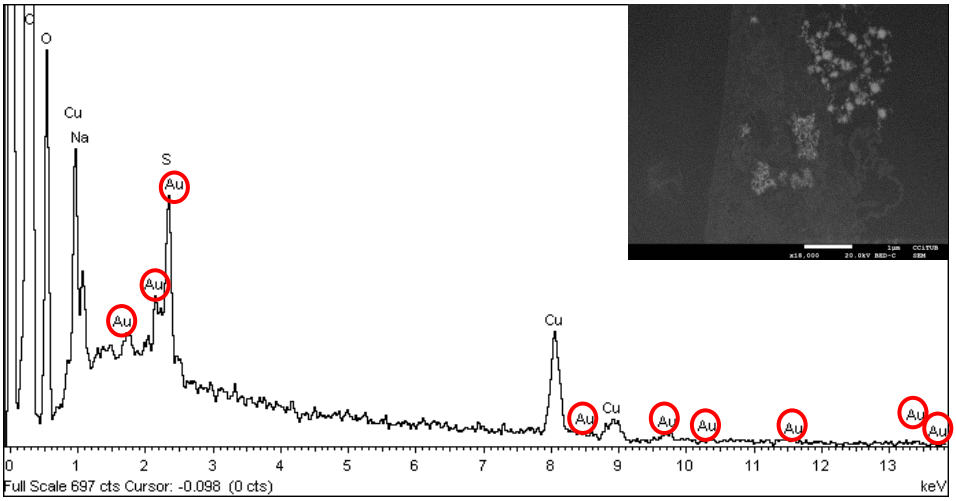


Figure 2.4.: EDX spectrum of $HAuCl_4$ precursor (250µM) incubated in HeLa cell for 24h. An image of the sample region analyzed is shown on the upper right corner of the spectrum. Peaks detecting gold element are labelled in red color.

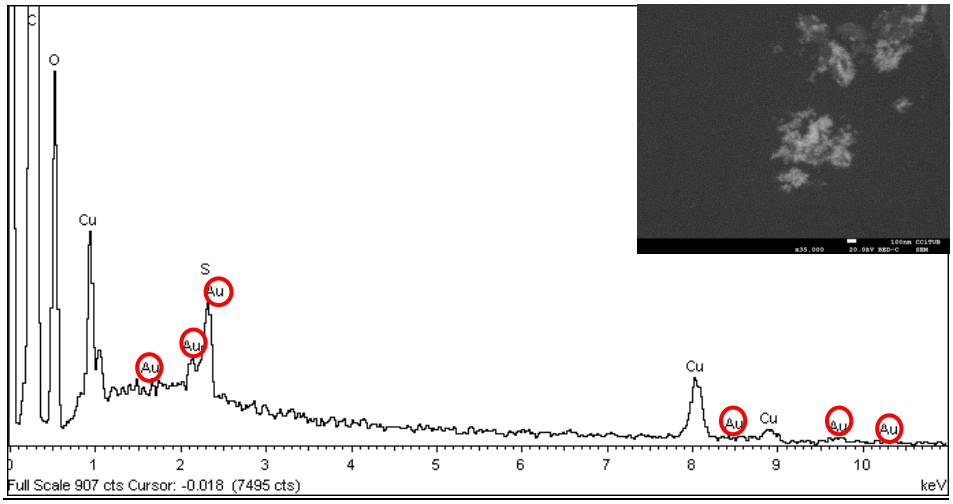


Figure 2.5.: EDX spectrum of ThioAu precursor (250µM) incubated in HeLa cell for 24h. An image of the sample region analyzed is shown on the upper right corner of the spectrum. Peaks detecting gold element are labelled in red color.

Although peaks corresponding to gold appear in both types of gold precursor spectra, other peaks detecting different elements (such as sodium, sulfur, oxygen and carbon) appeared overlapping the signal in the spectrum. These peak contributions correspond to the adhesive surface in which grids should be fixed for the FE-SEM characterization. An EDX control analysis of the adhesive layer spectrum without the microscope grid was carried out (Figure 2.6.).

Furthermore, the carbon peak is also a contribution of the microscope grid used for the fixation of samples. In the same way, the identification of copper peaks in both gold precursor samples spectra is also generated by the own carbon-cupper grid used for the fixation of samples.

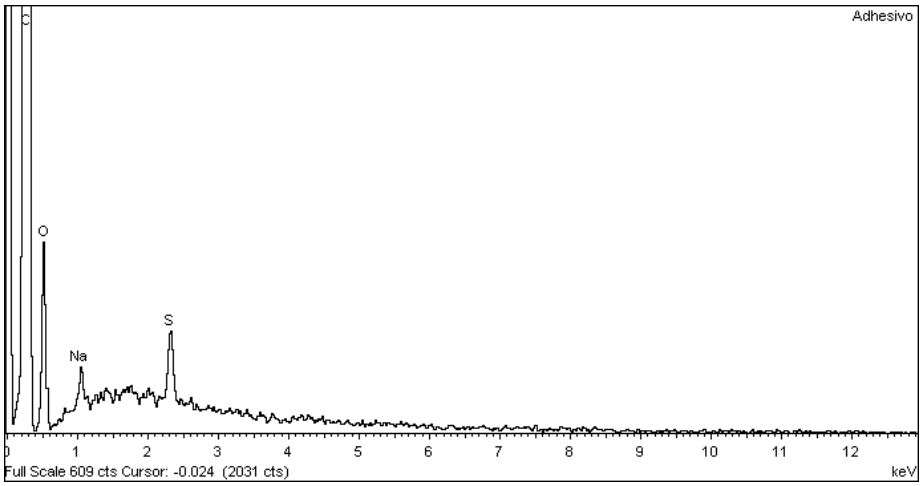


Figure 2.6.: EDX spectrum **of the adhesive surface in which samples had to be fixed for FE-SEM characterization.**

As a consequence, these peaks contributions from the adhesive layer have a strong influence in the resulting spectrum from gold precursors. Figure 2.7. shows the overlapping between both spectra indicating the clear distortion of the gold peaks due to the substrate contribution.

