

UNIVERSITÀ DEGLI STUDI DI MILANO

FACOLTÀ DI SCIENZE E TECNOLOGIE
DOCTORATE SCHOOL IN CHEMISTRY



DEVELOPMENT OF GOLD BASED NANO-SYSTEMS FOR BIOMEDICAL APPLICATIONS

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Cycle XXIX; Academic year 2016/2017

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Chapter 1:

Introduction

1. Nanotechnologies and nano-medicine

Nano-science is a widespread research field, characterized by strong multidisciplinary nature. It involves several disciplines like: quantistic physic, supra-molecular chemistry, material science and molecular biology. Nanotechnology is a branch of nano-science, which is recently attracting an ever-growing interest. It combines the knowledge deriving from nano-science with cutting-edge technologies, allowing the production of materials, systems and devices at the nanometric scale. The nano-materials production can be classified in two main approaches known as top-down and bottom-up. The top-down approach consists in the employment of physical methods to reduce macro-metric materials to the nanometric scale. Typical techniques of the top-down approach are the electron beam and X-ray lithography, widely employed in nano-electronics and nano-engineering. In the bottom-up procedures, instead, small components (e.g. atoms, molecules or aggregates of molecules) are used as building blocks and assembled to realize both organic and inorganic nanostructures. This approach is distinctive of the chemical based processes. The application of the nanotechnologies to the medicine is known as nano-medicine. Several diseases are due to modifications of biological processes which take place at the nanometric level. For example, they can be induced by genes mutation, uncorrected protein folding, viruses or bacteria. All these pathogens share the nano-metric scale or are protected by nano-structured barriers. By sharing the same dimension range, nano-materials demonstrated to have a fine interaction with these pathogens. The goal of nano-medicine is to exploit the chemico-physical properties of these materials for the diagnosis and therapy of disease at the molecular level. In fact, nano-materials can be designed to transport therapeutic agents through the biological barriers, to mediate the molecular interactions or to identify diseases and tumoral cells at early stages. Exploiting the advantages of nano-materials, a new generation of drug delivery agents, contrast media and diagnostic devices are in development (**Figure 1**). Up to now, several nano-materials has been subjected to preclinical experimentation and some of them have been approved by FDA for the clinical use. Nano-materials, differently from the bulk, own a large surface/volume ratio and peculiar optical, electronic and magnetic properties, which can be designed by finely tuning the size, shape and chemical composition. Moreover, nano-materials can be passively or actively targeted, depending on their application in nanomedicine. The passive targeting is based on the EPR (Enhanced Permeability and Retention) effect. For example, the blood vessels growing around the tumour posses an endothelial tissue characterized by enlarged pores and windows (up to 200 nm), that allows the extravasations of the nanoparticles and their accumulation in the tumoral tissue (**Figure 2**). Moreover, the lack of lymphatic drainage resulted in a prolonged permanence of the nanoparticles in these tissues. The active targeting, instead, consist in the functionalization of the nanoparticle with bioactive molecules that can be recognised by receptors over-expressed on the site of interest, allowing an accumulation in the target tissue. Some polymeric nano-materials have been used for the controlled release of drugs or active principles. By employing polymeric matrixes, it is possible to encapsulate millions of drug molecules inside a single particle, inducing a controlled release activated by endogenous and exogenous inputs.

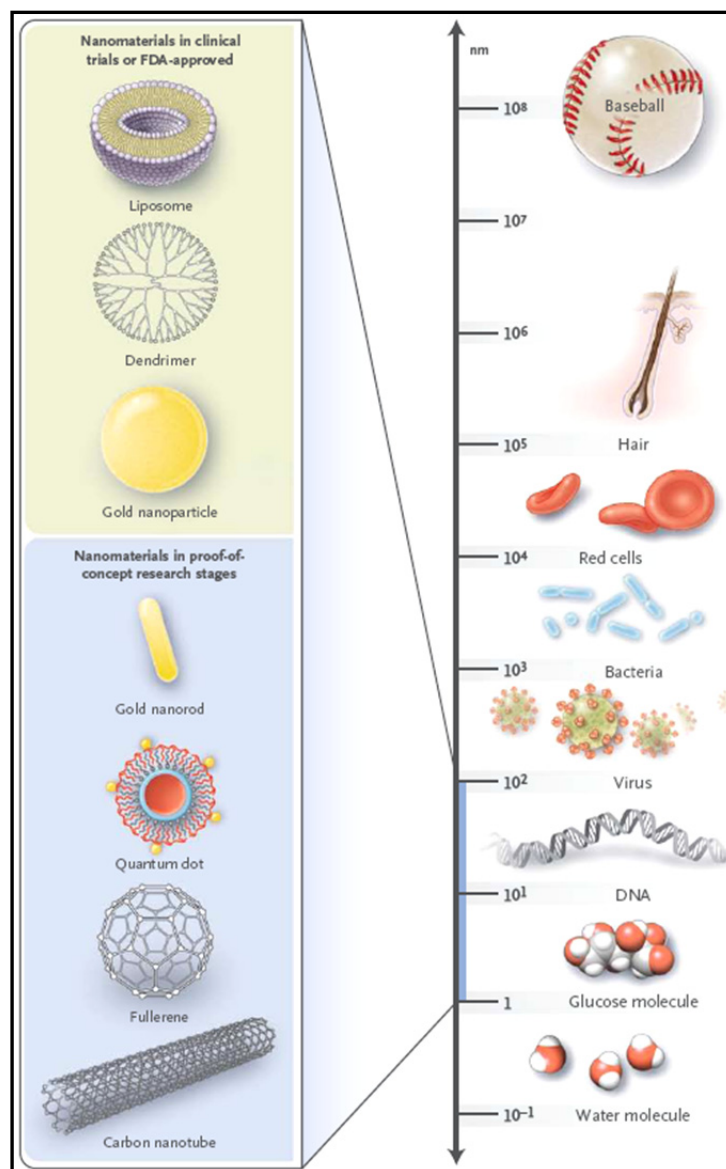


Figure 1. Examples of nano-materials employed in nano-medicine

Another field of great interest in nano-medicine is the nano-formulation of hydrophobic drugs. This practice gives several advantages, by avoiding the employment of toxic solvents or surfactants that can induce severe side effects. The most known example is relative to paclitaxel, which conventional formulation contains elevated concentrations of cromophor-EL (a solvent responsible of severe side effects at the neurological level). Nab-paclitaxel is one of the first approved drugs based on nano-materials, and it consists in paclitaxel molecules entrapped in albumin nanoparticles. In this specific case the employment of the nano-formulation allows, not only to avoid the employment of toxic solvent, but as well to double the maximum dose for a single treatment.

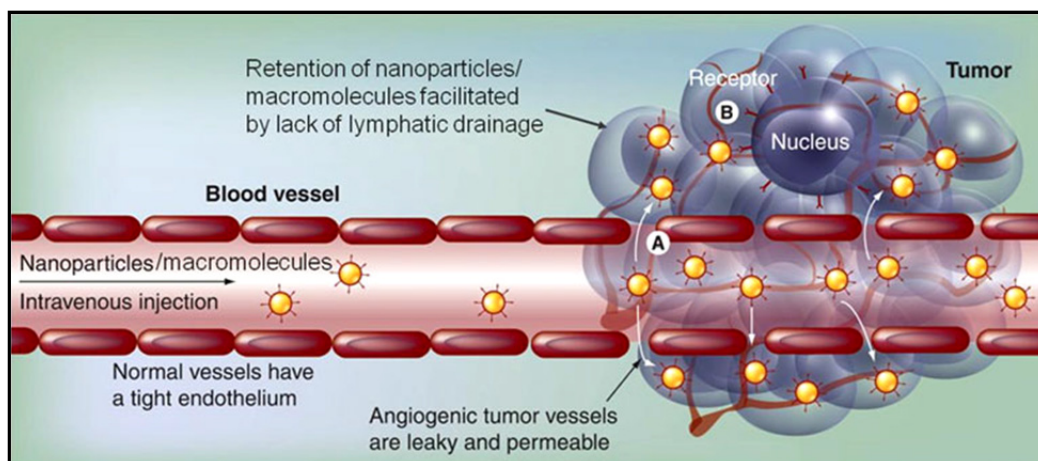


Figure 2. EPR (Enhanced Permeability and Retention) effect in carcinoma tissues

2. Gold: a precious metal for nano-medicine

Gold nanoparticles (Au NPs) always fascinated the scientific community, demonstrating several applications in nano-medicine. This is due to the peculiar properties that this metal owns at the nano-metric scale. The most known property is the localized surface plasmon resonance (LSPR). This phenomenon consists in a collective oscillation of the free electrons of the metal, induced by the light impinging on the Au NP. The LSPR process can be classified in two types of interaction between the light and the matter. The first one is the scattering, where the incident light is re-radiated in different direction with the same frequency. The second one is the absorption, in which the light is converted in heat. As effect of this process the incident light is strongly attenuated and intense electric fields are generated in the proximity of the Au NPs surface. This electric fields are of great interest because can be employed to enhance fluorescence of Raman signals close to the NPs surface. The surface plasmon resonance of Au NPs is dependent from a vast number of parameters like size, shape, morphology and the environment surrounding the NPs¹. By varying this parameters LSPR can be tuned though all the visible and near IR range. In biomedicine, the most interesting nanostructures posses a LSPR peak shifted in the near-infrared region. In fact at these wavelengths light can penetrate in the soft tissues, due to low absorption of water and blood and to the reduced scattering deriving from the soft tissues². A strategy to effectively tune the LSPR peak in the IR region is to carefully control the morphology and anisotropy of the nanoparticles. Gold nanostars, nanocages and nanorods are classical example of nanoparticles showing an intense absorption in this optical window. When NP dimension is comparable to the gold Fermi wavelength, the nanoparticles acquires photoluminescence properties². These small clusters have a diameter of approximately 0.5 nm. When they reach a diameter of 1.2 nm (comprised of 55 atoms), no longer exhibits photo-luminescence³. Bigger clusters (333 Au atoms, 2,2 nm of diameter) start to show a LPRS peak around 520 nm. Further increasing the dimension of the nanoparticles, the LSPR firstly increase its absorbance intensity and then starts to slowly move to higher wavelengths. Spherical nanoparticles with a diameter of 100 nm posses a maximum LSPR wavelength around 560 nm⁴. Beyond the optical properties gold nanoparticles possesses as well plenty of interesting properties for biomedical application. First of all, gold is characterized by a strong inert nature, resisting to the air oxidation and corrosion⁵. This chemical non-reactivity is correlated to a bio-inert nature of the metal that makes it an outstanding candidate for the

development of *in vitro* and *in vivo* devices^{2,4}. The reduced short term *in vitro* toxicity of Au NPs has been widely documented. Studies on different cellular lines highlighted the not toxicity of this nanoparticles when administrated in therapeutic doses and adequately coated with inert stabilizing molecules⁶⁻¹⁰. Majors concerning are related to the long term *in vivo* nanotoxicity of Au NPs. The toxicity is highly dependent from the administration route¹¹, NPs dimensions¹² and the coating^{13,14}. Numerous studies revealed that several days after the injections mice present accumulation of the NPs in spleen and liver, loss of body weight, spleen atrophy and anaemia¹⁵. Once again, this effects are strongly dependent from the coating of the NPs and more evident for “bare” and citrate coated NPs^{13,14}. Despite the great inertness of this noble metal, Au can form stable bonds (184 kJ/mol), with sulphur containing compounds, like thiols or disulfides^{16,17}. Exploiting this kind of chemistry is possible to easy and robustly functionalize Au NPs with different types of polymers, bio-molecules or targeting moieties. In this way engineered interfaces can be designed to specifically interact or to remain inert with the biological systems. One example is the employment of thiolated poly(ethylene glycol) (PEG) as biocompatible polymers allowing to prevent the nonspecific interactions with the biological matter, like proteins or lipids^{18,19}. The protein absorption, taking place during the *in vitro* and *in vivo* studies is a fundamental factor to consider during the design of the Au NPs. In fact, the adsorbed proteins can significantly modify the identity of the designed NPs and thus their interaction with biological systems. Moreover, PEG can minimize the *in vivo* uptake of Au NPs by the reticuloendothelial system (RES) allowing a prolonged circulation in the blood pool. The low interaction with bio-macromolecules and the reduced uptake by the RES organs are frequently correlated phenomena, generally known as “stealth” properties. Gold nanoparticles can be functionalized as well with active targeting moieties that can bind to specific receptors, over-expressed on the cancer cell membrane. In this way it is possible to increase the NPs concentration in a desired region, obtaining a more focused treatment or the early detection of the disease²⁰. Considering all these advantageous properties, it is easy to understand why gold attract a strong interest as multi-functional material applicable in biomedicine. In fact, its properties can be easily tuned modifying the size, shape, structure and superficial chemistry, maintaining an elevated biocompatibility.

3. Synthesis of Au NPs

3.1 In batch synthesis: Turkevich and Brust-Shiffrin methods

The most known and classical approach to synthesize Au NPs is the batch reduction of gold salts. These kinds of procedures are widely diffused because are easily approachable and does not require particular instruments to be performed. One of the most employed metal precursor is the tetrachloroauric acid (HAuCl_4), containing Au^{3+} ions, that can be reduced to Au^0 by a plenty of reducing agents. Some of these reducing agents are sodium citrate (Na_3Ct), sodium ascorbate, ascorbic acid (AA), hydroquinone and sodium borohydride (NaBH_4). The NPs synthesis is generally divided into nucleation and growth stages. In the early instants of the reaction the nucleation phase takes place, with the formation of the nanoparticle nucleus. During the growing stage, the reduction of the metal precursor is favoured on the surface of the preformed crystals, inducing their growth.

The first method for the production of colloidal gold was reported by Turkevich in the 1951.²¹ The method was further improved by Frens in the 70's, and consist in the reduction of tetrachloroauric salts in boiling water²². Experimentally the procedure is extremely simple, and consists in the addition of the Na_3Ct to a boiling aqueous solution of the gold precursor. Using this procedure, it

is possible to synthesize nanoparticles with diameters comprised between 10 and 50 nm, by varying the ratio between Na_3Cit and HAuCl_4 . In fact, sodium citrate not only plays the role of reducing agent, but caps as well the surface of the nanoparticles, resulting in the stabilization of the crystals and preventing uncontrolled growth and aggregation. High concentration of citrate allows to rapidly cap the nanoparticles by stopping their growth at dimensions comprised between 10 and 20 nm. Instead, a low concentration is reflected in an incomplete capping of the NP surface, leading to their aggregation and the formation of bigger and irregular NPs. In spite of the easy experimental procedure, the chemical mechanism at the base of the crystal formation in the Turkevich method is quite complicated (**Figure 3**)²³.

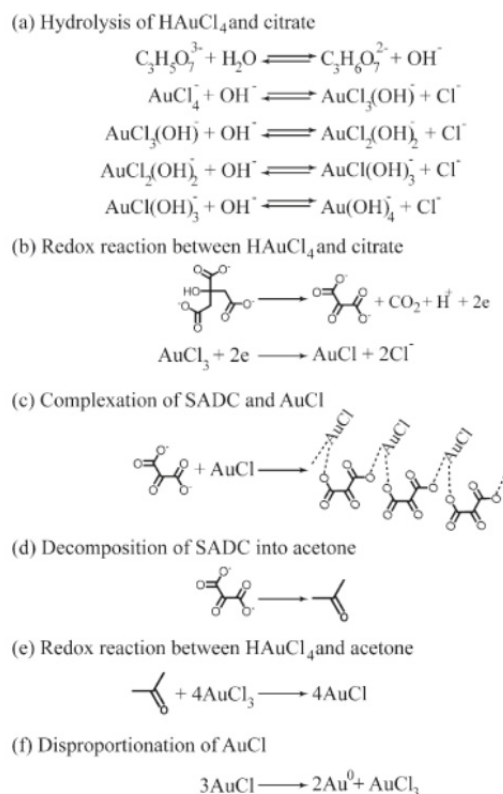


Figure 3. Mechanism involved in the Turkevich method for the synthesis of Au NPs. Reprinted with permission from {Wang, D., Xia, H. & Bai, S. *Langmuir* **26**, 3585–3589 (2010)}. Copyright {2010} American Chemical Society

When dissolved in water the AuCl_4^- ions are hydrolysed in 3 different types of ionic precursors. Depending on the pH, it is possible to obtain: $\text{AuCl}_3(\text{OH})^-$ (pH 6.2), $\text{AuCl}_2(\text{OH})_2^-$ (pH 7.1), $\text{AuCl}(\text{OH})_3^-$ (pH 8.1), and $\text{Au}(\text{OH})_4^-$ (pH 12.9) ions. The reactivity of these intermediates decreases in the order: $\text{AuCl}_4^- > \text{AuCl}_3(\text{OH})^- > \text{AuCl}_2(\text{OH})_2^- > \text{AuCl}(\text{OH})_3^- > \text{Au}(\text{OH})_4^-$. After the addition to the boiling solution, sodium citrate is oxidized to sodium acetone dicarboxylate (SADC) and, contemporaneously, the auric precursors are reduced to AuCl . As already presented by Turkevich in the 50's, SADC and AuCl can be coordinates in macromolecular complexes, which are colloidal stable precursors of the nanoparticles. The disproportionation of AuCl allows the formation of Au^0 . The subsequent collision among free gold atoms is responsible of the nuclei formation. Instead, during

the growing stage an auto-catalytic disproportionation of AuCl is catalysed by the just formed nuclei. In parallel to this mechanism, a side reaction takes place. At elevated pH, SADC can rapidly decompose in acetone, which can reduce the gold ionic precursors leading an undesired secondary nucleation. This secondary pathway should be avoided, as responsible of the broadening in the nanoparticle size distribution. From the mechanistic studies, the pH of the reaction resulted to be a fundamental parameter in the synthesis outcome, improving the sphericity and size distribution of the NPs. After the addition of Na₃Ct to HAuCl₄ the pH increases, due to the formation of a buffer effect. This factor can induce a un-homogeneous nucleation leading to a temporal superimposition between the nucleation and growing stages. To minimize the citrate buffer effect, Xia et al. modified the Frens procedure, by adding a premixed solution of citrate and tetrachloroauric acid to the boiling water. In this way, at the addition time, only the most reactive gold precursor (AuCl₃(OH)⁻) is formed, due to the lower pH of the reaction solution²³. In these conditions is avoided the un-homogeneous nucleation due to the presence of different ionic gold species. Moreover, at this pH the secondary nucleation due to the reduction of AuCl by acetone is avoided resulting in an overall sharper NP size distribution. Ji et al. studied, as well, the influence of the pH on the gold nano-crystals formation, founding out that, at pH higher then 6.5, the mechanism seems to be consistent with the classical crystal growth²⁴. Instead, at pH values lower then 6.5 (coinciding with the Frens reaction conditions) the formation of the nanoparticles is comprised of three steps: nucleation, casual aggregation of the nuclei, and finally intra-particle ripening or smoothing (**Figure 4**).

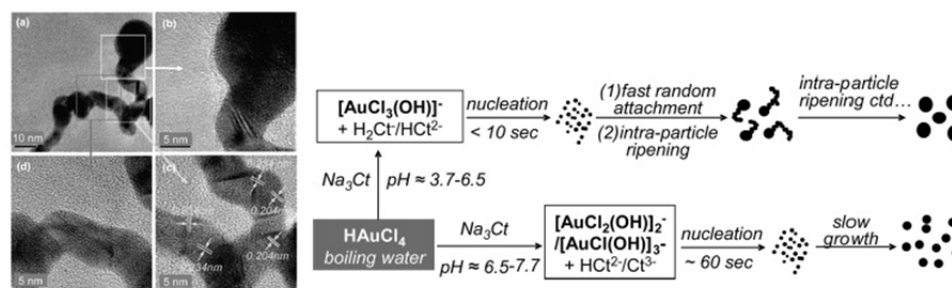


Figure 4. On the left: TEM micrographs of the nano-wires obtained during the synthesis at pH lower then 6.5. On the right: The two pH dependent pathways involved in the Au NPs synthesis. Reprinted with permission from {Ji, X. *et al. J. Am. Chem. Soc.* **129**, 13939–13948 (2007)}. Copyright {2007} American Chemical Society

At this pH, an aggregation of the formed nuclei in nano-wires can be observed. The formation of this structure is responsible of the greyish colour assumed by the colloidal solution during the initial stage of the reaction. By heating, the nanoparticles are rearranged to a spherical shape by mean of a ripening process. This process is leaded by the minimization of the superficial energy and to the obtainment of the most thermodynamically stable specie.

Recent studies demonstrated that Ag⁺ ions can catalyse the oxidation of the citrate to SADC in the presence of light²⁵. In this way the time resolution between the nucleation and growing steps can be enhanced and it is possible to obtain a sharper NP size dispersion²³. Moreover, the nanoparticles prepared in presence of AgNO₃ shows a uniform quasi-spherical shape. The enhanced isotropy of the nanoparticles can be explained by the silver under potential deposition shift on the gold crystal faces, that is respectively of 0.12, 0.17 e 0.28 eV for the [111], [100] and [110] faces²⁶. Basing on this theory, the silver ions should be preferentially reduced on the [100] and [110] faces of the NPs²⁷. Once deposited, silver can be oxidized again and exchanged with gold atoms, slowing down the growth of the crystal and enhancing its uniformity and spherical shape.

Exploiting the under potential deposition process (UDP), it is possible to modify the Turkevich method to obtain the production of anisotropic star shaped Au NPs. In fact, the employment of different reducing agents (like ascorbic acid or hydroquinone) and the increased concentration of the silver nitrate, can enhance the UDP effect inducing a more selective and stronger shielding of the [110] faces. The growing of the free faces can consequently lead to the formation of branches and star shaped Au NPs (**Figure 5**)^{28,29}.

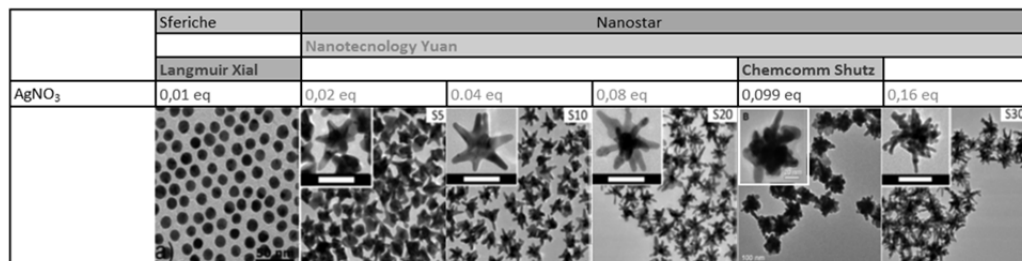
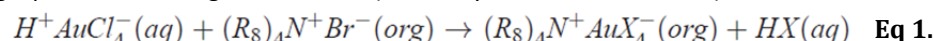
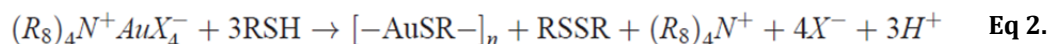


Figure 5. Dependence of the shape from the AgNO₃/HAuCl₄ ratio.

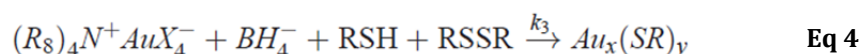
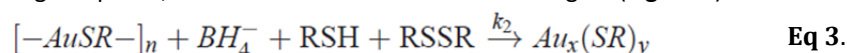
Another widely diffused method for the production of gold nanoparticles is the Brust-Shiffrin method (BSM), ideated in the 90'³⁰. With this method, using a stronger reducing agent (NaBH₄), it is possible to obtain spherical Au NPs with a diameter comprised between 3 and 8 nm. The produced nanoparticles are directly capped by mean of aliphatic thiols, showing elevated colloidal stability. In this case, the NP dimensions are modulated by the amount of aliphatic thiol present in solution. For example, in absence of thiols, the nanoparticles can reach a diameter of 8 nm. Instead, with a 2 : 1 proportion of thiol : HAuCl₄, 2 nm nanoparticles can be obtained. The BMS is performed in a biphasic system were HAuCl₄ and the gold ions are transferred in toluene employing a phase transfer agent like TOAB (Tetraoctylammonium bromide).



Then the two phases are separated and the aliphatic thiol is added to the organic one, reducing Au³⁺ to Au¹⁺.



The organic phase is now comprised of: TOAB, disulphide, Au¹⁺ in the polymeric form $-(AuSR)_n-$, and AuX₄⁻ complexes (with the TOA ions and the excess of RSH). A second aqueous phase containing the reducing agent is mixed with the organic phase. Sodium boride is transferred from the aqueous to the organic phase, where reduces Au¹⁺ ions to metallic gold (**Figure 6**).



As well, gold nanoparticles have been successfully prepared, in organic solvents, by using oleylamine (OAm) as reducing and stabilizing agent³¹. The protocol is relatively simple and involves the use of only two reagents: the oleylamine and the gold precursor (Gold(III) acetate) reacting at elevated temperatures. This procedure leads to the formation of 6-10 nm Au NPs. Although OAm-capped Au NPs are hydrophobic, they can be turned into hydrophilic by a ligand-exchange procedure that involves the replacing of OAm with thiolated water soluble ligands.

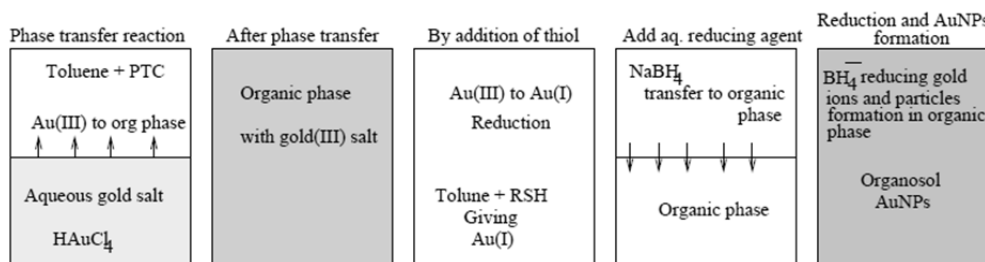


Figure 6. Synthesis of gold nanoparticles with Brust-Schiffrin method in biphasic system.

3.2 Synthesis in flow condition

Typically, batch processes are strongly dependent from operational parameters, like the sequence of addition and the mixing efficiency³². Hence, their major drawbacks are the lack of reproducibility and the problematic scale-up. Instead, flow chemistry provides an excellent control over reagent mixing and the reaction parameters. This is a promising technology to produce Au NPs in a reliable, reproducible and scalable way. Unlike conventional stirred tank reactors, the mixing among reactants takes place in micro-second time scale. This gives the opportunity to implement a fine control over the mixing process allowing to increase the reproducibility. Other advantages of the fluidic chemistry are: the efficient mass-and-heat transport, the control on the reaction time and the spatial separation between the nucleation and growing stages. By using fluidic chemistry, it is also possible to achieve a high-throughput production of the nano-materials employing several in parallel reactors. In the end an in real-time and on-line quality control is feasible, interfacing the fluidic reactor with analytical techniques.

In the last 10 years a plenty of scientific reports has been published on the in-flow synthesis of gold nanoparticles.³² The first reported case was published by Wagner et al in 2004.³³ The process consisted in the growth of preformed seeds inside a micro-reactor. The chip was constituted by two micro-mixers and a reaction zone. In the first micro-mixer gold seeds with an average diameter of 12 nm were blended with an ascorbic acid (AA) solution, acting as reducing agent. After the first mixing, a solution of HAuCl_4 was added, starting the reduction of the gold salt on the seeds surface. At 1:1 molar ratio between seeds and gold salts, Au NPs with a mean diameter of 24 nm were obtained. However, the nanoparticles were characterized by poor homogeneity and dimensional distribution. One year later the same authors published the first complete Au NPs synthesis employing a micro-fluidic reactor.³⁴ In this case they employed a continuous flow micro-reactor to perform the synthesis of Au NPs directly from the gold precursor and the reducing agent (AA). Tuning the experimental parameters (flow rate, pH, AA and PVP concentrations) the authors were able to modulate the dimensions of the NPs from 5 to 50 nm. Starting from these first attempts of NPs micro-fluidic synthesis the techniques were improved and more complex synthesis afforded, obtaining several remarkable results. In particular, numerous hybrid nano-systems, comprising gold combined with other metals, were synthesized using micro-fluidic reactors. For example, Au@Ag core-shell NPs were obtained in micro-fluidic conditions by Kölher et al. in 2007.³⁵ The employed reactor was comprised of two micro-mixers: in the first one HAuCl_4 solution was mixed with AA to generate gold seeds. Then, when AgNO_3 solution was introduced in the second micro-mixer, a silver shell on the preformed seeds was created, thanks to the preferential reduction of the metal precursor on the gold surface. Gomez et al. in 2012 published the synthesis of SiO_2 @Au nanoparticles employing a Teflon milli-reactor with an internal diameter (ID) of 1.3 mm.³⁶ In first instance an ethanol solution of silica precursor was mixed with ammonia solution at 40 °C obtaining amino-functionalized silica particles. The obtained nanoparticles were

isolated and purified before the second step in which gold seeds, with a mean diameter around 2–4 nm, were attached onto the SiO₂ particles. The obtained gold decorated SiO₂ NPs were further treated with HAuCl₄ solution using formaldehyde as reducing agent in order to obtain a homogeneous gold shell on the NPs surface. Another remarkable example of nanoparticles fluidic production was described by Gomez et al. The authors described for the first time the continuous synthesis of hollow Au NPs employing a PTFE (polytetrafluoroethylene) reactor with tubes ID of 1.6 mm.³⁷ In a first step, a cobalt precursor was mixed with sodium borohydride producing cobalt NPs. In a second mixer, the gold salts were introduced and the metal precursor was reduced on the surface of cobalt particles which were contemporaneously dissolved by mean of a galvanic replacement process. The obtained hollow Au NPs were functionalized in-situ with a thiolated polyethylene glycol.

In the field of anisotropic and shape controlled nanoparticles, several works were published on the fluidic synthesis of gold nano-rods.^{38,39} Recently, Lohse et al. developed a milli-fluidic system for the controlled high throughput synthesis for a variety of functionalized nanoparticles (spherical and rods like Au).⁴⁰ However the investigation concerning the synthesis of star shaped gold nanoparticles in fluidic environment is still a lacking field. The only example of fluidic production of branched anisotropic nanoparticles was reported by Ishizaka et al.⁴¹ The authors obtained flower-like nanoparticles, by adjusting the pH of a gold precursor solution and employing glucose as reducing agent. However, up to now, the production of high aspect-ratio gold nano-stars has never been performed in fluidic conditions.

Recently a big innovation in the field of fluidic production of nano-materials has been introduced with the employment of the phase segmented flow. Multiphase flows can be created through mixing in a controlled way two or more immiscible fluids. Among the several advantages that phase segmented flow can ensure, the most interesting are the formation of transverse convection and recirculation inside the drops. Moreover the employment of this technique prevents the direct contact between the NPs and the inner wall of the micro-channel avoiding the undesired fouling and clogging of the reactor.^{42,43}

3.3 Metal Vapor Synthesis

Metal vapor synthesis (MVS) is a technique applicable to the production of metallic colloids, consisting in the evaporation of bulk metals (at reduced pressure) by mean of joule effect or electronic beam⁴⁴. The formed metal vapours are allowed to co-condensate with weakly coordinating organic solvents vapours, on the walls of a glass reactor cooled at -196 °C (**Figure 7**).

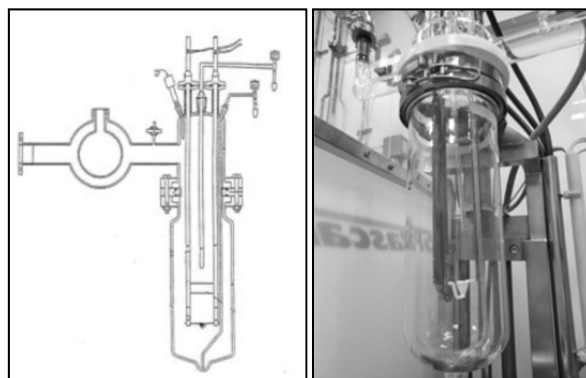


Figure 7. Schematic representation (left) and picture (right) of a static MVS reactor.

When the solid matrix is allowed to warm up to $-54\text{ }^{\circ}\text{C}$, the NPs nucleation and growing processes take place. Once the matrix is completely melted, a metal colloidal suspension is obtained. This suspension, called SMA (Solvated Metal Atom) solution, is comprised by extremely reactive NPs, stabilized only from the weak interaction with the solvent. This interaction is so weak that these nanoparticles can be considered “naked” or “ligand free”⁴⁵. Adding ligands with stronger affinity for the metal (like thiols in the case of Au SMA), it is possible to obtain nanoparticles that presents colloidal stability at room temperature. The SMA solution can be also deposited on a vast pool of organic and inorganic supports for the preparation of solid supported catalyst (**Figure 8**).

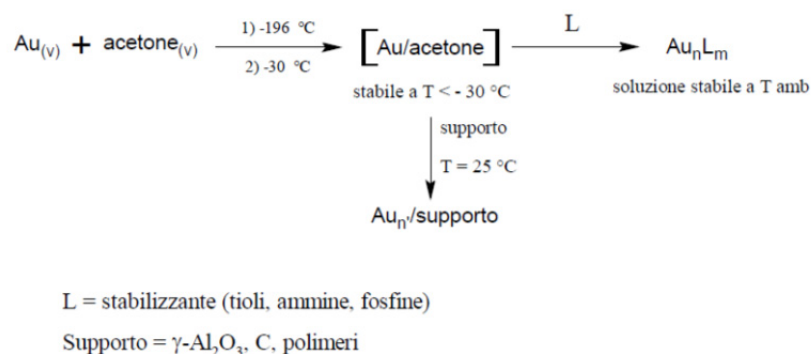


Figure 8. Schematic representation of the MVS synthetic process.

One of the advantages in employing this technique is the possibility to produce large amounts of SMA solution, evaporating up to 1g of gold for each day. A variation of the gold concentration in the SMA solution is reflected in a modification of the dimension of the nanoparticles^{46,47}. The colloids synthesized at lower gold concentration posses a sharp size distribution with diameters comprised between 2 and 10 nm. On the other side the solutions characterized by higher gold concentration own broader size distributions with diameters comprised between 4 and 20 nm and a significant fraction of metal clusters bigger than 10 nm.

It has been demonstrated by Klabunde and co-workers, that is possible to enhance the size distribution of concentrate Au SMA solution by using a digestive ripening process (know as well as nano-machining). This process consists in the heating or refluxing SMA solution in presence of an excess of organic soluble ligands with strong affinity for the metal (like long chain aliphatic thiols)⁴⁸. In fact, concentrated SMA solutions are often polycrystalline and characterized by the presence of crystal interfaces, edges and defective sites. In the digestive ripening conditions, it is thermodynamically possible that some ligands could remove atom or clusters of atoms from a NP surface moving and depositing them on other NPs. Under the effect of this process broad distributions of nanoparticles become narrow both by increasing of small NPs as reducing the larger ones, achieving finally an equilibrium size. This process allows to prepare monodisperse Au NPs in high amount. The choice of ligand, solvent, concentration, temperature and time are crucial for the success.

4. Biomedical applications of Au NPs

4.1 Optical sensing

As presented in **Section 3**, Au NPs shows a large number of optical properties that can be exploited in the development of a variety of biosensors for the detection of small molecules, bio-macromolecules (DNA or proteins) or metallic ions. In the following paragraphs a list of the most diffused sensing technologies based on gold nanoparticles is summarized.

4.1.1 Sensing based on photoluminescence

Gold clusters thanks to their peculiar photoluminescence properties can be employed as optical probes for the detection of bio-molecules. These metal clusters possess enhanced photo-stability and biocompatibility, in comparison to fluorescent dyes and quantum dots. Recently a vast number of sensors based on the gold cluster photoluminescence, were developed for the detection of metal ions^{49–51}, proteins^{52–55}, glucose and other small molecules^{56–58}.

A photoluminescence sensor can be based on two diverging mechanisms: the “turn off” and “turn on” detections. The “turn off” approach is characterized by a decreasing in the signal intensity in presence of the analyte. This decrease can be induced by two main factors: the interaction of the analyte with the ligand shell or the destruction of the gold clusters. An example of “turn off” approach based on the interaction with the ligand has been reported by Wen et al. Horse radish peroxidase (HRP) was employed as ligand for the preparation of sub-nanometric Au clusters, employable for the H_2O_2 detection⁵⁶. When H_2O_2 interacts with the HRP, is induced a change in the protein conformation. As result, the protein changes its interaction with the cluster and a decrease in fluorescence intensity can be registered. A similar sensor for proteases detection has been developed using proteins stabilized clusters⁵². When the enzyme breaks down the protein shell surrounding the cluster, the fluorescence intensity of the system decreases. This variation can be correlated to the analyte concentration (**Figure 9a**).

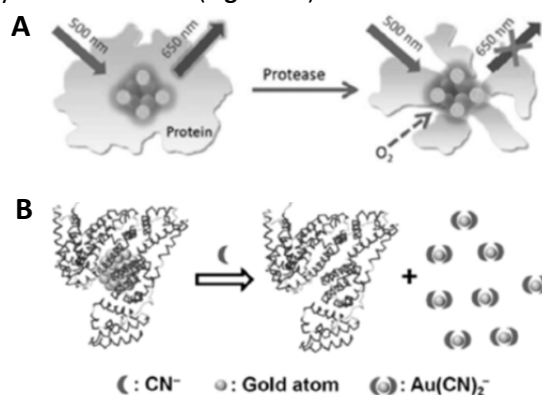


Figure 9. Two examples of biosensors based on the turn off photoluminescence process for the detection of protease (**a**) and cyanide ions (**b**). Reprinted with permission from {Xuan Y. *et al. Chem. Rev.* **115**, 10410–10488 (2015)}. Copyright {2015} American Chemical Society

A sensor, based on the “turn off” approach by destruction of the metallic core, was developed for the detection of cyanide ions (CN^-)⁵⁹. CN^- is able to dissolve the gold core, by complexing the metallic atoms and causing a diminishing in the photoluminescence signal (Figure 9b)⁶⁰. The detection of this ion is of great importance, as it presents in vivo elevated toxicity, inhibiting the

electron transport chain in the mitochondria, due to the strong affinity for the $\alpha 3$ cytochromes. The “turn on” approach, on the contrary, is based on the detection of an increasing in the signal intensity in presence of the analyte. In this case the increase in photoluminescence can be due to the interaction of the metallic cluster with strongly affine molecule that are adsorbed on the gold surface. For example, this approach has been employed for the development of glutathione (GSH) sensor that once bound can significantly enhance the fluorescence of histidine coated gold clusters⁶¹.

4.1.2 Sensing based on LSPR

The local surface plasmon resonance of gold nanoparticles can change as results of variation in the aggregation state of the NPs. In fact, when the inter-particle distance is bigger than the particle diameter the colloidal solution is characterized by red colour. Instead colloidal solutions with inter-particles smaller than the NPs diameter assume bluish shade. This phenomenon takes origin from the coupling of LSPR effects of individual nanoparticles, and can be exploited for the design of bio-sensor. Two different phenomena can be used for the detection: the controlled aggregation of discrete Au NPs, or the dissolution of preformed aggregates. If an aggregation process takes place after the addition of the analyte, the LSPR peak will shift to longer wavelengths, inducing a colour change to blue shades. In presence of some analytes, instead, aggregates of the Au NPs can be as well broken down shifting the plasmonic peak to lower wavelength. Even if this sensing method has been employed for vast numbers of analytes (e.g. metal ions, small molecules and proteins)^{62–64}, its main application is the detection of unfolded complementary DNA strands^{65–67}. This type of sensing is relative simple and versatile but presents two main draw backs: the elevated cost of the materials (gold and nucleic acids) and the low sensitivity.