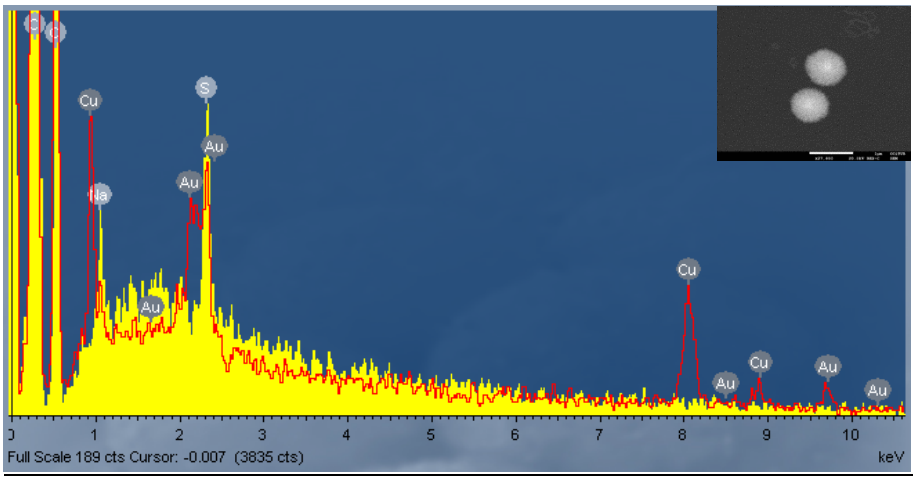


Figure 2.7.: EDX spectrum of the overlapping between ThioAu precursor (250µM) incubated in HeLa cell for 24h (red spectrum) and the substrate (yellow spectrum). An image of the sample region analyzed is shown on the upper right corner of the spectrum. Peaks detecting gold element are clearly overlapped by sulfur peak from the adhesive layer and are also shielded by copper peaks.

In order to explore and better characterize the formation of gold nanoparticles inside HeLa cells, High-Resolution Transmission Electron Microscopy (HRTEM), was used to obtain crystallographic structure images at atomic scale of the metallic gold nanostructure. The analyzed nanostructures were generated by the reduction of ThioAu precursor (250µM).

Results showed in (Figures 2.8. - 2.10.), indicated that gold nanostructures presented a hairy and rough morphology due to their composition formed by thin fibres of gold. These fibres of gold were further analyzed in detail at higher magnification in the microscope, showing that strands consisted of aligned and monodisperse gold nanoparticles. With a mean diameter of 2.7nm, gold nanoparticles presented a spheroidal and crystalline structure. The power spectrum of a single gold nanoparticle that contributed to the fibres of gold conforming the final nanostructure, could be analyzed by HRTEM, determining that the crystal structure of the small nanoparticle could be unambiguously assigned to cubic elemental Au (metallic).

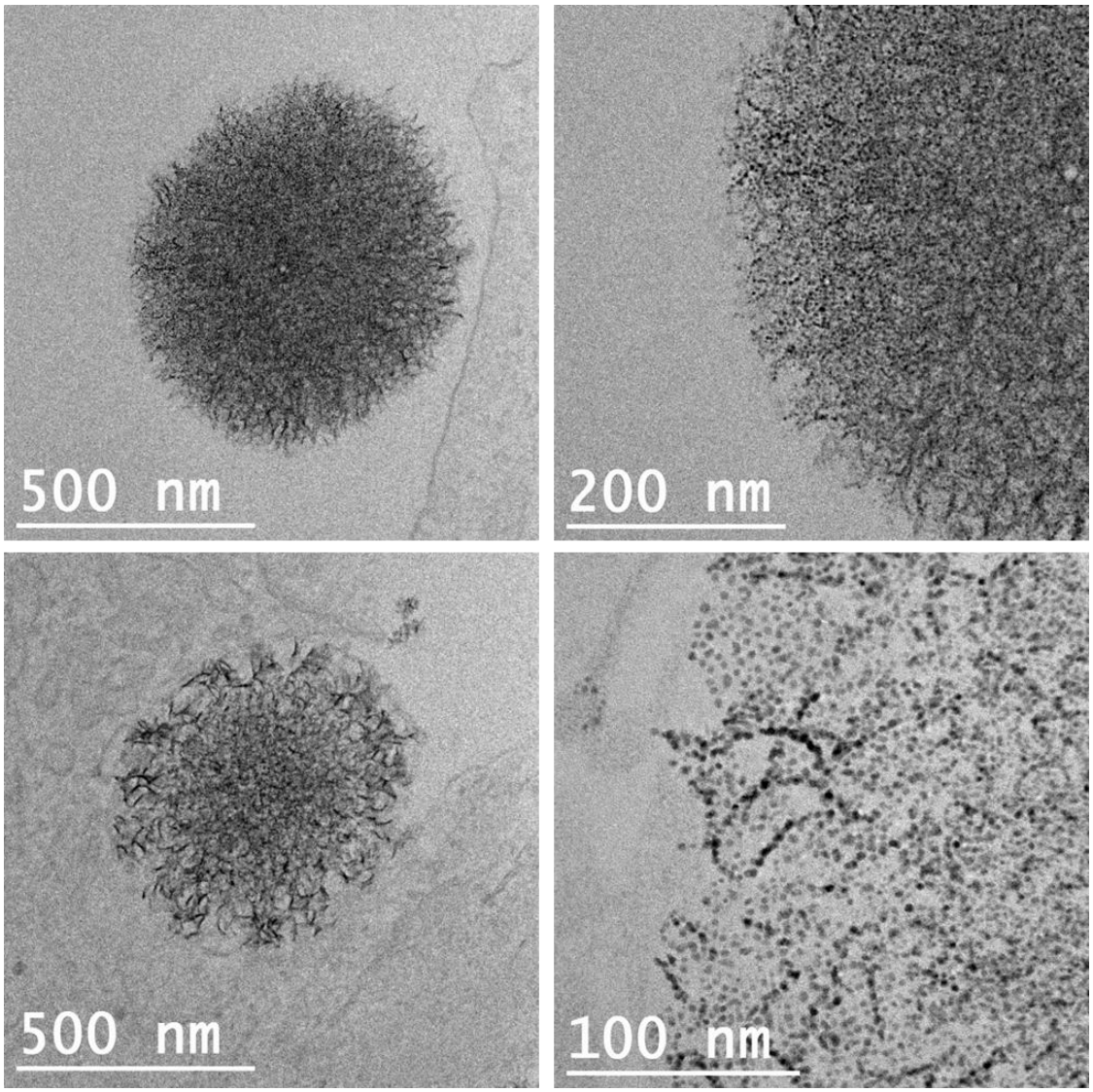


Figure 2.8.: TEM Images of gold nanostructures are shown on the left side. The nanoscale objects with a mean diameter of 500 nm exhibit a hairy, rough morphology caused by thin fibres of gold. Details of the nanospheres are depicted in higher magnification on the right side. The micrographs show the existence of strands consisting of aligned, monodisperse gold nanoparticles.