4.1.3 Sensing based on SERS

SERS based sensors can be classified as well in two different groups. The first one is comprised of label-free assays, where the analyte SERS peaks can be exploited for its identification and quantification. In the presence of gold nanoparticles, the Raman scattering intensity of the analyte can be amplified of 10^{14} order of magnitude. This amplification is even more accentuated when NP aggregates or branched crystals are employed, thanks to the formation of hot spots near to the metal surface⁶⁸. A SERS tag can be obtained attaching to the NP surface a molecule with an intense and distinguishable Raman pattern. Thanks to the distinctive Raman signature, these SERS tags can be employed for ultrasensitive and simultaneous detection of several bio-molecules. For example identification of different antigens was demonstrated employing a sandwich immunoassay format and Raman labelled Au NPs functionalized with the corresponding antibodies (Figure 10)⁶⁹.

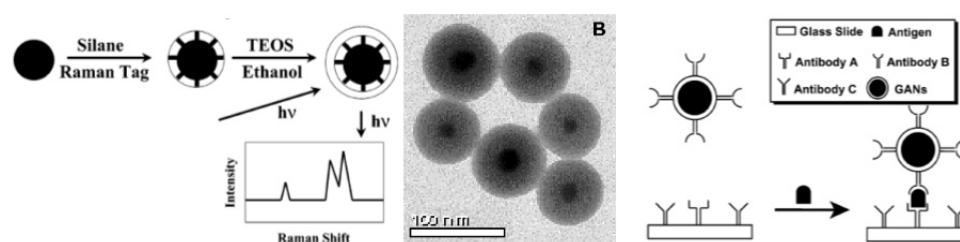


Figure 10. Sandwich based SERS assay for the multiplex detection of different antibodies {Mulvaney, S. P. *et al. Langmuir*. **19**, 4784-4790 (2003)}. Copyright {2003} American Chemical Society

The sensitivity of these types of sensors is higher if compared with the LSPR and photoluminescence based devices, but once again the cost for the production is high and, in addition, it is complex to apply SERS for quantitative analysis.

4.1.4 Sensing based on photoluminescence quenching

Gold nanoparticles are excellent fluorescent quenchers due to the broad energy bandwidth and the high molar extinction coefficient. The fluorescent quenching is dependent from the distance between the fluorescent specie and the gold particle; closer is the dye to the nucleus stronger is the quenching effect. By attaching organic chromophores (donors) to the Au NPs (acceptor) surface is possible to build Förster resonant energy transfer (FRET) systems. Murray and co-workers reported FRET based assay for the identification of different metal ions. When $[\text{Ru}(\text{bpy})]^{3+}$, a known fluorophore, is complexed with gold nanoparticles, its fluorescent is quenched⁷⁰. The employment of some types of electrolytes can allow to dissolve the complex, restoring the fluorescence signal deriving from $[\text{Ru}(\text{bpy})]^{3+}$. As well enzyme sensitive probes has been developed for FRET assays⁷¹. A gold nano-cage and a dye molecule were linked together by mean of an enzyme cleavable peptide. When associated to the nanoparticle, the dye fluorescence was efficiently quenched by mean of the FRET effect. In presence of protease, the peptidic linker is cleaved and the dye fluorescence is recovered to the original photoluminescence. By employing different combinations of peptide linkers and fluorescent dyes this approach can be transferred, in line of principle, to *in vivo* determination of enzymatic activity.

4.2 Imaging

4.2.1 Imaging based on photoluminescence

As discussed before gold clusters with sub-nanometric dimensions posses excellent photoluminescence properties. For these reason this type of nanoparticles are interesting candidates for cellular and *in vivo* imaging modalities based on fluorescence. Their bio-inert behaviour and the stable photoluminescence are great advantages exploitable for cellular imaging. Anyway such small clusters suffers of a reduced uptake by the cells⁷². Several studies have been focused on the enhancement of the internalization degree of these structures, by the superficial functionalization with active molecules^{73,74}. For example, gold clusters have been functionalized with cell-penetrating peptides (e.g. NLS, SV40 Nuclear-localization signal)⁷². The modified clusters were able to penetrate the cell membrane and were visualized, by mean of confocal laser microscopy, in the cytoplasm and the nucleus of the cellular structures. Other biomolecules exploited to enhance the internalization of gold nano-cluster were streptavidin, folic acid and D-penicillamine^{46,47}. In addition to the cellular imaging, the gold nanoparticles photoluminescence can be employed as well to visualize the NPs *in vivo* studies. Wang and co-workers demonstrated that BSA coated gold clusters can be employed *in vivo* photoluminescence, by distinguishing their signal from the organic matter background⁷⁵. Moreover, Zheng and co-workers developed glutathione (GSH) coated NPs for NIR photoluminescence imaging, studying their bio-distribution in the body and clearance pathways⁷⁶. The interesting photoluminescence and optical properties of gold nanoparticles allowed to develop a wide number of imaging modalities for their optimal visualization. These modalities can be classified in three main categories: single, two and three photons excitations. In single photon photoluminescence (SPPL) the gold nanoparticles are excited with UV or visible light. The low energy and power density applied in this technique allows to have a low photo-toxicity level, but this modality cannot be used *in vivo* imaging, due to the high

absorption of blood and tissues in the employed wavelength window. Two photon photoluminescence (TPPL) exploit a NIR laser to excite the gold nanostructure. The advantages of this modality are several. First of all, the non-linear dependence related to the excitation intensity allows to have a 3D spatial resolution. Moreover, the use of a NIR laser ensures a deeper tissue penetration and allows to easily separate the signal from the auto-fluorescence. Furthermore, this modality allows, to perform qualitative imaging and also to acquire information on the gold concentration in the region of interest. Nevertheless, this imaging modality exhibits some drawbacks. In order to generate signal with a detectable intensity, it is required the use of femto-seconds pulsed laser with a relative high power intensity. Additionally, when the laser excitation overlaps with the LSPR peak, photothermal conversion can take place, damaging the tracer and the cells. Finally, the background auto-fluorescence with this technique is frequently strong. Three photons photoluminescence (3PPL) can answer to the problems involving both the auto-fluorescence and photothermal toxicity employing an excitation source operating at even higher wavelengths (e.g. 720 nm). In this way is avoided the super imposition with the LSPR peak and the photoluminescence generation is more effective. Apart from the mentioned advantages, due to the participation of a three photons process, 3PPL suffer of a low quantum yield. Concluding, the choice of the right methodology for the photoluminescence imaging of gold nanostructures, derives from the right compromise between signal intensity, photothermal toxicity and penetration depth.

4.2.2 Imaging based on elastic light scattering

The nanoparticles scattering cross sections grows proportionally to the increasing of their dimensions⁷⁷ and NPs bigger than 15 nm present a strong scattered intensity. It is noteworthy that the light scattered by Au NP can be more than 1 million times intense than the fluorescence emitted by a dye⁷⁸. For this reason, the employment of the elastic scattered light for the visualization of the Au NPs is an attracting concept. Dark field microscopy can be used to record the light elastic scattered by gold nanoparticles, showing a single NP detection ability in the case of nanorods, nanoboxes and nanocages. A halogen lamp is used as source of light and the central area of illumination is selectively blocked, collecting only the light scattered from the sample. As the Au NPs possess the maximum scattering cross section at the LSPR peak wavelength, this technique can also be improved employing nanoparticles with different LSPR peaks, obtaining in this way a colour coding of the signals. Irudayaraj and co-workers applied this technique to map the biomarker distribution on the cellular membrane⁷⁹. Each biomarker has been targeted with different aspect ratio gold nanorods, simultaneously tracking 3 different types of signals. Generally, this type of imaging is suitable only for *in vitro* cellular studies as the absorption and scattering deriving from soft tissues is extremely high, preventing an *in vivo* application of the technique.

4.2.3 Imaging based on inelastic light scattering

The inelastic light scattering, known as well as Raman scattering, can give structural information on the involved analyte, furnishing a characteristic fingerprint for the identification of functional groups or molecular species. With the use of commercially available Raman confocal microscopes, the Raman spectra can be precisely registered in different position of 2D or 3D organized structures. The drawback of this technique are the long exposure times required to register a single Raman spectra, and the subsequent requirement of several hours to obtain a single mapping⁸⁰. As discussed in **Section 3**, by employing Au NPs, the Raman signal can be amplified of several orders of magnitude, enhancing the sensitivity and reducing the exposure time required

for the technique. Moreover, thanks to the progresses in the instrument design, SERS images can be now acquired at high speed, allowing an in real time tracking of drugs or Au NPs inside the cell. Kawata and co-workers, in 2008, developed a slit-scanning Raman microscope, and used it to study the macrophage endocytosis process for 50 nm spherical nanoparticles, with elevated spatial and temporal resolution^{81,82}. Conjugating the gold nanoparticles with functional groups is possible to obtain Raman active molecular probes. With this aim, Kneipp and co-workers used aggregates of p-MBA-coated Au NPs for pH sensing, monitoring the intensities of p-MBA bands in different intracellular compartments⁸³. Raman probes based on gold nanoparticles has been developed as well for *in vivo* imaging demonstrating tumor targeting abilities⁸⁴. Nie et al. labelled 60 nm PEGylated spherical Au NPs with malachite green (a widely employed Raman responsive molecule) and employed this nano-system as effective *in vivo* SERS imaging agent⁸⁵. The gold based nano-tags were subcutaneously injected in the flank of nude mice, registering a detectable signal by mean of Raman spectrophotometer. Conjugating these nano-tags with EGFR-antibodies fragment is possible to selectively target the anti-EGFR, over-expressed in a wide number of cancer cells. Gambhir and co-workers demonstrated the multiplex imaging of SERS nano-tags in live mice^{86,87}. They produced different types of nano-tags, labelling the gold surface with different Raman responsive molecules and subsequently covering them with SiO₂ and PEG covering shells. The *in vivo* biodistribution of the nano-tags has been monitored by mean of Raman spectroscopy, with the possibility to distinguish between the fingerprints of the different labelling molecules. As the shape of the nanoparticles is a fundamental factor in the efficiency of the SERS effect, recently several types of anisotropic nanoparticles (nanostars, nanorods, nanocages) are emerging as important candidates for the production of SERS active nano-tags.

4.2.4 X-ray CT imaging

Computed Tomography (CT) is a worldwide diffused imaging technique based on X-rays. It allows to obtain, in a rapid and economic way, anatomic and diagnostic information. CT is an advantageous technique to visualize bones and mineralized tissues in the body, but frequently its resolution is affected by the lack of natural contrast in soft tissues. Consequently a good contrast can be only appreciated between hard and soft tissues, and for distinguish among soft tissues is required the use of exogenous contrast agents⁸⁸. Nowadays the most diffused CT contrast agents are iodinated compounds, that are effective in enhancing the X-ray attenuation within the soft organs, but do not allow to have targeting capabilities resulting in perfusion of the contrast and feeble edges⁸⁸. Au possess high X-ray absorption coefficient, due to its elevated electron density and atomic number. For this reason, Au NPs play a key role among the candidates for the future CT contrast agent generation. Röntgen, soon after the discovery of X-rays, observed for the first time the attenuation properties of gold. In fact, from an X-ray image of his wife hand he noticed the strong contrast deriving from her golden ring. Smilowitz and co-workers, in the 2004, for the first time, used citrated stabilized Au NPs as *in vivo* CT contrast agents⁸⁹. Following this paper, a variety of functionalized Au NPs and more complex nanostructures has been employed to investigate the bio-distribution and contrast enhancement in tumoral models. For example, gold nanoparticles were entrapped inside PAMAM dendrimers⁸⁸, creating hybrid nanostructures with a high attenuation ability and several superficial groups available for the conjugation with active molecules. The efficiency of this systems has been further improved by entrapping in the dendrimeric structure ditriazolic acid (a iodinated aromatic compound)⁹⁰. Gold nanoparticles were as well superficially modified with heparin polysaccharides, to selectively target the epithelial Kupffer cells inside the liver⁹¹. As well, glucose⁹² and folic acid⁹³ were employed to functionalize the NPs, achieving a selective targeting of tumoral cells. The main drawback of X-ray CT, is its lack

of sensitivity, that requires the employment of elevated doses of Au NPs to obtain appreciable contrast enhancements. Actually, the research interest is focused in the development of multimodal contrast media or theranostic agents, combining the imaging ability with thermal and radiotherapies. Different multimodal agents for CT/MRI (magnetic resonance imaging) have been reported in literature. They can be classified in two main groups on the base of the magnetic responsive specie. In the first class the gold nanoparticles are conjugated to gadolinium chelating complexes. In comparison to the free gadolinium contrast agents (employed in MRI) these nanostructures presented reduced toxicity and the possibility to contemporaneously acquire MRI and CT images. The second class is comprised by hybrid nano-materials, characterized by the co-presence of gold and iron oxide nanoparticles. Moreover, the employment of core shell $\text{Fe}_2\text{O}_3@\text{Au}$ NPs allows to exploit the Au biocompatibility and its affinity with thiols to achieve a stable superficial functionalization. Furthermore, it is possible to target the gold nanoparticles with I^{25} , a radioactive specie emitting γ radiations at low energy with a half-life of 60 days. These systems can be contemporaneously visualized by PET and CT, monitoring the long-term bio-distribution. Finally, anisotropic nanoparticles can be used as SERS/CT contrast agents, combining the elevated X-ray absorption with the previously described peculiar optical properties.

4.3 Drug delivery

Different types of drugs have been loaded on the Au NPs directly exploiting the Au-S and Au-N chemistry⁹⁴, grafting it to the capping polymer⁹⁵, by electrostatic interaction⁹⁶, hydrogen bonding⁹⁷ or van der Waals forces⁹⁸. The most direct way to load a drug on a gold nanoparticle is to exploit the strong soft-soft interaction between Au and S or N, introducing NH_2 or SH groups in the drug structure. Linking silicon phthalocyanin, a drug under clinical investigation for photodynamic therapy, to PEGylated Au NPs has been obtained an efficient vector that can strongly improve the delivery efficacy of this hydrophobic drug⁸⁵. Both nitro and thiol linked phthalocyanin have been tested, pointing out a more efficient drug release in the first case, thanks to the lower stability of the Au-N bond⁹⁹. Drugs can be loaded on the NPs as well by grafting on the capping ligands. Paclitaxel was loaded on gold nanoparticles grafting the drug to the 4-mercapto-phenol¹⁰⁰. Mirkin and co-workers as well attached a Pt(IV) pro-drug to the carboxylic groups of DNA strands, acting both as active principle and as stabilizing ligand¹⁰¹. The inactive pro-drug, once internalized by the cell, can be reduced to release cisplatin and transported by the DNA strands in the nucleus, killing the cancer cells. The electrostatic interaction has been frequently employed to charge on CTAB gold nanorods (positively charged) negatively charged single strand DNA (ss-DNA) or small interfering DNA (si DNA)¹⁰¹. Layer by layer (LbL) approach is a more sophisticated version of the electrostatic interaction used to create multiples layer on the Au NPs surfaces^{102–105}. This approach is easy and versatile and can allow to incorporate different types of charged molecules. Typically the LbL technique can be employed to deliver si-RNA entrapped between shells of reversed polyelectrolytes like PEI (poly(allylamine hydrochloride)) and PAH-Cit (Citraconic anhydride)⁸⁷. This kind of nanoparticles could protect the si-RNA from the enzymatic degradation, improving its uptake in HeLa cells thank to the PEI shell acting as polycationic transfection agent. Thanks to the versatility of the methodology a vast number of polyelectrolytes has been screened to cover the Au NPs. It is also possible to exploit the van der Waals forces to load drugs on a gold nanoparticle. For example, Murphy and co-workers used CTAB coated nanorods to deliver 1-naphthol, exploiting the bi-layer formed by the capping agent to sequester the hydrophobic model drug¹⁰⁶. As well doxorubicin has been loaded on DNA coated Au NPs using the van der Waals forces between the drug and nucleic acid¹⁰⁷. Finally, the hydrogen bonds have been mainly used to load and deliver DNA or RNA strands using nanoparticles functionalized with oligonucleotides. Gold hollow

nanostructures, like nanocages, are ideal candidates for the drug delivery because of the possibility to increase the loading efficiency exploiting the hollow interior. Temperature sensitive polymers, like poly(N-isopropylacrylamide), can be employed to entrap the drug inside the NPs cavity preventing its leakage^{108,109}. At temperatures lower than the critical point the polymer is hydrophilic and soluble in water, and the fully extended chains will prevent the leakage of the drug. When the temperature is higher than the critical value, the chains will collapse, allowing the drug to diffuse out from the cavity.

5. Aim of the thesis

Considering the attractive Au NPs properties and their vast number of application in the biomedical field, it is easy to understand the interest of the scientific community toward this type of nanoparticles. By tuning the Au NPs size, shape, coating, labelling and active targeting it is possible to obtain designed platforms acting as therapeutic, diagnostic or theranostic agents. In the present thesis are investigated three fundamental aspects correlated with the employment of gold nanoparticles in biomedicine.

As described in **Section 4.2.4**, Au NPs, thanks to the high density and atomic number, present an elevated X-ray absorption coefficient. These NPs, if properly functionalized, can act as effective CT contrast agent and can be easily visualized by mean of *in vivo* micro-CT analysis. In **Chapter 2** is reported a methodology for the synthesis of highly stable and functionalized Au. The presented method allowed us to tune the surface coatings and the morphology of the Au NPs, designing a “one-pot” synthesis of engineered isotropic and anisotropic nanoparticles¹¹⁰. The present study is devoted to elucidate the major factors involved in the *in vivo* biodistribution of PEGylated Au NPs. The effects of NPs structural parameters (eg. charge, shape and dimension) on the circulation time in the blood pool were analysed. From this study, we derived interesting information, generally applicable to the design of different types nano-structured contrast agents. Furthermore, other two fundamental topics involved in the design of effective nano-structured contrast agents has been investigate: the nanoparticles renal clearance and the active targeting toward inflamed tissues.

In **Chapter 3** is investigated the interaction of surface functionalized Au NPs with the biological matter. When nanoparticles come in contact with biological materials, bio-molecules are adsorbed on their surface. This phenomenon causes a modification the identity of the systems and a uncontrolled aggregation of the NPs. The investigation of this phenomenon is fundamental to understand both the intracellular dynamics and the physiological behaviour of the nanoparticles. It is essential to consider these factors in the design of nano-systems effectively applicable in the biomedical field. In particular, in the present study, Fluorescence Correlation Spectroscopy (FCS) has been exploited to acquire information on the diffusion times of surface functionalized Au NPs, both in protein solution and in living cells.

In **Chapter 4** is investigated a more technical issue, related to the nanoparticles synthesis. In order to think to a reliable application of the nanotechnologies in the biomedical field, innovative synthetic procedure needs to be identified, to ensure a scale up and higher reproducibility of the production. A fluidic manufacturing method for the production of structurally controlled Au NPs was developed. Fluidic chemistry is a promising technology that can address to the scale up and reproducibility issues that affect the nano-materials production. The presented manufacturing

method demonstrated to be strongly versatile, allowing the one-pot production of nano-materials with controlled shape, and engineered surface, readily applicable in a vast number of fields.

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Chapter 2:

Development of gold based hybrid nano-systems as functional CT contrast agents.

1. Introduction

Nanoparticles are an important and emerging class of materials with peculiar features suitable for biomedical imaging¹⁻³. NPs can be employed to strongly amplify endogenous signals (e.g. SPIONS in magnetic resonance imaging)⁴ or induce exogenous ones (e.g. fluorescent NPs in fluorescent imaging or Au NPs in Computed Tomography)^{5,6}. Moreover, properly functionalized nanoparticles, could selectively enhance the uptake in target cells like macrophages or carcinoma cells, allowing an early stage localization of tumor-genesis and inflammatory processes⁷.

Among the most common clinical imaging techniques Computed Tomography (CT) is one of the most diffused, thanks to its low cost, good resolution, high efficiency and not invasive nature⁸. Nowadays, the wide diffusion of this technique can be attested by the high number of the instruments globally present in the clinical centres (45'000 units) and the impressive number of CT scan performed yearly in the U.S.A. (70 millions). The idea to exploit computed tomography as medical and diagnostic tool was adopted soon after the discovery of X-rays by W.C. Roentgen in 1895. Although this, the technologies needed to realize this intuition were reached only in 1972 when G.N. Hounsfield developed the first machine actually able to perform effective CT scans. Computed tomography is an advantageous technique to visualize bones and mineralized tissues in the body, but frequently its resolution is affected by the lack of natural contrast in soft tissues. In this case, exogenous contrast media are required to fill this lack of contrast, allowing to acquire detailed anatomical information of the region of interest. It is commonly known that materials, that own high density (ρ) and atomic number (Z), can efficiently attenuate the X-ray radiations. This relationship is expressed in equation 1, where μ is absorption coefficient of X ray, A the atomic mass of the element and E is the X-ray energy.

$$\mu = \frac{\rho Z^4}{AE^2} \quad \text{eq.1}$$

This relationship points out that the absorption coefficient is directly proportional to the fourth power of the atomic number. Consequently, to be efficient, CT contrast agent candidates need to be comprised of heavy atoms. Iodine ($Z=53$) is the historically employed atom for CT imaging applications. Sodium and lithium iodides were the first water soluble contrast media employed, but the free iodine toxicity and the high concentration required prevented their clinical diffusion. Currently, the most diffused CT contrast media are aromatic poly-iodinated compounds, characterized by improved vascular permeability and rapid renal clearance⁹. Nevertheless, also this type of contrast agent presents some drawbacks. This disadvantages are mainly referred to allergic reactions, renal toxicity and other side-effects¹⁰. Moreover, high amounts of contrast agent are required in CT imaging compared to techniques like MRI or nuclear imaging. Consequently, the research of an improved class of contrast agents that can be used in reduced amounts is a focal

topic in the last years. With this aim, a series of requirement relative to the design of an optimal contrast media has been identified:

- The contrast media should enhance the attenuation coefficient of the target organ at least of a factor 2 in respect to the surrounding environment.
- The contrast media should contain an elevated amount of the X-ray attenuating element for each unity (molecule, macromolecule or particle) with the aim to reduce the volume and the concentration employed for a single analysis.
- The circulation time and retention time of the contrast media inside the organs should be long enough to allow a complete CT scan of the region of interest.
- The contrast agents should be characterized by an excellent solubility or create a stable suspension with low viscosity in physiological environment
- The contrast agent metabolites should not be toxic
- The contrast media should be eliminated by the organism in a relative short time (indicatively 24 h)

With the attempt to address these requisites, in the last years, several papers described the development of nanostructured CT contrast agents^{11,12}. The chance to tune the nano-materials features, makes nanotechnology a remarkable field for the development of smart contrast agents, characterized by a prolonged circulation time and active targeting through specific sites. Au NPs play a key role among the candidates for the future CT contrast agents generation. In fact, these nanoparticles present high X-ray absorption coefficient, easy surface functionalization and good biocompatibility. To date, a vast number of example of potential CT contrast media based on gold nano-structures has been reported in literature, indicating the great potential of this material^{6,13,14}. The particle size, shape and superficial chemistry strongly affect the performance of these types of contrast media, affecting the circulation time, renal clearance and accumulation in endothelial reticulum (RES) organs. For example, ultra-small Au NPs can be cleared by means of renal excretion, thanks to their dimensions, smaller than the renal glomerulus, avoiding the metal accumulation in the organs^{13,15}. Moreover, thanks to the strong soft-soft interaction between thiols and gold, Au NPs can be stably functionalized on their surface. Exploiting this chemistry, the NP vast surface area can be loaded with several moieties and active compounds, affording selective contrast agents and eventually minimizing the amount of NPs employed.

2. Results and discussion

2.1 Aim of the work

Several are the requirements that an effective nano-structured contrast agent needs to address. In fact, the interaction of nanoparticles with the organism and their *in vivo* behaviour are ruled by factors like: the circulation time in the blood pool, the uptake by the RES organs (etc. liver and kidney), the fate, the NP elimination pathway and their eventual active targeting toward sites of interest³. Methodologies to address these fundamental aspects are currently at the centre of the scientific interest. Up to now different factors emerged as fundamental in this behaviour and among them the dimension, shape, coating and surface chemistry of the NPs are crucial³. A careful design of injectable nano-system should consider all these parameters to identify the best ones. The choice of the synthetic strategy is a fundamental part in the process allowing to obtain the desired nano-material. A good synthetic strategy should be versatile: let to modulate the dimension and morphology of the core and a rapid and to easy functionalize of the surface of the

nanoparticles. Moreover, the synthetic method should be robust of should afford a large amount of nanoparticles for each batch.

In the first part of this chapter it is reported a study devoted to elucidate the major factors involved in the *in vivo* biodistribution of PEGylated nanoparticles. A synthetic method for the synthesis of highly stable and functionalized Au NPs is reported. The presented method allowed us to tune the surface coatings and the morphology of the Au NPs, designing a “one-pot” synthesis of engineered isotropic and anisotropic nanoparticles¹⁶. In particular, next to symmetrical Au NPs, we were interested in the *in vivo* behaviour of anisotropic star shaped gold nanoparticles, which are emerging as innovative material for multifunctional imaging and theranostic applications. In fact, combining the peculiar properties of gold with the optical ones deriving from the morphology, Au nano-stars are an example of multimodal contrast media, responding both to CT imaging and surface enhanced Raman spectroscopy (SERS)¹⁷. The chosen class of coating molecules for this study is hetero bifunctional thiolated PEG, in particular HS-PEG₅₀₀₀-COOH and HS-PEG₅₀₀₀-OMe. This type of polymer thanks to the thiol moiety can be tightly linked to the gold surface ensuring an improved stability to the nano-system. Moreover PEG are known to be highly biocompatible, able to give stealth properties to the nanoparticles, hiding them from the RES system and prolonging their circulation in the blood pool¹⁸.

In the second part of the chapter is described the development of CT nano-tracer based on glucosamide-coated Au NPs. We demonstrated, as proof of concept, that this functional probe is characterized by an active targeting towards inflamed regions and tissues, which present an increased metabolic activity. It is generally known that inflammatory and malignant cells present an increased cellular uptake of glucose (even in aerobic conditions), resulting in an enhanced dependence from the glycolitic pathway¹⁹. Consequently the metabolism of 2-deoxy-D-glucose is an extensively topic studied²⁰ and 2-([¹⁸F]fluoro)-2-deoxy-D-glucose (2FDG) a radioactive analogue widely employed as PET-CT radiotracer for the detection of inflammatory and sites tumours^{21,22}.

Another hot topics regarding biomedical application of metal-based nanoparticles is their fate in the organism after the body injection and the pathway of elimination of the nano-material²³. Trying to address to this issue in the third part of the chapter, is described a synthetic procedure for the production of gold nanoparticles with a reduced hydrodynamic diameter. As the renal glomerulus wall has a pore size of 10 nm, nanoparticles smaller than this size can pass through them, allowing to expel the contrast agents through the urines. In order to improve the renal clearance of the contrast agent, nanoparticles with the 8 nm hydrodynamic diameter were synthesized using a combination between Metal Vapor Synthesis (MVS) technique and aqueous digestive ripening process. This synthetic strategy allows not only to obtain nanoparticles with the desired hydrodynamic diameter but as well to produce them in elevated amount with a good control over the dimensional dispersion. In the end, we demonstrated that this type of contrast agent thanks to the combination between surface functionalization and small hydrodynamic dimensions allows an efficient renal clearance, still maintaining a prolonged circulation blood and a stealth capability.

2.2 Design of functionalized gold nanoparticles for computed tomography imaging

2.2.1 Synthesis and characterization of functionalized gold nanoparticles

A key factor to obtain a reliable gold based contrast agent is the synthetic procedure employed for the production of the Au NPs. In fact, the selected synthetic strategy needs to be easy approachable, economic and robust and should afford large amounts of stable nano-material. In literature is present a vast number of protocols dedicated to the gold nanoparticle synthesis²⁴. The most diffused and employed strategy is the chemical reduction of gold salts in water medium to the zero valence metal, forming a colloidal solution^{23,25}. Among the reductive strategies, the Turkevich method is the most common and flexible protocol, and has been subjected to a wide number of revision and improvements^{26,27}. One of these modified protocols has been published by Schütz et al., reporting the addition of hydroquinone and silver nitrate, to the classical method, in order to modulate the shape of the NPs²⁸. Taking inspiration from the protocol described in this paper, we developed a “one-pot” strategy to synthesize functionalized Au NPs tuning the core morphologies (**Figure 1**). The procedure requires the use of sodium citrate and hydroquinone as reducing agents to convert HAuCl_4 into metallic gold. Employing this protocol is possible to implement a control over the nanoparticles morphology avoiding the use of toxic templating agent. In fact the shape of the nanoparticles is controlled by mean of catalytic amounts of AgNO_3 and no cetyl trimethyl-ammonium bromide (CTAB) has been used, as frequently reported in literature²⁹. Moreover, employing the proposed procedure it is possible to obtain large quantities of functionalized nanoparticles without intermediate purifications of the Na_3Ct stabilized NPs. In fact, the selected ligands can be directly added to the reaction mixture not only to stabilize the nanoparticles but to cap and stop the NPs growing phase too. We choose to employ a seeded-growth strategy to ensure a higher control over the morphology and the dimensional dispersion of the NPs. The seeds synthesis was performed adding to boiling water a pre-incubated mixture of gold salts, sodium citrate and silver nitrate. The nucleation of the NPs was immediate, but to allow the complete reduction of the gold precursor and the homogenization of the gold crystals the reaction mixture was kept under stirring at 100 °C for additional 45 min.

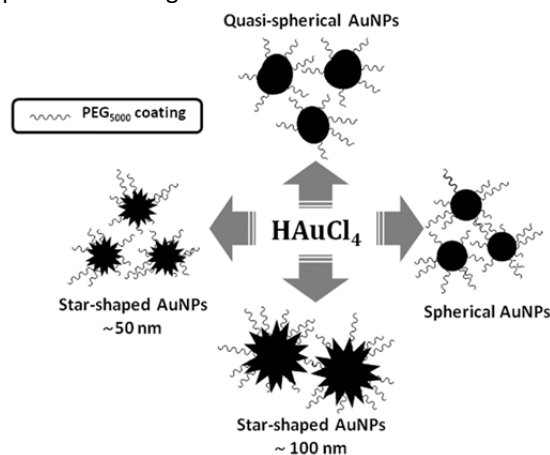
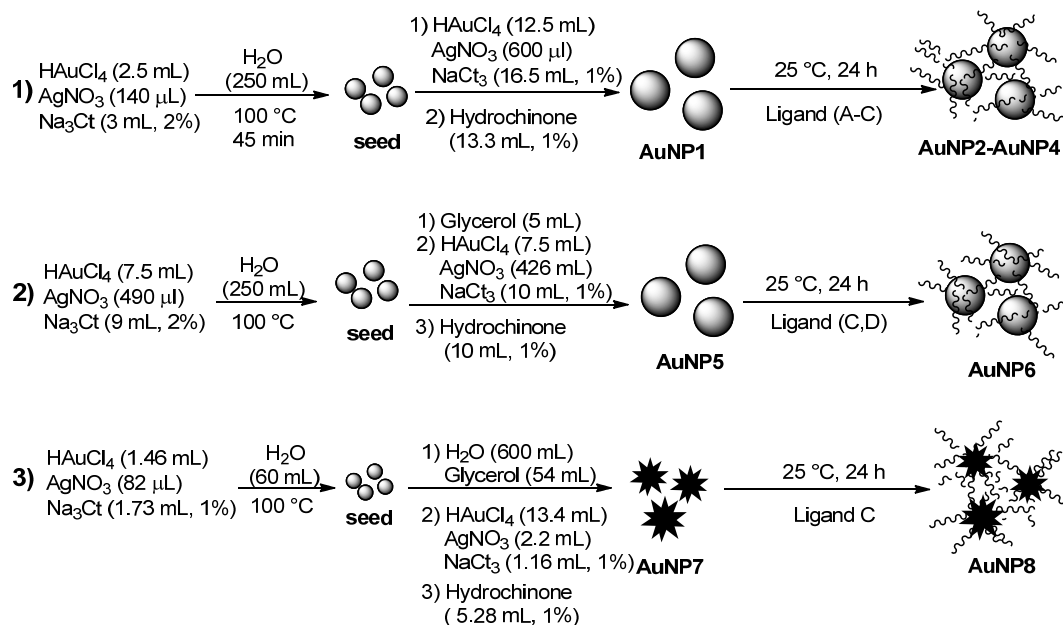


Figure 1. Schematic summary of the synthesized Au NPs morphologies. The NPs were obtained by reduction gold salts (HAuCl_4) and were coated with different kinds of thiolated PEG. The reported dimensions are referred to the size of the cores. Reprinted with permission from {Silvestri A. et al. *Contrast media and molecular imaging*, 405-414 (2016)}. Copyright {2016} Wiley.

The second stage consisted in the grown of the preformed seeds. The seeds solution was cooled down and a pre-incubated mixture of HAuCl_4 , AgNO_3 and sodium citrate was added to the reaction mixture at R.T. Immediately after, a solution of hydroquinone (acting as reducing agent) and Na_3Ct (as temporary stabilizing agent) were added to the reaction mixture. After 1 h of stirring at R.T. the nanoparticles were capped by mean of thiolated PEGs.



Scheme 1. Synthetic procedures employed to obtain the described Au NPs. The concentrations of the working solutions are $[\text{HAuCl}_4] = 10 \text{ mM}$; $[\text{AgNO}_3] = 5.9 \text{ mM}$; The coating employed are: **A**: HS-PEG₅₀₀₀COOH (**Au NP 2**); **B**: HS-PEG₅₀₀₀COOH/HS-PEG₅₀₀₀OMe 1/1 (**Au NP 3**); **C**: HS-PEG₅₀₀₀OMe (**Au NP 4**, **Au NP 6**, **Au NP 9**). Reprinted with permission from [Silvestri A. et al. *Contrast media and molecular imaging*, 405-414 (2016)]. Copyright [2016] Wiley.

For our purpose, positively charged and neutral polymers were screened as ligands (HS-PEG₅₀₀₀-COOH, HS-PEG₅₀₀₀-OMe). The employment of the PEG coating can guarantee improved colloidal stability imparting to our NPs stealth abilities and a prolonged circulation time in the blood pool³⁰. With this type of coating the obtained nanoparticles are stable enough to be concentrated at the level of several mg/mL, concentration needed to perform *in vivo* CT analysis. Moreover, even at these high concentrations, the colloidal solution resulted stable for several months. The proposed synthetic method resulted extremely versatile, in fact, an accurate modulation of reagents amounts, allowed us to obtain Au NPs characterized by different core morphologies: quasi-spherical (**Au NP 2-Au NP 4**), spherical (**Au NP 6**, **Au NP 7**) or anisotropic (**Au NP 9**). A summary of the synthesized nanoparticles comprising of morphology and superficial functionalization is reported in **Scheme 1** and **Table 1**.

Au NP 2, **Au NP 3** and **Au NP 4** are characterized by quasi-spherical shape (**Figure 23 a, b, c**, Section 4.3) differing in the nature of the coating ligand: **Au NP 2** has been coated with HS-PEG₅₀₀₀-COOH, **Au NP 3** with a 1:1 mixture of HS-PEG₅₀₀₀-COOH: HS-PEG₅₀₀₀-OMe, and **Au NP 4** with HS-PEG₅₀₀₀-OMe. The NPs core corresponds to crystalline metallic Au, and has been confirmed by the Electron Diffraction (ED) (**Figure 23 g**, Section 4.3). The addition of glycerol during the

nanoparticle growing stage, ensured an improved control over Au NPs uniformity and dimensional distribution. With this modification in the synthetic procedure it was possible to obtain effectively spherical NPs (**Au NP 6**) coated with HS-PEG₅₀₀₀-OMe. The TEM micrograph of this nanoparticles, characterized by an average diameter of 15.8 nm $\pm$ 3.1 nm, is reported in **Figure 2.a**. Star shaped anisotropic nanoparticles (**Au NP 9**; **Figure 2 b**), were synthesized modulating the reagents ratio during the growing of the preformed seeds as described in **Scheme 1** and have been functionalized with HS-PEG₅₀₀₀-OMe (**Table 1**).

<i>Samples</i>	<i>Morphology</i>	<i>Coating</i>	<i>D_H</i> (nm)	<i>ζ-potential (mV)</i>	<i>[Au]</i> mg/mL	<i>HU in vitro</i>
AuNP2	Quasi-spherical	HS-PEG ₅₀₀₀ -COOH	27.04	-37.64	35.00	3378
AuNP3	Quasi-spherical	HS-PEG ₅₀₀₀ -COOH/ HS-PEG ₅₀₀₀ -OMe	39.03	-29.76	28.12	2304
AuNP4	Quasi-spherical	HS-PEG ₅₀₀₀ -OMe	40.87	-23.00	28.75	2376
AuNP6	Spherical	HS-PEG ₅₀₀₀ -OMe	39.82	-20.00	23.01	2062
AuNP8	Star	HS-PEG ₅₀₀₀ -OMe	52.69	-18.79	22.04	1834
AuNP10	Star	HS-PEG ₅₀₀₀ -OMe	176.30	-17.54	7.67	424

Table 1. Summary of the Au NPs comprehensive of: morphology, nature of the coating, hydrodynamic diameter (D_H), Zeta-potential (ζ -potential), Au concentration and X-rays attenuation values of the colloidal solutions.

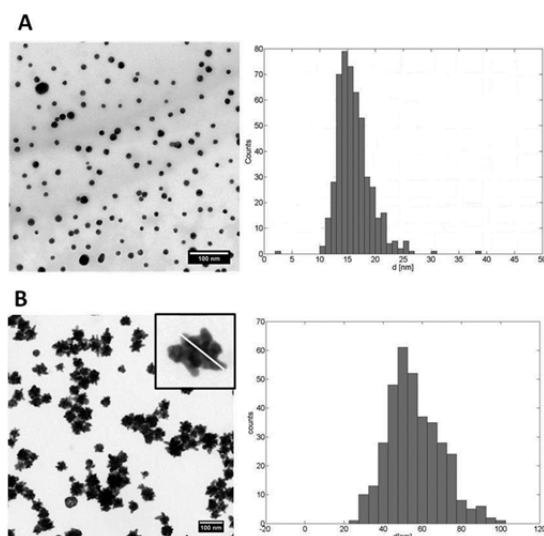


Figure 2. A. Left: TEM image of **Au NP 6**. Right: statistical distribution of nanoparticles diameters: $d_m = 15.8 \pm 3.1$ nm; B. Left: TEM image of **Au NP 8**. Right: statistical distribution of nanoparticles dimension measured as branch to branch distance (see inset) $d_m = 54.2 \pm 13.6$ nm. Reprinted with permission from {Silvestri A. et al. *Contrast media and molecular imaging*, 405-414 (2016)}. Copyright {2016} Wiley.

In the end we evaluated the effect of the dimensions of star shaped **Au NPs**, synthesizing “one-pot” functionalized gold nano-stars employing exclusive hydroquinone as reducing agent³¹. The obtained nanoparticles were characterized by multi-branched shape and enhanced core dimension (**Au NP 11**, Table 1, **Figure 26**, Section 4.9). Finally, the synthesized Au NPs were purified and their morphology, composition, coating functionalization and X-rays contrast ability fully characterized (**Table 1**). The X ray absorption efficiency of the system, reported in **Table 1**, is measured Hounsfield Units (HU), and is referred to *in vitro* values of water colloidal solutions.

2.2.2 *In vivo CT imaging*

The evaluation of the biodistribution of Au NPs in healthy mice was performed by mean of micro CT imaging. The nanoparticles were administrated by mean of tail intravenous injection and the X-rays attenuation values were monitored in different organs. A particular attention was given to the liver and hearth attenuations, evaluating and comparing the stealthiness and blood pool circulation time among the different types of engineered NPs (**Figure 3** and **Figure 4**). Each of the proposed colloidal solutions presented high stability and strong *in vitro* X-rays absorption but once injected in the mouse model it was possible to notice different behaviours related to the structural features of the nanoparticles.

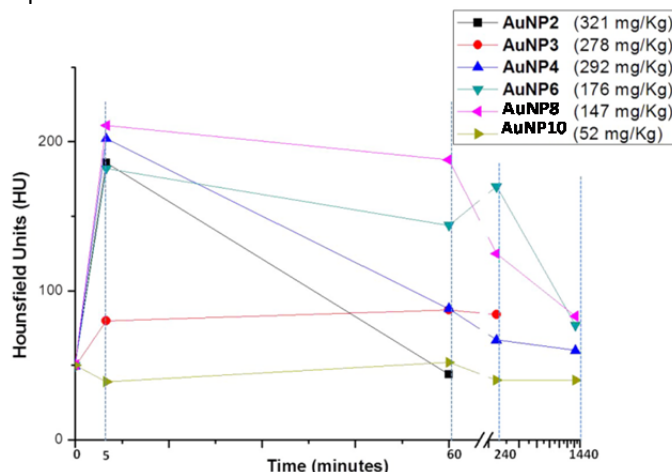


Figure 3. Trend in attenuation values measured within the heart in function of the observation time. In round brackets are reported the doses (mg Au/Kg bw) injected for each experiment. The HU values at 0 minutes correspond to the basal value measured before the injection. Reprinted with permission from {Silvestri A. et al. *Contrast media and molecular imaging*, 405-414 (2016)}. Copyright {2016} Wiley.

In **Figure 4** are summarized the results related to all the nanoparticles comprised in the study expressed in terms of heart/liver X-ray attenuation ratio. Different observations can be extrapolated from the present study. First of all, it has been generally observed that the gold nanoparticles displaying negative charges on the surface of the system (**Au NP 2** and **Au NP 3**), presented a quickly and intense internalization in the liver, due to a rapid recognition of the NPs by the macrophages. In particular, mice treated with these set of nanoparticles clearly presented increasing attenuation values within the liver, 1 hour after the injection. Moreover, the nano-system characterized by the highest hydrodynamic dimension (**Au NP 10**), despite the lack of free charges on its surface, presented a copious uptake by the liver. Our studies confirmed that the

dimension of the nanoparticles to present a reduced recognition from the macrophages and a prolonged circulation time is comprised between 10 and 60 nm. In the end, no effect deriving from the shape and anisotropy of the nanoparticles has been detected. In fact, both spherical and anisotropic nanoparticles presented a prolonged circulation time and very good X-Rays absorption, paving interesting application for anisotropic nanoparticles as theranostic agents or multimodal contrast media. In conclusion, **Au NP 6** and **Au NP 8** were identified as the best systems in terms of prolonged blood pool circulation and low uptake by the liver.

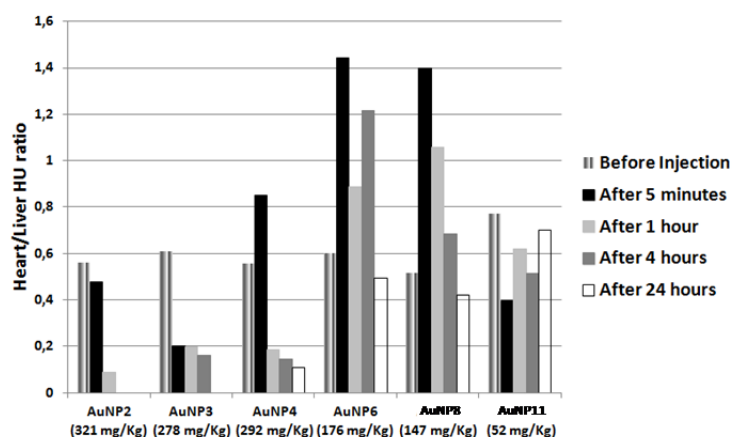


Figure 4. Heart/liver HU ratio measured after 5 minutes, 1, 4 and 24 hours from Au NPs injection. In round brackets are reported the doses (mg Au/Kg bw) injected during each experiment. Reprinted with permission from {Silvestri A. et al. *Contrast media and molecular imaging*, 405-414 (2016)}. Copyright {2016} Wiley.

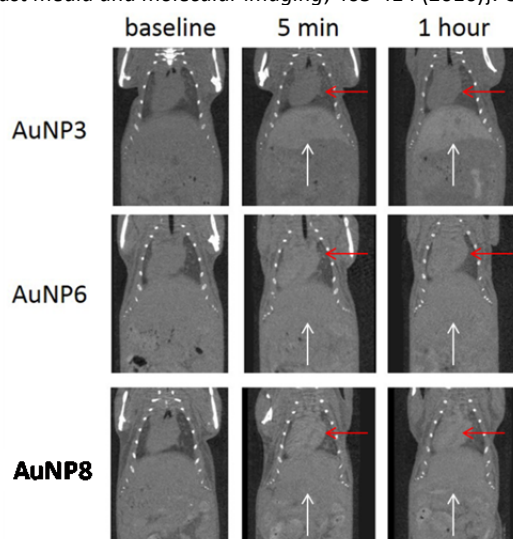


Figure 5. CT scans (coronal section) of mice treated with **Au NP 3**, **Au NP 6** and **Au NP 8**. Baseline: images obtained before infusion. White arrow: liver; red arrow: heart. Reprinted with permission from {Silvestri A. et al. *Contrast media and molecular imaging*, 405-414 (2016)}. Copyright {2016} Wiley.

In **Figure 5** are reported the contrast distribution in coronal sections of live mice, over a time frame of 1 hour. In the scans are represented three mice that received i.v. infusions respectively of: **Au**

Au NP 3, **Au NP 6** and **Au NP 8**. It is possible to appreciate that **Au NP 3** induced a rapid increase of the liver attenuation values without any appreciable variation in the heart contrast. This suggests that these kind of charged nanoparticles are rapidly sequestered by the liver. On the contrary, **Au NP 6** and **Au NP 8** led immediately to an increase within the heart contrast that remained constant up to 4h after the injection, suggesting a persistent circulation of these NPs in the blood pool.

2.3 Development of glucosamine functionalized nano-tracer

One of the most compelling goals in the nano-medicine field is the development of innovative smart agents, able to selectively target specific sites. The active targeting could be advantageously applied to nano-structured contrast agent allowing the production of nano-tracers able to afford not only anatomical and morphological information but also metabolic ones⁷. Once having in our hands an effective and reliable procedure for the synthesis of stable Au NPs, we decided to synthesize Au NPs displaying on their surface glucosamine moieties. We synthesised a thiolated polyethylene glycol bearing on the ω extremity a glucosamine (see Section 4.10) and employed it to coat spherical nanoparticles (**Figure 6b**). These nanoparticle were employed for the active targeting of lung inflamed sites. First of all, the *in vitro* attenuation ability of the system has been measured, resulting of 2453 HU for a gold concentration of 31.20 mg/ml.

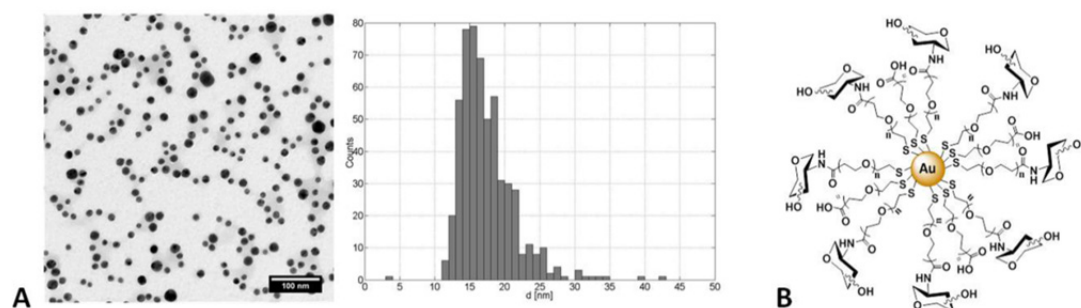


Figure 6. Gold nano-tracer used as CT contrast agent **A)** Left: TEM image of the Au NPs. Right: statistical distribution of nanoparticle diameters: $d_m = 16.6 \pm 3.9$ nm. **B)** Scheme representative of the nano-tracer structure. Reprinted with permission from {Silvestri A. et al. *Contrast media and molecular imaging*, 405-414 (2016)}. Copyright {2016} Wiley.

The *in vivo* behaviour of the synthesized nano-tracer has been tested on mice treated with a HCl solution, to induce a chemical pneumonia and subsequently infused with a colloidal solution of Au NPs. A first mouse has been injected with 200 μ l of nano-tracer solution ([Au]=23 mg/ml). In **Figure 7** are reported the X-rays attenuation values registered within different organs of a representative mouse, describing the biodistribution of the nano-systems. We registered a rapid increasing in the attenuation values within the heart, values that are kept constant up to 4 h after the injection, indicating once again a prolonged circulation time of NPs. The contrast within the lung lesion was continuously increasing during the observation time, reaching at the fourth hour the same attenuation values registered within the heart.

Four hours after the injection, the mouse was sacrificed in order to perform *ex vivo* CT scans on the lungs and the heart of the animal. In **Figure 8 a**. It is clearly visible the accumulation of nanoparticles in the damaged site (red circled region). Three consecutive bronchoalveolar lavages were performed to confirm the presence of the nanoparticles within the damaged site and cells. **Figure 8 b** are reported the TEM micrographs of the biological sample, showing the presence of isolated nanoparticles together with lung tissue fragments. It is remarkable that these

nanoparticles maintained their individuality and shape even after the passage in the blood stream and through the mouse tissues, pointing out the outstanding stability of our nanoparticles. We performed on the TEM images a statistical measurement of the core diameter of the recovered NPs, obtaining an average diameter of 32.5 ± 5.9 nm.

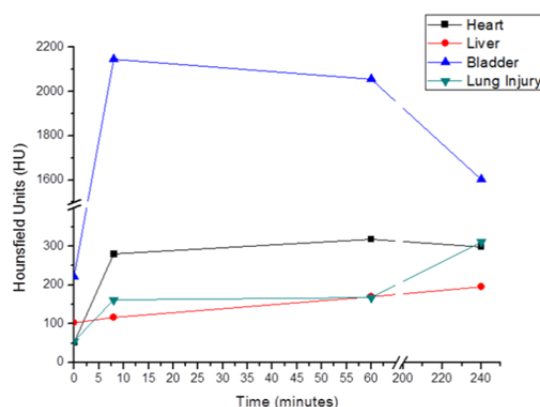


Figure 7. Nano-tracer contrast values registered within the heart, liver, bladder and lung injury in function of the incubation time. The HU values at 0 minutes correspond to the basal value measured before the injection. Reprinted with permission from {Silvestri A. et al. *Contrast media and molecular imaging*, 405-414 (2016)}. Copyright {2016} Wiley.

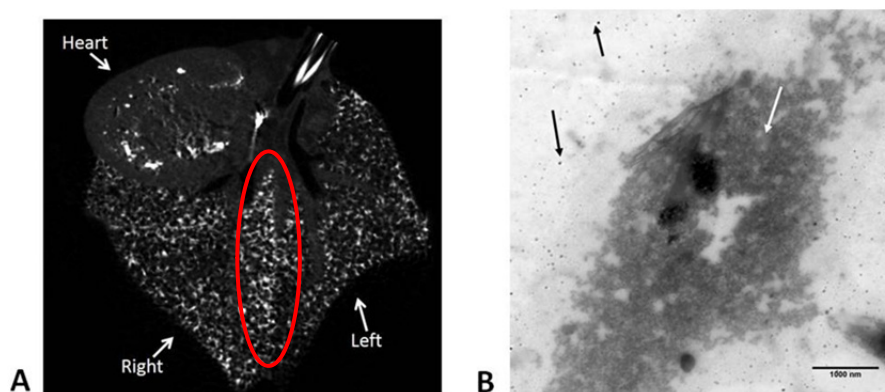


Figure 8. **a)** *Ex vivo* CT imaging of the injured lung. The lesion at the right lung is highlighted with the red circle (HU right lung = 217; HU left lung = 42). **b)** TEM micrograph of the collected material from the bronchoalveolar lavage: Au NPs are identified by the black arrow. The fragment of pulmonary tissue is identified by the white arrow. Reprinted with permission from {Silvestri A. et al. *Contrast media and molecular imaging*, 405-414 (2016)}. Copyright {2016} Wiley.

After this preliminary study a new set of experiments was performed on a group of 10 injured mice with chemically induced pneumonia. In **Figure 9** is shown a histological proof of the inflicted damage: in **Figure 9 a** is reported an image of the tissue deriving from the right injured lung, and in **Figure 9 b** the comparison with the health tissue deriving from the left lung. 6 of the 10 mice were treated with the glucosamine targeted nano-tracer (coated by HS- PEG₅₀₀₀-CONHGlucose). The remaining 4 were infused with not targeted Au NPs (coated by HS-PEG₅₀₀₀-OMe). The nano-tracer

presented a selective accumulation in the lung inflammation, due to the glucose moieties displayed on their surface, furnishing both anatomical and metabolic information (**Figure 10**).

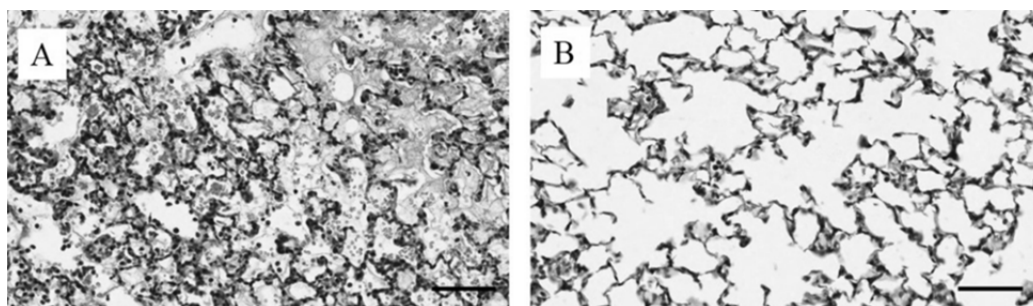


Figure 9. Images of eosin and hematoxylin stained lung tissue sections shows serofibrinous and inflammatory exudate and hemorrhage in the injured right lung (a). Instead no pathologic signs are observed in the left lung (b). (scale bar = 100 μ m). Reprinted with permission from {Silvestri A. et al. *Contrast media and molecular imaging*, 405-414 (2016)}. Copyright {2016} Wiley.

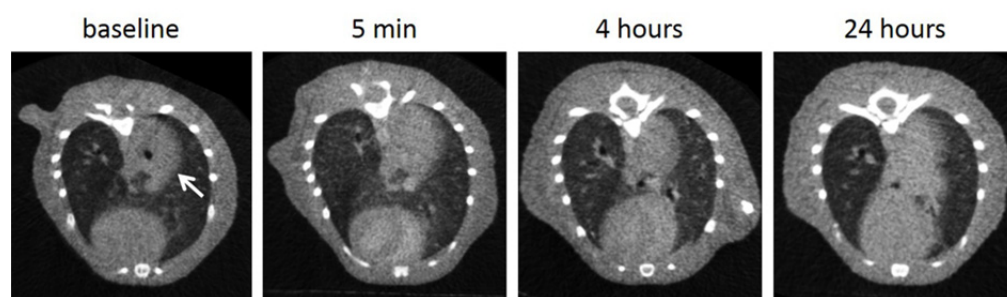


Figure 10. CT imaging (transaxial sections) obtained in live mice with chemically induced pneumonia, located in the right lung (dorsal region), showing the accumulation of the nano-tracer during the observation time. Reprinted with permission from {Silvestri A. et al. *Contrast media and molecular imaging*, 405-414 (2016)}. Copyright {2016} Wiley.

The X-rays attenuation values in different organs were monitored during a time frame of 24 hours for each mouse (the doses are reported in **Table 2**, Section 4.21). The attenuation values for heart, liver and bladder present similar values and the same trend for both the mice populations. Instead, noteworthy, the levels of nano-tracer in the lung injury are higher if compared to the control nanoparticles, even if statistic relevance was not reached. Moreover, it is interesting to notice that the attenuation values registered in the mice treated with the nano-tracer remained constant in the observation time, suggesting an effective selectivity of the nanoparticle for the damaged site and probably an uptake in the damaged cells. Instead the level referred to the control nanoparticles rapidly decreased after the first five minutes of observation, returning comparable to the baseline value at 24 hours (**Figure 11**). The gold nanoparticles uptake within the liver remained moderate for all the animals, confirming the stealth properties of both nano-systems. The increasing of the attenuation values in the bladder can be ascribed to a small fraction of ultra-small Au NPs, which can pass through the renal glomerulus, and be concentrated in the small bladder volume³². In order to confirm this point, a sample of urine was collected 1 hour after the injection and analysed with TEM microscopy. From the TEM micrographs is possible to appreciate the presence of small Au NPs with diameter smaller than 5 nm deriving from the lower part of the

original nanoparticles dimensional distribution (**Figure 12**). The mice were sacrificed 24 hours after the injection and the organs removed to quantify the amount of Au accumulated within them. Subsequently the organs were digested in aqua regia in order to quantify the amount of metal by means of ICP-OES. In **Figure 13** are reported the results of the analysis, that confirmed the trend already seen in the CT scans. The right injured lung of mice treated with the nano-tracer, presented a higher metal accumulation in respect to the control. Instead the amount of gold presented in the left lungs was comparable for both the NPs, confirming the selectivity of the glucose coated nanoparticles toward the inflammation site.

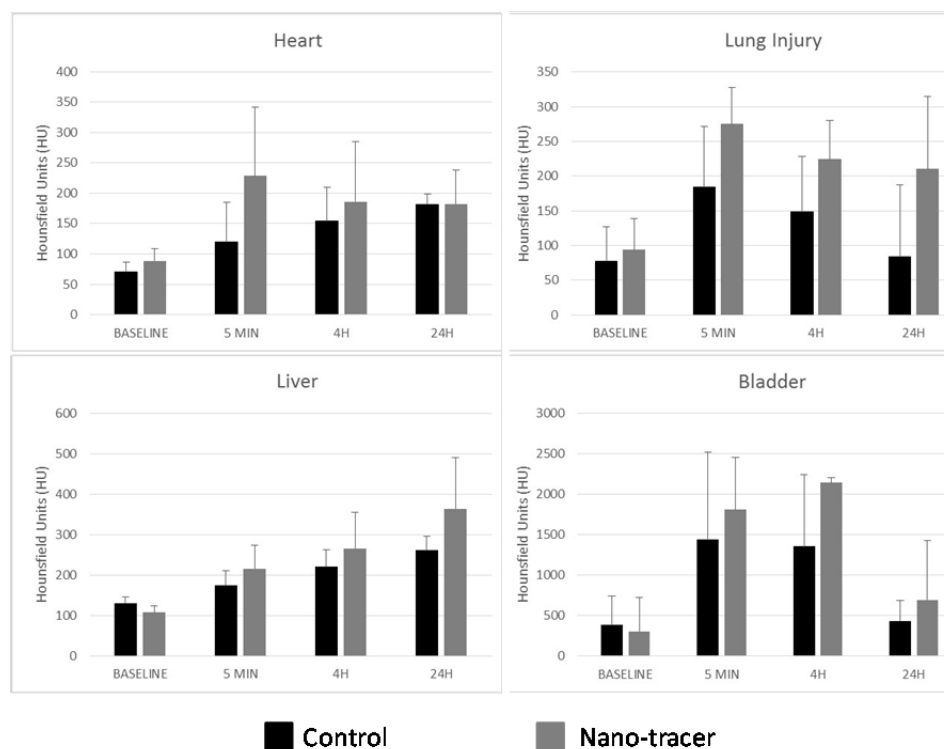


Figure 11. Control nanoparticles (n=4) and nano-tracer (n=6) contrast values collected within the heart, lung injury, liver and bladder in function of the observation time. Reprinted with permission from {Silvestri A. et al. *Contrast media and molecular imaging*, 405-414 (2016)}. Copyright {2016} Wiley.

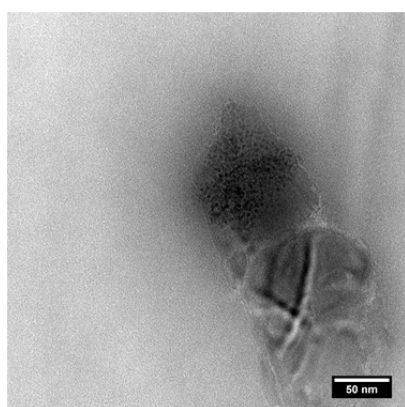


Figure 12. TEM micrograph of urines collected 1 hour after the injection. The Au NPs are visible inside the biological organic matter (indicated by white arrow) and shows have small core diameter ($d < 5\text{nm}$). Reprinted with permission from {Silvestri A. et al. *Contrast media and molecular imaging*, 405-414 (2016)}. Copyright {2016} Wiley.

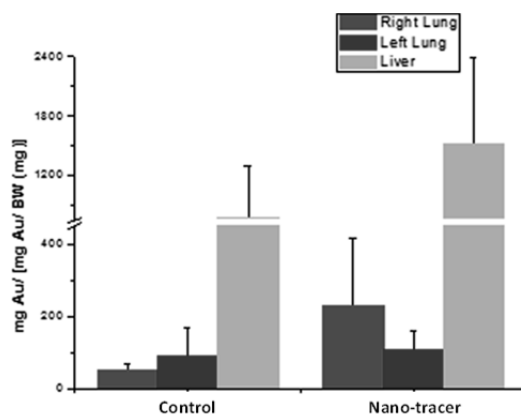


Figure 13. Au amounts were determined by ICP-OES in liver and lungs of sacrificed mice 24 hours after the injection of the control NPs (n=4) and the radiotracer (n=6). The amount of gold found in the organs is reported in milligrams normalized for the injected dose in each mouse (mg Au/mg BW). Reprinted with permission from {Silvestri A. et al. *Contrast media and molecular imaging*, 405-414 (2016)}. Copyright {2016} Wiley.

2.4 Synthesis of gold based contrast agent with enhanced renal clearance

Another hot topic in the design of nano-structured contrast media is the fate and the clearance of the nanoparticles, once injected in the organism.^{23,32} As explained before, inorganic NPs own a huge potential in nano-medicine both as imaging media and therapeutic agents.^{3,6} Anyway the long-term tissue retention of nanoparticles containing heavy metals is a serious concern for the toxicity of these types of systems. The production of NPs with enhanced clearance performances can minimize the toxicity risks correlated to the long exposure to these agents.²³ For this reason we concentrated our efforts in the investigation of innovative synthetic methodologies for the production of renal clearable nano-structured CT contrast agents. In particular, we wanted to develop a synthetic procedure that could allow to produce elevated amounts of Au NPs in a reproducible way, implementing a control over the dimension and size distribution of the particles. In fact, to possess enhanced renal clearance, the designed nanoparticles need to have a hydrodynamic dimension smaller than 10 nm, to freely pass through the sieves of the renal glomerulus. We identified this synthetic procedure in a combination of MVS and aqueous digestive ripening³³. The production of Au NPs by mean of MVS technique is described in **Chapter 1 (Section 3.13)** and allows to obtain highly concentrated SMA solution evaporating up to 1g of gold each day. Instead the combination of this technique with digestive ripening process has been reported for the first time by Klabunde and coworkers³⁴, demonstrating that this procedure can allow to obtain organic soluble monodispersed gold nanoparticles in large amount and reproducible way^{35,36}. The approach, described by Klabunde, consisted in the refluxing of the colloidal suspension, comprising of poly-dispersed Au solvated metal atoms (SMA), in non-polar organic solvents in presence of an excess of aliphatic thiols or different capping molecules (e.g. amines, silanes, phosphines). The product of this treatment is a nearly monodisperse Au colloidal solution. This phenomenon could be ascribed to an inter-particle transfer of gold atoms diffusing from larger particles to small ones obtaining a narrower particle distribution³⁷. However, this phenomenon has never been reported in aqueous environment with the employment of polymeric water soluble chains. Here for the first time we report the combination of MVS and aqueous digestive ripening for the production of a renal clearable CT contrast media.

2.4.1 Synthesis of Au NPs by mean of MVS and aqueous digestive ripening

In **Figure 14** is schematically represented the synthetic procedure employed to obtain the Au-PEG-NPs. First of all a highly concentrated ($[Au] > 4 \text{ mg/ml}$) Au SMA (Solvated Metal Atom) solution has been prepared employing acetone as solvent and preliminary stabilizing agent^{34,38}. The Au/acetone SMA solution was added to a water solution containing an excess of $\text{CH}_3\text{O}-\text{PEG}_{5000}-\text{SH}$. At this point is obtained a suspension of Au aggregates stabilized by the strong interaction between the thiols and the gold atoms³³ characterized by a dark purple shade. These aggregates are mainly comprised of highly defective particles with a broad dimensional distribution and average diameter of 7.5 nm (**Figure 15 a**). From higher magnifications of the TEM images is possible to distinguish both individual crystallites and nanoparticles held together forming aggregates (**Figure 15 a inset**).

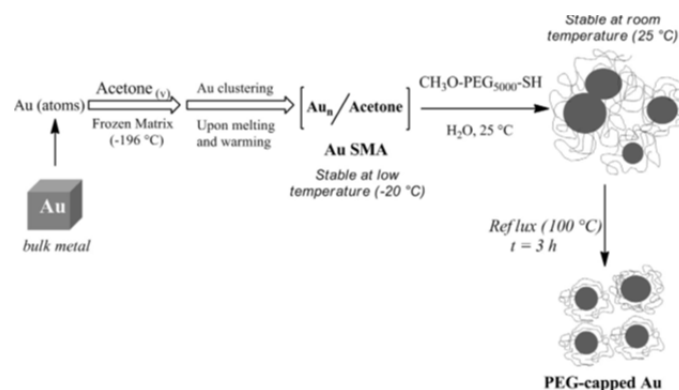


Figure 14. Schematic representation of the synthesis of Au-PEG NPs combining MVS and aqueous digestive ripening. Reprinted with permission from {Silvestri A. et al. *Journal of colloid ad interface science*, 28-33 (2016)}. Copyright {2016} Elsevier.

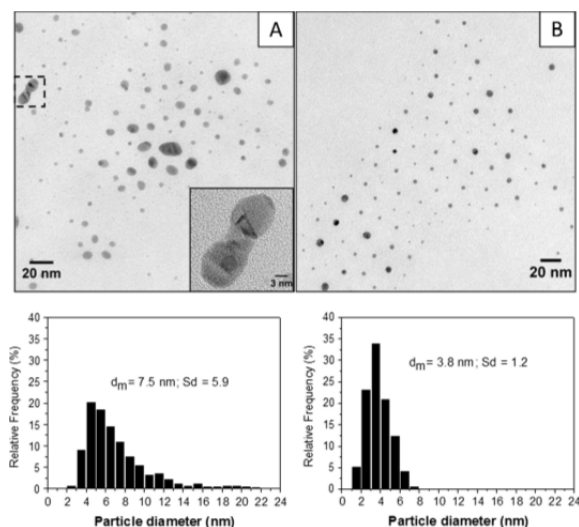


Figure 15. TEM imaging of Au-PEG NPs: before (a) and after (b) 3 hours of aqueous digestive ripening. Reprinted with permission from {Silvestri A. et al. *Journal of colloid ad interface science*, 28-33 (2016)}. Copyright {2016} Elsevier.

We evaluated the role of the warming up on the Au NPs size and dimensional distribution, refluxing different samples for 1, 3, 6 and 18 hours. Each of the refluxed solution was analysed by

TEM (**Figure 16**). Already after 1 hour of refluxing in water the nanoparticles underwent to notable effect of the digestive ripening, affecting the morphology and size distribution of the gold crystals. However, few gold aggregates are still present in the sample, indicative of an incomplete process during this time frame. The particle distribution of the samples refluxed for 3 and 6 hours (**Figure 16 c, d**) are very similar and the gold aggregates were completely turned into mono-disperse spherical Au NPs.

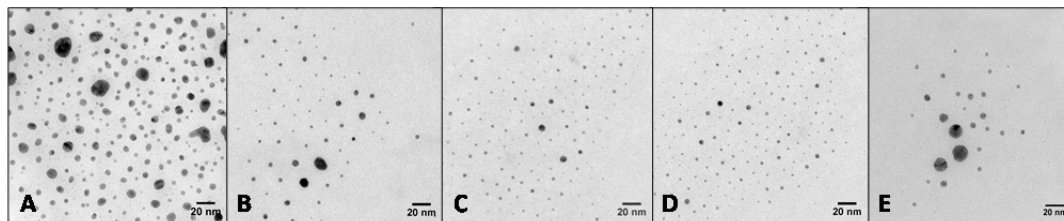


Figure 16. Representative TEM micrographs of the Au NPs **a**) before the digestive ripening **b**) after 1h **c**) 3h **d**) 6h **e**) 18 h. The scale bars correspond to 20 nm. Reprinted with permission from {Silvestri A. et al. *Journal of colloid ad interface science*, 28-33 (2016)}. Copyright {2016} Elsevier.

The nanoparticles obtained at the end of this process are characterized by an average core size of 3,8 nm with a particle size range comprised between 2.5–7.5 nm. In the described conditions, it is thermodynamically possible that some ligands could remove atom or clusters of atoms from a NP surface moving and depositing them on other NPs.³⁷ Under the effect of digestive ripening broad distributions of nanoparticles become narrower and more homogeneous, achieving an equilibrium size. With longer refluxing time (18 hours) the NPs poly-dispersion and dimensions increased, indicating the beginning of an unfavourable Ostwald ripening process³³. It is interesting to notice that, by a careful analysis of TEM micrographs of the sample obtained after 3h of refluxing, the nanoparticles resulted regularly packed in a pattern with a medium inter-particle distance of 15 nm. This value is consistent with the length of a fully extended chain of $\text{CH}_3\text{O}-\text{PEG}_{5000}-\text{SH}$ ³⁹, suggesting the presence of a uniform PEG coating surrounding each gold nanoparticle. The success of the digestive ripening procedure can be observed as well from the UV-vis absorption spectra. The maximum of the plasmonic peak of the gold nanoparticles after 3 hour of refluxing resulted shifted at lower wavelength (519 nm) and reduced in amplitude (**Figure 17**).

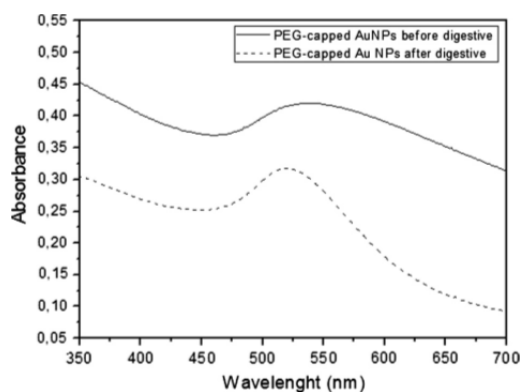


Figure 17. UV-Vis spectra of Au-PEG NPs before (—) and after (- -) 3 hours of aqueous digestive ripening. Reprinted with permission from {Silvestri A. et al. *Journal of colloid ad interface science*, 28-33 (2016)}. Copyright {2016} Elsevier.

The hydrodynamic dimension in aqueous solution is a fundamental information to have a deeper comprehension of the *in vivo* behaviour of the nano-system and an essential part of its characterization. We derived the hydrodynamic dimension of Au-PEG NPs employing two different techniques: dynamic light scattering (DLS) (**Figure 18**) and ^1H NMR diffusion ordered spectroscopy (DOSY) (**Figure 19**).

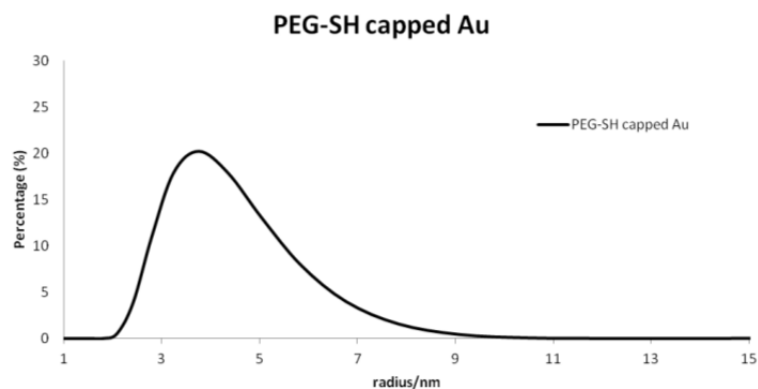


Figure 18. Dynamic light scattering (DLS) of PEG-capped Au after 3h of refluxing in water. Reprinted with permission. Reprinted with permission from {Silvestri A. et al. *Journal of colloid ad interface science*, 28-33 (2016)}. Copyright {2016} Elsevier.

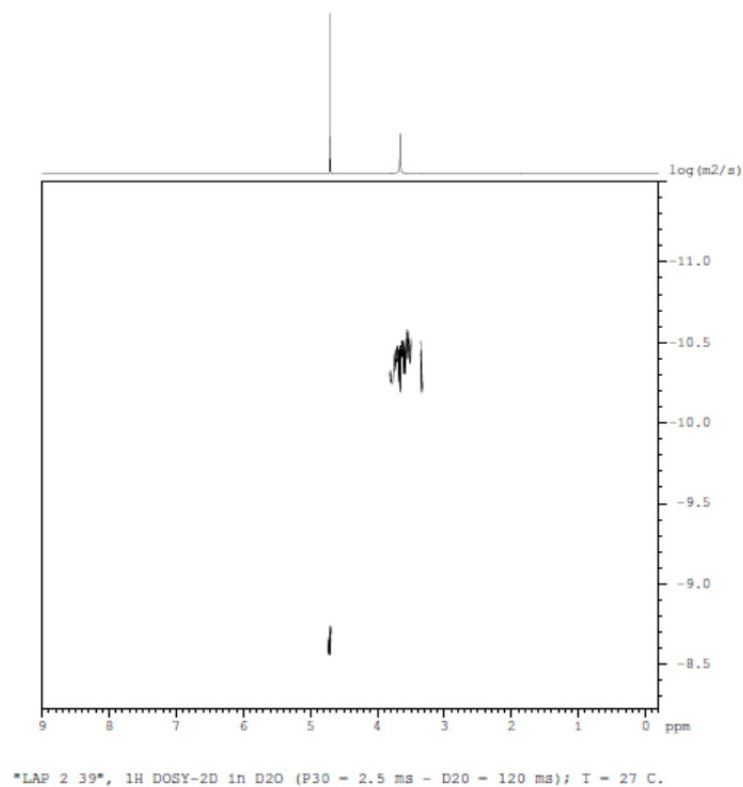


Figure 19. ^1H -NMR diffusion ordered spectroscopy (DOSY) of PEG-capped Au (after 3h of refluxing) in D_2O

The diffusion times obtained by the two techniques were substituted in the Stokes–Einstein equation to derive the hydrodynamic radius:

$$D = \frac{KT}{6\pi\eta R_H} \quad \text{eq. 2}$$

where K is the Boltzmann constant, T the solution temperature, and η the viscosity. Employing ^1H NMR DOSY experiments^{40,41} the hydrodynamic radius was estimated to be 4 nm, in good accordance with the R_H determined by DLS (3.7 nm). Consequently, with respect to the core dimensions, an average radius increase of 2.1 nm was detected. According to the reported theories^{42,43} PEG can be anchored to the nanoparticles surface mainly in two conformation: “mushrooms” or “brush”. The “brush” conformation is characterized by a dense package on the metallic surface with fully extended PEG chains able to enhance the average radius of the nanoparticles of 8 nm. “Mushrooms” conformation instead, is characterized by random oriented polymer chains that present a reduced packing density. It is reported that a PEG coating disposed with a “mushrooms” conformation can produce an increasing of the R_H comparable to the Flory radius of the polymer (5.9 nm)^{35,36}. Our experiment furnished a value smaller than the expected ones, suggesting that the PEG chains assumed a sort of “shrunk mushroom” conformation on the naked gold nanoparticles surface. This value, different from the ones reported in literature, can be ascribed to the fact that previously studies were performed on citrate stabilized surfaces, and the PEG was attached by mean of a ligand exchange procedure. Instead in our system the anchoring of the PEG takes place for a direct interaction of the polymer with a naked surface.

2.4.2 *In vivo CT imaging*

The nanoparticles obtained after 3 hours of refluxing presented narrow dimensional distribution, high uniformity and hydrodynamic dimensions of 8 nm. These characteristics confer to the nano-system the optimal requirements to act as an effective CT contrast agent with a high renal clearance. Prior to the injection, we performed a micro-CT of Au NPs solution, in order to evaluate their attenuation ability. The X-rays scan of a tube containing a water solution of Au-PEG NPS ([Au]=7 mg/ml) is reported in **Figure 20 a**. From the image is possible to appreciate the difference between the gold colloidal solution and the pure solvent (water); the nanoparticles presented an X-ray attenuation of 608 ± 18 HU, 8 times higher than the contrast of soft tissues (80 HU). Then, the *in vivo* biodistribution of Au-PEG NPs, was evaluated by mean of micro-CT imaging experiments (**Figure 20 b**). Mice well tolerated the intra venous administration of PEG Au NPs, without any signal of toxicity within the 24 hours after the injection.

In **Figure 21** is reported the Au NPs distribution in function of the observation time. The 100% relative increase in the attenuation values within the heart demonstrated that these nanoparticles were able to circulate in blood stream up to 4 hours of observation, in spite of the reduced hydrodynamic dimensions. Any relevant accumulation was detected in the liver, where the X-ray attenuation values underwent to a minimal increase, remaining stable over time. Moreover, their renal clearance of was monitored in real time, observing a rapid increment in the contrast values within the bladder from 50 to 1900 HU few minutes after the injection. The urines of the mouse were collected 4 hours after the injection and their X-Ray attenuation evaluated by mean of a CT scan (**Figure 22 a**). The high attenuation value (1955) proved the presence of the gold in the urines and the effective clearance of the system, that freely passed through the kidneys, the renal glomerulus and the bladder. Moreover, from TEM micrographs, performed on the urine samples,

registered the presence of Au NPs in the biological fluid with a particles size comparable to the original system (**Figure 22 b**).