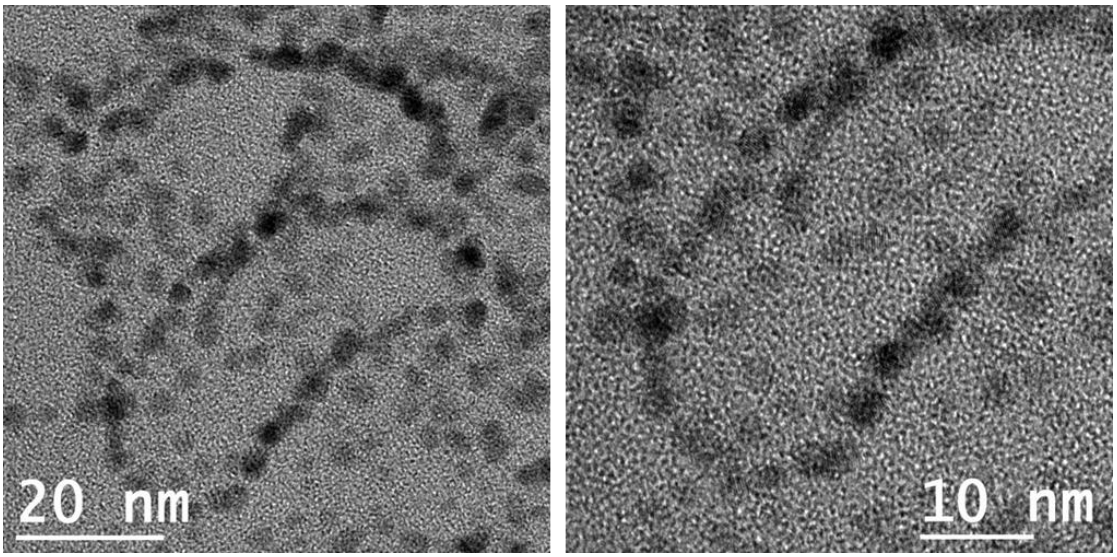


Figure 2.9.: HRTEM images show details of the gold nanostructures. The big nanostructures consist of strands of spheroidal, crystalline nanoparticles with a mean diameter of 2.7 nm.

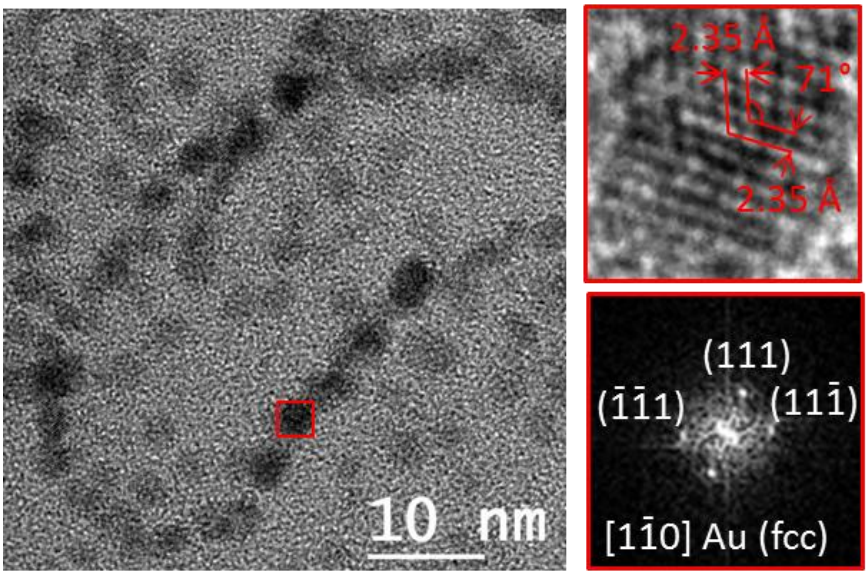


Figure 2.10.: The HRTEM image shows a detail of the gold nanostructures. One of the small gold nanoparticles indicated by the red square is shown in more detail on the right side along with its power spectrum. The crystal structure of the small nanoparticle could be unambiguously assigned to cubic elemental Au (metallic). The atomic planes distance between (111) of Au is 2.35Å and the angle between them is 71°.

HRTEM analysis could be correlated with Selected Area Electron Diffraction (SAED) technique which is able to identify crystal structures (Figure 2.11.). One of the gold nanostructures (composed of thousands of small gold nanoparticles) was analyzed. As it was previously determined by HRTEM, SAED also confirmed that the diffraction rings

observed in the pattern could be unambiguously assigned to the metallic gold phase. These rings suggest disordered distribution of crystal orientations, consequently no indication of selective attachment.