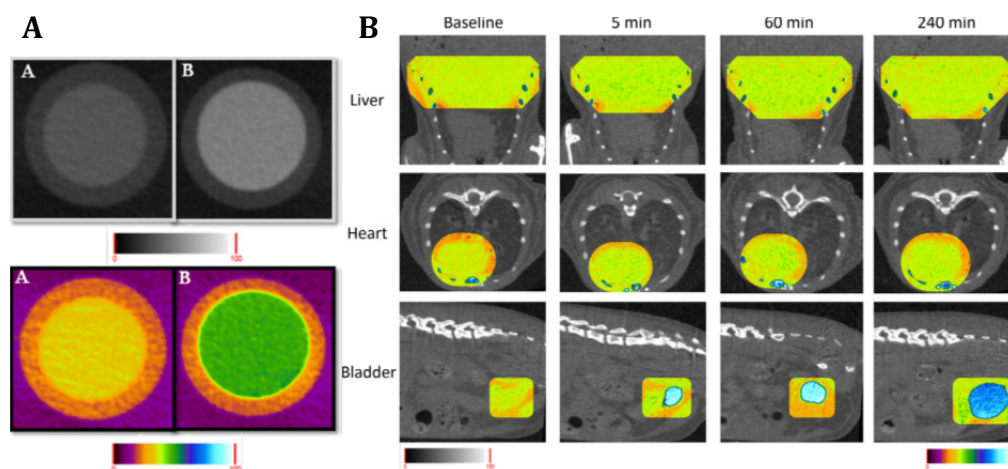


Figure 20. a) Micro-CT images of water (left side) and Au-PEG NPs (right side) in grey and coloured scale. b) *In vivo* CT images reconstructed under different projections (from top to bottom: coronal, axial and sagittal) The images were registered at different time points: baseline, 5 min, 1 hour 4 hours after Au NPs injection). Reprinted with permission from {Silvestri A. et al. *Journal of colloid ad interface science*, 28-33 (2016)}. Copyright {2016} Elsevier.

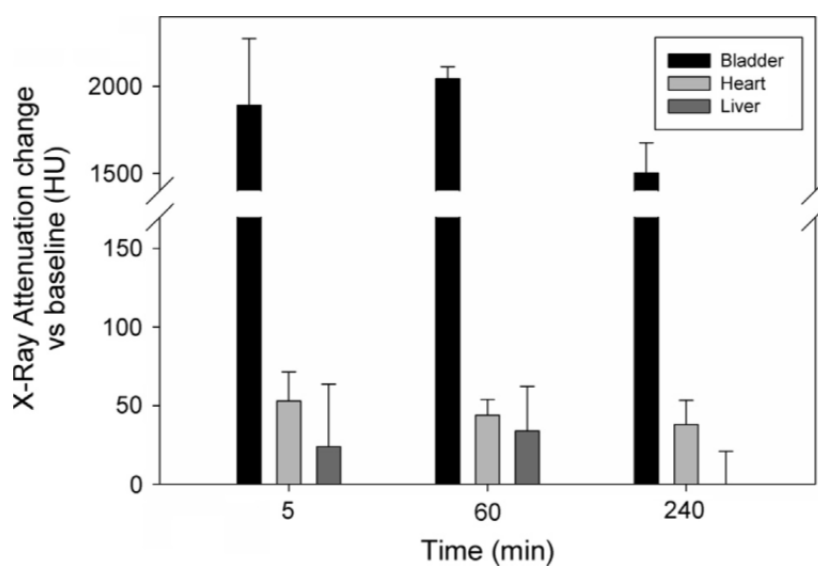


Figure 21. X-ray attenuation versus the baseline value plotted in function of the observation time and organ accumulation of Au-PEG NPs. Reprinted with permission from {Silvestri A. et al. *Journal of colloid ad interface science*, 28-33 (2016)}. Copyright {2016} Elsevier.

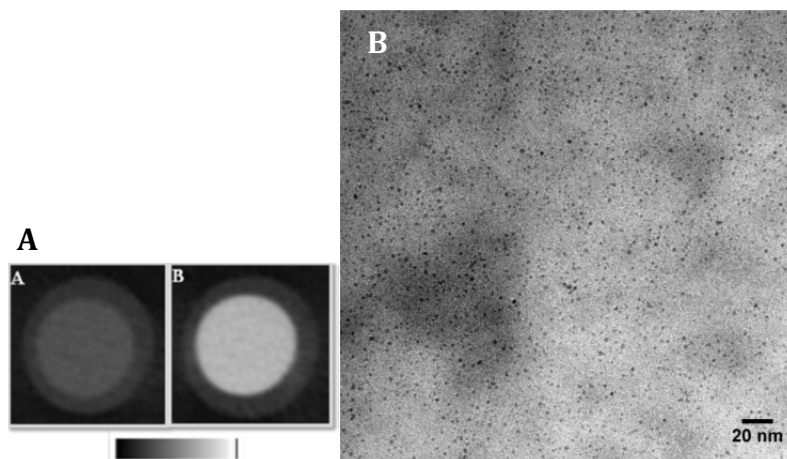


Figure 22. **a)** Grey scale micro-CT images of urine at the baseline (left side) and 4 hours after the injection of Au-PEG NPs injection (right side). **b)** TEM image of the collected urine 4 hours after the injection of Au-PEG NPs. Reprinted with permission from {Silvestri A. et al. *Journal of colloid ad interface science*, 28-33 (2016)}. Copyright {2016} Elsevier.

3. Conclusions

In this chapter, different fundamental aspects in the design of a successful CT nano-structured contrast agent have been investigated. In first instance, it has been presented a protocol for the “one-pot” production and functionalization of shape-controlled nanoparticles, that allows to obtain reproducible and stable engineered nanoparticle. **Au NP 6** and **Au NP 9** proved to be excellent candidates as CT contrast agents, having an increased blood-time permanence and a low uptake from RES organs. The best performing nanoparticles have in common the complete absence of free superficial charges and the hydrodynamic dimension comprised between 10 and 60 nm. Moreover, no remarkable differences have been observed employing anisotropic NPs instead of spherical ones.

The same protocol for the production of highly stable Au NPs has been employed to prepare a glucosamine-functionalized nano-tracer, characterized by 20 nm spherical nanoparticles as gold core. This nano-tracer has been proved to act as an efficient functional contrast agents, able to selectively accumulate in the lung injuries of mice affected by chemically induced pneumonia. Moreover, we developed an alternative protocol to produce large amounts of monodispersed water soluble Au-PEG NP. These NPs thanks to the reduced hydrodynamic diameter can freely pass through the renal glomerulus to be expelled by the urines. The procedure consisted in a combination of MVS technique and aqueous digestive ripening process to afford functionalized NP surface with controlled shape and dimension. The described regime, is an efficient and reliable way to synthesize Au-PEG NPs with a main core diameter of 8 nm acting as effective CT contrast media in mice models. This reduced hydrodynamic dimensions permitted to achieve a CT contrast media characterized by an enhanced renal clearance.

4. Experimental

4.1 Materials and methods

The reagents were furnished by Sigma-Aldrich and employed without further purification. $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ was stored at 4 °C shielded by light as 10 mM aqueous solution. AgNO_3 aqueous solution (5.9 mM) was prepared before each synthesis and kept under dark atmosphere. HS-PEG₅₀₀₀-COOH and HS-PEG₅₀₀₀-CH₃O, were furnished by Rapp Polymer GmbH, employed as received and stored at -20 °C under dry argon atmosphere. All glassware employed for Au NPs synthesis was cleaned using aqua regia ($\text{HCl}:\text{HNO}_3/3:1$). MilliQ water, was employed for the aqueous solutions preparation. The purification and concentration of Au NPs was achieved by ultrafiltration employing Millipore Amicon Centrifugal Filter Units (30 KDa cut-off). The concentration of the Au NPs solution was completed, using Eppendorf tubes at 14 K rpm, up to 0.7 ml. All the operations concerning the MVS products were carried out under a dry argon atmosphere. Gold shots (99.999%) were purchased from Strem Chemicals. Acetone was dried using molecular sieves and stored under dry argon atmosphere. Dynamic Light Scattering (DLS) measurements were performed employing a 90 Plus Particle Size Analyzer (Brookhaven Instrument Corporation; Holtsville, NY). The instrument operates with a 15 mW solid-state laser ($\lambda=661$ nm), with a scattering angle of 90°. In order to remove large particulate DLS specimen were filtrated with a cellulose acetate syringe filter (0.45 μm). Each sample was equilibrated for 3 min prior to measurement. At least ten independent measurements of 60 s were performed for each sample. Zeta-potential was evaluated 90 Plus Particle Size Analyzer (Brookhaven Instrument Corporation; Holtsville, NY) at 25 °C, equipped with an 120 V AQ-809 electrode. Agilent 8453 spectrometer was employed to register UV/Vis spectra. A 1 cm optical path length cuvette was employed for the measurements. FTIR spectra were registered employing Thermo Nicolet NEXUS 670 FTIR instrument. The samples were prepared by titration as KBr pellets. Transmission Electron Microscopy images were collected employing TEM-Zeiss LIBRA 200FE. Inductively Coupled Plasma-Optical Emission Spectrometers (ICP-OES; iCAP 6300 Duo, Thermofisher) was employed to determine the concentration of gold in the different samples. ICP-OES was employed to determine the metal content. 1 ml of each sample was digested inside a glass vial, heating with 2 ml of aqua regia, and the operation was repeated for four times. The residues were dissolved in a 0.5 M HCl aqueous solution and diluted to 10 ml with MilliQ water. High resolution MALDI were recorded employing a MALDI tof-tof Autoflex II instrument. TEM samples were prepared dropping an Au NPs aqueous solution on a carbon-coated copper grid (300 mesh) and subsequently evaporating the solvent. Software Pebbles was employed to analyse, from TEM images, the particle size distribution, for spherical and quasi spherical samples⁴⁴. For Au nanostars the dimensions were estimated measuring the distance between two opposite branches employing ITEM-TEM Imaging platform (Olympus Soft Imaging Solutions). At least 250 measurements were performed for each sample. The biological matter recovered from the lung of injured mouse has been reduced to a pellet by centrifugation, incorporated into a resin and finally cut with a microtome to perform TEM analysis.

4.2 Synthesis of Au NP 2, Au NP 3 and Au NP 4

A water solution of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (2.5 ml, 10 mM), sodium citrate (3 ml, 68 mM), and AgNO_3 (140 μl , 5.9 mM) was prepared and mixed at R.T. for 6 minutes. The pre-incubated mixture was, then, blended to 250 ml of water at 100 °C. The mixture was stirred at 750 rpm for 1 h. Afterwards the reaction solution was left to cool to R.T. Once again, a mixture of HAuCl_4 (12.5 ml, 10 mM), sodium citrate (16.5 ml, 34 mM) and AgNO_3 (600 μl , 5.9 mM) was pre-mixed for 6 minutes and then added to the reaction solution, immediately followed by a hydroquinone solution (13.3 ml, 91 mM). Then, the colloidal solution was left to age for 1h, stirring at 750 rpm. The obtained **Au NPs 1** were directly employed without any further concentration or purification. **Au NPs 2** were obtained by adding (under argon atmosphere) 30 mg of thio-PEG $\text{HOOC-PEG}_{5000}\text{-SH}$. **Au NPs 3** were obtained adding 1:1 mixture of $\text{MeO-PEG}_{5000}\text{-SH}$ and $\text{HOOC-PEG}_{5000}\text{-SH}$. Finally, **Au NPs 4** were obtained adding the same amount of $\text{MeO-PEG}_{5000}\text{-SH}$. The PEGs were dissolved in 5 ml of MilliQ water basified with NaOH (9 mg). The reaction mixture was allowed to stir for further 48 hours at R.T. The functionalized **Au NPs 2-4** were purified and concentrated to a final volume of 0.7 ml using Amicon centrifugal filter units.

4.3 TEM characterization of Au NP 2, Au NP 3 and Au NP 4

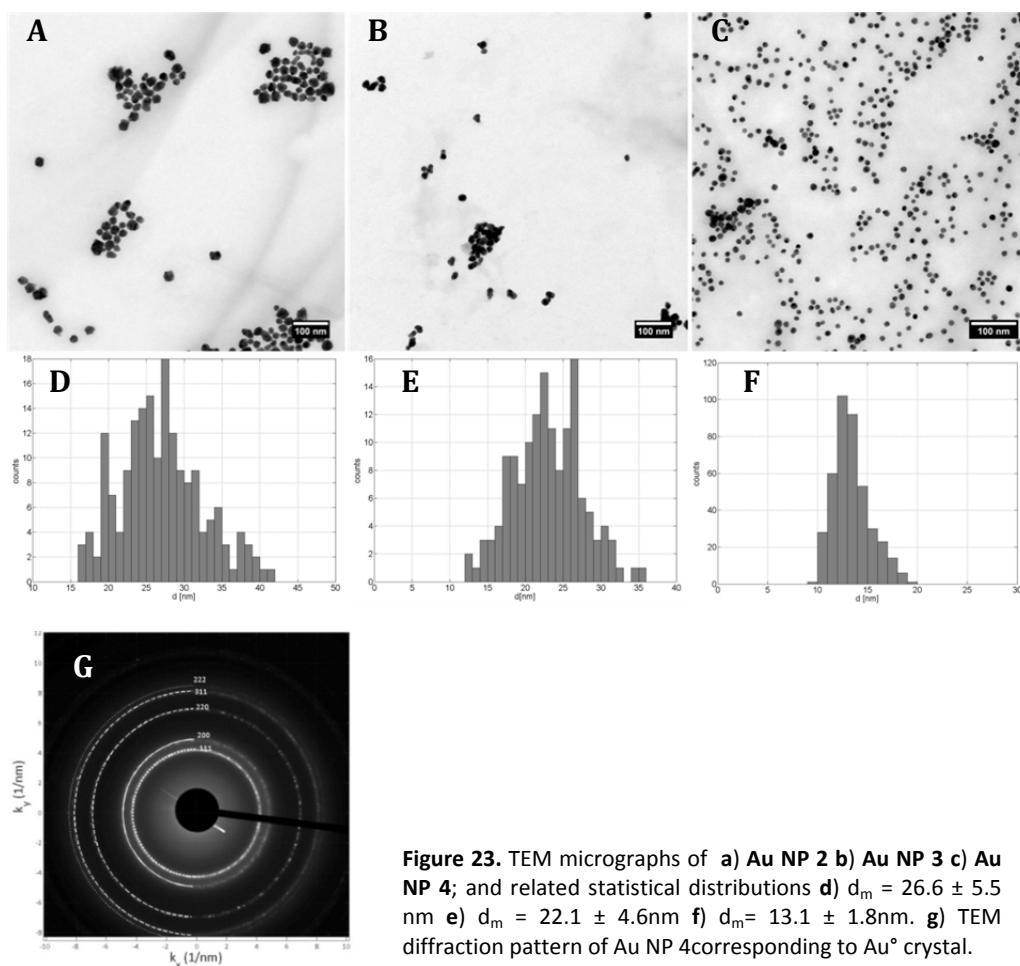


Figure 23. TEM micrographs of a) Au NP 2 b) Au NP 3 c) Au NP 4; and related statistical distributions d) $d_m = 26.6 \pm 5.5$ nm e) $d_m = 22.1 \pm 4.6$ nm f) $d_m = 13.1 \pm 1.8$ nm. g) TEM diffraction pattern of Au NP 4 corresponding to Au^o crystal.

4.4 Synthesis of Au NP 6

A water solution of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (7.5 ml, 10 mM), sodium citrate (9 ml, 68 mM), and AgNO_3 (490 μl , 5.9 mM) was prepared and mixed at R.T. for 6 minutes. The pre-incubated mixture was, then, blended to 250 ml of water at 100 °C. The mixture was stirred at 750 rpm for 1 h. Afterwards the reaction solution was left to cool to R.T. and 5 ml of glycerol were added. After 10 minutes a second mixture of HAuCl_4 (7.5 ml, 10 mM), sodium citrate (10 ml, 34 mM) and AgNO_3 (426 μl , 5.9 mM) was pre-mixed for 6 minutes and then added to the reaction solution, immediately followed by a hydroquinone solution (8 ml, 91 mM). Then, the colloidal solution was left to age for 1h, stirring at 750 rpm. The obtained **Au NPs 5** were directly employed without any further concentration or purification. **Au NPs 6** were obtained by adding (under argon atmosphere) 30 mg of MeO-PEG₅₀₀₀-SH. The PEG was dissolved in 5 ml of MilliQ water basified with NaOH (9 mg). The reaction mixture was allowed to stir for further 48 hours at R.T. The functionalized **Au NPs 6** were purified and concentrated to a final volume of 0.7 ml using Amicon centrifugal Filter units.

4.5 TEM characterization of Au NP 6

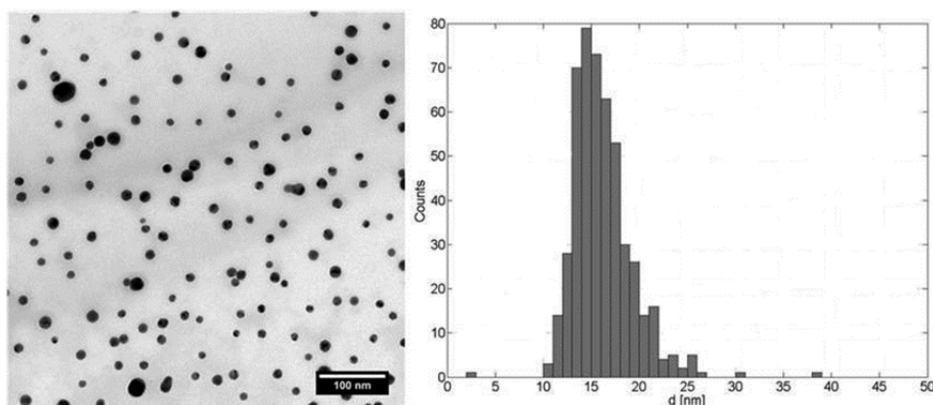


Figure 24. Left: TEM micrograph of **Au NP 6**. Right: statistical distributions $d_m = 15.6 \pm 3.1$ nm. Reprinted with permission from {Silvestri A. et al. *Contrast media and molecular imaging*, 405-414 (2016)}. Copyright {2016} Wiley.

4.6 Synthesis of Au NP 8

A water solution of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (1.73 ml, 68 mM), sodium citrate (1.46 ml, 10 mM), and AgNO_3 (82 μl , 5.9 mM) was prepared and mixed at R.T. for 6 minutes. The pre-incubated mixture was, then, blended to 60 ml of water at 100 °C. The mixture was stirred at 750 rpm for 1 h. Afterwards the reaction solution was left to cool to R.T. and 54 ml of glycerol and 600 ml of water were added. After 10 minutes a second mixture of HAuCl_4 (13.4 ml, 10 mM), sodium citrate (1.16 ml, 34 mM) and AgNO_3 (2.2 ml, 5.9 mM) was pre-mixed for 6 minutes and then added to the reaction solution, immediately followed by a hydroquinone solution (5.3 ml, 91 mM). Then, the colloidal solution was left to age for 1h, stirring at 750 rpm. The obtained **Au NPs 7** were directly employed without any further concentration or purification. **Au NPs 8** were obtained by adding (under argon atmosphere) 30 mg of MeO-PEG₅₀₀₀-SH. The PEG was dissolved in 5 ml of MilliQ water basified with NaOH (9 mg). The reaction mixture was allowed to stir for further 48 hours at R.T. The

functionalized **Au NPs 8** were purified and concentrated to a final volume of 0,7 ml using Amicon centrifugal filter units.

4.7 TEM characterization of Au NP 8

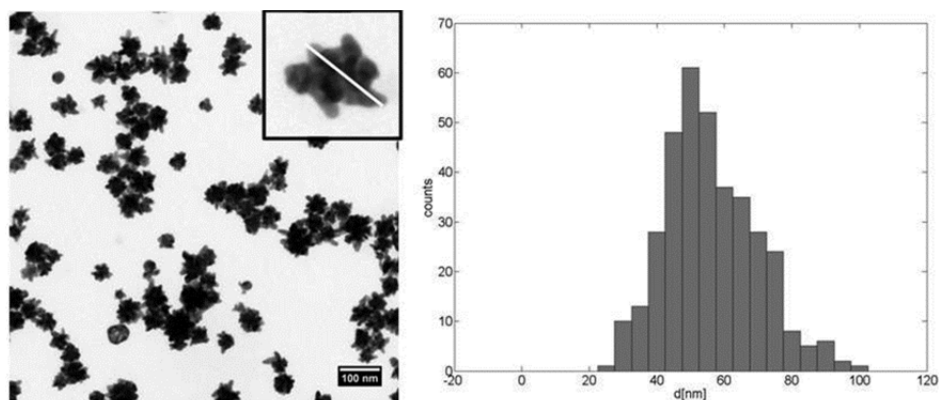


Figure 25. Left: TEM micrograph of **Au NP 8**. Right: statistical distributions $d_m = 54,2 \pm 13,6$ nm. Reprinted with permission from {Silvestri A. et al. *Contrast media and molecular imaging*, 405-414 (2016)}. Copyright {2016} Wiley.

4.8 Synthesis of Au NP 10

A HAuCl_4 solution (7.5 ml, 10 mM) was diluted in 200 ml of MilliQ water. Then aqueous hydroquinone solution (6 ml, 11 mg/ml) was rapidly injected. The solution instantaneously turned from light yellow to blue indicating the formation of Au nanostars. 30 mg of $\text{MeO-PEG}_{5000}\text{-SH}$ were added to the reaction mixture to cap the AuNPs. Functionalized **Au NPs 10** were then purified and concentrated employing of Amicon centrifugal Filter units up to a volume of 0.7 ml.

4.9 TEM characterization of Au NP 10

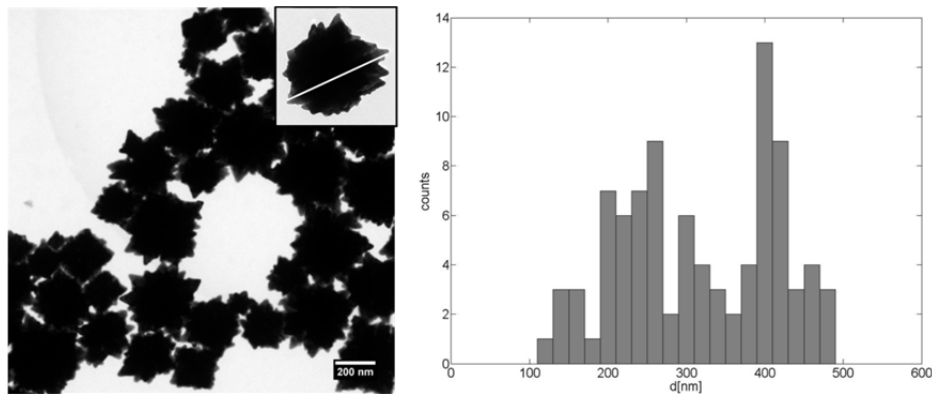


Figure 26. Left: TEM micrograph of **Au NP 10**. Right: statistical distributions ($d_{\max} = 474.0$ nm $d_{\min} = 123.9$). Reprinted with permission from {Silvestri A. et al. *Contrast media and molecular imaging*, 405-414 (2016)}. Copyright {2016} Wiley.

4.10 Synthesis of HS-PEG₅₀₀₀-CONHGlucose^{45,46}

50 mg of $\text{HS-PEG}_{5000}\text{-COOH}$ (0.01 mmol) were solubilised in 5 ml of MilliQ water. N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) (11.5 mg, 0.06 mmol) and N-Hydroxysuccinimide (NHS) (6.9 mg, 0.06 mmol) were employed to activate the carboxylic moiety and stirred for 1 hour at R.T. Glucosamine hydrochloride (11 mg, 51 mmol) was treated at pH 8 to restore free amine moieties and subsequently added to the reaction mixture. The reaction mixture was left to react for 18 hours at 65°C and subsequently for further 48 hours at R.T. The solvent was evaporated at reduced pressure and the crude product was solubilised in 1 ml dichloromethane (DCM) and added, drop wise, to 20 ml of diethyl ether to precipitate the PEG chains. The white fluffy solid was recovered by filtration, washed with ice-cold diethyl ether and then solubilised in 6 ml of phosphate buffer (pH 7.4) and left to react with dithiothreitol (DTT) (23 mg, 0.15 mmol) at 50°C for 3 h. The solution containing the crude product was concentrated up to 1 ml, and the product purified by size exclusion chromatography (Sephadex® G-25) and directly employed for the nanoparticles functionalization.

4.11 UV-Vis quantification of un-reacted glucosamine^{47,48}

5 plugged tubes were thermo-stated at 95° C in a water bath. In 4 tubes standard solutions were prepared introducing in each tube 380/250/188/125 µl of citric acid buffer (prepared employing 60 mL of NaOH 1M, 5.26 g of citric acid, in 190 ml of milliQ water, with a final pH of 5.26) and 3/2/1.5/1 ml of glucosamine hydrochloride (0,02%, previously treated with NaOH to restore free amine moiety). In the last tube 1 mL of the reaction sample (to prepare it 2 mL of the reaction solution were picked up and diluted to 5 mL using milliQ water) was introduced with 202 µl of citrate buffer. Then, 380/250/188/125 µl of ninhydrin (0.8% in mass) were introduced in the standards and 202 µl in the unknown sample. The mixtures in the different tubes were left to react for 30 min at 90° C and then rapidly cooled in an ice bath. Each solution was diluted to 10 ml with ice-cold MilliQ. The UV-vis spectra were immediately recorded. The calibration line was prepared employing the absorbance at 570 nm and the sample absorbance was fitted employing a linear trend function.

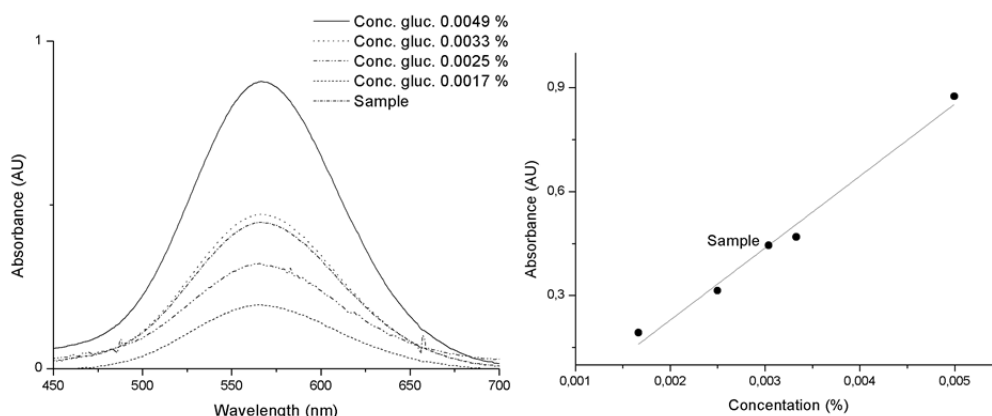


Figure 27. a) UV-vis spectra and b) calibration line employed to quantify the un-reacted glucosamine. Reprinted with permission from {Silvestri A. et al. *Contrast media and molecular imaging*, 405-414 (2016)}. Copyright {2016} Wiley.

The amide formation yield can be derived using the following formula:

$$y_{\text{funct}} = \frac{m_{\text{gluc}} - (C_{\text{gluc}} \times V_{\text{reaz}})}{m_{\text{gluc}}/eq_{\text{gluc}}} \times 100$$

Where m_{gluc} is the mass of glucosamine employed in the reaction (g), C_{gluc} is the concentration of not reacted glucosamine at the end of the reaction (g/ml), V_{reaz} is the reaction volume (ml) and eq_{gluc} are the glucosamine equivalent employed.

The functionalization yield in the reported case is 61 %.

4.12 FTIR Characterization of PEG derivative

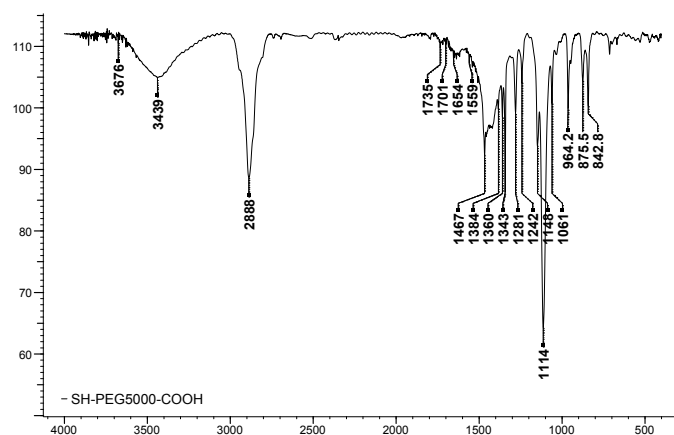


Figure 28. FTIR of SH-PEG₅₀₀₀-COOH. Reprinted with permission from {Silvestri A. et al. *Contrast media and molecular imaging*, 405-414 (2016)}. Copyright {2016} Wiley.

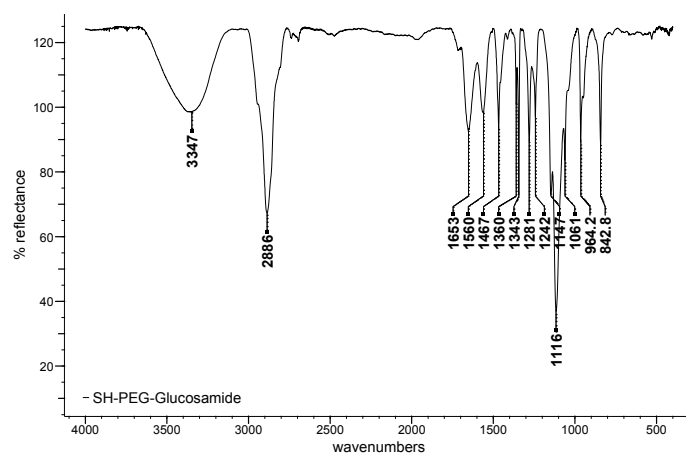


Figure 29. FTIR of SH-PEG₅₀₀₀-CONHGlucose. Reprinted with permission from {Silvestri A. et al. *Contrast media and molecular imaging*, 405-414 (2016)}. Copyright {2016} Wiley.

In Figure 2 is reported the FTIR spectra of HS-PEG₅₀₀₀-CONHGlucose showing two bands (1630 nm, 1560 nm) in the of C=O amides absorption zone, and the CH₂-O band (1116 nm) and C-H band (2886 nm) characteristic of PEG⁴⁹.

4.13 MALDI-ToF MS Characterization of PEG derivative

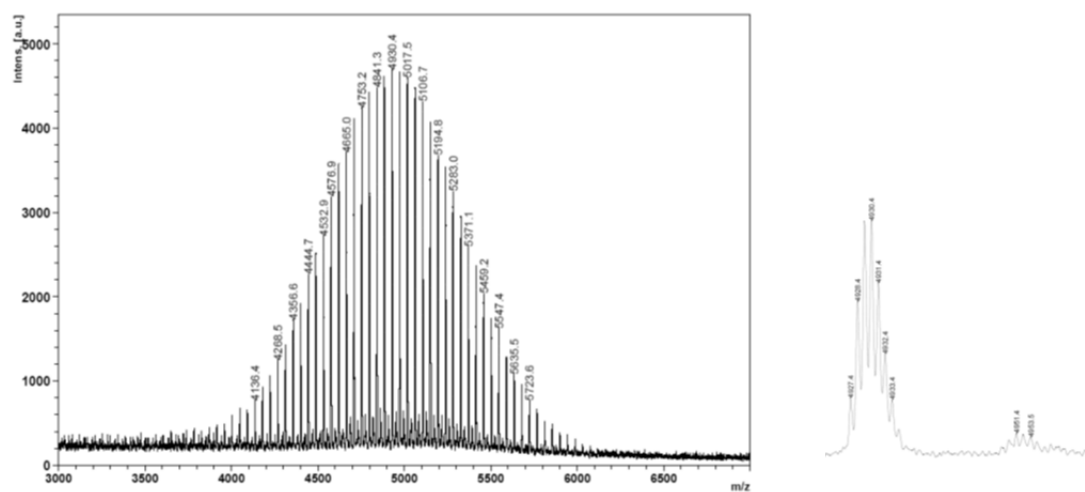


Figure 30. MALDI-ToF MS of HS-PEG₅₀₀₀-COOH. Reprinted with permission from {Silvestri A. et al. *Contrast media and molecular imaging*, 405-414 (2016)}. Copyright {2016} Wiley.

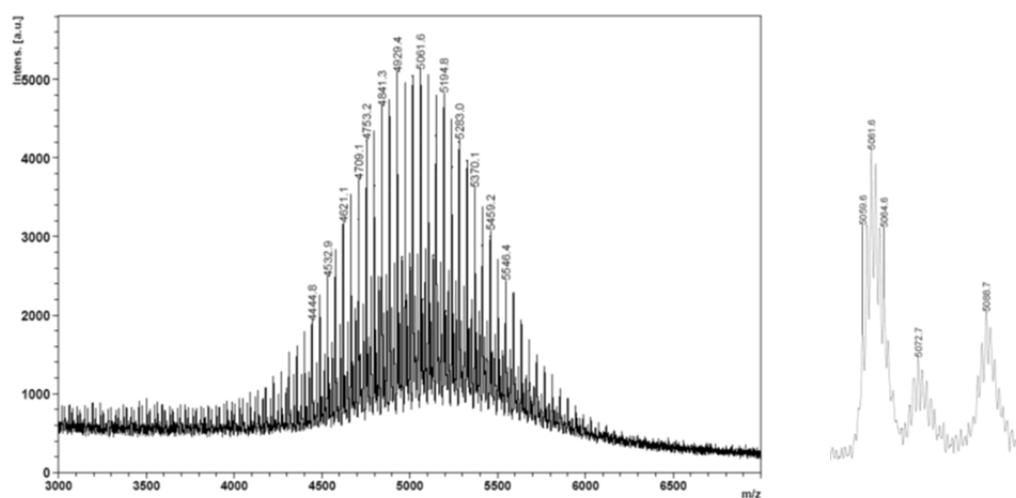


Figure 31. MALDI-ToF MS of HS-PEG₅₀₀₀-CONHGlucose. Reprinted with permission from {Silvestri A. et al. *Contrast media and molecular imaging*, 405-414 (2016)}. Copyright {2016} Wiley.

4.14 *Synthesis of the glucosamine functionalized nano-tracer*

A water solution of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (7.5 ml, 10 mM), sodium citrate (9 ml, 68 mM), and AgNO_3 (490 μl , 5.9 mM) was prepared and mixed at R.T. for 6 minutes. The pre-incubated mixture was, then, mixed to 250 ml of water at 100 °C. The mixture was stirred at 750 rpm for 1 h. Afterwards the reaction solution was left to cool to R.T. and 5 ml of glycerol were added. After 10 minutes a second mixture of HAuCl_4 (7.5 ml, 10 mM), sodium citrate (10 ml, 34 mM) and AgNO_3 (426 μl , 5.9 mM) was pre-mixed for 6 minutes and then added to the reaction solution, immediately followed by a hydroquinone solution (8 ml, 91 mM). Then, the colloidal solution was left to age for 1h, stirring at 750 rpm. The obtained Au NPs were directly employed without any further concentration or purification. The nano-tracer was obtained by adding (under argon atmosphere) 30 mg of HS-PEG₅₀₀₀-CONHGlucose. The PEG was dissolved in 5 ml of MilliQ water basified with NaOH (9 mg). The reaction mixture was allowed to stir for further 48 hours at R.T. The functionalized nano-tracer was purified and concentrated to a final volume of 0,7 ml using Amicon centrifugal Filter units.

4.15 *FT-IR of the nano-tracer*

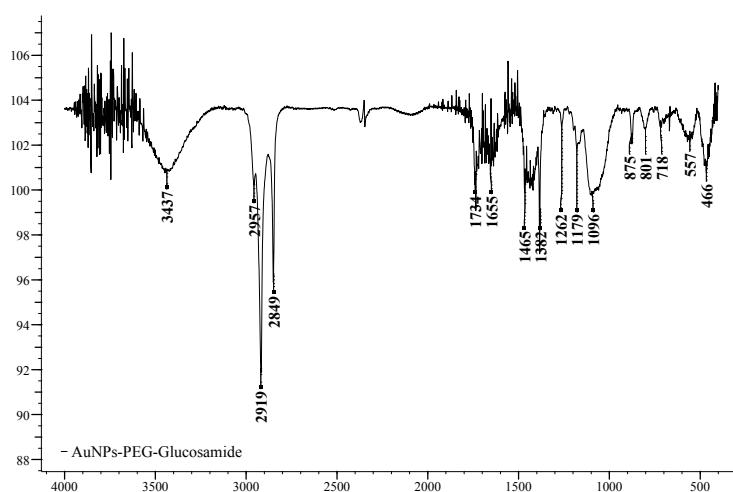


Figure 32. FT-IR spectra of the glucosamide functionalized nano-tracer. Reprinted with permission from {Silvestri A. et al. *Contrast media and molecular imaging*, 405-414 (2016)}. Copyright {2016} Wiley.

4.16 TEM characterization of the nano-tracer

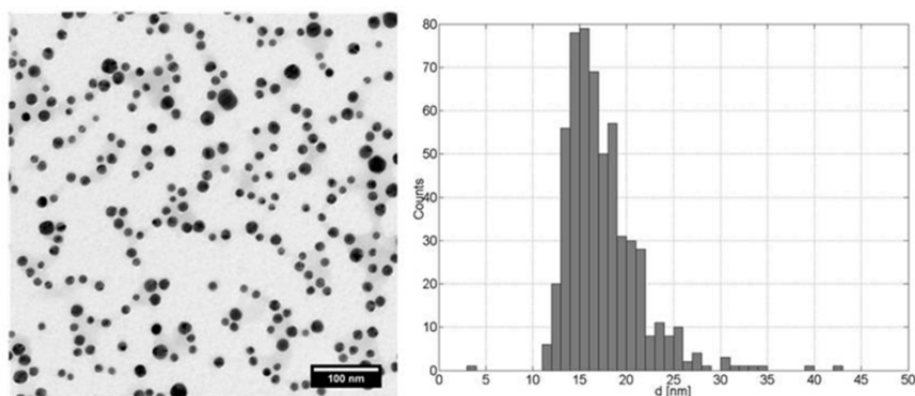


Figure 33. Left: TEM micrograph of nano-tracer. Right: statistical distributions ($d_m = 16.6 \pm 3.9$ nm). Reprinted with permission from {Silvestri A. et al. *Contrast media and molecular imaging*, 405-414 (2016)}. Copyright {2016} Wiley.

4.17 UV-vis stability of the nano-tracer

The colloidal stability of the nano-tracer was monitored by mean of UV-vis spectroscopy. During the concentration step (from 250 ml to 600 μ l) any alteration of the plasmonic peak was noticed. The concentrated solution presented a broader plasmonic peak only after 14 days of fridge storage, anyway no modifications were observed in of the maximum wavelength. This phenomenon could be ascribed to a slightly aggregation of the nanoparticles in this highly concentrated conditions.

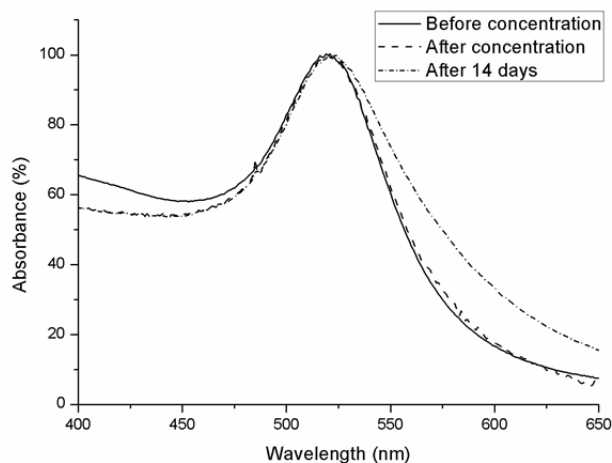


Figure 34. UV-vis evaluation of nano-tracer stability. Reprinted with permission from {Silvestri A. et al. *Contrast media and molecular imaging*, 405-414 (2016)}. Copyright {2016} Wiley.