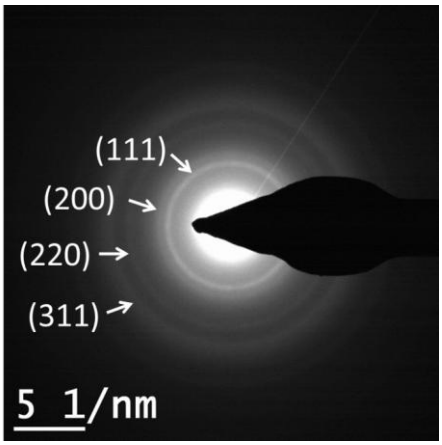
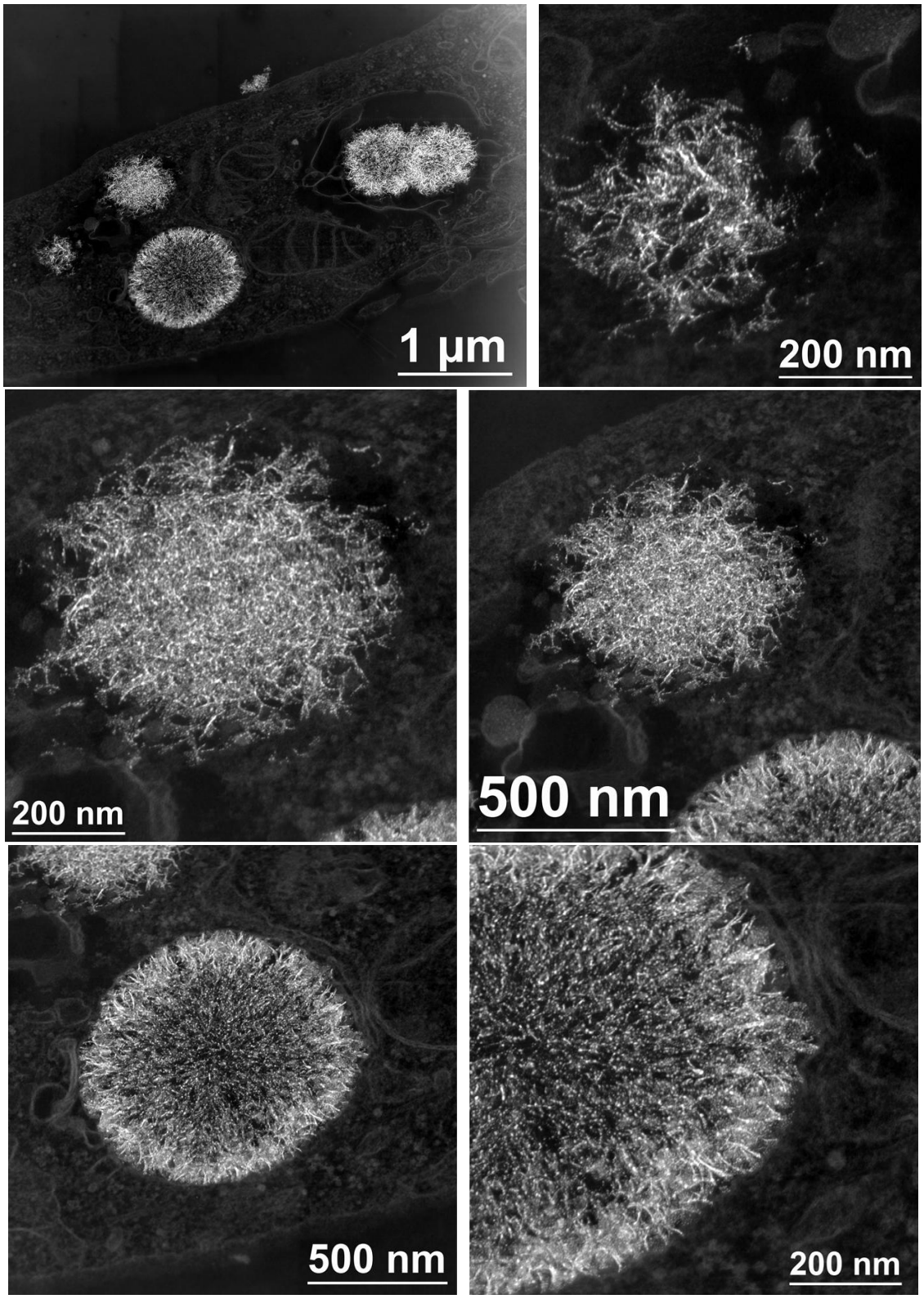


Figure 2.11.: SAED pattern of one gold nanostructure (composed of thousands of small gold nanoparticles). The diffraction rings observed in the pattern can be unambiguously assigned to the metallic gold phase.

In addition, another method of mapping samples as Annular Dark-Field imaging in a Scaning Transmission Electron Microscope (ADF-STEM) was used to determine the morphology of nanoparticles. ADF-STEM was performed in parallel with Energy-Dispersive-X-Ray Spectroscopy (EDX) acquisition in order to determine the elemental nature (gold) of the selected fraction of sample containing the nanostructures under study.

ADF-STEM micrographs of gold nanostructures (Figures 2.12.) corroborated the aligned gold nanoparticles morphologies previously shown by HRTEM, providing an EDX map of the exact portion of the image being studied. In this selected region, colour composite map can be detected for Au-C where elemental gold can be clearly identified in red colour in contrast with the carbon which is shown in green (Figures 2.13. and 2.14.).

When analyzing images by ADF-STEM, contrast distribution suggest some degree of selective attachment indicating a preferential orientation of nanoparticles.


1 µm
200 nm
200 nm
500 nm
500 nm
200 nm

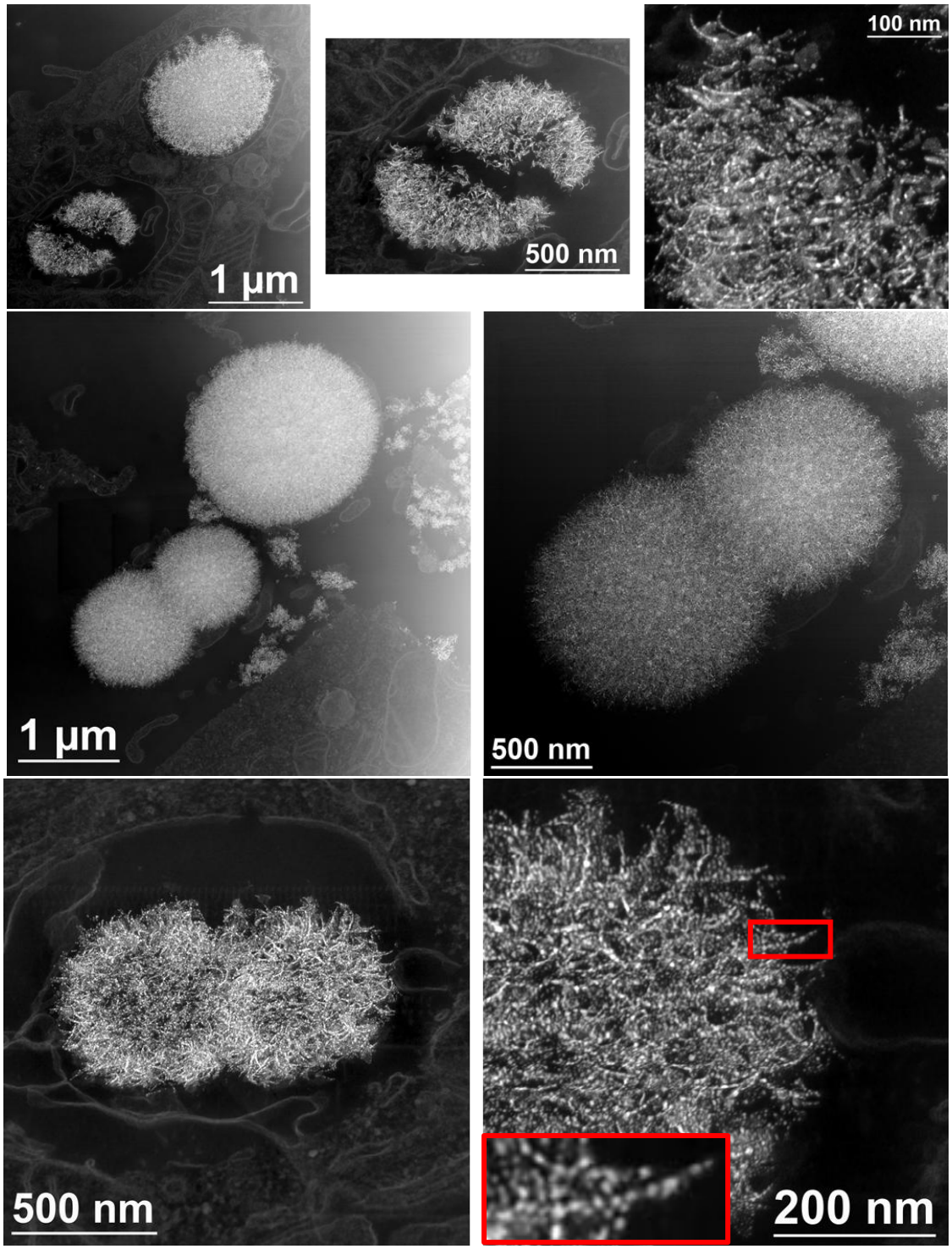


Figure 2.12.: ADF-STEM micrographs of the gold nanostructures in the cell. The inset is showing a magnified detail from the red squared region. It is interesting to point out that the claw-like morphologies are in fact composed of aligned Au-nanoparticles embedded in some kind of membrane.

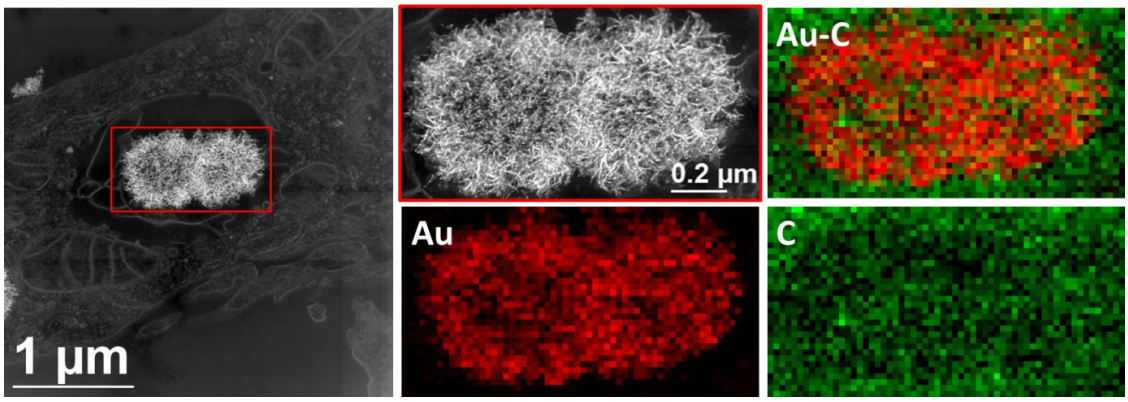


Figure 2.13.: ADF STEM micrograph of two gold nanostructures. EDX maps of the red rectangular region: Elemental Au (in red) and C (in green) maps and colour composite map for Au – C.

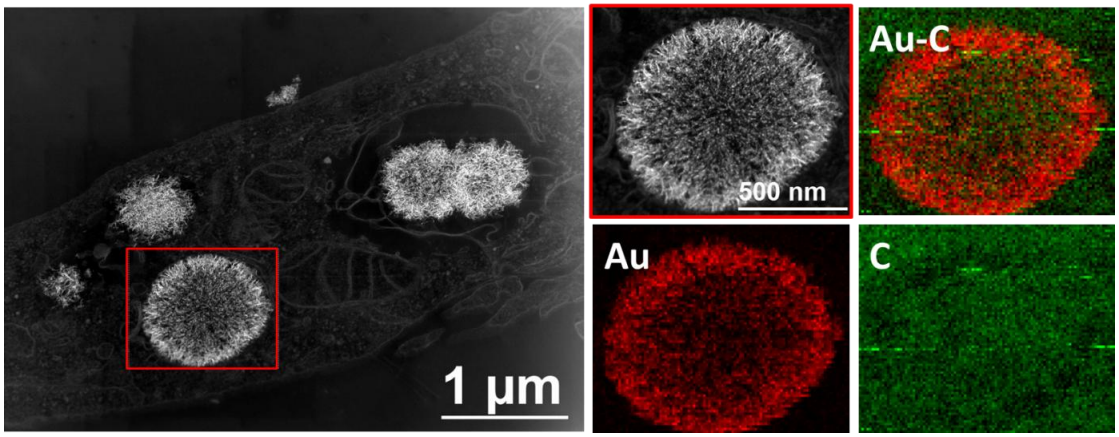


Figure 2.14.: ADF STEM micrograph of several gold nanostructures within the cell. EDX maps of the red rectangular region: Elemental Au (in red) and C (in green) maps and colour composite map.