From the UV-vis spectrum of **AuNP7** concentrated at 600 μ l it is possible to determine the hydrodynamic diameter of the nanoparticles, using the following formula:

$$D_H = \exp(B_1 \frac{A_{spr}}{A_{450}} - B_2)$$

Where A_{spr} is the absorbance of the plasmonic peak A_{450} is the absorbance at 450 nm and B_1 and B_2 are experimentally determined values ($B_1= 3.55$ and $B_2=3.11$)⁵⁰. The value obtained (31 nm) is in accordance with the results obtained by using dynamic light scattering (DLS) ($D_H= 33$ nm)

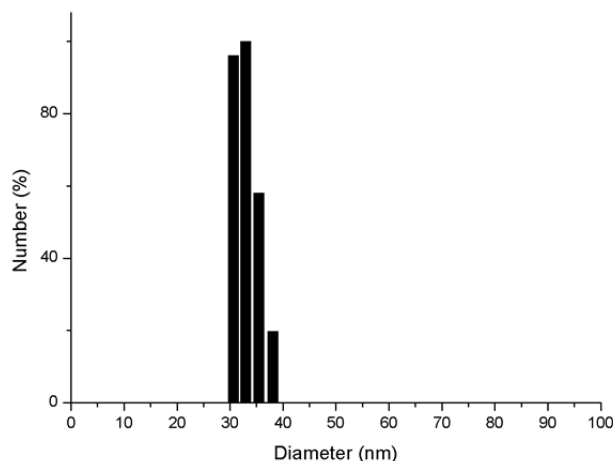


Figure 34. Hydrodynamic diameter distribution of the nano-tracer obtained by DLS measurements. Reprinted with permission from {Silvestri A. et al. *Contrast media and molecular imaging*, 405-414 (2016)}. Copyright {2016} Wiley.

4.18 *Synthesis of Au/Acetone SMA*

The synthesis of Au/acetone Solvated Metal Atom (SMA) solution was performed in a static MVS reactor. Gold vapours are generated by mean of resistive heating of 500 mg of gold shots inside an alumina crucible. The metal vapours were co-condensed with acetone (100 mL) at liquid nitrogen temperature inside the glass reactor of the MVS apparatus. Subsequently, the reactor chamber was allowed to warm up to the melting point temperature of the solid matrix (-80 °C). The resulting solution was transferred in a Schlenk tube and stored at -20 °C in a refrigerator. The Au content in the SMA solution was determined by ICP-OES (4.65 mg Au/mL).

4.19 *Synthesis of Au-PEG NPs by mean of aqueous digestive ripening*

A portion of the Au/acetone SMA solution (5 mL, 0.12 mmol of Au) was dropped inside 150 ml of a 1 mg/ml solution of HS-PEG₅₀₀₀-OCH₃ (0.03 mmol) at 0 °C. The mixture was stirred 1 h in a ice bath and, subsequently, 1 h at R.T. Then the mixture was refluxed for different time frames: 1, 3, 6 or 18 hours. CH₃O-PEG₅₀₀₀-SH-capped Au NPs were concentrated and purified by centrifuge using Amicon™ centrifugal filters (30 KDa cut-off).

4.20 Correlation HU vs Au [mg/ml]

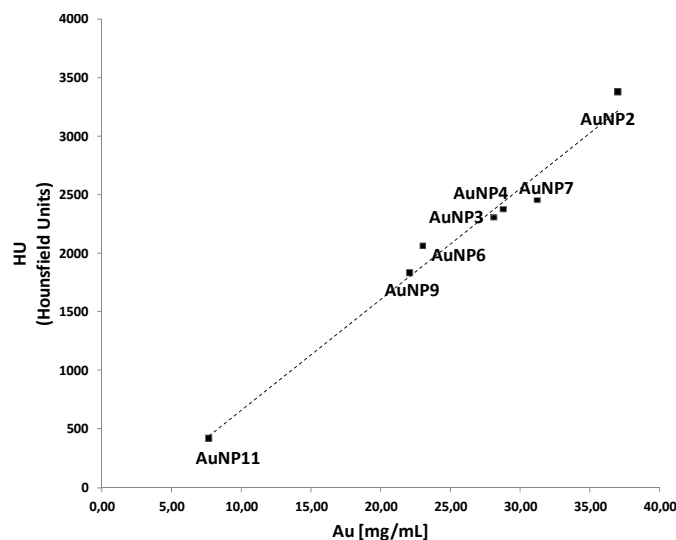


Figure 36. *In vitro* attenuation values of the different colloidal solutions plotted against the Au concentration, demonstrating the linearity of the relationship. Reprinted with permission from {Silvestri A. et al. *Contrast media and molecular imaging*, 405-414 (2016)}. Copyright {2016} Wiley.

4.21 *In vivo* CT imaging

CD1 mice (20-25 g) were furnished by Harlan Laboratories (Udine, Italy) and ministered to laboratory condition. Institutional guidelines, complied with national and international laws and policies, were followed during taking care of the animals and performing the preclinical procedures. The ethical committee of UNIMIB (Università degli Studi di Milano Bicocca) approved the presented study. We studied two sets of healthy and injured animals. The anesthetization of the animals was performed employing ketamine and xilazine. Lung injury was induced into the right bronchus by instillation of 1.5 ml/kg HCl 0.1 M, passing through a small tracheal incision (34). After the removing of the bronchial catheter the tracheal incision was sutured. Animals were awakened and NP injected. The micro-CT imaging was performed 24 hours after the induction of the damage, to allow the development of the inflammatory reaction. For imaging, anesthetized mice received PEG-capped Au NPs injection via tail vein (i.v.) (Au concentration described to **Table 2**, volume 200 μ l) and underwent to micro-CT scans at different time points (baseline, 5, 60 and 240 minutes after injection). Each CT scanning took around five minutes to be accomplished (voltage 58 kV, current 431 μ A). Images reconstruction was performed using a smoothing filter and were corrected for beam hardening and ring artefact. Before to perform the image analysis, the Hounsfield Units (HU) scale was calibrated, by scanning an air and water samples, which values were respectively ascribed to 0 HU and -1000 HU. The density (in HU) was then manually measured on selected Regions of Interest (ROIs) in the monitored organs.

Bronchoalveolar lavage sample preparation: 0.6 ml of lavage solution comprising of cells were recovered from the right lung and fixed with 2.5% glutaraldehyde and 4% para-formaldehyde. The collected material was centrifuged and the resulting pellet suspended in sodium alginate solution.

15 μ l drops of this suspension were extruded through an Eppendorf-type micro-pipette into a 0.1M calcium chloride buffer at pH 7.2. An Epoxy/Araldite resin was employed to contain this drops and the resulting pellet was cut in a series of for TEM analysis. For the histological examination, 4% para-formaldehyde was used to fix the lungs, then the organs were treated with paraffin and stained with eosin and hematoxylin. For the ICP-OES determination of the metal content inside the organ, aliquots of organs (1 ml each), homogenized in PBS solutions, were digested with aqua regia (2 ml). the operation was repeated for 10 times. The dried residues were dissolved in a 0.5 M HCl aqueous solution and then diluted to 10 ml.

Mouse	AuNPs	Dose (mg/mg BW)
#1	Nano-tracer	0.00023
#2	Nano-tracer	0.00012
#3	Nano-tracer	0.00024
#4	Nano-tracer	0.00023
#5	Nano-tracer	0.00022
#6	Nano-tracer	0.00025
#7	Nano-tracer	0.00024
#8	AuNP6	0.00013
#9	AuNP6	0.00020
#10	AuNP6	0.00024
#11	AuNP6	0.00025

Table 2. Doses of Au NPs infused in the different mice during the study.

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Chapter 3:

Application of Fluorescence Correlation Spectroscopy to the study of protein corona formation and intracellular dynamics of surface modified Au NPs

1. Introduction

1.1 Proteins corona and NPs cellular uptake

Nanoparticles (NPs) are characterized by a large surface to volume ratio that is reflected in a large superficial free energy. This potential favours the interaction of NPs with biological matters (e.g. proteins, lipids etc.) leading to the reduction of this superficial energy. The result of this interaction is the formation of a proteins corona (PC) around the surface. PC creates a complex interface that confers to the NPs a novel biological identity.^{1,2} The characterization of these coronae is fundamental in order to understand the interaction of the NPs with the biological matter and consequently with cells. In fact the formation of a PC affects the NPs' colloidal stability impacting on the biological response and cellular fate^{3,4}

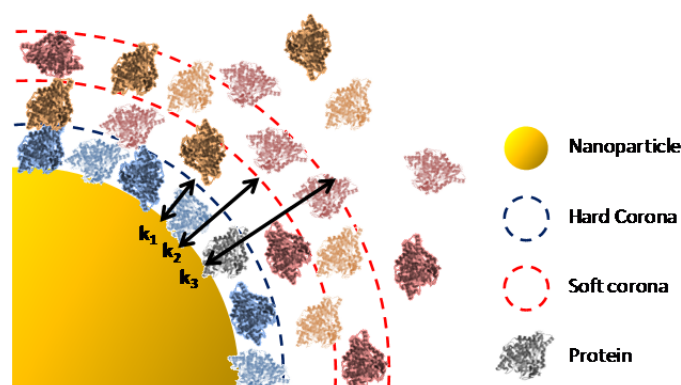


Figure 1. Graphical representation of the model of PC formation, showing the hard and soft corona layers. Each of several proteins layers is characterized by a specific dissociation constant (k_d).

The protein corona is generally described as composed by a “hard corona” (HC) and a “soft corona” (SC) (**Figure 1.**). This distinction is defined on the base of the binding strength between the NPs and the proteins. HC is formed of proteins strongly bound to the surface that presents a very slow exchange rate with the surrounding environment. This shell is stable over the time and able to preserve the fingerprint of the environment to which the NPs have been exposed. HC is generally composed by few tens of proteins that are not necessarily the most abundant in the

plasma. SC is formed by two or more layers of proteins weakly bound to the HC layer or to the NP surface. For this reason, SC presents a faster protein exchange rate with the surrounding environment.

The protein corona formation process is thermodynamically driven and has been widely investigated in the last years. Several models have been elaborated in order to describe the experimental evidences observed during the study of this phenomenon. In the first model, described by Salvati et al. in the 2012, the PC formation is driven by relative proteins binding affinities and concentrations that cause their adsorption and desorption from NP surface.⁵ According to this model, in a first instance, most abundant proteins bind to the NP surface. Then, proteins with higher affinities for the NP surface will displace the first adsorbed ones. This phenomenon, known as “Vroman effect”, was observed for the first time on flat surfaces in 1964.⁶ This model gives a valid explanation to the dependence of HC and SC from the binding strength of the proteins. Treuel et al. recently developed a new model to explain the protein corona evolution.^{7,8} In this model, the authors describe PCs enriched with numerous proteins which evolve over time in quantity rather than quality.⁹ Moreover they found new kinetic trends for some proteins that do not follow Vroman effect. In their model, the PC is formed within 30 seconds from the exposure to complex biological environment and is made up of multi-layers of proteins. Also in this case the inner layer is described as more stable while the outer layers, more dynamic, can exchange proteins with the surrounding environment. The greatest difference is that these exchanges can happen following kinetics both dependent or not from the Vroman effect.

The PC formation can be strongly affected by the NPs characteristics and by the surrounding environment. Regarding the NPs, the main structural properties that can affect the formation of the protein corona are: the coating, the size, the curvature radius of NPs and the superficial chemistry of the systems. Concerning the size, is known that smaller NPs adsorb more proteins, having a larger total surface area. In particular, the PC for small NPs is constituted for the 60-80% by proteins with MW below 50 kDa because more suitable for the curvature radius. On the contrary, PCs associated to bigger NPs are enriched with larger proteins.¹⁰ The surface charge of the NPs can affect the amount of adsorbed proteins and the composition of the protein corona,⁹ however other interactions, beyond the electrostatic ones, can have a role in the PC nature and composition. Among them are included hydrogen bonding, London dispersion and hydrophobic/hydrophilic interactions. The surface chemistry of NPs can be modified, with the aim to minimize these interactions, achieving a reduced proteins adsorption and a stealth effect (i.e. limitation of the interactions with macrophages resulting in a prolonged NPs circulation in blood pool). Sakulkhu et al. demonstrated that the chemistry of the coating can significantly affect the amount and types of adsorbed proteins.^{11,12} The authors compared the PCs of SPIONs coated with dextrane and PVA (polyvinyl alcohol). They found out that the two PCs differed in composition with proteins specific only for PVA or for dextrane.

Environmental parameters can affect the formation of the PC. The main factors to consider are the exposure time, the temperature of incubation and the dynamic or static state of the plasma solutions. Regarding the exposition time, a study performed by Tenzer and coworkers revealed that a complex PC is already formed within half a minute of exposure to the media.⁹ Moreover is reported that the major modifications happen at early times, while the compositions of the PC will be constant once reached an equilibrium state. The temperature is another fundamental factor: it has been shown that temperature variations, above 37°C, can affect corona composition and proteins abundance^{13,14}. Between the environmental factors, the most interesting one is the dynamic of the fluid that contains the NPs. Recently Pozzi et al.¹⁵ used a peristaltic pump to

reproduce the abdominal aortic flow rate and they found differences between the PC size and composition formed in static or dynamic conditions. In particular, the dynamic incubation enriched corona with apolipoproteins with a significant impact on the interactions with macrophages.

The PC impact on active targeting properties and the cellular internalization of the NPs can be either positive or negative depending on the nature and composition of the PC. For example serum albumin, when adsorbed on nanoporous-polymeric NPs, induces a change in the NPs dimension and conformation, increasing the uptake in macrophages but not in monocytes¹⁶. As well it has been proved that the adsorption of IgG on the NP surface can promotes macrophage uptake and NPs removal from blood stream.¹⁷ Moreover, apolipoproteins are involved in lipid and cholesterol transport in the membrane. As a consequence of the interactions of apolipoproteins enriched coronae with cells, reorganization of the cellular membrane proteins and lipids can be speculated.¹⁰ Recently, Ritz et al. demonstrated that the composition of the protein corona can effectively favour or hinder the cellular uptake of polystyrene NPs. In the specific proteins ApoA4 and ApoC3 were identified to decrease the cell uptake, instead ApoH has been demonstrated to increase the internalization¹⁸. An innovative concept is the modification of NPs surface with ligands to favour the formation of a corona enriched with certain proteins to control cell internalization, as published the last year by Schöttler et al.¹⁹ The main concept is that the protein corona is necessary for the stealth properties of polymers like poly ethylene glycol (PEG) and poly phophoether (PEEP). More in detail, they proved that PEG and PEEP are stealth polymers and can, not only reduce the amount of proteins adsorbed on the NPs, but also affect the composition of protein corona formed around them, favouring the absorption of proteins that are effectively necessary to prevent the non-specific cellular uptake. Moreover, they demonstrate that clusterin protein plays a crucial role in the process, and is an interesting candidate for future studies on stealth effects.

1.2 Fluorescence Correlation Spectroscopy

Fluorescence correlation spectroscopy (FCS) is a high spatial and temporal resolution correlation technique for the analysis of fluorescent molecules and species.²⁰ Differently from other techniques based on fluorescence, FCS analyses the time-dependent intensity fluctuations of the system, resulting from the dynamic processes of the molecules. Typically, these fluctuations are originated by translation diffusion across an observation volume. When the fluorescent specie enters into the observation volume, a burst of emitted photons takes place, due to the excitation of the fluorophore. A rapid diffusion of the specie outside from the volume is reflected in a short duration of the photon burst. Instead, with a slower diffusion, the photon burst will show a longer life-time. The diffusion time of the fluorophore can be determined applying a correlation analysis of the intensity fluctuations. Other phenomena that can cause these intensity fluctuations are: rotational diffusion, bindings between ligands and macromolecules, intersystem crossing, internal macromolecule dynamics and excited-state reactions.²⁰⁻²³ In a typical FCS analysis setup (**Figure 3a**.) the fluorophore excitation is usually achieved by mean of a limited spot focused laser. A dichroic filter selects the emission and the signal coming outside from the desired volume is rejected by the confocal pinhole. The volume of observation is called confocal volume and can be approximated to an ellipsoid with elongated z axis. The volume of the ellipsoid is in the order of 1 fL (**Figure 3b**) and the concentration of the fluorophores, needed to perform the analysis, in the range of 1 nM. In fact, differently from other fluorescence techniques, FCS requires low fluorophore concentrations, in order to have only few molecules inside the confocal volume during the measurment.²⁰⁻²³

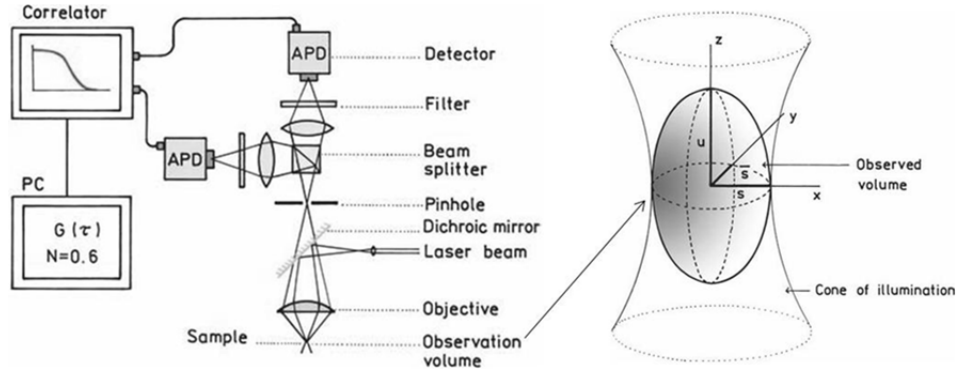


Figure 3. a) Typical instrumentation for FCS **b)** Confocal volume with the peculiar ellipsoidal shape. Reprinted with permission from {Principles of Fluorescence Spectroscopy (ed. Lakowicz, J. R.) 797–840}. Copyright {2006} Springer Science + Business Media, LLC.

Changes in signal intensity can be correlated to variations in occupation number. When the fluorophore has slow diffusion, it will move slowly across the confocal volume. Instead if the diffusion is faster the occupation number and intensity will change more rapidly. A statistical analysis of the time-dependent intensity can provide the values of frequency distribution and amplitude of the fluctuations. The frequency distribution is obtained comparing the intensity at time t ($F(t)$), with the intensity measured after a delay time τ ($F(t + \tau)$). With intensity fluctuations slower than τ , $F(t)$ and $F(t + \tau)$ have higher probability to be similar in magnitude (**Figure 4 a**). When the diffusion is faster than the delay time τ , $F(t)$ and $F(t + \tau)$ are more probable to be different and their magnitudes not related (**Figure 4 b**). In order to determine this correlation in a large range of delay times (τ), FCS instruments are equipped with a dedicated correlation board. The resulting autocorrelation function ($G(\tau)$) provides information on the fluorescent species concentration (occupation number) and their diffusion times and coefficients.^{20,24–27} The autocorrelation function can be expressed as the product between the intensity at time t , ($F(t)$) and intensity after the delay time τ ($F(t + \tau)$). This product needs to be averaged over a wide number of measurements:^{20,24–27}

$$R(\tau) = F(t)F(t + \tau) = -\frac{1}{T} \int_0^T F(t)F(t + \tau)dt \quad \text{eq. 1}$$

where T is the data collection time and the products $F(t)F(t + \tau)$ are normalized over the factor $1/T$. The range of data collection can be comprised between few seconds and several minutes with a delay time τ usually in the range of milliseconds.

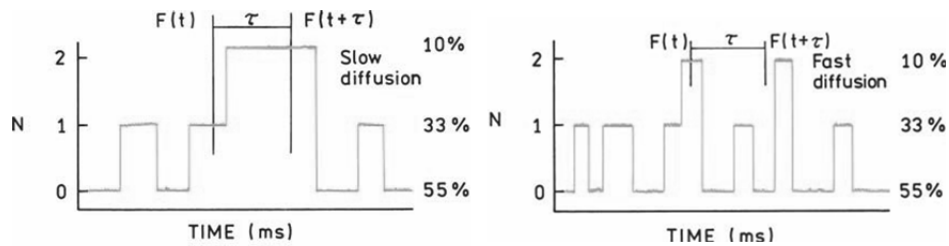


Figure 4. Intensity fluctuations of fluorophores in the focal volume in: a) case of fast diffusion b) case of slow diffusion. Reprinted with permission from {Principles of Fluorescence Spectroscopy (ed. Lakowicz, J. R.) 797–840}. Copyright {2006} Springer Science + Business Media, LLC.

A derivative function can be introduced in order to consider the fluctuations of $F(t)$ around a mean value that is actually the quantity of interest of the analysis.

$$\delta F(t) = \langle F \rangle - F(t) \quad \text{eq. 2}$$

Substituting **eq.2** in **eq.1** and normalizing fluorescence intensities by average square intensity is possible to obtain the following expression of the correlation function

$$G(\tau) = \frac{\langle F(t)F(t+\tau) \rangle}{\langle F \rangle^2} = 1 + \frac{\langle \delta F(0)\delta F(\tau) \rangle}{\langle F \rangle^2} \quad \text{eq. 3}$$

In the expression, for simplicity, t has been replaced with 0. In this way only the τ value is relevant. FCS is commonly employed to measure translational diffusions. The diffusion rate can be affected by the molecules size and their interaction with other species. For the case of translational diffusion in three dimensions the correlation function is given by

$$G(\tau) = G(0) \left(1 + \frac{\tau}{\tau_D}\right)^{-1} \left(1 + \frac{\tau}{\gamma^2 \tau_D}\right)^{-1/2} \quad \text{eq. 4}$$

Where $G(0)$ is the amplitude at $\tau=0$ and $\gamma = \frac{z_0}{\omega_{xy}}$ is the structural parameters of the instruments that represent the ratio of the Gaussian used to approximate the focus.

Eq 4. describes the dependence between the correlation function and the diffusion coefficient of the free fluorescent diffusing specie. In **Figure 5** are depicted some examples of autocorrelations functions. The typical trend shows that $G(\tau)$ amplitude decreases at the increasing of τ values. At long delay times $G(\tau)$ comes close to zero since the fluorophore loses the memory of its initial position. The value of τ_D can be determined from the autocorrelation function, employing a least-squares fitting of the experimental data. As is possible to see from **Figure 5** a shift of the correlation function, along τ , is reflected in slower fluctuations correlated to a slower diffusion coefficient of the fluorophores. The diffusion time of the fluorescent specie is inversely proportional to the diffusion coefficient following the equation:

$$\tau_D = \frac{\omega_{xy}^2}{4D} \quad \text{eq. 5}$$

The correlation function depends from the concentration of dye in solution. In fact, from the autocorrelation function is possible to provide estimation of the molecules present in the confocal volume (N). The number of fluorescent molecules is inversely proportional to the intercept at $\tau = 0$. This value, called correlation function amplitude or $G(0)$, is equal to $1/N$. This relation easily explains why in FCS is recommended to operate at low fluorophores concentrations: smaller is the number of molecules larger will be the amplitude of the autocorrelation function. Another fundamental aspect of $G(\tau)$ is that the correlation function for single species is independent from the brightness of the fluorophore.

Employing FCS is also possible to derive the diffusion of coefficients of two or more species contemporaneously diffusing in the same solution. Each diffusing species will have a specific diffusion coefficient D_i and diffusion time τ_i . If all the species have the same brightness, the correlation function can be described as:

$$G(\tau) = \frac{1}{N} [\sum_{i=1}^n \rho_i G_i(\tau)] = \frac{1}{N} \left[\sum_{i=1}^n \rho_i \left(1 + \frac{\tau}{\tau_i}\right)^{-1} \left(1 + \frac{\tau}{\gamma^2 \tau_i}\right)^{-\frac{1}{2}} \right] \quad \text{eq. 7}$$

where ρ_i is the fraction of each diffusing specie and $\sum_{i=1}^n \rho_i = 1$.

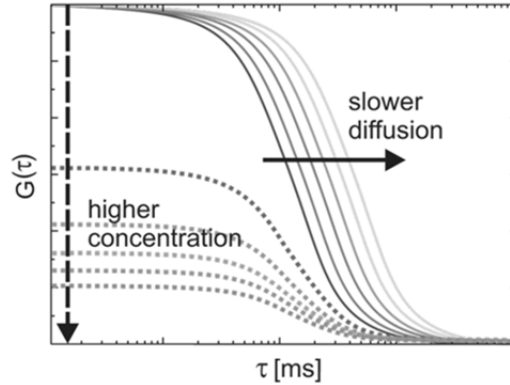


Figure 5. Examples of autocorrelation functions plotted against the delay time τ , showing the dependence of the slopes from the diffusion times and fluorophore concentration.

Other factors to be considered in the description of the autocorrelation function are the non-fluorescent states of the dyes. Due to the high illumination intensities employed in FCS, a “flickering” of the fluorescence intensity is frequently observed. This phenomenon is typically due to the electrons transition from first excited singlet state to the triplet state. When this relaxation pathway takes place, the fluorophore needs a longer time to return back to the ground state. For the duration of these gaps, the molecule cannot emit fluorescence photons, appearing dark. This phenomenon is called intersystem crossing (IC) and can be imagined like a series of dark intervals interrupting the fluorescence emission of the dye. This intersystem crossing gives rise to faster fluctuation than the movement of the molecules or particles, and a mathematical separation of the contributions is possible. The IC can be fitted by an exponential decay and the resulting correlogram possess an additional shoulder at short time scales. A complete cross correlation function, taking in consideration all the described parameters, can be expressed as:

$$G(\tau) = \frac{1}{N} \sum_{i=1}^n \rho_i G_i(\tau) = \frac{1}{N} \left(\frac{1 - \theta_{non} + \theta_{non} e^{-\tau/\tau_{non}} - \theta_{trip} + \theta_{trip} e^{-\tau/\tau_{trip}}}{1 - \theta_{non} - \theta_{trip}} \right) \left[\sum_{i=1}^n \rho_i \left(1 + \frac{\tau}{\tau_i}\right)^{-1} \left(1 + \frac{\tau}{\gamma^2 \tau_i}\right)^{-1/2} \right] \quad \text{eq. 8}$$

Numerous are the applications of FCS technique in the biomedical field. For example, this correlation technique is frequently employed to measure binding interaction and affinities between biomolecules. Some examples are the of binding of small ligands to proteins,^{28,29} the DNA hybridization,^{30,31} the protein–protein interactions,³² the DNA– protein interactions,^{33,34} the polymerase chain reactions,^{35,36} and the interactions of substrates with receptors.^{37,38} Moreover the estimation of the mobility parameters and diffusion coefficient of free species in solution is one of the main applications of FCS, and the technique is widely employed to monitor the behaviour of biological active molecules in water solutions. The high spatial resolution of the technique makes it a functional technique for intracellular measurements.³⁹ FCS allows to reach an increased sensitivity and high dynamic performance using low dye concentrations and laser powers, which are less stressful conditions for cells during the investigation. In the field of NPs characterization, in the last years FCS is acquiring an increasing importance. The possibility to perform measurement in complex matrixes like proteins solution and intracellular compartment

allowed several research group to study the hydrodynamic radius increase and the aggregation of the NPs in different biological matters. In particular, Parak and co-workers published several articles monitoring the formation of protein corona, around metallic nanoparticles, with FCS technique. The authors were able to extrapolate from the analysis, information like the affinity constants between proteins and NPs,⁴⁰ the effects of different ligands on the PC formation⁴¹ and even the disposition of the proteins around the NP surface.⁴² Recently Murray et al. published an original paper describing a quantitative study on the intracellular dynamics of gold NPs realized by FCS. The authors obtained interesting information on the state of aggregation of NPs inside cells revealing the presence of single NPs or NPs aggregates and estimating their punctual concentration.⁴³

2. Results and discussion

2.1 Aim of the work

A fundamental aspect in the rational design of NPs is the choice of the superficial chemistry. The outer shell of NPs is the most exposed part of the material and plays a fundamental role in the interaction with the surrounding environment. Therefore, the behaviour in biological fluids, in cell or *in vivo* is strictly related to the nature of the surface of the nano-material. The ligands and surfactants typically employed to stabilize and cover the nanoparticles can be divided in two main classes: charged molecules (that will stabilize the NPs by electrostatic interactions) and neutral molecules (in which the main stabilizing factor is the steric repulsion). Among the neutral ligands, PEG has a prominent role, thanks to the stealth and water dispersability properties that can confer to NPs. These advantages make this polymer an excellent stabilizing agent in the design of nano-materials in biosciences.

The aim of the work presented in this chapter (performed in collaboration with Soft Matter Nanotechnology Group – CIC biomaGUNE, San Sebastian, Spain) is to exploit the FCS technique to acquire information on the behaviour of fluorescent Au NPs with different coatings in complex matrixes, like enriched proteins media and in cells.

2.2 Design and synthesis of the Au NPs

Au NPs, among the metallic based NPs, are the most biocompatible and are widely employed in the field of the nano-medicine and biosciences. As pointed out during our *in vivo* studies on the bio-distribution of PEGylated Au NPs (**Chapter 2**), the optimal dimension of NPs for biomedical applications is comprised in the range between 10 and 60 nm. In fact, NPs with a diameter smaller than 10 nm can be easily secreted from the human body because can freely pass through the renal glomerulus. Nanoparticles with diameters bigger than 60 nm can be, instead, easily detected by RES (reticulum-endothelial system) organs leading to the accumulation in the liver and kidney or can remain stacked in the lungs alveolus. Taking into consideration these aspects, we choose as model for our studies the same Au NPs that presented the best *in vivo* behaviour in **Chapter 2 (Au NP 6, ~15 nm spherical gold NPs)**. As already explained in the previous chapters, another advantage given by the employment of Au NPs is that the gold atoms on the surface can easily create strong bonds with sulphur atoms. In this way, we can guarantee a rapid and stable superficial modification of our systems. In order to evaluate the effect of the superficial chemistry

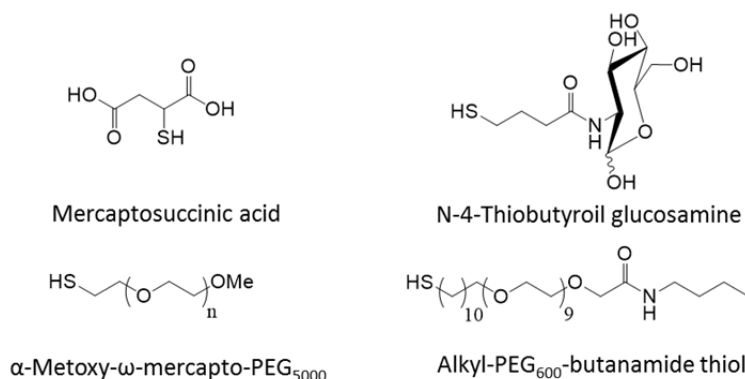
on the interaction with proteins and in the intracellular dynamic of the NPs we chose 4 different types of stabilizing ligands (**Scheme 1**):

- **Mercapto succinic acid**: is a small charged molecule widely employed to stabilize gold NPs in water solution. Is comprised of a SH group for the bounding to the gold surface and two carboxylic moieties that allows to have electrostatic repulsion between NPs avoiding aggregation. In some works is reported that negative charged NPs can induce stealth abilities reducing the uptake by the RES organs *in vivo* models⁴⁴. This aspect makes this ligand an interesting candidate for our studies in order to perform a comparison with the neutral coatings.

- **N-4-thiobutyroil glucosamine**: Glucose can efficiently suspend and stabilize NPs in aqueous environment thanks to the high number of hydroxyl groups present in his structure. Moreover glucose coated NPs are reported in literature for their stealth abilities *in vivo* models^{45,46}.

- **α -Metoxy- ω -mercapto PEG₅₀₀₀**: This type of polymer is one of the most used for the design of biocompatible and stealth NPs for biomedical application. Among the several advantages, PEG can: improve the water dispersability of the NPs, decrease the aspecific interaction with biomolecules and mask the particles from the host immune system (resulting in a prolonged circulatory time and a reduced renal clearance).

- **HS-Alkyl-PEG₆₀₀ ([1-mercaptoundec-11-yl]PEG₆₀₀)-acetic acid butanamide** : this amphiphilic molecule is constituted by a aliphatic chain (undecanthiol) conjugated with a PEG chain of 600 Da and possesses on the other edge a butanamide functionality. With this kind of surfactant the PEG properties coexist with the advantages of a more compact packing of the molecules on the NPs surface, due to the presence of the aliphatic chain⁴⁷.



Scheme 1. Summary of molecules selected for the coating of Au NPs

The Au NPs were synthesized employing the ‘one-pot’ strategy described in **Chapter 2**. The selected ligands were added directly to the reaction mixture, to stop and cap nanoparticles growing. In this way a panel of four Au NPs characterized by different coatings was made. For all particles 10% of the surface was coated introducing a α -amino- ω -thiol PEG₅₀₀₀ to allow the subsequent labelling of the system with a fluorescent molecule. We selected the long amino PEG chain in order to have a large distance between the metallic core and the fluorophore molecule, reducing the quenching effect deriving from the presence of the noble metal. We choose to cover

a 10% of the surface with α -amino- ω -thiol PEG₅₀₀₀ to have enough fluorescent signal from the NPs but at the same time to do not affect too much the surface properties of the molecule.

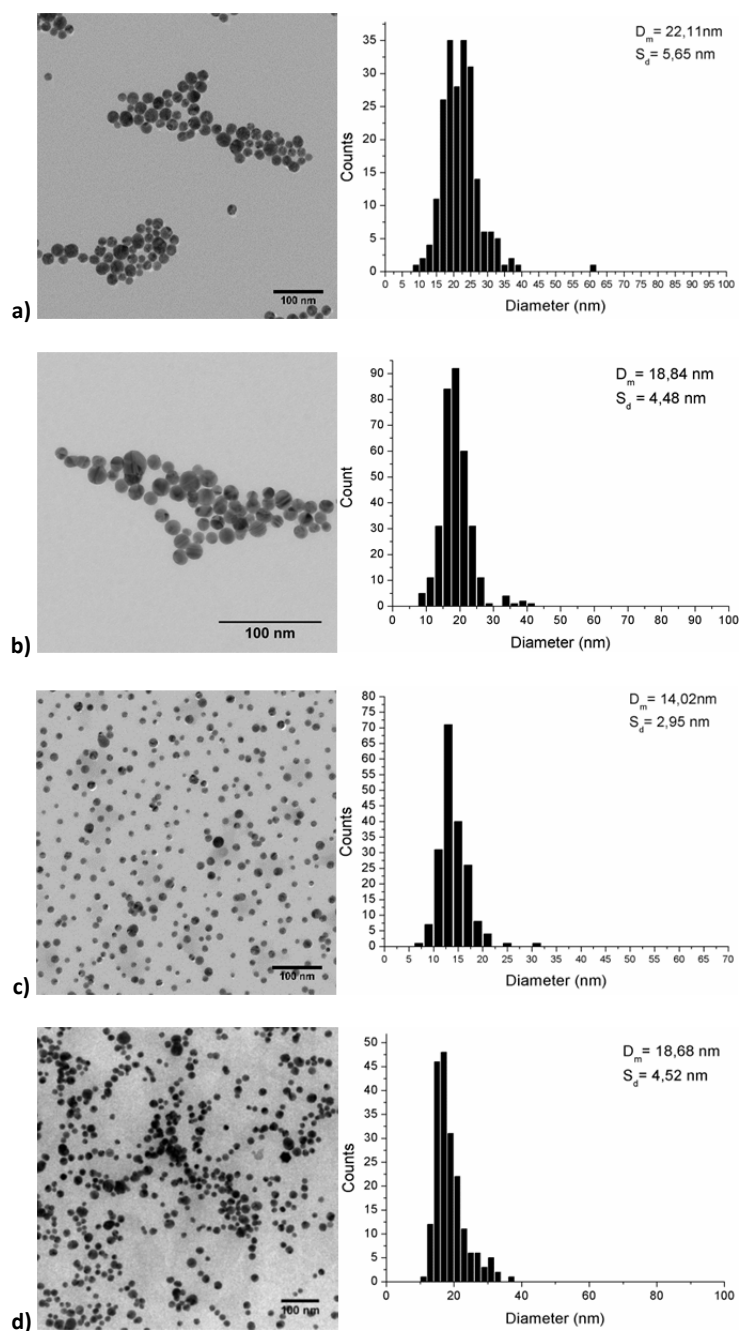


Figure 6. TEM micrographs of the nanoparticles composing the panel. In the order: **a)** Au-MSA NPs ($d_m=22.11 \pm 5.56\text{ nm}$), **b)** Au-Glucosamine NPs ($d_m=18.84 \pm 4.48\text{ nm}$), **c)** Au-PEG₅₀₀₀ NPs ($d_m=14.02 \pm 2.95\text{ nm}$) and **d)** Au-Alkyl-PEG₆₀₀ NPs ($d_m=18.68 \pm 4.52\text{ nm}$).

In **Figure 6** are represented the TEM micrographs and dimensional distributions of the obtained Au NPs. From this moment on, for simplicity, the non-fluorescent nanoparticles will be named Au-MSA NPs, Au-Glucosamine NPs, Au-PEG₅₀₀₀ NPs and Au-Alkyl-PEG₆₀₀ NPs. The obtained nanoparticles were concentrated on Amicon 30 kDa centrifuge filter units and purified by mean of dialysis (10 kDa cut-off). Once isolated and purified, the free amino moieties present on the surface were coupled with ATTO 550 NHS ester, employing a buffer solution at pH 8,7 to favour the reactivity of the groups in water. The nanoparticles were let to react for 1 hour at R.T. and then purified. First the excess of fluorophore was removed using amicon 30 kDa centrifuge filter units and finally purified by mean of 24 hours of dialysis with 100 kDa membranes, in order to ensure the elimination of all free diffusing dye. Despite this strong purification treatment, a fraction of free fluorophore was still present in the solution. To enhance the purity of our system we sonicated the nanoparticles for 10 min, then we double filtered the solution on 0,22 μ m cellulose syringe filters. The first step of this purification procedure should allow to detach the dye adsorbed on the NPs surface or entrapped in the PEG. Then the filtration process should allow to pass only the hydrophilic nanoparticles blocking the free ATTO 550, that is a hydrophobic molecule. The nanoparticles obtained were stocked as 1 mg/ml solution and used for the characterization, FCS and confocal measurements. From this moment on the fluorescent Au NPs will be named Au-MSA* NPs, Au-glucosamine* NPs, Au-PEG₅₀₀₀* NPs and Au-Alkyl-PEG₆₀₀* NPs (**Figure 7**).