Regarding the analyzed nanoparticles morphology and the localization in cells, it can be predicted a cellular pathway of nanoparticles inside HeLa cells. As it is shown in the following image (Image 1), gold nanoparticles are generated inside cytoplasm of the cell where nanoparticles of ~20nm have been observed. It is expected that they follow a secretory pathway inside HeLa cells. Consequently, small gold nanoparticles will be collected from the cytoplasm inside vesicles. Because of the vesicular conditions, the small nanoparticles will adquire a disposition in thin fibres that will ultimately generate a hairy nanostructure of gold nanoparticles. Vesicles carrying the nanostructures will move outwards, secreting nanoparticles outside the cells via the secretory pathway.

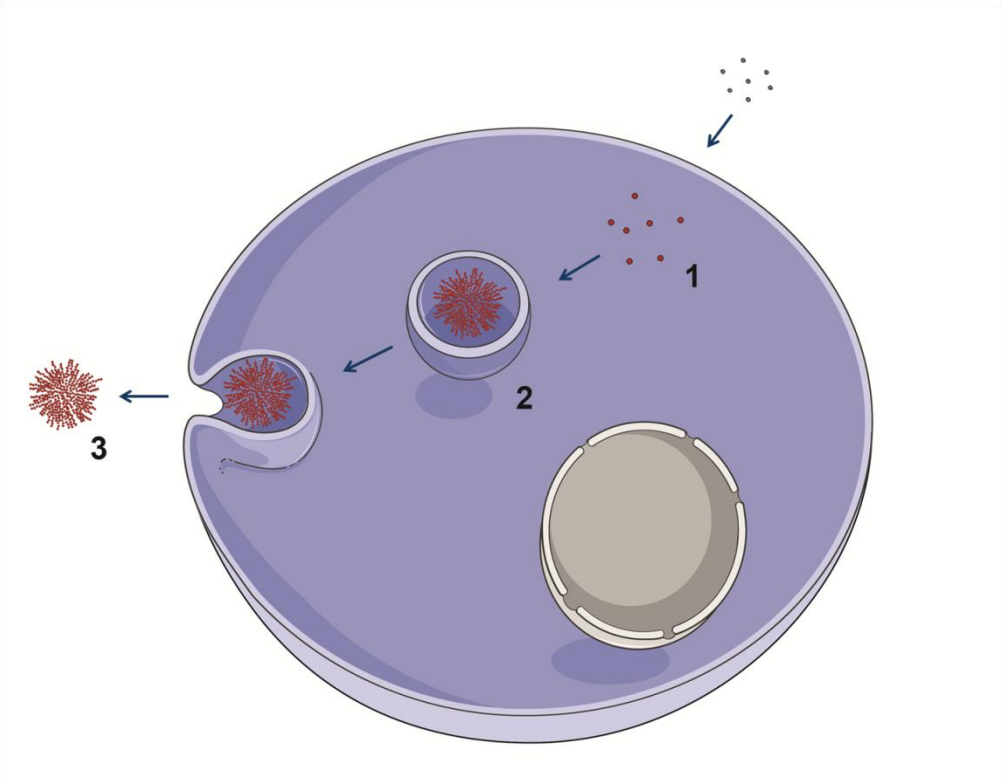


Image 1: Schematic representation of gold nanoparticles pathway inside HeLa cells. Gold precursors enter in cells and small nanoparticles will be generated inside cytoplasm (1) by the gold precursors reduction. Nanoparticles will be collected from the cytoplasm and accumulated inside vesicles (2). Finally, nanoparticles will be secreted outside cells via vesicular and cellular membrane fusion (3). Nucleus of the cell is represented by the grey circle inside the cell. Image was designed thanks to the creative collaboration of Muriel Freixanet, MS.

Cytotoxicity assay

The analysis of the biological effects of gold nanoparticles generated by biological reduction of $HAuCl_4$ gold precursor in HeLa cells and the comparison with the pre-synthesized citrate-stabilized gold nanoparticles (Figure 2.15.) was done by studying the cell viability after gold salt exposure at different incubation time (24h, 48h and 72h). Note that these are rather long times, more appropriated to study cell response than nanoparticles synthesis kinetics.

Besides, control monodisperse citrate-stabilized gold nanoparticles with an uniform quasi-spherical shape of ~10 nm and a narrow size distribution were synthesized following a kinetically controlled seeded growth strategy via the reduction of $HAuCl_4$ by sodium citrate[(50)].

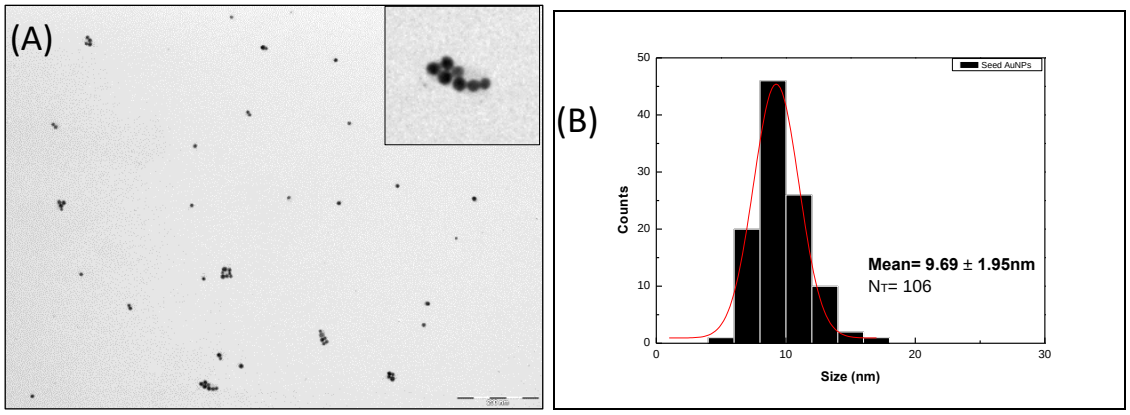


Figure 2.15.: TEM characterization of pre-synthesized gold nanoparticles **(A)** and their narrow size-distribution **(B)** showing an uniform quasi-spherical shape of around 10nm. The bar in TEM image indicates 200nm.