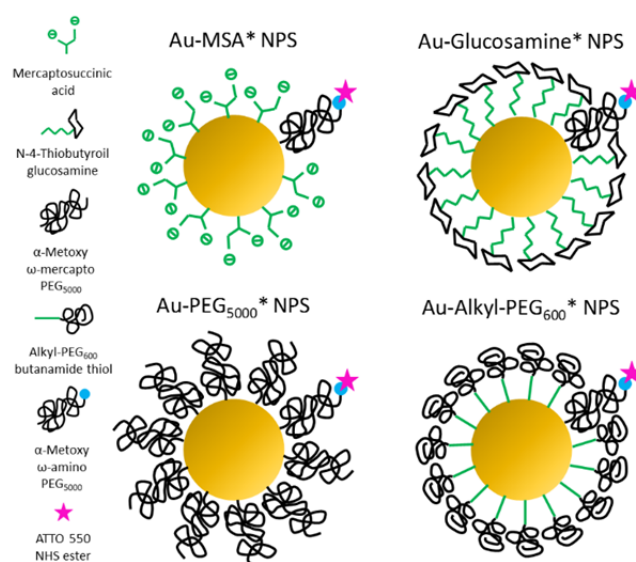


Figure 7. Summary of Au NPs designed to perform the FCS measurements

2.3 FCS for the determination of the Au NPs hydrodynamic radius

The formation of PC can be followed by the variation of the hydrodynamic diameter of the nanoparticles. FCS is an interesting technique employable for the determination of the hydrodynamic dimension of free diffusing species. In order to evaluate the formation of the PC around our Au NPs we developed an experimental procedure to perform FCS experiments on our samples that could allow us to derive with good confidence the hydrodynamic radius of our NPs. The samples were excited with a 561 nm laser and the employed optical pathway is described in

Figure 8. In first instance, we developed procedure for the determination of Au NPs hydrodynamic radius in water, comprised of 3 steps (**Figure 9**):

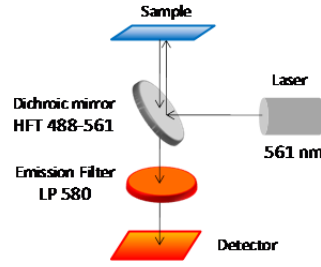


Figure 8. Schematic representation of the light path employed for the FCS measurements

Step 1: consist in the calibration of the instrument. Prior to each session of measurements 50 nM solution of a dye with known diffusion coefficient has been employed for the determination of the structural parameter of the system. The correlogram of the solution was registered and the autocorrelation functions $G(\tau)$ was fitted following a 3D diffusion model that can be denoted as:

$$G(\tau) = G_{\infty} + \frac{1}{N} X_{back} \cdot \left(\frac{1 - \theta_{non} + \theta_{non} e^{-\tau/\tau_{non}} - \theta_{trip} + \theta_{trip} e^{-\tau/\tau_{trip}}}{1 - \theta_{non} - \theta_{trip}} \right) \cdot [(1 - \rho_2 - \rho_3) \cdot g_1(\tau) + \rho_2 \cdot g_2(\tau) + \rho_3 \cdot g_3(\tau)] \quad \text{eq. 9}$$

Where the factor $g_1(\tau)$ is correspondent to:

$$g_i(\tau) = \left(1 + \frac{\tau}{\tau_{diff,i}} \right)^{-1} \cdot \left(1 + \frac{\tau}{\gamma^2 \tau_{diff,i}^2} \right)^{-1/2} \quad \text{eq. 10}$$

And the background correction is calculated like:

$$X_{back} = \frac{(I-B)^2}{I^2} \quad \text{eq. 11}$$

I is the intensity of the signal and B is background intensity.

The parameters involved in the function are:

- G_{∞} : offset of the correlation function
- N : overall particle number (including currently dark particles, for example in triplet state)
- $\theta_{trip}, \theta_{non}$: fractions of the particles in one of the first two non-fluorescent states
- τ_{trip}, τ_{non} : decay times of the first two non-fluorescent states
- ρ_1, ρ_2, ρ_3 ($\rho_1 = 1 - \rho_2 - \rho_3$): fractions of the three diffusing components
- $\tau_{diff,i}$: diffusion decay time of the i^{th} diffusing component
- $\gamma = \frac{z_0}{\omega_{xy}}$: structural parameters of the instruments that represent the ratio of the Gaussian used to approximate the focus
- ω_{xy} : lateral half axis of the focus
- z_0 : longitudinal half axis of the focus
- cps : average background corrected intensity during the measurement

Autocorrelation functions $G(\tau)$ were analysed by Quickfit 3.0 software (DKFZ, Germany) and employing a Levenberg Marquard least squares algorithm.

We choose Rhodamine B to perform the calibration, that is reported in literature to have a diffusion coefficient of $420 \pm 30 \mu\text{m}^2/\text{s}$. Knowing that the diffusion coefficient is equal to:

$$D = \frac{\omega_{xy}^2}{4\tau_D} \quad \text{eq. 12}$$

and imposing this value inside a first order fitting model, we derived the values of γ and ω_{xy} that describes the geometrical properties of the focal volume. For our system the γ value was typically comprised between 5 and 7, ω_{xy} between 200 and 300 nm.

Step 2: Once obtained the structural parameters of the confocal volume is possible to proceed with the measurement of the free diffusing dye (50 nM). The fitting of the autocorrelogram of free diffusing ATTO 550 has been performed employing the same model and algorithm as in step 1. Fixing the previously obtained values of γ and ω_{xy} in the fitting model is possible to derive the values of τ_{trip} , τ_D typical of the fluorophore. Performing more than 20 measurements on free ATTO 550 NHS ester we obtained an average diffusion coefficient of the fluorophore of $405 \pm 23 \mu\text{m}^2/\text{s}$.

Step 3: finally, the auto-correlogram of the fluorescent nanoparticle solution (100 $\mu\text{g}/\text{ml}$) can be registered. The fitting of the autocorrelation function was performed employing a 3D diffusion model and considering 2 diffusing species: in fact, despite the purification procedures a fraction of free diffusing dye was still detected in the samples. Is important to underline that this free fraction was always overestimated. In fact gold nanoparticles quench the dye molecules directly attached to their surface while enhance the fluorescence of the free dye fraction diffusing in their proximity⁴⁸. The parameters of γ , ω_{xy} , τ_{trip} and the τ_D of the first specie were fixed to the values experimentally determined from steps 1 and 2. The fitting function will return the value of τ_{D2} , corresponding to the diffusion time of the second specie present in solution (in our case the labelled gold nanoparticles). From the fitting function is possible to calculate also the fractions of the two populations composing the sample (residual free dye and labelled Au NPs). At the end, applying **eq. 13** is possible to derive, from the diffusion time, the diffusion coefficient and substituting the last one in Stoke-Einstein equation the hydrodynamic radius of the NPs (**Figure 9**).

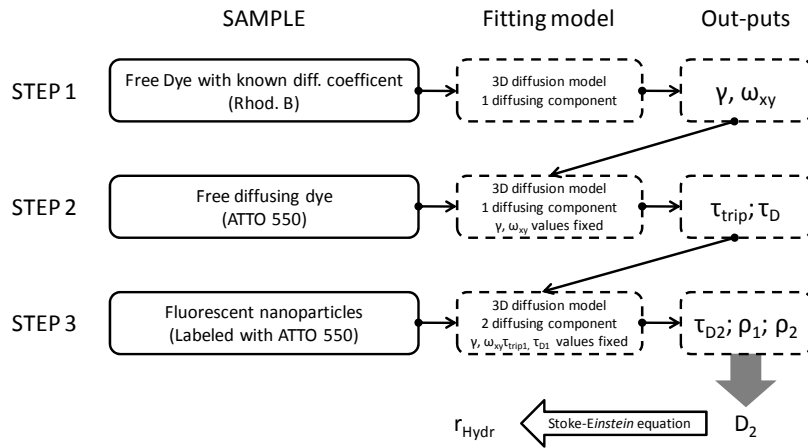


Figure 9. Flow chart of the procedure for the FCS data elaboration

In **Table 1** are reported the hydrodynamic diameters obtained using the described procedure, mediated over three independent measurements. The error correlated to the measure is the

standard deviation over the three measurements. Comparing the hydrodynamic diameters calculated from FCS with the core diameters deriving by TEM statistics we obtained a approximation of the radius increase and the hydrodynamic shell of our NPs. For Au-MSA NPs the calculated hydrodynamic shell increase is around 4 nm. Even if MSA is a small molecule the charge on the NPs surface brings to the formations of several hydration e and ionic shells, explaining such increase of the hydrodynamic radius. Instead glucose that is a small neutral molecule brings to a little increment in the hydrodynamic shell that is no significant compared with the experimental error. In Au-PEG₅₀₀₀ NPs the increment of the radius is of 7 nm and is in good correlation with previously reported experimental data for a 5 kDa PEG arranged in brush conformation⁴⁹. Finally, the hydrodynamic shell of Au-Alkyl-PEG₆₀₀ NPs was about 4.6 nm resulting comprised between the increments obtained MSA and PEG₅₀₀₀. To validate the FCS obtained hydrodynamic diameters we performed UV-vis and DLS analysis on batches of the correspondent non-fluorescent NPs. From the UV visible spectra of Au NPs is possible to calculate the hydrodynamic radius employing the absorbance of the plasmonic peak resonance (Abs_{spr}) and at 450 nm (Abs_{450})⁵⁰.

	TEM		FCS		
	Core diameter (nm)	S_d (nm)	D_h (nm)	S_d (nm)	Radius Increase (nm)
Au-MSA NPs*	22.1	5.7	30.3	2.9	4.1
Au-Glucosamine NPs*	18.8	4.5	24.8	5.4	3.0
Au-PEG₅₀₀₀ NPs*	14.0	3.0	28.0	5.0	7.0
Au-Alkyl-PEG₆₀₀ NPs*	18.7	4.5	27.8	1.7	4.6

Table 1. Table resuming the diameters obtained by of TEM and FCS relative radius increases

To obtain the hydrodynamic radius values we applied the equation described by Haiss et al. defining a correlation between the plasmonic peak high and the dimension of the nanoparticles:

$$D = \exp(B_1 \frac{Abs_{spr}}{Abs_{450}} - B_2) \quad \text{eq. 13}$$

The DLS data were analysed with Malvern software and the reported data are referred to the average diameter of the NPs evaluated in volume terms. The results obtained with the different techniques (**Table 2**) are comparable, even if not identical, validating the results and the FCS data elaboration procedure.

	TEM		UV-vis			DLS		
	Core diameter (nm)	S_d (nm)	D_h (nm)	S_d (nm)	Radius Increase (nm)	D_h (nm)	S_d (nm)	Radius Increase (nm)
Au-MSA NPs	22.1	5.7	28.0	1,5	2.9	32.7	17,2	5,3
Au-Glucosamine NPs	18.8	4.5	28.1	1,1	4.6	31.0	14,0	6.1
Au-PEG₅₀₀₀ NPs	14.0	3.0	22.8	1.7	4.4	25.0	13.1	5.5
Au-Alkyl-PEG₆₀₀ NPs	18.7	4.5	25.3	1.3	6.6	33.6	20.0	7.5

Table 2. Table resuming the diameters obtained by of TEM, Uv-visible, DLS with relative radius increases

2.4 FCS for the study of the interactions between Au NPs and proteins

In the previous paragraph, we validated the evaluation of hydrodynamic radii of labelled Au NPs by FCS. We decided to apply this method to derive the variation of the NPs hydrodynamic radii in the presence of proteins. In this way we obtained information on NPs-proteins interactions and on eventual PC formation as a function of the coatings. We focused our studies on the first hour of incubation, monitoring the samples at several time points, because it is a time window described as the most dynamic and interesting, where the main processes of interaction take place^{5,7,44}. We employed a commonly used cellular media, RPMI, to disperse our NPs. Furthermore, to accurately reproduce cell *in vitro* experiments we enriched it with 10% fetal bovine serum (FBS). The procedure employed for the determination of the hydrodynamic radius has been slightly modified respect the above described. When proteins are mixed to the NP solution, in fact adsorb in electrostatic way the part of free dye remaining in solution, creating a new fluorescent specie with a third diffusion time (**Figure 10**). The modified procedure is constituted by 4 steps, where the first two are exactly as the previously reported.

Step 1: calibration of the instrument. Registration of the correlograms of a 50 nM Rhodamine B solution with known diffusion time and determination of the structural parameter of the system (γ and $\omega_{x,y}$).

Step 2: measurement of the free diffusing dye (ATTO 550). Fixing the previously obtained values of γ and $\omega_{x,y}$ in the fitting model is possible to derive the values of τ_{trip} , τ_D typical of the fluorophore.

At this point a third step is inserted in order to identify a medium diffusion time of the proteins presents in FBS:

Step 3: the auto-correlogram of a mixture of ATTO 550 solution in RPMI with a 10% of FBS has been registered. The fitting of the autocorrelation function is performed employing a 3D diffusion model and considering 2 diffusing species. The parameters of γ , ω_{xy} , τ_{trip} and the τ_D of the first specie were fixed to the values experimentally determined from steps 1 and 2. The fitting function will return the value of τ_{D2} , corresponding to the diffusion time of the second specie present in solution (in this case the protein mixture). From the fitting function is possible to calculate also the fractions of the two populations composing the sample (residual free dye and proteins).

The last step was modified employing a 3 components model in order to consider in the fitting also the contribution derived from the fluorescent proteins:

Step 4: finally, the auto-correlogram of the fluorescent nanoparticle solution (100 $\mu\text{g/ml}$) in 10% of FBS (RPMI solution) can be registered at different time points. The fitting of the autocorrelation function is performed employing a 3D diffusion model and considering 3 diffusing species. The parameters of γ , ω_{xy} , τ_{trip} , τ_{D1} , and τ_{D2} of the first and second species were fixed to the values experimentally determined from steps 1, 2 and 3. The fitting function will return the value of τ_{D3} , corresponding to the diffusion time of the third specie present in solution (corresponding to the adducts between proteins and Au NPs). From the fitting function is possible to calculate also the fractions of the three populations composing the sample (residual free dye, free proteins and Au NPs-Protein adducts). Employing **eq. 12** is possible to derive, from the diffusion time, the diffusion coefficient and substituting the last one in Stoke-Einstein equation the hydrodynamic radius.

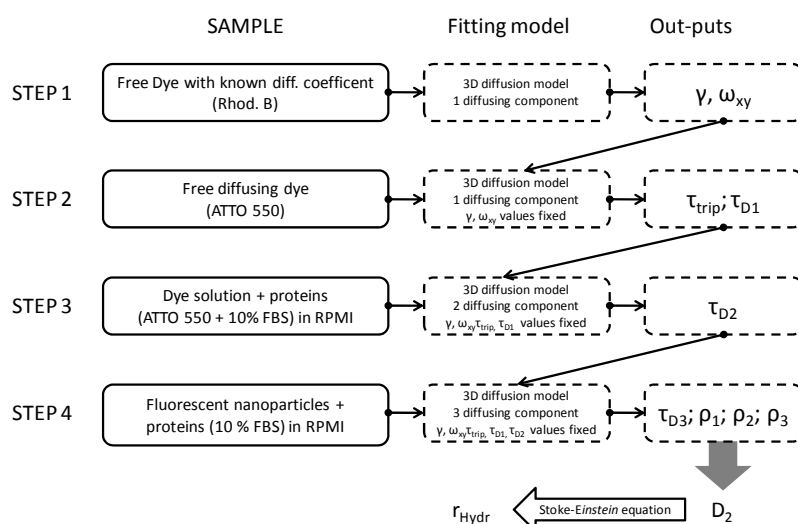


Figure 10. Flow chart of the procedure for the FCS data elaboration in protein solutions.

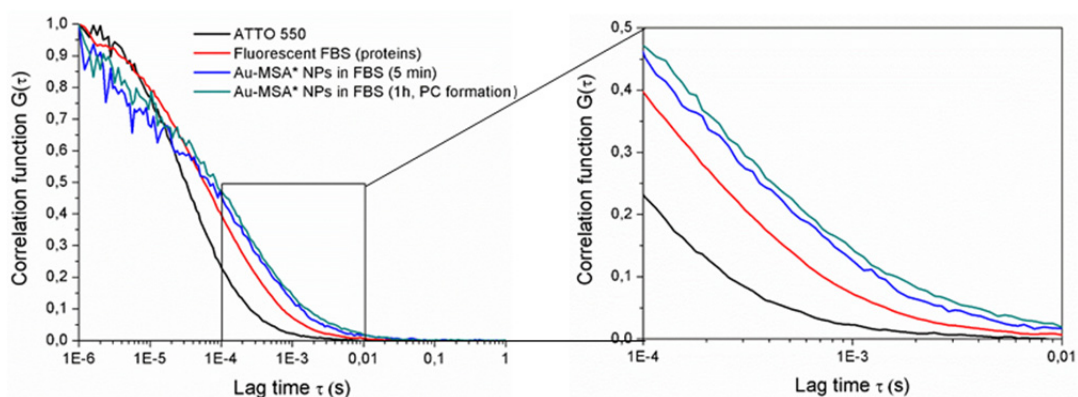


Figure 11. Representative normalized correlograms of the free dye, FBS fluorescent proteins solutions, Au-MSA* NPs with and without the protein corona. In the inset is possible to better appreciate the shift to higher lag times correlated with the increasing of the decay times of the species.

In **Figure 11** are reported some representative FCS normalized correlograms of free dye (ATTO 550), fluorescent FBS proteins, Au-MSA* NPs in RPMI and Au-MSA* NPs in RPMI after the formation of the PC (1h of incubation time). Is possible to appreciate the shift between the different slopes directly correlated to the differences in diffusion time and, consequently, in size of the species. The free dye is the smaller specie and presented the faster diffusion time (in the range of 20 μ s), with a rapidly decaying autocorrelation function. The labelled proteins contained in FBS presented a slope shifted to higher lag times and a diffusion time comprised between 100 and 300 μ s, coherent with the proteins dimensions. At the end the Au-MSA* NPs presented even bigger diffusion times ($\sim$ 700 μ s) and when the protein corona is fully formed (after 1 h) is possible to appreciate a slightly shift of the correlogram to higher lag times.

The hydrodynamic diameters of the fluorescent nanoparticles have been compared in different solutions: water, RPMI and full RPMI (10% FBS) at different time points up to 1 h (**Figure 12**). Each

of the reported data has been obtained by the medium of three independent measurements. In all the cases, with the exception of Au-PEG₅₀₀₀* NPs, the NPs hydrodynamic diameters increase in RPMI respect to water. This effect can be ascribed to a higher RPMI ionic strength, inducing the aggregation of the NPs if not stabilized enough. This phenomenon is emphasized for the Au-MSA* NPs and Au-Glucosamine* NPs, where the increments of D_{Hydr} are bigger than 20 nm. These data and their wide standard deviations, suggest the formation of aggregates with heterogeneous dimensions. The effect observed on Au-Alkyl-PEG₆₀₀* NPs is almost negligible with an average increment of 5 nm that suggests a modification of the hydrodynamic shell more than a real nanoparticles aggregation. Finally, concerning the Au-PEG₅₀₀₀* NPs a reduction of the hydrodynamic diameter has been registered.

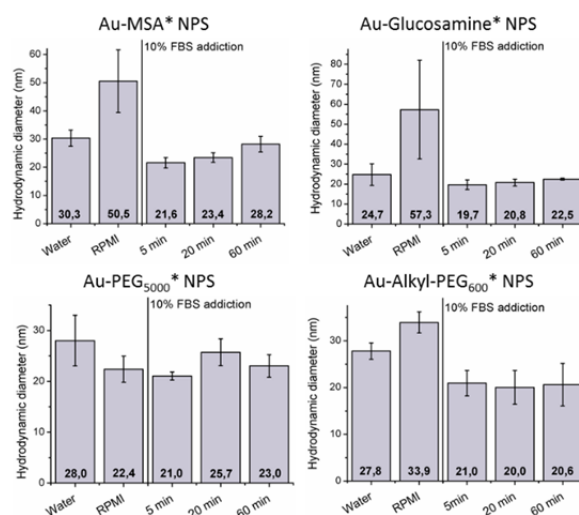


Figure 12. Hydrodynamic radius of the Au NPs * in water, RPMI and RPMI enriched with the 10% of FBS (at 5, 20 and 60 min after the initial incubation time).

When the FBS solution is added to the RPMI suspension of NPs a reduction of the hydrodynamic dimension is denoted. The addition of a new specie in the solution seems to modify the equilibrium previously reached, promoting a de-assembling of the NPs aggregates in favour of smaller and more stable NP-protein complexes. The effect is appreciable just after 5 minutes of incubation in accordance to what is suggested in literature.⁷

After the addition of FBS proteins, the samples have been monitored at 3 different time points: 5, 20 and 60 minutes after the start of the incubation. Au-MSA* NPs and Au-Glucosamine* NPs showed a constant increment in the hydrodynamic diameter during the observation time. As expected, this phenomenon is more evident for charged nanoparticles, with a diameter increment at the end of the hour of 6.6 nm. The half of this value is comparable with the diameter of albumin, the most abundant protein in serum, and let us think that in this case a monolayer of proteins is formed around the NP. The diameter increment registered for Au-Glucosamine* NPs is smaller and could be explained by the formation of an incomplete protein corona. Au-PEG₅₀₀₀* NPs registered an increment in the hydrodynamic diameter of 4.8 nm, 20 minutes after the start of the incubation, and a subsequent reduction to 2 nm after 1 hour. This data show that the interaction with proteins is stronger after 20 min, leading to the formation of a thicker PC. At 1 hour, the equilibrium changes and a desorption of the proteins takes place. This data is in good agreement

with the studies recently performed by Pelaz et al. where has been registered the same hydrodynamic radius increment for FePt NPs coated with a shell of poly(maleic anhydride-alt-dodecene) (PMA) and PEG₅₀₀₀⁴¹. This finding has been explained by the authors in two different ways: it can be due to fewer proteins bond to PEGylated NPs, or to the fact that proteins can partially penetrate into the PEG layers of the NPs. The dynamic behaviour of PEGylated Au NPs is an appealing subject for further future investigations. The last behaviour was identified for Au-Alkyl-PEG₆₀₀* NPs. The nanoparticles coated with this type of surfactant do not present any increment in the hydrodynamic diameter in the observation time. This finding suggests that this type of molecule is able to limit the interaction between the FBS proteins and the nanoparticles surfaces avoiding the formation of a significant PC.

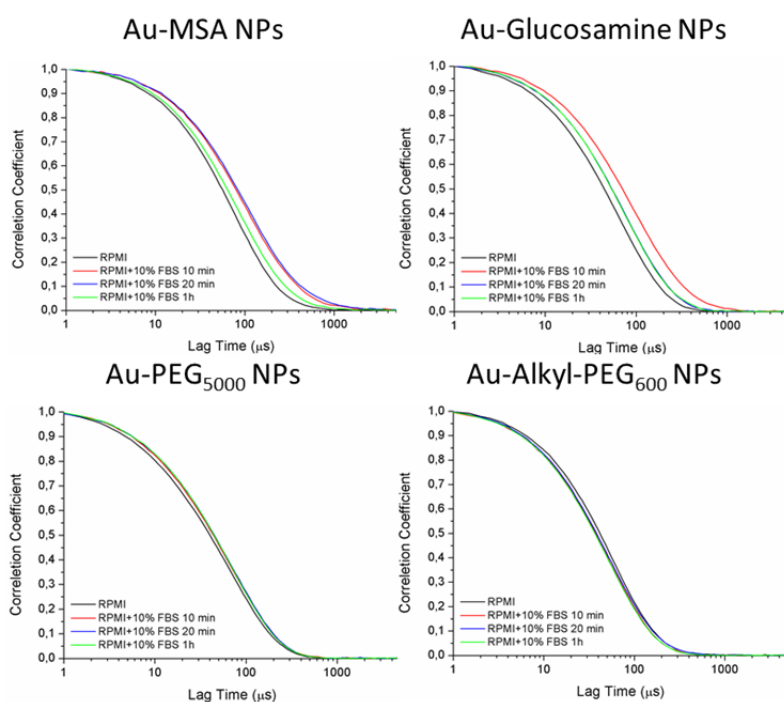


Figure 13. DLS correlograms of the Au NPs In RPMI and RPMI enriched with the 10% of FBS (at 10, 20 and 60 min after the initial incubation time).

Dynamic Light Scattering (DLS) was employed to measure hydrodynamic diameters of non-fluorescent nanoparticles, in the same conditions used for FCS measurements (incubation in full RPMI up to 1 h at 37°C). Simply comparing the normalized correlograms of the NPs at different time points is possible to distinguish which of them are less inclined to interact with the proteins (**Figure 13**). In fact it is possible to appreciate that the correlograms of Au-MSA NPs and Au-Glucosamine NPs in FBS solutions show large shifts in the lag time during the incubation period indicating hydrodynamic dimensions increase. Instead for Au-PEG₅₀₀₀ NPs and Au-Alkyl-PEG₆₀₀ NPs correlograms the shifts are reduced indicating a lower affinity between the coating and the proteins.

In addition, the NPs size and their aggregation were monitored by UV-vis spectroscopy. In particular, we monitored the variation of the maximum wavelength and the intensity of the plasmonic peak. The intensity and shape of the peak for gold nanoparticles with a diameter

smaller than 30 nm can be directly correlated to their dimensions⁵⁰. In literature⁴⁴, the UV-vis spectroscopy has been already employed as technique to evaluate the stability of Au NPs in cell culture media. In the previously reported work the plasmonic peak of the Au NPs underwent to strong variation in shape and wavelength. In the present study, after one hour, all the nanoparticles presented really small variation in the plasmonic peak indicating the high colloidal stability of our NPs in protein solution. For this reason, we prolonged the observation time to 24 hours. Au-MSA NPs and Au-Glucosamine NPs present a constant increasing of the diameter up to 24 hours. Au-PEG₅₀₀₀ NPs show an increasing in the hydrodynamic dimensions after one hour to decrease again at 24 hours, a trend that reminds of the ones obtained with FCS (**Figure 14 a**). No evidence of the formation of a protein corona around the Au-Alkyl-PEG₆₀₀ NPs has been highlighted confirming the FCS results. Trends obtained analysing the plasmonic peaks shift suggest the same conclusions. Again, the NPs that seem to be less affected by the proteins are Au-Alkyl-PEG₆₀₀ NPs that show a shift of just 0,5 nm after 24 hours of incubation (**Figure 14 b**).

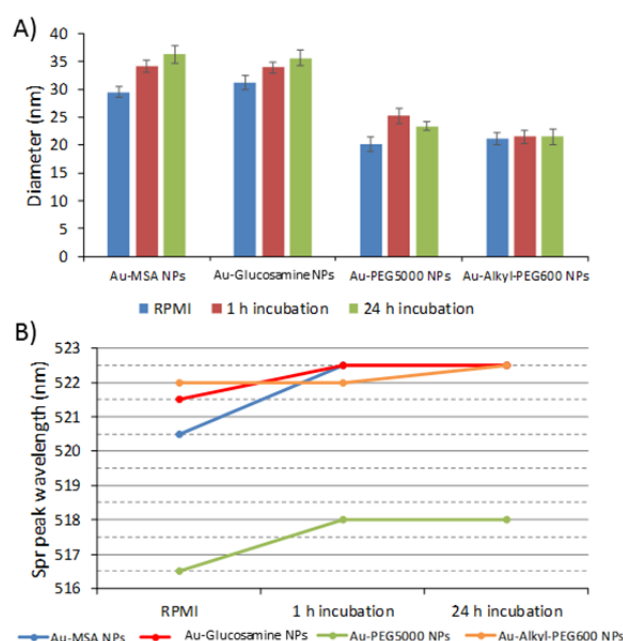


Figure 14. a) Variation in the plasmonic peak wavelength and **b)** of the hydrodynamic diameter calculated by UV-vis spectroscopy during the incubation of the Au NPs with RPMI and RPMI enriched with 10% of FBS.

In the end, in order to evaluate the affinity of the proteins for the different coating it was performed a colorimetric quantification of the proteins adsorbed on the NPs surface (**Figure 15 a.**). To perform this colorimetric test the nanoparticles were incubated for 24 hours in pure FBS At 37°C. Then the solution was centrifugated over a layer of sucrose to allow the separation between the NPs with PC (that sediment) and the free proteins (at the top of the sucrose layer). The NPs were suspended in PBS and mixed with PierceTM 660 Protein Assay and the UV-vis spectra of the solutions registered (further information on the procedure are reported in **Section 4.20**). From the collected data is clearly visible that the affinity of Au-Alkyl-PEG₆₀₀ NPs is definitely lower when compared with the other NPs of the panel. Au-MSA NPs and Au-Glucosamine NPs clearly show a higher affinity with the proteins and the formation of a consistent protein corona while the Au-PEG₅₀₀₀ NPs showed limited proteins absorption. The data reflects the same trend individuated

with FCS, DLS and UV-vis analysis, confirming that HS-Alkyl-PEG₆₀₀ thiol is the most effective coating molecule to inhibit protein absorption on the NPs surface.

Once determined that Au-MSA NPs and Au-Glucosamine NPs forms a detectable protein corona around their surface, we focused our attention on the “stealth nanoparticles”. Native agarose gel electrophoresis has been performed on Au-Alkyl-PEG₆₀₀ NPs and Au-PEG₅₀₀₀ NPs, both the bare form and with PC. If a protein corona is formed around the NP a difference in size and superficial charge of the particle is expected. In gel electrophoresis, this differences can be easily visualized as a diverse electrophoretic mobility. This difference is evident in **Figure 15b** for Au-PEG₅₀₀₀ NPs and Au-PEG₅₀₀₀ NPs, where the band of the NPs with PC runs more through the gel indicating a variation in the charge and dimension. On the other side, Au-Alkyl-PEG₆₀₀ NPs do not present big difference in the bands before and after the incubation with proteins giving an additional proof of the inert behaviour of these NPs.

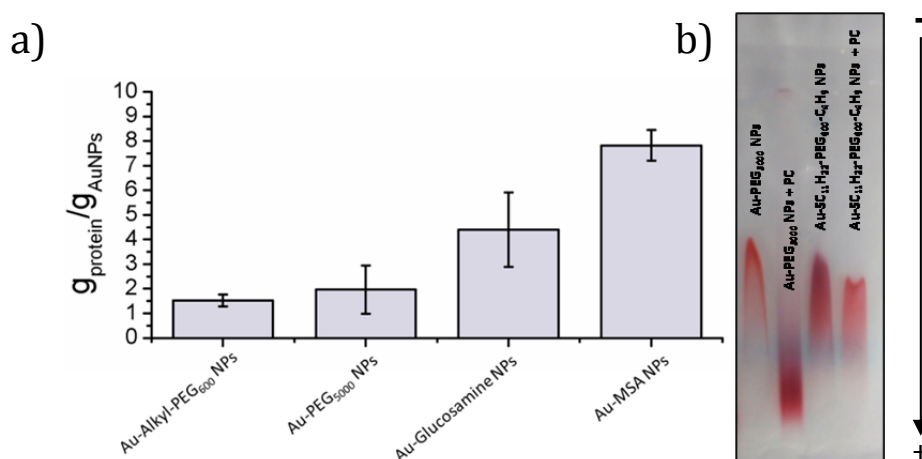


Figure 15. a) Colorimetric quantification of the proteins adsorbed on the NPs surface, b) agarose gel electrophoresis performed on the bare NPs and on the NPs with PC

2.5 FCS for the study of the intracellular dynamics of Au NPs

Recently the scientific community presented an interest in understanding intracellular fate of nano-materials. In fact, information, relative to the intracellular behaviour of NPs are fundamental for improving NPs performances and evaluate eventually the toxicity. FCS is an appropriate technique to measure the concentration, mobility and size of the nanoparticles in different cellular compartments. In the present study, the dynamic behaviour of the nanoparticles presented in **Section 2.2 (Figure 6)** has been studied, collecting and comparing meaningful data on the aggregation and diffusion times of the different NPs. A549 cells were incubated with the different NPs sample, with a concentration of 15 µg/ml. The observation time, like in the study of the PC formation, was set at 1h with an intermediate point at 30 minutes. For each nanoparticle sample, confocal images were registered at 30 min and 1h (**Figure 16**) on living cells. From the collected images the nanoparticles seems to be internalized by mean of endocytosis with the formation of vesicles. Some of these vesicles, already visible at 30 min, present intense fluorescence signal indicating elevated concentration of NPs in the endosomes. The vesicles seem to lead the nanoparticles in a specific region near to the nucleus. This region presents a visible fluorescence at 1h and has been identified as the endothelial reticulum, where the NPs seem to accumulate. On

the base of the observed internalization pathway we identified four regions of interest to perform a statistical number of FCS measurements, in order to compare the intracellular dynamics of the NPs in different cellular compartments. These regions of interest are: cytosol, endoplasmic reticulum, endocytic vesicles (nearer to the cell membrane and recently internalized) and intracellular vesicles (nearer to the nucleus in a later stage) (**Figure 17**). FCS experiments were performed as well on/close to membrane and nucleus, but a low signal was frequently registered for these regions (**Figure 18**), paving a negligible presence of NPs accumulation in these compartments. FCS measurements were done on at least 3 cells recording 20 runs of 10 second. At least 2 experiments were performed in two different points of each region of interest: 2 in the cytoplasm; 2 in the endoplasmic reticulum and, whenever visible, 2 in brighter spots near to the membrane (endocytic vesicles) and 2 in brighter spots near to the nucleus (intracellular vesicles). Moreover 1 experiment was performed on/close to the membrane and 1 on/close the nucleus. Tracks were at first screened one-by-one to remove tracks showing bleaching (AC_a), low signal (AC_b) and visible aggregates (AC_c) (**Figure 19**).

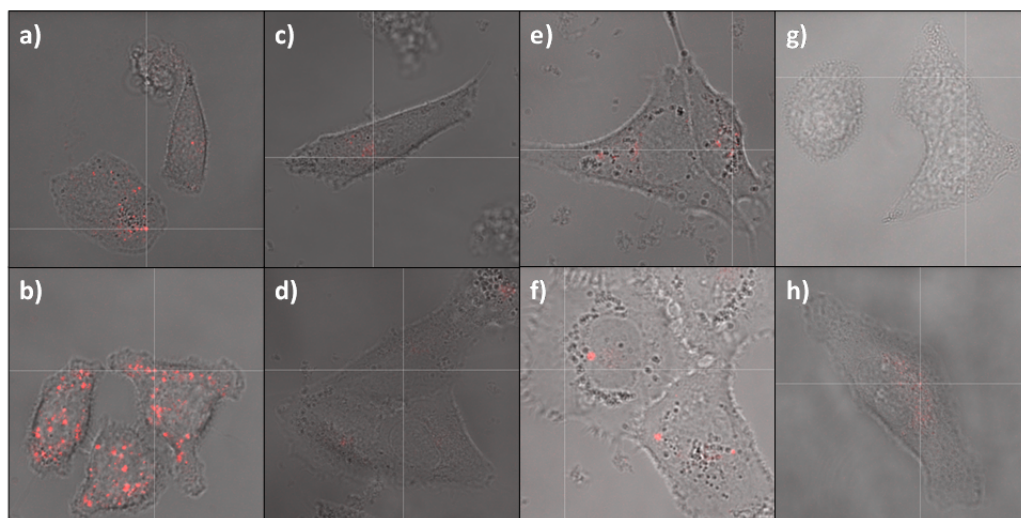


Figure 16. Fluorescence confocal scanning microscope micrographs of **a)** and Au MSA* NPs after 30 min, **b)** Au MSA* NPs after 1 h, **c)** Au-Glucosamine* NPs after 30 min, **d)** Au-Glucosamine* NPs after 1h, **e)** Au-PEG₅₀₀₀* NPs after 30 min, **f)** Au-PEG₅₀₀₀* NPs after 1 h, **g)** Au-Alkyl-PEG₆₀₀* NPs after 30 min and **h)** Au-Alkyl-PEG₆₀₀* NPs after 1 h. In red is represented the fluorescent signal deriving from the labelled NPs.

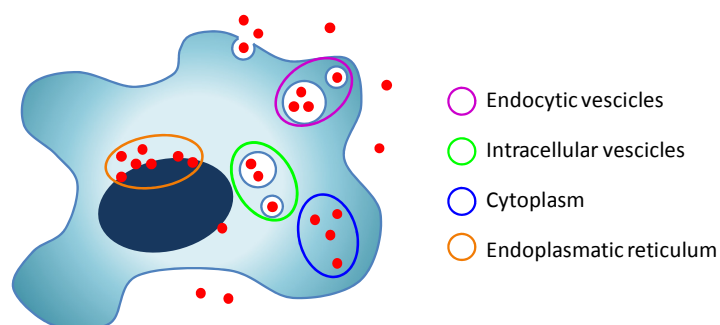


Figure 17. Schematic representation of the proposed NPs internalization pathway and of the identified regions of interest for the statistical FCS analysis.

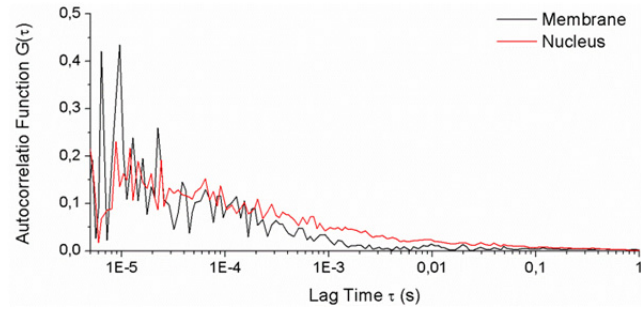


Figure 18. Representative FCS autocorrelation function for the membrane and nucleus of the cell (Au-MSA* NPs). The intensity signal registered in these regions was too low to be included in the statistical analysis.

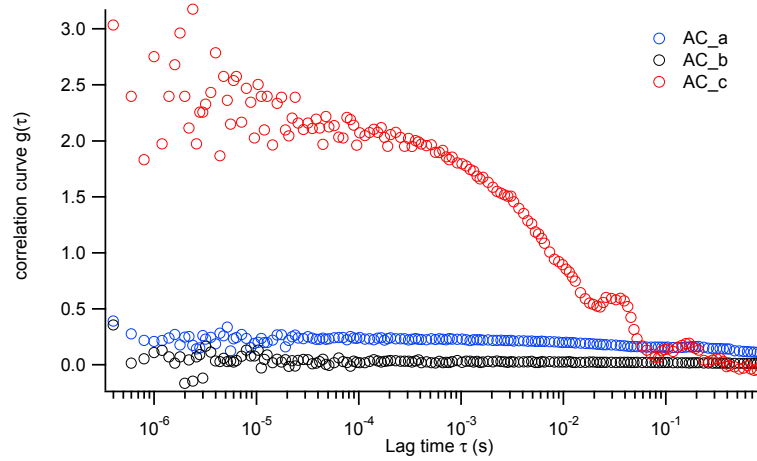


Figure 19. Representative correlation function of the excluded track showing bleaching (AC_a), low signal (AC_b) and visible aggregates (AC_c).

The remaining tracks were fitted one-by-one using a 3D normal diffusion fit model. Considering the decay time (τ_D , μs) of the most abundant specie (fraction $\Phi > 0.5$), the tracks were grouped as follow:

- 1) **$500 < \tau_D (\mu s) < 1500$** : comparable to the diffusion time of isolated free diffusing NPs
- 2) **$1500 < \tau_D (\mu s) < 3000$** : comparable to small aggregates of few nanoparticles, or to a nanoparticle interacting with bio-macromolecules
- 3) **$3000 < \tau_D (\mu s) < 6000$** : comparable aggregates of NPs
- 4) **$3000 < \tau_D (\mu s) < 6000$** : comparable to large aggregates of nanoparticles or to nanoparticles stacked in the cellular matrix.

For each group the average track was fitted again and the average decay time with the correspondent standard deviation calculated. The procedure was repeated for each measurement of each cell compartment. Finally decay times were averaged per compartment considering all cell replicates referring to the above-mentioned decay times grouping. The percentage of tracks

representative of the species decaying with the latter diffusion times were calculated over the total of the good tracks analysed in all cell replicates. In all cases, Au NPs were present as two or more distinct diffusing species, a fast diffusing specie (D_{fast}) and a slower diffusing specie (D_{slow}).

In **Figure 20** are reported four representative FCS correlograms (one for each region of interest) fitted with a double exponential fitting model. In the confocal micrograph are shown the punctual positions where the correlograms were registered.

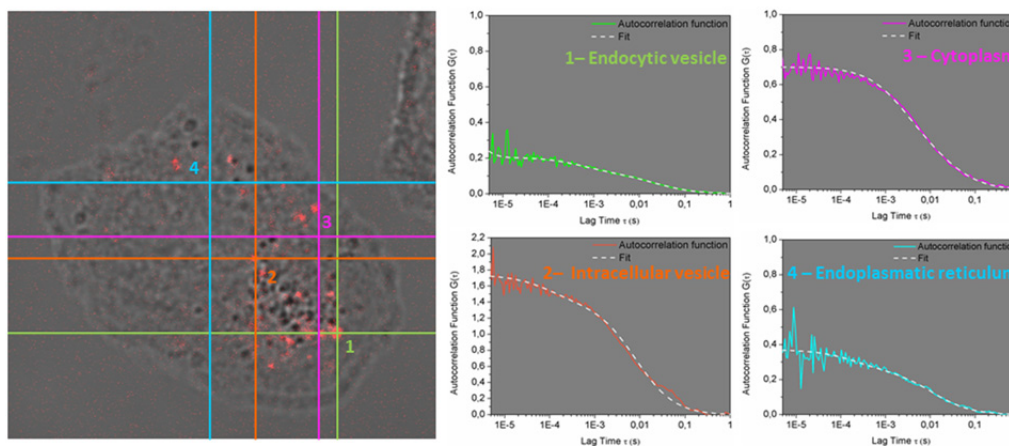


Figure 20. Representative confocal image recorded as a combination of the transmission and fluorescence channels. In the confocal micrograph the areas where FCS measurements were performed are indicated. FCS correlograms registered in the region of interest, were fitted with a 3D double exponential model.

In **Figure 21** and **Figure 22** are reported the statistical analysis of the D_{fast} , typically the most abundant specie, for the different coated Au NPs (Au-MSA* NPs, Au-Glucosamine* NPs, Au-PEG₅₀₀₀* NPs and Au-Alkyl-PEG₆₀₀* NPs) in the 4 regions of interest at 30 and 60 min of incubation time. In **Figure 21** are reported the percentages of the species measured, whose diffusion times fall within the groups described above ($500 < \tau_D (\mu s) < 1500$; $1500 < \tau_D (\mu s) < 3000$; $3000 < \tau_D (\mu s) < 6000$ and $\tau_D (\mu s) > 6000$), for each of the Au NPs in the different cellular compartments. In **Figure 22**, are reported the histograms describing the correspondents mean diffusion times for each group reported in **Figure 21**. From this statistical analysis, it is possible to significantly differentiate between the NPs that in section 2.4 we identified as stealth (Au-PEG₅₀₀₀* NPs and Au-Alkyl-PEG₆₀₀* NPs), and Au-MSA* NPs and Au-Glucosamine* NPs. During the observation time, the stealth nanoparticles maintained generally constant the diffusion times values and percentages distribution, paving a higher colloidal stability in the intracellular environment. Instead, for Au-MSA* NPs and Au-Glucosamine* NPs, going from 30 to 60 min of incubation, the percentage of traces describing species with $\tau_D (\mu s) > 6000$ increased, and of the faster diffusion time populations disappeared, suggesting a aggregation of the NPs in quite all the cellular compartments. Moreover, at 30 min of incubation, Au-MSA* NPs, Au-PEG₅₀₀₀* NPs and Au-Alkyl-PEG₆₀₀* NPs presented a variegated distribution of diffusing times. Instead Au-Glucosamine* NPs were identified as the less intracellularly stable NPs, presenting an elevated fraction of slow diffusing species in quite all the cellular compartments just after 30 min of incubation. From the analysis of the diffusion times inside the vesicles is possible to derive meaningful information about the stability of the NPs constrained in a reduced volume, enriched of bio-macromolecules. A drastic change in the diffusion time has been highlighted for Au-MSA* NPs and Au-Glucosamine* NPs, passing from 30

to 60 min, when the majority of the traces present diffusion times higher than 6000 μs . Instead even if confined inside endocytic vesicles Au-PEG₅₀₀₀* NPs and Au-Alkyl-PEG₆₀₀* did not present great changes in the τ values and distributions. Inside the intracellular vesicles, the ones nearer to the nucleus, these differences are even more accentuated, as the NPs has been constrained inside the vesicles for longer time frames. In this region of interest after 1 h of incubation times the totality of the Au-MSA* NPs and Au-Glucosamine* NPs diffusion time is bigger than 6000 μs indicating the formation of big aggregates or the presence of Au NPs stacked in the biological matter. Inside the intracellular vesicles is possible to distinguish different behaviour also between Au-PEG₅₀₀₀* NPs and Au-Alkyl-PEG₆₀₀*. In fact in the first case, after 1 hour of incubation, only remained species diffusing with times higher than 3000 μs . Instead for Au-Alkyl-PEG₆₀₀* are still present populations diffusing with $500 < \tau_D (\mu\text{s}) < 3000$ indicating the presence of isolated free diffusing particles. Moreover, the changes in diffusion times values and percentage distributions are minimal. In conclusion, the intracellular dynamics of the NPs seem to reflect the coatings stealth properties observed *in vitro*. Nanoparticles that in full RPMI presented a stronger affinity with the proteins, once internalized in the cell, showed a reduced stability and more evident formation of aggregates. Once again, Au-Alkyl-PEG₆₀₀* NPs presented the best colloidal stability and intracellular behaviour, even if compared with Au NPs coated with a longer PEG chain (5 kDa). In fact, at 30 min of incubation, three of the regions of interest presented a significant fraction of Au NPs with a diffusing time comprised between 500 and 1500 μs , comparable to the diffusion time of isolated free diffusing particles. The dynamic behaviour remained constant up to 1 h of incubation time, highlighting the elevated colloidal stability and reduced interaction with biomolecules of these NPs.

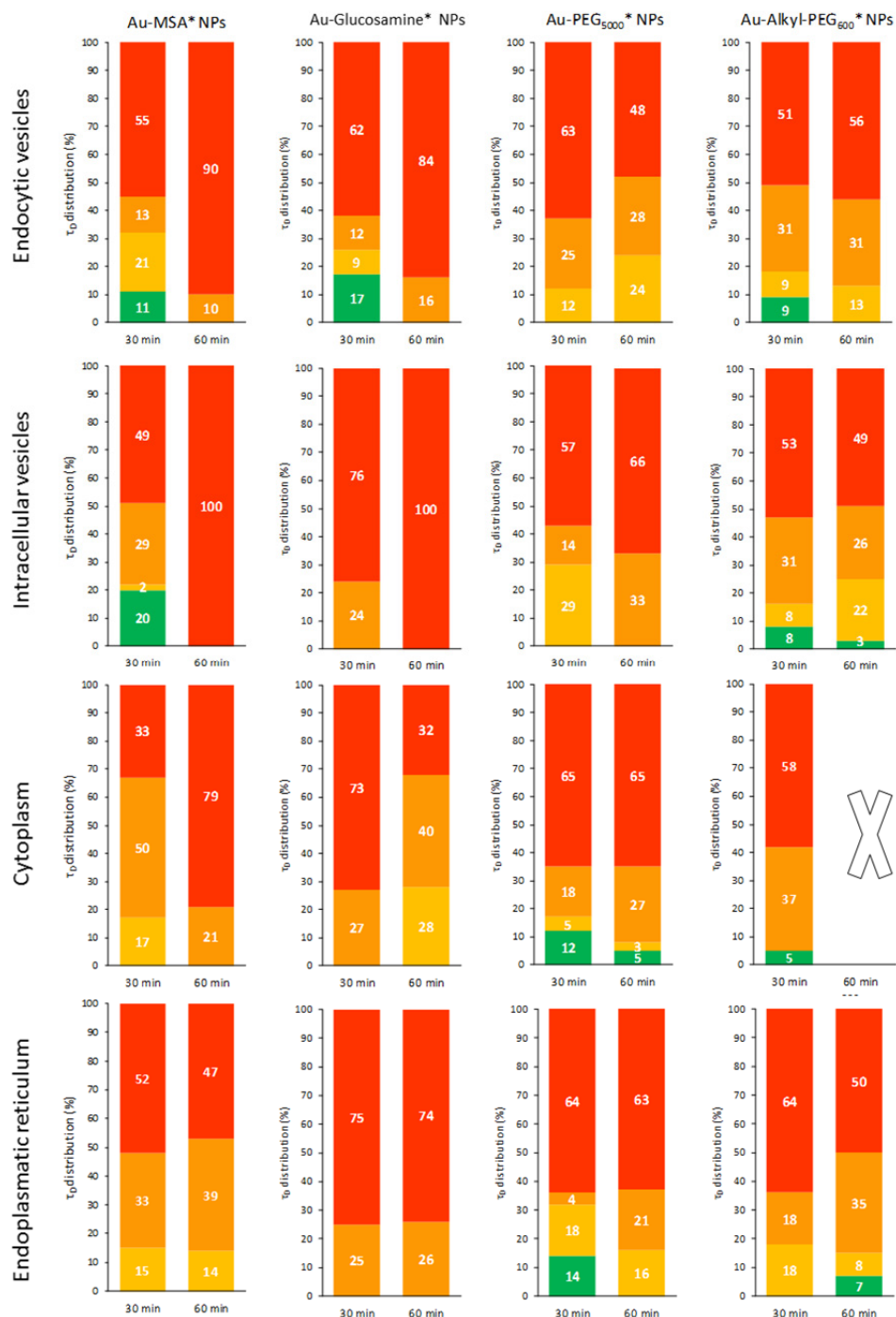


Figure 21. Percentage distribution of the D_{fast} in the different compartments of the cell grouped in four classes of diffusion times ($500 < \tau_D (\mu s) < 1500$; $1500 < \tau_D (\mu s) < 3000$; $3000 < \tau_D (\mu s) < 6000$ and $\tau_D (\mu s) > 6000$). In each section of the table are reported the results for Au-MSA* NPs, Au-Glucosamine* NPs, Au-PEG₅₀₀₀* NPs and Au-Alkyl-PEG₆₀₀* NPs at 30 minutes and 1 hour of incubation. EV=Endocytic vesicles; IV=intracellular vesicles; ER=Endoplasmatic reticulum; CYT=Cytoplasm.

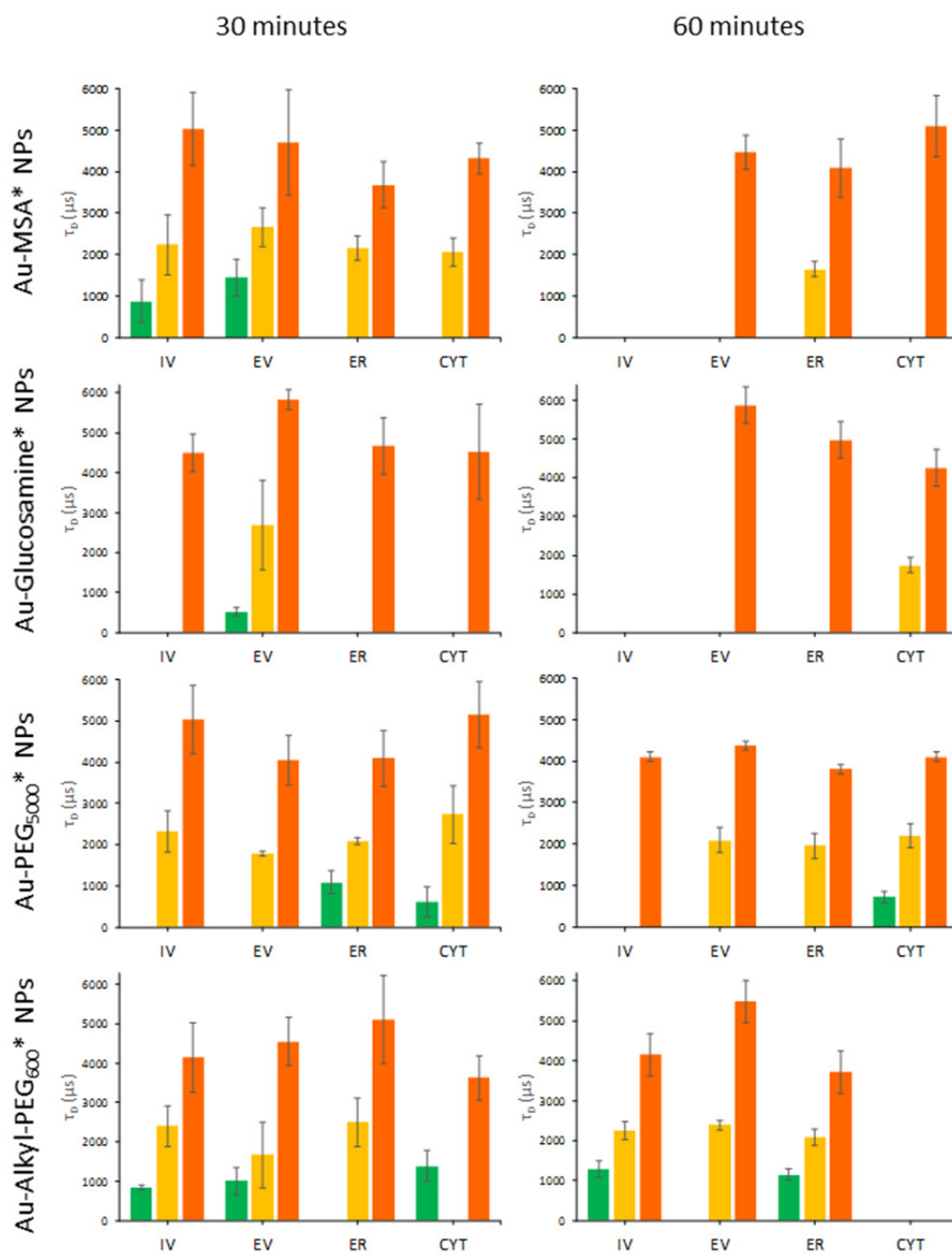


Figure 22. Histograms of the mean diffusion times for each region of interest obtained after the grouping of the trades in the four classes of diffusion times. Each histogram reports the results for Au-MSA* NPs, Au-Glucosamine* NPs, Au-PEG₅₀₀₀* NPs and Au-Alkyl-PEG₆₀₀* NPs at 30 minutes and 1 hour of incubation. EV=Endocytic vesicles; IV=intracellular vesicles; ER=Endoplasmic reticulum; CYT=Cytoplasm.

3. Conclusion

The temporal dynamics of the formation of PC around gold functionalized nanoparticles has been studied by mean of FCS, incubating Au NPs, coated with different ligand (Au-MSA* NPs, Au-Glucosamine* NPs, Au-PEG₅₀₀₀* NPs and Au-Alkyl-PEG₆₀₀* NPs), in RPMI enriched with 10% FBS. A solid protocol to derive the hydrodynamic radius of fluorescent nanoparticles in different media has been presented, allowing to follow the variation of the dimension of the NPs correlated with the interaction with proteins. The tested nanoparticles clearly presented two different behaviours, highlighting that Au-MSA* NPs and Au-Glucosamine* NPs present a stronger affinity with FBS proteins and a higher propensity to form a PC. Au-PEG₅₀₀₀* NPs and Au-Alkyl-PEG₆₀₀* NPs instead presented stealth properties with a reduced tendency to interact with proteins reflected in constant hydrodynamic size. In particular, Au-Alkyl-PEG₆₀₀* NPs presented the best performances in terms of colloidal stability, low interaction with proteins and stealth abilities. The FCS data were supported and confirmed by DLS, UV-vis, colorimetric proteins quantification and native agarose gel electrophoresis.

Moreover, the intracellular dynamics of the above cited NPs have been investigated, by mean of live cell FCS, in different cellular compartments. The results show an interesting correlation with the data obtained during the study of the protein corona. The Au NPs that *in vitro* presented a higher affinity with proteins (Au-MSA* NPs and Au-Glucosamine* NPs), when incubated in living cells show an evident aggregation of the NPs that grows with time. Instead, stealth NPs (Au-PEG₅₀₀₀* NPs and Au-Alkyl-PEG₆₀₀*) maintained quite constant the amount of diffusing species and relative average diffusion times. Au-Alkyl-PEG₆₀₀* NPs, after 30 minutes of incubation, showed a significant fraction of species with diffusion time comprised between 500 and 1500 μ s in quite all the regions of interest, comparable with the τ_D of isolated free diffusing particles. Moreover, only small changes can be detected passing from 30 minutes to 1 hour of incubation, even when the NPs are constrained in small volumes like the intracellular vesicles. These results underline excellent performances of HS-Alkyl-PEG₆₀₀ in terms of colloidal stability conferred to the NPs, even better than a longer PEG chain, paving his wide diffusion as coating molecule in the field of biotechnology.

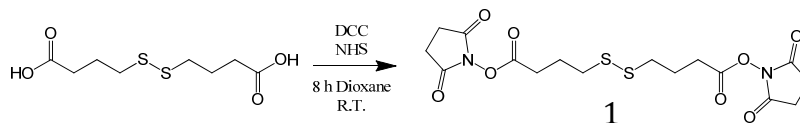
4. Experimental

4.1 Materials and methods

HAuCl₄·3H₂O, AgNO₃, hydroquinone, 4,4'-dithiodibutyric acid, glucosamine hydrochloride, butylamine, EDC, DCC, NHS, DTT and NaOH were purchased from Sigma-Aldrich, and used without further purification. HAuCl₄·3H₂O was stored at 4°C, shielded from light, as 10 mM solution and NaOH was stored as 1M water solution. AgNO₃ 10 mM and hydroquinone solutions were freshly prepared before every synthesis (avoiding the exposition to the light). HS-PEG₅₀₀₀-OCH₃ and HS-PEG₅₀₀₀-NH₂, purchased from Rapp Polymer GmbH, were used as received and stored under dry argon atmosphere at -20 °C. HS-Alkyl-PEG₆₀₀-COOH was synthesized by Choris srl. following a literature reported procedure⁴⁷. Au NPs were characterized by transmission electron microscopy (Zeiss LIBRA 200FE, equipped with: 200 kV FEG, in column second-generation omega filter for energy selective spectroscopy (EELS) and imaging (ESI), HAADF STEM facility, EDS probe for chemical analysis, integrated tomographic HW and SW system). The samples for TEM analysis were prepared depositing a drop of nanoparticle solution on a carbon-coated copper grid (300 mesh) and evaporating the solvent. The particle size distribution was obtained by measuring at least 300 nanoparticles. UV-vis spectroscopy (Spectrophotometer Bio UV-Vis V630 Jasco) was performed using disposable cuvette with 1 cm optical path length. Dynamic Light Scattering (DLS) and ζ potential measurements were performed employing a Malvern Zetasizer Nano ZS90. In order to remove large particulate DLS specimens were filtrated with a cellulose acetate syringe filter (0.22 μm) before to load the cuvette. Each sample was equilibrated for 2 min prior to measurement. At least three independent measurements of 10 runs (10 s each one) were performed for each sample. A reduced volume plastic cuvette was employed for DLS experiments charged with 450 μl of sample. A capillary zeta cell was used for ζ-potential measurements loaded with 1 ml of sample. Fluorescence spectra were registered employing Fluorometer Fluorolog-TSPC (Horiba-Jovine Ivone). The sample was excited at a 564 nm wavelength with a 2 nm slit and 5 mediated accumulations, to enhance the signal to noise ratio. The fluorescence signal was acquired starting from 570 nm. A disposable cuvette with 1 cm optical path length was used for the measurements.

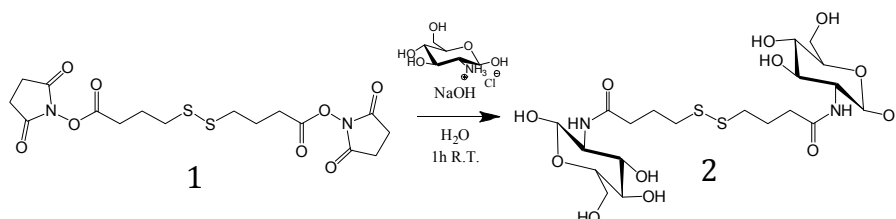
4.2 Synthesis of *N*-4-thiobutyroil glucosamine

4.2.1 Synthesis 4,4'-Dithiodibutyric NHS ester



The reaction was performed under dry conditions and Argon atmosphere. Dioxane was filtered on an alumina column to remove the peroxides and degassed under vacuum before to start the reaction. DTBA (Dithiodibutyric acid, 400 mg, 1.67 mmol, 1 eq) and NHS (422 mg, 3.67 mmol, 2.2 eq) were dissolved in 12 ml of dioxane. 4 ml of a DCC (756 mg, 3.67 mmol, 2.2, eq) solution in dioxane were slowly dropped inside the reaction mixture and was left under vigorous stirring at R.T. for 8 hours. The solvent was evaporated at reduced pressure and the obtained product dissolved in Et₂O-Acetone (1:1) solution. The mixture was filtrated to remove the white insoluble solid and product 1 (dissolved in the liquid phase) was recovered evaporating the solvents at reduced pressure. The product was directly used for the next step without any further purification.

4.2.2 Synthesis of 4,4'-Dithiodibutyroil glucosamine



Glucosamine hydrochloride (825 mg, 3.84 mmol 2.3 eq) was dissolved in 4 ml of NaOH 1M and 22 ml of MES (0,1M) buffer were added. Compound 1 (691 mg, 1.59 mmol, 1 eq) was dissolved into 42 ml of DMF anhydrous and added to the glucosamine solution. After 1 h of reaction the solvent was evaporated at reduced pressure. The crude was suspended in 2 ml of water, and the white insoluble solid removed by filtration. Product 2 was purified by mean of size exclusion chromatography.

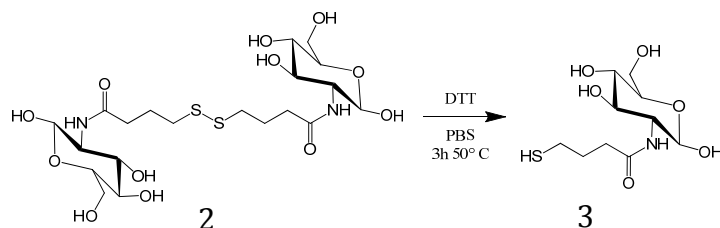
Yield = 63%

m/z = 560,17

¹H NMR (400 MHz, D₂O) δ = 8.41 (s, 2H, NH), 5.16 (d, J = 3.5 Hz, 1H, H-1 α), 4.67 (d, J = 8.4 Hz, 1H, H-1 β), 3.90 – 3.60 (m, 8H, H-2, H-3, H-5, H-6), 3.41 (m, 2H, H-4), 2.71 (t, 4H, CH-S), 2.39 (m, 4H, CH-CO), 1.97 (m, 4H, CH₂-CH-CH₂).

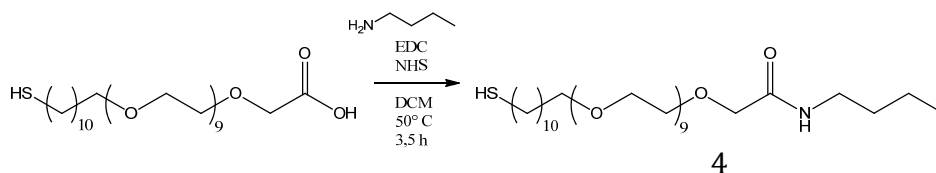
¹³C NMR (101 MHz, D₂O) δ = 171.52 (C=O), 95.70-90.91 (C-1), 71.60 (H-2), 70.87 (H-5), 70.21 (H-6), 60.81 (H-4), 37.25 (H₂C-S), 34.35 – 34.16 (H₂C-C=O), 24.67 (CH₂-CH₂-CH₂).

4.2.3 Synthesis of N-4-thiobutyl glucosamine



Compound 2 (40 mg, 0.07 mmol, 1 eq) has been dissolved into 10 ml of PBS solution at pH 7.4. DTT (54 mg, 0.35 mmol, 5 eq) was added and the reaction mixture left under stirring at 50 °C for 3 hours, under argon atmosphere. The water was removed at reduced pressure and DTT removed from the crude by several washes with Et₂O. The obtained product 3 (white solid) was directly used to coat the Au NPs

4.3 Synthesis of ([1-mercaptoundec-11-yl]PEG₆₀₀)-acetic acid butanamide



The reaction has been performed in dry conditions under argon atmosphere. HS-Alkyl-PEG₆₀₀-COOH (125 mg, 0.15 mmol, 1 eq) was dissolved in DCM dry. EDC (71.3 mg, 0.3 mmol, 2 eq) was added, followed, five minutes later, by NHS (43.1 mg, 0.3 mmol, 2eq). The reaction was stirred at R.T. for 30 min, then butylamine (92.5 mg, 0.75 mmol, 5 eq) were added. The reaction mixture was stirred for 3 hours at 50 °C. The solvent was evaporated at reduced pressure and product 4 was purified by flash chromatography (DCM:MeOH=95:5).

Yield = 78 %

m/z=780,53

¹H NMR (400 MHz, CDCl₃) δ = 7.29 (s, 1H, NH), 4.08 (s, 2H, H₂C-C=O), 3.67 (s, 30H, O-CH₂-CH₂-O), 3.62 – 3.57 (m, 2H, O=C-CH₂-O-CH₂), 3.47 (t, J = 6.8 Hz, 2H, CH₂-CH₂-CH₂-O), 3.32 (dd, J = 13.2, 6.9 Hz, 2H, CH₂-NH), 2.69 (t, 2H, S-S-CH₂), 2.54 (dd, J = 14.7, 7.4 Hz, 2H, S-CH₂), 1.74 – 1.49 (m, 6H CH₃-CH₂-CH₂, HS-CH₂-CH₂-CH₂-CH₂-CH₂-O), 1.44 – 1.34 (m, 2H, CH₃-CH₂-CH₂), 1.29 (s, 7H, CH₂-CH₂-CH₂), 1.01 – 0.92 (m, 3H, CH₃).

¹³C NMR (101 MHz, CDCl₃) δ = 158,14 (C=O) 71.56 (CH₂-NH), 71.04 (O=C-CH₂-O), 70.59 (O-CH₂-CH₂-O), 70.06 (O=C-CH₂-O-CH₂), 38.97 (S-S-CH₂), 34.05 (S-CH₂-CH₂), 31.50 (CH₂-CH₂-CH₂-O), 29.50 (CH₂-CH₂-CH₂), 28.38 (CH₂-CH₂-CH₂-O), 26.09 (CH₂-CH₂-CH₃), 24.65 (S-CH₂), 20.08 (CH₂-CH₃), 13.75 (CH₃).

4.4 Au NPs synthesis

A water solution of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (7.5 ml, 10 mM), sodium citrate (9 ml, 68 mM), and AgNO_3 (490 μl , 5.9 mM) was prepared and mixed at R.T. for 6 minutes. The pre-incubated mixture was, then, mixed to 250 ml of water at 100 °C. The mixture was stirred at 750 rpm for 1 h. Afterwards the reaction solution was left to cool to R.T. and 5 ml of glycerol were added. After 10 minutes a second mixture of HAuCl_4 (7.5 ml, 10 mM), sodium citrate (10 ml, 34 mM) and AgNO_3 (426 μl , 5.9 mM) was pre-mixed for 6 minutes and then added to the reaction solution, immediately followed by a hydroquinone solution (8 ml, 91 mM). Then, the colloidal solution was left to age for 1h, stirring at 750 rpm. The obtained Au NPs were directly functionalized without any further concentration or purification.

4.5 Synthesis of Au-MSA NPs

Au-MSA NPs were obtained by adding (under argon atmosphere) 22 mg of mercapto succinic acid and 8 mg of $\text{H}_2\text{N-PEG}_{5000}\text{-SH}$, dissolved in 5 ml of MilliQ water, to the gold colloidal solution. The ligands proportions were calculated to obtain 10% of the NPs surface covered with PEG, considering a foot print of 5 nm^2 for $\text{H}_2\text{N-PEG}_{5000}\text{-SH}$ and a footprint of 0,5 nm^2 for mercapto succinic acid. The reaction mixture was allowed to stir for further 48 hours at R.T. The functionalized Au NPs were purified and concentrated to a final volume of 10 ml using Amicon centrifugal filter units. The purification of the system was completed using dialysis tubes with a cut-off of 10 kDa (48 hours, 6 changes of water).

4.6 Synthesis of Au-Glucosamine NPs

Au-Glucosamine NPs were obtained by adding (under argon atmosphere) 25 mg of compound 3 and 5 mg of $\text{H}_2\text{N-PEG}_{5000}\text{-SH}$, dissolved in 5 ml of MilliQ water, to the gold colloidal solution. The ligands proportions were calculated to obtain 10% of the NPs surface covered with $\text{H}_2\text{N-PEG}_{5000}\text{-SH}$, considering a foot print of 5 nm^2 for $\text{H}_2\text{N-PEG}_{5000}\text{-SH}$ and a footprint of 0.5 nm^2 for compound 3. The reaction mixture was allowed to stir for further 48 hours at R.T. The functionalized Au NPs were purified and concentrated to a final volume of 10 ml using Amicon centrifugal filter units. The purification of the system was completed using dialysis tubes with a cut-off of 10 kDa (48 hours, 6 changes of water).

4.7 Synthesis of Au-PEG₅₀₀₀ NPs

Au-PEG₅₀₀₀ NPs were obtained by adding (under argon atmosphere) 27,5 mg of $\text{CH}_3\text{O-PEG}_{5000}\text{-SH}$ and 2.5 mg of $\text{H}_2\text{N-PEG}_{5000}\text{-SH}$, dissolved in 5 ml of MilliQ water, to the gold colloidal solution. The ligands proportions were calculated to obtain 10% of the NPs surface covered with $\text{H}_2\text{N-PEG}_{5000}\text{-SH}$, considering a foot print of 5 nm^2 for both the PEG molecules. The reaction mixture was allowed to stir for further 48 hours at R.T. The functionalized Au NPs were purified and concentrated to a final volume of 10 ml using Amicon centrifugal filter units. The purification of the system was completed using dialysis tubes with a cut-off of 10 kDa (48 hours, 6 changes of water).

4.8 Synthesis of Au-Alkyl-PEG₆₀₀ NPs

Au-Alkyl-PEG₆₀₀ NPs were obtained by adding (under argon atmosphere) 23,5 mg of compound 4 and 6.5 mg of H₂N-PEG₅₀₀₀-SH, dissolved in 5 ml of MilliQ water, to the gold colloidal solution. The ligands proportions were calculated to obtain 10% of the NPs surface covered with H₂N-PEG₅₀₀₀-SH, considering a foot print of 1.5 nm² for compound 4 and of 5 nm² for H₂N-PEG₅₀₀₀-SH. The reaction mixture was allowed to stir for further 48 hours at R.T. The functionalized Au NPs were purified and concentrated to a final volume of 10 ml using Amicon centrifugal filter units. The purification of the system was completed using dialysis tubes with a cut-off of 10 kDa (48 hours, 6 changes of water).

4.9 UV-visible spectra of Au NPs

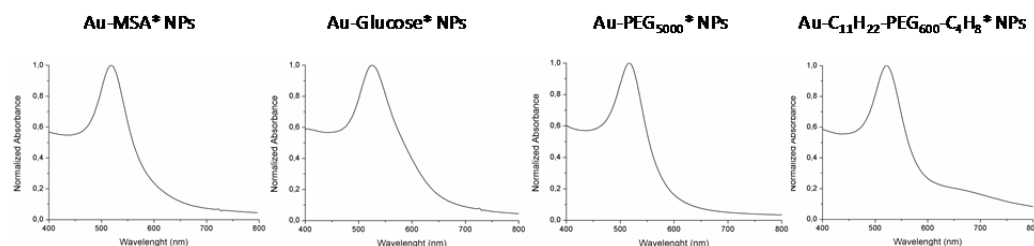


Figure 23. UV-visible spectra of Au NPs.

4.10 ζ -potential and hydrodynamic diameter of Au NPs

	Au-MSA NPs	Au-Glucosamine NPs	Au-PEG ₅₀₀₀ NPs	Au-Alkyl-PEG ₆₀₀ NPs
ζ -pot (mv)	-24.6 $\pm$ 10.8	-21.7 $\pm$ 19.0	-21.8 $\pm$ 9.1	-19.8 $\pm$ 11.7
Hydrod. Diam. (nm)	32.7 $\pm$ 12.2	30.6 $\pm$ 14.0	25.0 $\pm$ 13.1	33.6 $\pm$ 20

Table 3. ζ -potential and hydrodynamic diameter of the Au NPs.

4.11 Labelling of Au NPs with ATTO 550 NHS ester

The buffer employed to perform the fluorescent labelling has been obtained mixing 20 parts of a PBS buffer (Phosphate-Buffered Saline, pH 7.4) with 1 part of 0.2 M NaHCO₃ solution, adjusted to pH 9.0 with 2 M NaOH. The obtained labelling buffer should have a pH of 8.3, optimal for the reaction. 3mg of Au NPs has been dissolved into 2 ml of buffer. 1.5 eq of ATTO 550 NHS ester (stocked in 1 mg/ml DMSO Dry solution) was added for each free amino moiety present on the Au NPs surface. The mixture was sonicated for the first 10 minutes and then let to react for 1 hour at R.T under vigorous stirring. The excess of ATTO 550 NHS ester was removed by centrifugal filtration on Amicon centrifugal filters (30 kDa cut-off). The purification of the labelled Au NPs was completed using dialysis tubes with a cut-off of 100 kDa (48 hours, 6 changes of water).