In order to study if cell viability could be compromised by the exposure of gold salts, a cell proliferation and cytotoxicity assay was carried out by tetrazolium dye MTT reduction. The cytotoxic effects of gold drugs, nanoparticles generated by the biological reducing potential of cells as well as those pre-synthesized before exposure to HeLa cells, can be determined through this cell proliferation and toxicity assay.

The colour change associated with the reduction of yellow tetrazolium salt to purple insoluble formazan by oxidoreductase enzymes inside the cytosol of cell, is directly related to cell viability. The absorbance of this colour formazan can be quantified by a spectrophotometer reflecting the number of viable cells present. Therefore, cytotoxicity (loss of viable cells) due to gold drugs can be measured by a decrease of the colorimetric absorbance trough this dye assay.

The cytotoxic effects of $HAuCl_4$ gold precursor were studied in HeLa cells and compared with the cytotoxicity generated by pre-synthesized citrate-stabilized gold nanoparticles after their exposure to HeLa cells.

Additionally, at the different incubation times, cells with the considered gold drugs were exposed to white light lamp for one hour. A control of gold-treated cells without light exposure was carried out at each incubation time.

Comparison of cytotoxic effects between pre-synthesized gold nanoparticles and the ones generated inside cells, can be shown in Figure 2.16. (A, B). It was observed that the $HAuCl_4$ gold precursor that will lead to the generation of gold nanoparticles by cytoplasmic reduction, presents a higher cytotoxicity against cells than pre-synthesized citrate-stabilized gold nanoparticles. The former (Figure 2.16.-A) results in a significant decrease of cell viability for high concentrations of $HAuCl_4$ precursor (500µM and 1000µM) at all incubation times (24h, 48h and 72h); whereas the latter (Figure 2.16.-B) maintain a much more stable cell viability around 70-100% for all the studied gold nanoparticles concentrations and incubation times.

This could be related to the reduction/oxidation process in the presence of gold in the cytoplasm. At high gold precursor concentrations makes us to think in $HAuCl_4$ precursor as toxic and gold nanoparticles as non toxic. It should be remarked that the same gold precursor ($HAuCl_4$) was used for these pre-synthesized gold nanoparticles. Therefore, the cytotoxicity of both gold drug conditions can be correlated. As a consequence, it can be concluded that gold nanoparticles generated inside HeLa cells by cytoplasmic reduction of $HAuCl_4$ precursor, produce a higher cytotoxicity against HeLa cells than the pre-synthesized citrate-stabilized gold nanoparticles at high concentrations of gold drug.

Furthermore, gold-treated HeLa cells were exposed to white light for one hour in order to analyze if cytotoxicity could be altered as a consequence of light absorption by gold nanoparticles.

As a result of light exposure, a decrease of cell viability was appreciated in the case of gold nanoparticles generated by cytoplasmic reduction of $HAuCl_4$ precursor at concentrations of 500µM and 1000µM (Figure 2.16.-C). However, no significant decrease of cell viability was noticed in the case of pre-synthesized gold nanoparticles where HeLa cells still remain between 70-100% of cell viability (Figure 2.16.-D). Similar results to the ones obtained in the case of non-light exposure were observed for the pre-synthesized gold nanoparticles. Hence, it could be considered that -in this case- there is no redox effect taking place.

Consequently, the gold nanoparticles generated inside HeLa cells by cytoplasmic reduction of $HAuCl_4$ precursor, were identified as nano-objects that are active. This is a result of their capacity to produce cell damage as a consequence of light absorption and hyperthermia[28, 51].

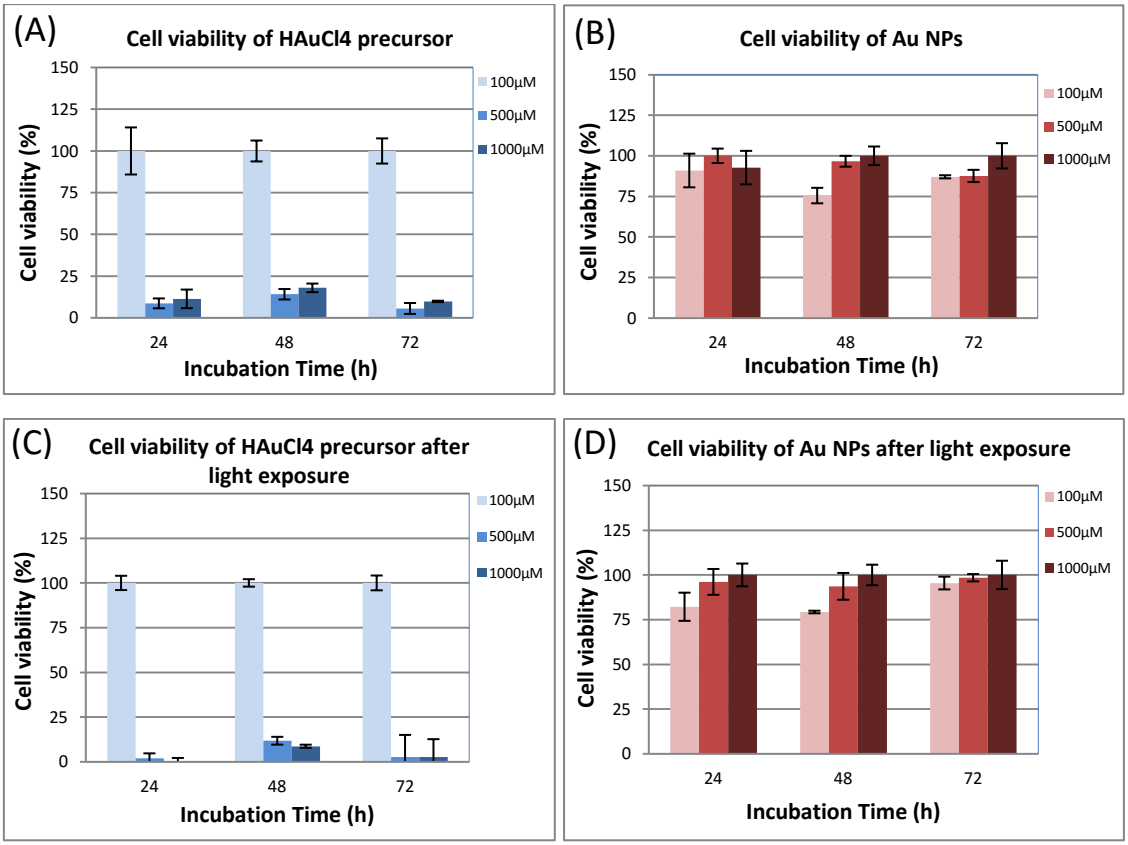


Figure 2.16.: Cytotoxicity assay of $HAuCl_4$ precursor **(A)** and the corresponding pre-synthesized citrate-stabilized gold nanoparticles **(B)** at identical concentrations (100µM, 500µM and 1000µM) and incubation times in HeLa cells (24h, 48h and 72h). Figure **(C,D)** show the cytotoxic effects of gold drugs after one hour of white light exposure of gold-treated HeLa cells. Bars in graphs are result of tree replicas measurements. Graph D is marked with a signal (*) that means a non-legible measurement of the condition of 250µM of gold nanoparticles incubated for 24h in HeLa cell.

Previously to tetrazolium dye MTT reduction, analysis of HeLa cells can be done by optical microscopy of samples (Figures 2.17.). Anomalies in cell morphology can be detected and correlated to the cytotoxicity results above explained in Figure 2.16.

In the case of $HAuCl_4$ precursor, a colour change of the cell medium is experienced at 24h of incubation. It occurs before and after exposure of HeLa cells to light, changing the colour medium from brownish to purple at concentrations of 500µM and 1000µM

of gold precursor (Figure 2.17.-A). This colour change of cell medium, can be correlated with the loss of cell viability experienced by MTT assay at the same concentrations.

Related to HeLa cells exposed to pre-synthesized gold nanoparticles, a similar brownish colour is maintained in cell medium at 24h of incubation for all concentrations of gold nanoparticles tested (100µM, 500µM and 1000µM). It occurs before and after 1hour of light exposure to cells. Results, presented in Figure 2.17.-B., can be directly correlated to the cytotoxicity assay performed (Figure 2.16.) where cell viability maintained stable around 70-100% for all conditions.

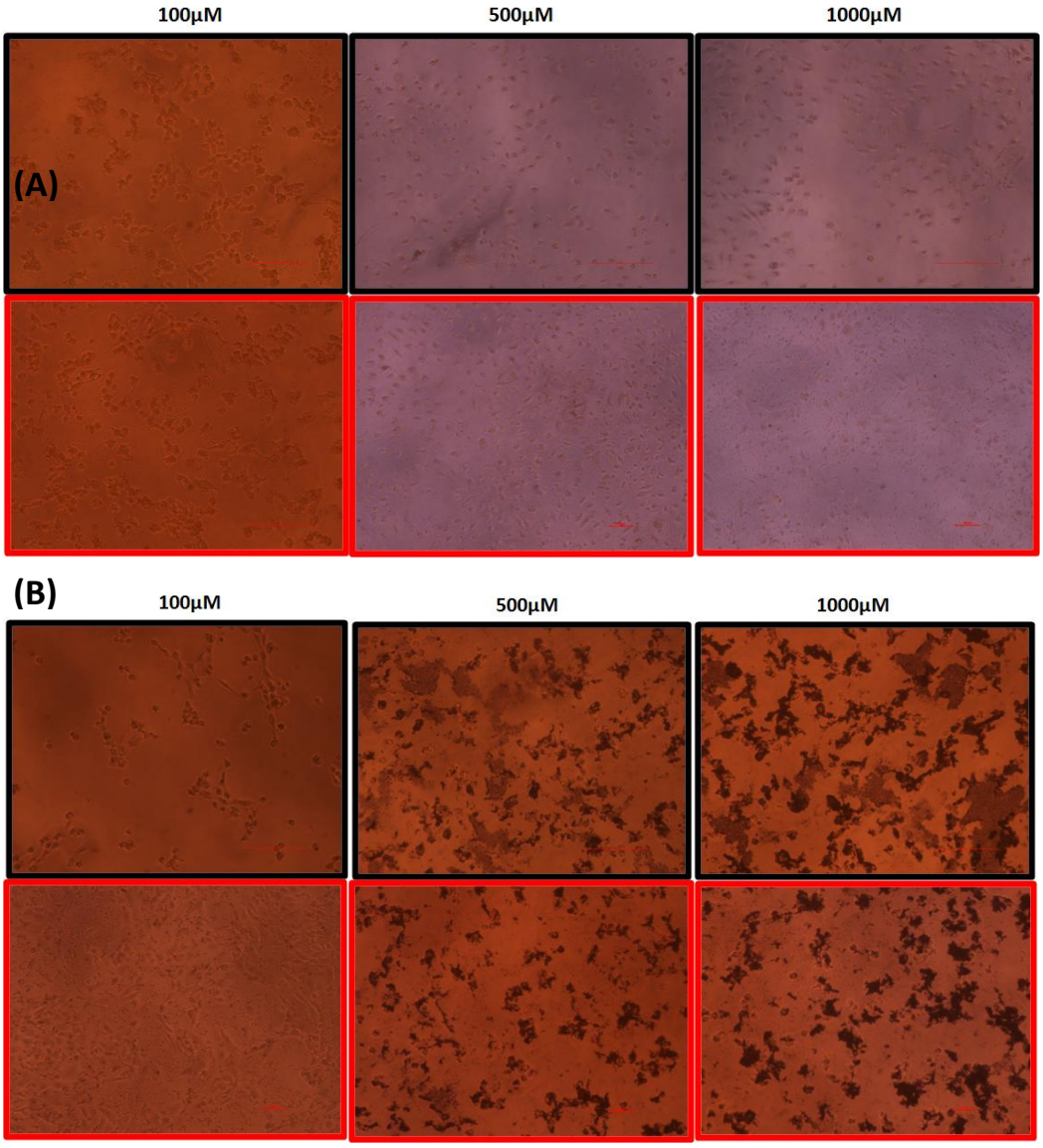


Figure 2.17.: HeLa cells exposed to $HAuCl_4$ precursor **(A)** and gold nanoparticles **(B)** at 24h of incubation and concentrations of 100µM, 500µM and 1000µM. Black and red frameworks means before and after light exposure respectively.