4.12 Spectroscopic characterization of labelled Au NPs

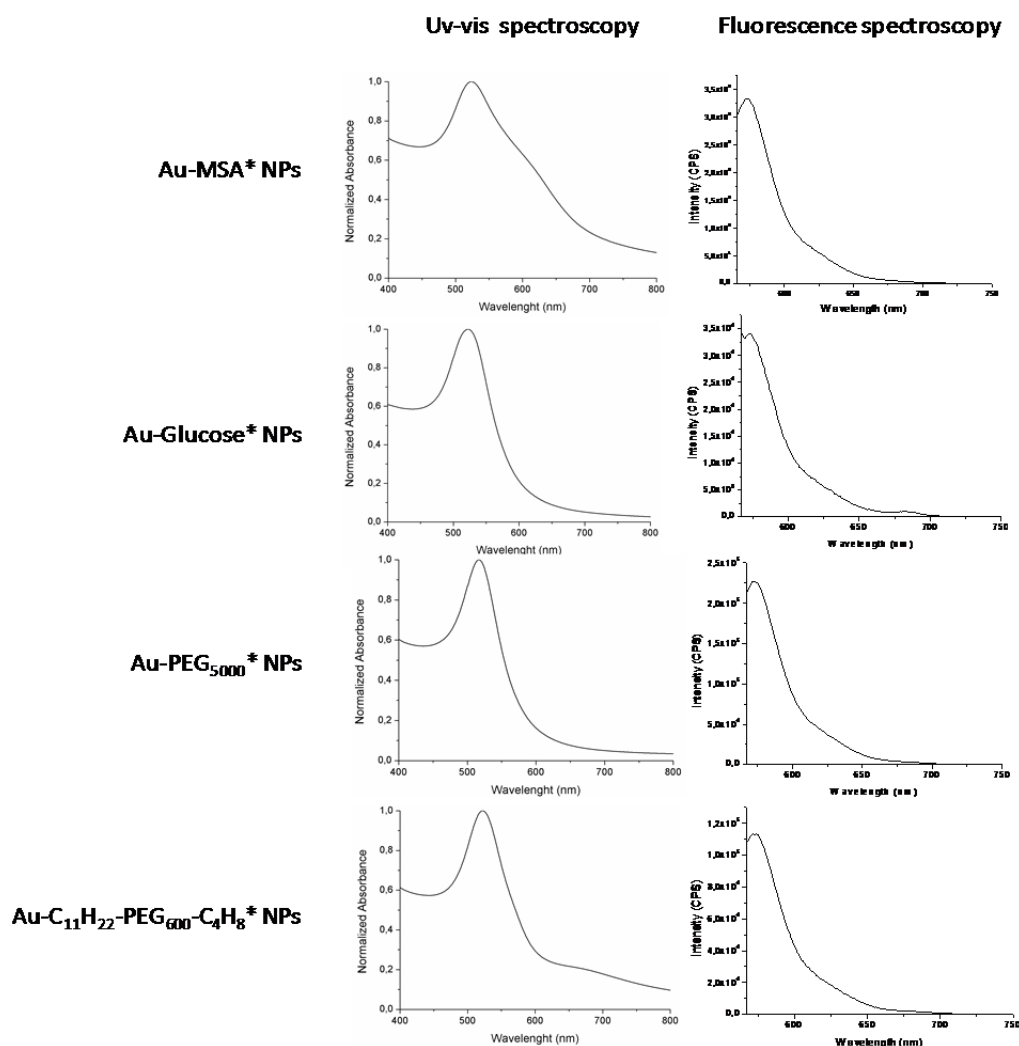


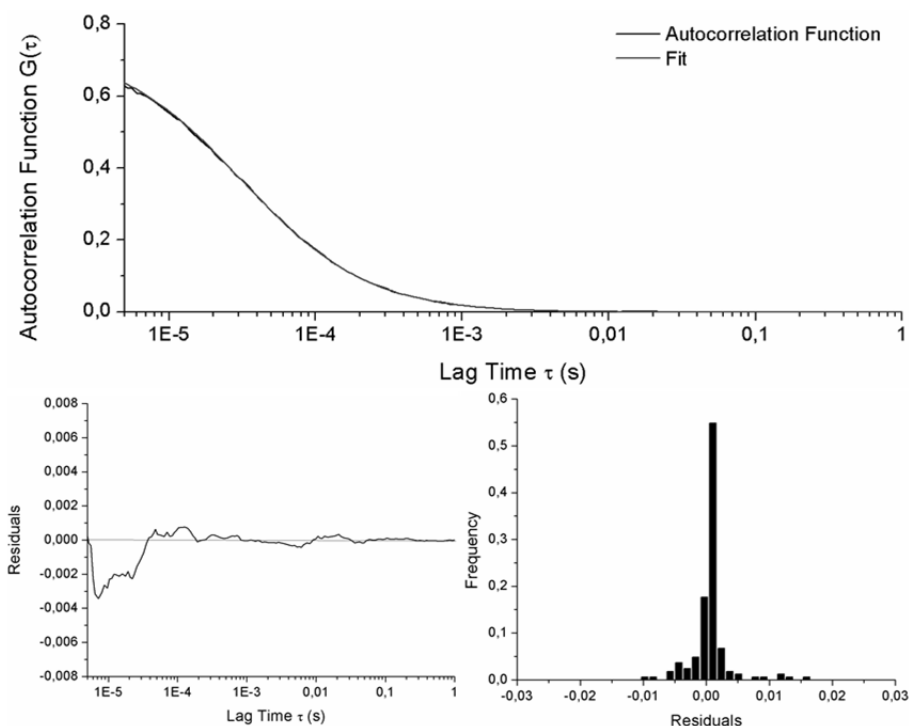
Figure 24. UV-visible and Fluorescence spectra of the labelled Au NPs

4.13 Procedure to perform the FCS experiments in solution

Stock solutions of ATTO 550 labelled Au NPs with a concentration of 1mg/ml were prepared. Prior to each FCS measurement the solutions were sonicated for 5 min and double filtered on 0.22 μ m regenerate cellulose syringe filters. This operation helps to remove the excess of free fluorophore that can remain electrostatically attached to the surface of the Au NPs. To perform the FCS measurements 25 μ l of Au NPs were dissolved in 225 μ l of a solution of phenol red free RPMI, enriched with a 10% in volume of FBS. The FCS measurements were registered 10 min, 20 min, and 1h after the mixing of the solutions. As comparison, a FCS measurement was performed on bare NPs in RPMI and in water. Moreover, for each measurement session 50 nM water solutions of Rhodamine B and of ATTO 550 were measured to determine the structural parameter and the

diffusion coefficient of the free dye. The diffusion coefficient of proteins was determined mixing 25 μg of FBS with a 50 nM solution ATTO 550 in RPMI. The measurements were performed employing scanning Confocal microscope (Zeiss LSM 510) equipped with Argon laser (covering the wavelengths of 458, 477, 488, 514 nm), DPSS 516nm and HeNe 633nm and set up with a ConfoCor 3 spectroscopy. Acquisition and analysis of LSM image and Confocor is controlled by Zen software. The DPSS laser at 516 nm has been employed as excitation source. Autocorrelation functions $G(\tau)$ were analysed by Quickfit 3.0 software (DKFZ, Germany) employing a Levenberg Marquard least squares algorithm. All the fittings were performed using a three-dimensional normal diffusion model (more detailed descriptions of the model and fitting procedures are reported in Sections 2.3 and 2.4)

4.14 Fitting of FCS data for Atto 550 NHS water solution



Model Parameters (fit results):

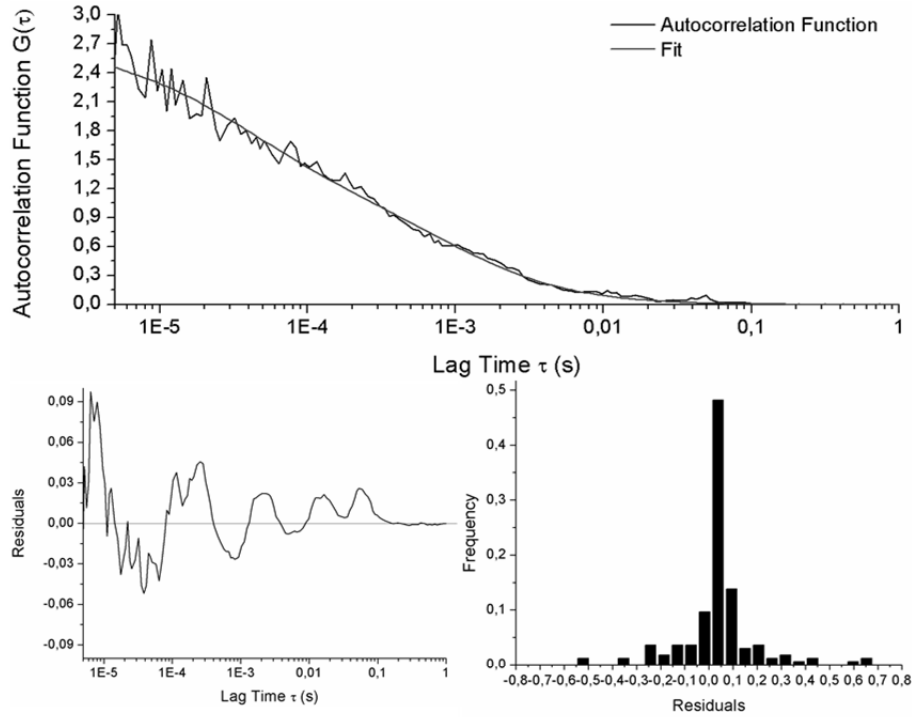
Parameter	Value	Range	Parameter	Value	Range
# non-fluorescent =	0	0...2	w_{xy} =	229.6179 ± 6.22	nm 0...10 ⁴
components =	1	1...3	V_{eff} = C	0.5285 ± 0.0594	fl 0...10 ⁵⁰
N = F	1.35326 ± 0.00671	10 ⁻¹⁰ ...10 ⁵	C_{all} = C	4.2523 ± 0.479	nM 0...10 ⁵⁰
1/N = C	0.73896 ± 0.00367	10 ⁻¹⁰ ...10 ⁵	D_1 = C	420.83 ± 23.5	$\mu\text{m}^2/\text{s}$ 0...10 ⁵⁰
$T_{D,1}$ = F	31.3216 ± 0.409	μs 1...10 ⁵	count rate =	$0 \pm \text{nan} \times 10^{-2147483648}$	Hz 0...10 ⁵⁰
G_{∞} = FX	0 ± 0	-10...10	background =	0 ± 0	Hz 0...10 ⁵⁰
γ = F	7.8391 ± 0.61	0.01...100	cnt/molec = C	$0 \pm \text{nan} \times 10^{-2147483648}$	Hz 0...10 ⁵⁰

legend: F: fit parameter, X: fixed parameter, C: calculated parameter

Fit Statistics:

$\chi^2 = 0.00197157$	χ^2 (weighted) = 35.6153
$\langle E \rangle = 9.75153\text{e-}05$	$\langle E \rangle$ (weighted) = -0.0161074
$\sqrt{\langle E^2 \rangle} = 0.00346587$	$\sqrt{\langle E^2 \rangle}$ (weighted) = 0.465733
NP = 3	NR = 164
DF = 160	TSS = 10.1577
$R^2 = 0.999806$	R^2 (weighted) = -2.50624
$R^2_{\text{adjusted}} = 0.999803$	R^2_{adjusted} (weighted) = -2.54979
AICc = -1851.77	AICc (weighted) = -244.293
BIC = -1842.62	BIC (weighted) = -235.143
	$\max_p(\sigma_p/ P) = 7.78151\%$
$\det(\text{COV}) = 4.00533\text{e-}07$	$p_{\text{Bayes}}(\text{model} \text{data}) = 1.71655\text{e-}15$ (= 10 ^{-14.7653})

4.15 Fitting of FCS data for Au-MSA* NPs water solution



Model Parameters (fit results):

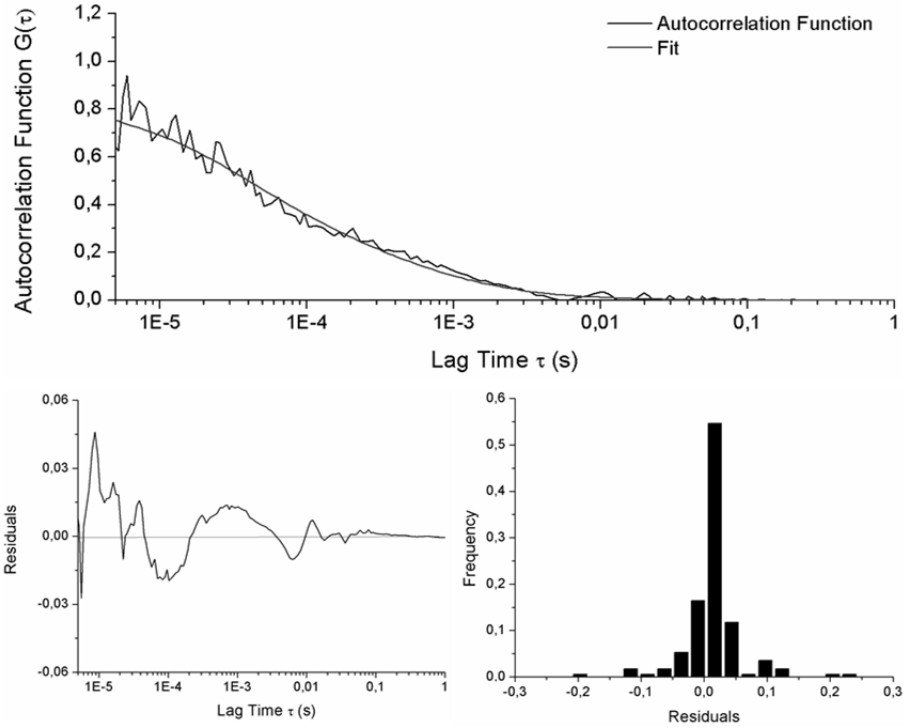
Parameter	Value	Range	Parameter	Value	Range
# non-fluorescent =	1	0...2	$G_{\text{off}} = \text{FX}$	0 ± 0	-10...10
components =	2	1...3	$\gamma = \text{FX}$	6.0105 ± 1	0.01...100
$\tau_{\text{trip}} = \text{FX}$	1.23 ± 0	μs 0...10	$w_{xy} =$	240.674 ± 9.32	nm 0...10 ⁴
$\theta_{\text{trip}} = \text{F}$	0.2952 ± 0.104	0...0.99999	$V_{\text{eff}} = \text{C}$	0.4666 ± 0.0947	fl 0...10 ⁵⁰
$N = \text{F}$	0.3806 ± 0.0118	10 ⁻¹⁰ ...10 ⁵	$C_{\text{off}} = \text{C}$	1.3546 ± 0.278	nM 0...10 ⁵⁰
$1/N = \text{C}$	2.6272 ± 0.0814	10 ⁻¹⁰ ...10 ⁵	$D_1 = \text{C}$	408.3755 ± 31.6	μm ² /s 0...10 ⁵⁰
$\rho_1 = \text{C}$	0.5576 ± 0.0299	0...0.99999	$D_2 = \text{C}$	14.8396 ± 1.64	μm ² /s 0...10 ⁵⁰
$\tau_{0,1} = \text{FX}$	35.46 ± 0	μs 1...10 ⁵	count rate =	7.36867 × 10 ²⁸⁰ ± 2.66 × 10 ²⁸⁴ Hz	0...10 ⁵⁰
$\rho_2 = \text{F}$	0.4424 ± 0.0299	0...0.99999	background =	0 ± 0	Hz 0...10 ⁵⁰
$\tau_{0,2} = \text{F}$	975.8338 ± 77.3	μs 1...10 ⁸	cnt/molec = C	1.93593 × 10 ²⁸¹ ± 6.99 × 10 ²⁸⁴ Hz	0...10 ⁵⁰

legend: F: fit parameter, X: fixed parameter, C: calculated parameter

Fit Statistics:

$\chi^2 = 0.623219$	$\chi^2 \text{ (weighted)} = 51.1462$
$\langle E \rangle = 0.00604155$	$\langle E \rangle \text{ (weighted)} = -0.0125117$
$\sqrt{\langle E^2 \rangle} = 0.0593696$	$\sqrt{\langle E^2 \rangle} \text{ (weighted)} = 0.54047$
NP = 7	NR = 175
DF = 167	TSS = 94.9918
$R^2 = 0.993439$	$R^2 \text{ (weighted)} = 0.461572$
$R^2_{\text{adjusted}} = 0.993205$	$R^2_{\text{adjusted}} \text{ (weighted)} = 0.442343$
AICc = -971.917	AICc (weighted) = -200.597
BIC = -950.434	BIC (weighted) = -179.114
	max _P (σ _P / P) = 87.5%
det(COV) = 4.26821e-12	$p_{\text{Bayes}}(\text{model} \text{data}) = 1.32742e-30 \text{ (} = 10^{-29.877} \text{)}$

4.16 Fitting of FCS data for Au-Glucosamine* NPs water solution



Model Parameters (fit results):

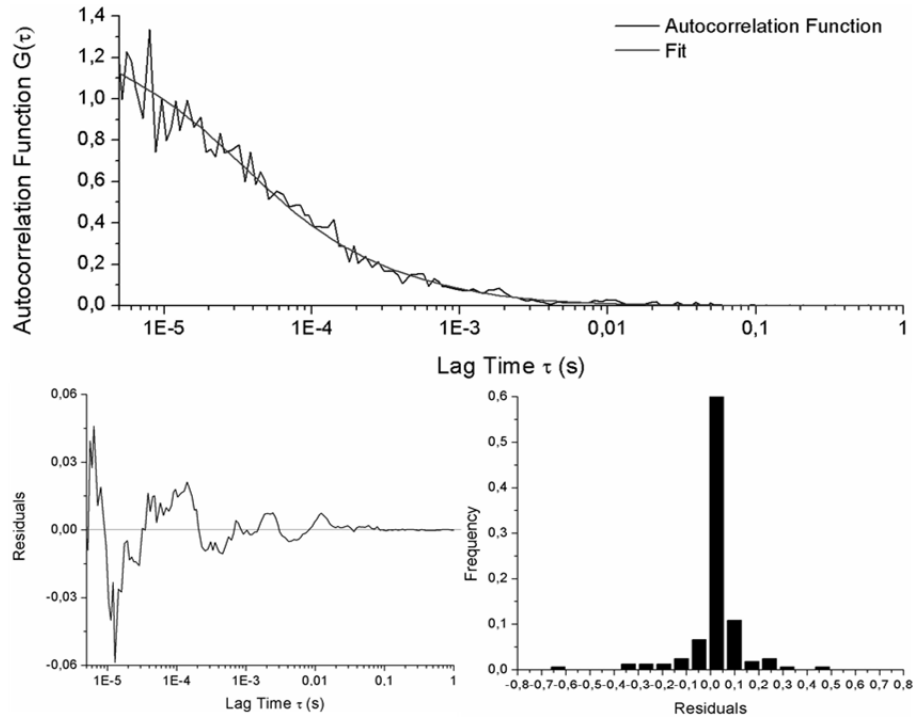
Parameter	Value	Range	Parameter	Value	Range
# non-fluorescent =	1	0...2	G_{∞} = FX	0 ± 0	-10...10
components =	2	1...3	γ = FX	7.9 ± 1	0.01...100
T_{trip} = FX	0.4 ± 0	μs 0...10	$w_{x,y}$ =	244 ± 6	nm 0...10 ⁴
θ_{trip} = F	0.8286 ± 0.0392	0...0.99999	V_{eff} = C	0.639 ± 0.0936	fl 0...10 ⁵⁰
N = F	1.1872 ± 0.0864	10^{-10} ...10 ⁵	C_{all} = C	3.0849 ± 0.505	nM 0...10 ⁵⁰
$1/N$ = C	0.8423 ± 0.0613	10^{-10} ...10 ⁵	D_1 = C	402.2703 ± 19.8	$\mu m^2/s$ 0...10 ⁵⁰
ρ_1 = C	0.7273 ± 0.0871	0...0.99999	D_2 = C	25.5532 ± 8.13	$\mu m^2/s$ 0...10 ⁵⁰
$T_{0,1}$ = FX	37 ± 0	μs 1...10 ⁵	count rate =	$443.3933 \pm 2.04 \times 10^{236}$	Hz 0...10 ⁵⁰
ρ_2 = F	0.2727 ± 0.0871	0...0.99999	background =	0 ± 0	Hz 0...10 ⁵⁰
$T_{0,2}$ = F	582.4707 ± 183	μs 1...10 ⁸	cnt/molec = C	$373.4882 \pm 1.72 \times 10^{236}$	Hz 0...10 ⁵⁰

legend: F: fit parameter, X: fixed parameter, C: calculated parameter

Fit Statistics:

$\chi^2 = 0.843796$	χ^2 (weighted) = 248.023
$\langle E \rangle = 0.00646012$	$\langle E \rangle$ (weighted) = 0.0409328
$\sqrt{\langle E^2 \rangle} = 0.0693374$	$\sqrt{\langle E^2 \rangle}$ (weighted) = 1.19321
NP = 4	NR = 174
DF = 169	TSS = 25.2284
$R^2 = 0.966554$	R^2 (weighted) = -8.83108
$R^2_{adjusted} = 0.965964$	$R^2_{adjusted}$ (weighted) = -9.00457
AICc = -918.992	AICc (weighted) = 69.9135
BIC = -906.592	BIC (weighted) = 82.313
	$\max_p(\sigma_p/ P) = 31.9399\%$
$\det(\text{COV}) = 2.53384e-05$	$p_{Bayes}(\text{model} \text{data}) = 4.19732e-64 (= 10^{-63.377})$

4.17 Fitting of FCS data for Au-PEG* NPs water solution



Model Parameters (fit results):

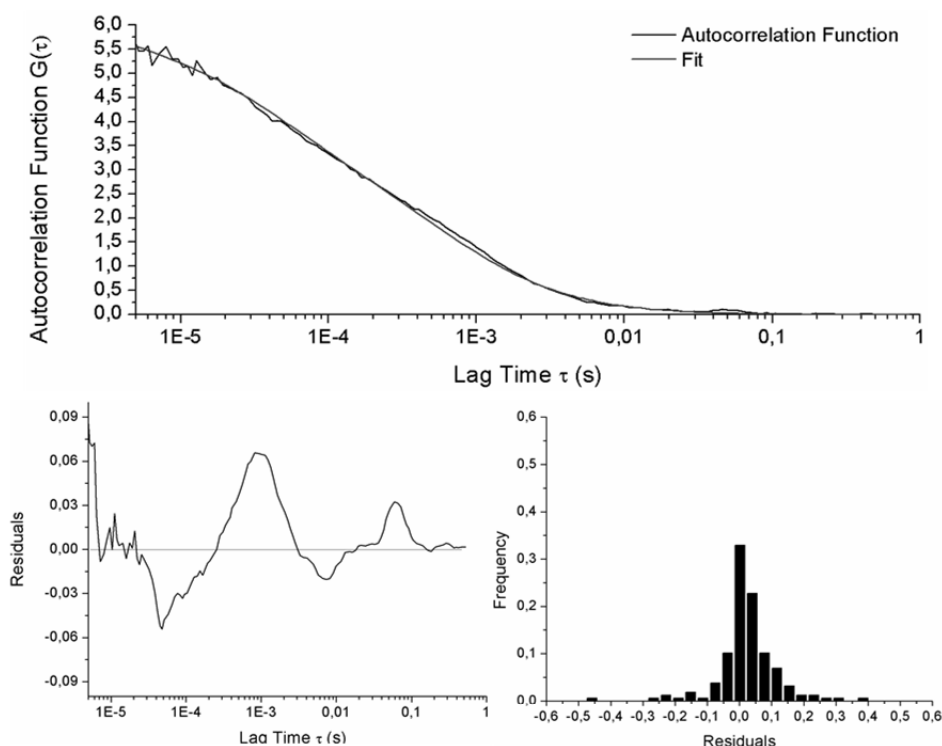
Parameter	Value	Range	Parameter	Value	Range
# non-fluorescent components =	1	0...2	$G_{\infty} = FX$	0 ± 0	-10...10
$\tau_{trip} = FX$	1.19 ± 0	μs 0...10	$\gamma = FX$	6.54 ± 1	0.01...100
$\theta_{trip} = F$	0.4376 ± 0.14	0...0.99999	$w_{x,y} =$	228.3 ± 10.8	nm 0...10 ⁴
$N = F$	0.7913 ± 0.0523	10^{-10} ...10 ⁵	$V_{eff} = C$	0.4333 ± 0.0904	fl 0...10 ⁵⁰
$1/N = C$	1.2638 ± 0.0836	10^{-10} ...10 ⁵	$C_{\text{all}} = C$	3.0322 ± 0.664	nM 0...10 ⁵⁰
$\rho_1 = C$	0.8952 ± 0.0448	0...0.99999	$D_1 = C$	394.8552 ± 37.4	$\mu m^2/s$ 0...10 ⁵⁰
$\tau_{D,1} = FX$	33 ± 0	μs 1...10 ⁵	$D_2 = C$	17.8783 ± 9.92	$\mu m^2/s$ 0...10 ⁵⁰
$\rho_2 = F$	0.1048 ± 0.0448	0...0.99999	count rate =	$1.58629 \times 10^{224} \pm 8.69 \times 10^{306}$	Hz 0...10 ⁵⁰
$\tau_{D,2} = F$	728.83 ± 398	μs 1...10 ⁸	background =	0 ± 0	Hz 0...10 ⁵⁰
			cnt/molec = C	$2.00475 \times 10^{224} \pm 1.1 \times 10^{307}$	Hz 0...10 ⁵⁰

legend: F: fit parameter, X: fixed parameter, C: calculated parameter

Fit Statistics:

$\chi^2 = 3.12242$	$\chi^2 \text{ (weighted)} = 48.4896$
$\langle E \rangle = -0.00249388$	$\langle E \rangle \text{ (weighted)} = -0.0153988$
$\sqrt{\langle E^2 \rangle} = 0.137126$	$\sqrt{\langle E^2 \rangle} \text{ (weighted)} = 0.540249$
NP = 4	NR = 166
DF = 161	TSS = 44.2743
$R^2 = 0.929475$	$R^2 \text{ (weighted)} = -0.0952086$
$R^2_{\text{adjusted}} = 0.928169$	$R^2_{\text{adjusted}} \text{ (weighted)} = -0.11549$
AICc = -651.332	AICc (weighted) = -196.038
BIC = -639.133	BIC (weighted) = -183.838
	$\max_P(\sigma_P/ P) = 54.6081\%$
$\det(\text{COV}) = 0.000866215$	$p_{\text{Bayes}}(\text{model} \text{data}) = 1.02336e-20 (= 10^{-19.99})$

4.18 Fitting of FCS data for Au-Alkyl-PEG₆₀₀* NPs water solution



Model Parameters (fit results):

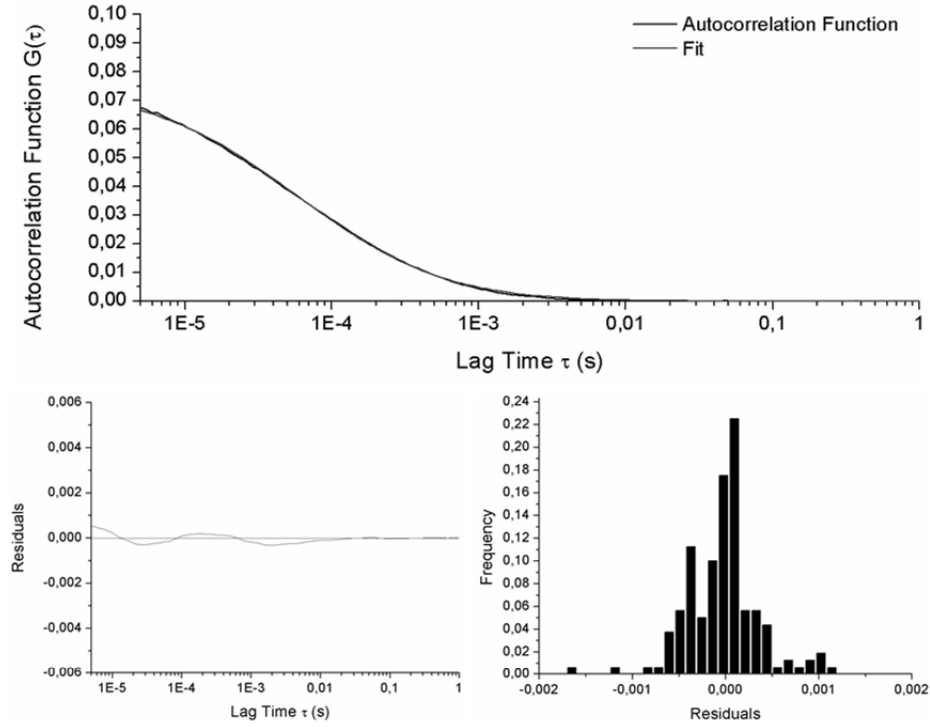
Parameter	Value	Range	Parameter	Value	Range
# non-fluorescent =	1	0...2	$G_{\infty} =$ FX	0 ± 0	-10...10
components =	2	1...3	$\gamma =$ FX	7 ± 1.35	0.01...100
$\tau_{trip} =$ FX	1.57 ± 0	μs 0...10	$w_{x,y} =$	216 ± 10	nm 0...10 ⁴
$\theta_{trip} =$ F	0.233 ± 0.0461	0...0.99999	$V_{eff} =$ C	0.3928 ± 0.0934	fl 0...10 ⁵⁰
$N =$ F	0.17075 ± 0.00361	10^{-10} ...10 ⁵	$C_{all} =$ C	0.7218 ± 0.172	nM 0...10 ⁵⁰
$1/N =$ C	5.8564 ± 0.124	10^{-10} ...10 ⁵	$D_1 =$ C	302.961 ± 28.1	$\mu m^2/s$ 0...10 ⁵⁰
$\rho_1 =$ C	0.4925 ± 0.0425	0...0.99999	$D_2 =$ C	16.9701 ± 2.27	$\mu m^2/s$ 0...10 ⁵⁰
$\tau_{D,1} =$ FX	38.5 ± 0	μs 1...10 ⁵	count rate =	$3.92711 \times 10^{256} \pm 2.55 \times 10^{257}$	Hz 0...10 ⁵⁰
$\rho_2 =$ F	0.5075 ± 0.0425	0...0.99999	background =	0 ± 0	Hz 0...10 ⁵⁰
$\tau_{D,2} =$ F	687.3274 ± 66.5	μs 1...10 ⁸	cnt/molec = C	$2.29989 \times 10^{257} \pm 1.49 \times 10^{258}$	Hz 0...10 ⁵⁰

legend: F: fit parameter, X: fixed parameter, C: calculated parameter

Fit Statistics:

$\chi^2 = 1.59387$	χ^2 (weighted) = 25.3303
$\langle E \rangle = 0.00822654$	$\langle E \rangle$ (weighted) = 0.0824572
$\sqrt{\langle E^2 \rangle} = 0.100101$	$\sqrt{\langle E^2 \rangle}$ (weighted) = 0.391815
NP = 4	NR = 158
DF = 153	TSS = 843.903
$R^2 = 0.998111$	R^2 (weighted) = 0.969984
$R^2_{adjusted} = 0.998075$	$R^2_{adjusted}$ (weighted) = 0.9694
AICc = -717.974	AICc (weighted) = -280.972
BIC = -705.985	BIC (weighted) = -268.983
	$\max_{\sigma}(\sigma_P/ P) = 19.7854\%$
$\det(COV) = 1.03072e-08$	$p_{Bayes}(\text{model} \text{data}) = 1.04948e-15$ ($= 10^{-14.979}$)

4.19 Fitting of FCS data for FBS labelled proteins in RPMI solution



Model Parameters (fit results):

Parameter	Value	Range	Parameter	Value	Range
# non-fluorescent =	1	0...2	$G_{\infty} = FX$	0 ± 0	-10...10
components =	2	1...3	$\gamma = FX$	7.83 ± 0.61	0.01...100
$\tau_{\text{diff}} = FX$	1.35 ± 0	$\mu\text{s } 0...10$	$w_{\text{diff}} = C$	229.62 ± 6.22	nm $0...10^4$
$\theta_{\text{diff}} = F$	0.2075 ± 0.0221	$0...0.99999$	$V_{\text{eff}} = C$	0.5279 ± 0.0594	fl $0...10^{50}$
$N = F$	13.6717 ± 0.0555	$10^{-10}...10^5$	$C_{\text{all}} = C$	43.0086 ± 4.84	nM $0...10^{50}$
$1/N = C$	$0.07314 \pm 2.97 \times 10^{-4}$	$10^{-10}...10^5$	$D_1 = C$	420.86 ± 22.8	$\mu\text{m}^2/\text{s } 0...10^{50}$
$\rho_1 = C$	0.573 ± 0.0162	$0...0.99999$	$D_2 = C$	90.2933 ± 6.02	$\mu\text{m}^2/\text{s } 0...10^{50}$
$\tau_{D,1} = FX$	31.32 ± 0	$\mu\text{s } 1...10^5$	count rate =	460.392 ± 620	Hz $0...10^{50}$
$\rho_2 = F$	0.427 ± 0.0162	$0...0.99999$	background =	0 ± 0	Hz $0...10^{50}$
$\tau_{D,2} = F$	145.9835 ± 5.68	$\mu\text{s } 1...10^6$	cnt/molec = C	33.6749 ± 45.3	Hz $0...10^{50}$

legend: F: fit parameter, X: fixed parameter, C: calculated parameter

Fit Statistics:

$\chi^2 = 2.59463\text{e-}05$	$\chi^2 \text{ (weighted)} = 141.109$
$\langle E \rangle = -7.40499\text{e-}05$	$\langle E \rangle \text{ (weighted)} = -0.302123$
$\sqrt{\langle E^2 \rangle} = 0.00039583$	$\sqrt{\langle E^2 \rangle} \text{ (weighted)} = 0.889187$
NP = 4	NR = 160
DF = 155	TSS = 0.105447
$R^2 = 0.999754$	$R^2 \text{ (weighted)} = -1337.2$
$R^2_{\text{adjusted}} = 0.999749$	$R^2_{\text{adjusted}} \text{ (weighted)} = -1362.94$
AICc = -2493.29	AICc (weighted) = -11.8443
BIC = -2481.24	BIC (weighted) = 0.198333
	max $\rho(\sigma_P/ P) = 10.6506\%$
det(COV) = 7.06359e-11	$p_{\text{Bayes}}(\text{model} \text{data}) = 2.62982\text{e-}41 \text{ (} = 10^{-40.5801} \text{)}$

4.20 DLS

To perform the DLS measurements of Au NPs in water, 50 µl of Au NPs solution (1mg/ml) were diluted with 450 µl of MilliQ water, sonicated and filtered on 0.22 µm syringe filters units. The experiments were performed registering for each NP 3 tracks at 25 °C, each one composed of 10 runs of 10 seconds. The system was allowed to equilibrate for 2 min prior to each measurement. The data were analysed employing Zetasizer software from Malvern and reported in terms of volume abundance.

To perform the DLS study of PC formation 50 µl of Au NPs solution (1mg/ml) were dissolved a solution of phenol red free RPMI enriched with 10% in volume of FBS. The nanoparticles were incubated for 10 min, 20 min, and 1h at 37 °C. The experiments were performed registering for each NP 3 tracks at 37 °C, each one composed of 10 runs of 10 seconds. The system was allowed to equilibrate for 2 min prior to each measurement. The data were analysed employing Zetasizer software from Malvern and reported in terms of volume abundance. As comparison, a correlogram of bare NPs in RPMI has been registered.

4.21 UV-visible

To perform the DLS measurements of Au NPs in water, 50 µl of Au NPs solution (1mg/ml) were diluted with 450 µl of MilliQ water, sonicated and filtered on 0.22 µm syringe filters units. The experiments were performed registering for each NP 3 different spectra at 25 °C. MilliQ water was employed as baseline. From the UV-vis spectra is possible to determine the hydrodynamic diameter of the nanoparticles, using the following formula:

$$D_H = \exp(B_1 \frac{A_{spr}}{A_{450}} - B_2)$$

Where A_{spr} is the absorbance of the plasmonic peak A_{450} is the absorbance at 450 nm and B_1 and B_2 are experimentally determined values ($B_1=3,55$ and $B_2=3,11$)⁵⁰.

To perform the UV-vis study of PC formation 50 µl of Au NPs solution (1mg/ml) were dissolved a solution of phenol red free RPMI enriched with a 10% in volume of FBS. The nanoparticles were incubated for 1 and 24 hours at 37 °C. The experiments were performed registering for each NP and time point 3 different spectra at 25 °C. As comparison, a UV-visible spectrum of bare NPs in RPMI has been registered. A solution of RPMI enriched with a 10% in volume of FBS was employed as baseline.

4.22 Proteins quantification assay

The protein's calibration line has been obtained employing different concentration solutions of a BSA standard solution from 25ug/ml to 1000ug/ml. 100 µl of protein solution was incubated with 1.5 ml of Pierce™ 660 Protein Assay for 1 h. The absorption spectra of the sample were registered employing a mixture of 100 µl of PBS and 1.5 ml of Pierce™ 660 Protein Assay as baseline. The absorbance values at 660 nm were employed to create the calibration line.

For the quantification of the PC, 915 µg of nanoparticles was incubated in 1ml of pure FBS for 1h at 37 °C. The sample was divided in 3 equal parts (in order to perform 3 replicates). The nanoparticles with PC was separated from the excess of proteins employing centrifugation (15000

RPM for 1h) over a layer of sucrose 0,7 M (700ul). The supernatant was removed and a pellet of NPs isolated. Each pellet was suspended in 100 μ l of PBS and 1.5 ml of PierceTM 660 Protein Assay was added. The nanoparticles with PC were incubated with the colorimetric reagent for 1h. The absorption spectra of the sample were registered employ a solution of 100 μ l of PBS and 1,5 ml of PierceTM 660 Protein Assay as baseline. The best results were obtained for a dilution 1:4 of the samples. A spectrum of barely Au NPs was previously registered and employed to perform a deconvolution of the signal in order to subtract the contribute of Au NPS from the absorbance at 660 nm.

4.23 *Agarose gel electrophoresis*

100 μ l of Au NPs solution (1mg/ml) were incubated in 100 μ l of pure FBS at 37 °C overnight. The NPs with PC were separated from the excess of proteins employing centrifugation (15000 RPM for 1h, 4°C) over a layer of sucrose 0.7 M (700ul). The formed pellet was re-suspended in 10 μ l of PBS. The Agarose gel was prepared with 0.5% w/w of agarose in tris-borate buffer 90 mM. The running buffer was 90 mM tris-borate + 0.1% SDS. The electrophoresis was run for 30 min at 100V.

4.24 *Confocal imaging and FCS measurements in living cell*

A549 cells were grown in RPMI media enriched with 10% of fetal bovine serum (FBS) and 1% of penicillin-streptomycin (PS) in a humidified atmosphere at 37 °C with 5% CO₂. To perform a Raman mapping experiment 10'000 A549 cells were seeded on NuncTM Lab-Tek Chambered Coverglass (purchased from Thermo Fisher Scientific) and grown in a humidified atmosphere at 37 °C with 5% CO₂ for 24 hours. Then the cells were incubated with 500 μ l of 15 μ g/ml solution of Raman nano-probe (dissolved in full RPMI media) for 30 and 60 minutes. The nanoparticle was removed and the cells washed twice with warm PBS. To perform the experiments on living cells the sample was covered with 500 μ l of Hepes % = mM.

FCS measurements were done on at least 3 cells recording 20 runs of 10 second each in 8 distinct area inside the cell: 2 in the cytoplasm; 2 in the endoplasmatic and, whenever visible, 2 in brighter spots near to the membrane (endocytic vesicles) and 2 in brighter spots near to the nucleus (intracellular vesicles). Moreover 1 experiment was performed on/close to the membrane and 1 on/close the nucleus. Tracks were at first screened one-by-one to remove tracks showing bleaching, low signal and visible aggregates. The remaining tracks were fitted one-by-one using a 3D normal diffusion fit model. The fit algorithms used were in order, Simulated Annealing with box constraints and the Levenberg-Marquardt Algorithm with box constraints with one non-fluorescent state and 2(or 3) diffusing components. Considering the decay time (τ_D , μ s) of the most abundant specie (fraction $\Phi > 0.5$), the tracks were grouped as follow: $500 < \tau_D (\mu s) < 1500$; $1500 < \tau_D (\mu s) < 3000$; $3000 < \tau_D (\mu s) < 6000$; $\tau_D (\mu s) > 6000$. The average track was fitted again and the average decay time with the correspondent standard deviation calculated. The procedure was repeated for each measurement of each cell compartment. Finally decay times were averaged per compartment considering all cell replicates referring to the above-mentioned decay times grouping. The percentage of tracks representative of the species decaying with the latter diffusion times were calculated over the total of the good tracks analysed in all cell replicates.

5. Bibliography

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Chapter 4:

Fluidic manufacture of morphologically controlled gold nanoparticles.

1. Introduction

1.1 Fluidic synthesis of NPs

Since the development of the first micro-fluidic reactors, in the early 1990's, their employment in the chemical synthesis has found a wide diffusion and a plenty of application in pharmaceutical, biotechnological and chemical fields.¹ The use of fluidic reactors for chemical processes (both in their meso and micro-fluidic setups) gives large advantages in terms of high-throughput production, control of reaction parameters, and safer handling of toxic and hazardous reagents.² Moreover, by means of this technology the scale up of the production is accessible and has been widely demonstrated by employing several in-parallel fluidic reactors.³ The application of fluidic processes to the production of nano-materials can provide answers to some of the main drawbacks that affect this field. First of all, the micro-fluidic approach gives the opportunity to produce NPs in gram scale, without modifying the reaction conditions. This is particularly true for the cases of anisotropic nanoparticles, that are currently synthesized in the amounts of few micro-grams in batch conditions. Then, the easier control of the reaction parameters in flow continuous production, can guarantee a higher NP reproducibility. The issue of the reproducibility is close to heart of the nanotechnologies and fundamental for their reliable application in the daily life. Furthermore, the faster and precise mixing achieved in micro and meso-fluidic reactors, allows to better control the nucleation and growth steps that are timely and spatially separated inside the reactor channel. Finally, the use of fluidic devices allows to directly interface analytical techniques with the nanoparticles synthesis, achieving a real-time quality control. A brief description of published researches on the fluidic production of Au NPs is reported in **Chapter 1 (Section 3.1.2)**

1.2 Star shaped and multi-branched Au NPs

Gold nano-stars present peculiar optical behaviour and plasmonic bands tuneable in the near infrared region.⁴ The optical properties of Au NPs are primarily ruled by collective oscillations of the conduction electrons, taking place when the system is restoring its equilibrium distribution, after a perturbation due to an external electromagnetic radiation. This phenomenon gives origin to an intense absorption band, known as LSPR (localized surface plasmon resonances) bands. The LSPR wavelength varies in function of nanoparticle's size and shape, and also with the dielectric properties of the medium.⁴ LSPR is responsible as well of the generation of enhanced electromagnetic fields close to the Au NP surface. The localization and intensity of these fields can be influenced by the morphology of the nanoparticles and advantaged by the presence of an irregular surface and tips⁴. The electromagnetic enhancement caused by LSPR is the main effect explaining the phenomena of surface enhanced Raman scattering (SERS)⁴. Raman spectroscopy is a

diffused and sensible analytical techniques for molecular species identification, providing vibrational information correlated with the chemical composition of molecular systems. SERS spectroscopy can show even stronger sensitivity due to the enhanced fields present close to the metal surface. Gold nano-stars are ideal candidates for SERS applications, due to the focalization of the electromagnetic field near to the tips of the NPs. Even if the most attractive applications of gold nano-stars are related to their optical properties, these types of NPs can be employed in catalysis. In fact, the large surface area-to-volume ratio, the surface defects, and the exposition of peculiar crystal facets, are appreciated benefits in the field⁷.

Regarding the synthesis of multi-branched and star like Au NPs, a large pool of wet synthetic methods is available in literature. The principal parameters that can affect the outcome of this kind of synthesis are: the metal precursor reactivity⁵, the employment of capping/shielding agents⁶, the reducing agent strength⁷, the reaction temperature⁸ and the presence of catalysts or additives that modify the reaction kinetics⁹. The known synthetic methods can be divided in two main categories: seeded-growth and one-pot strategies. The employment of preformed seeds is a popular choice that allows to obtain nano-stars with a high degree of uniformity and monodispersity. One of the most diffused synthetic method involves the reduction of HAuCl_4 with AA in the presence of cetyl-trimethylammonium bromide (CTAB), employing preformed seeds¹⁰⁻¹³. CTAB seems to display preferential adsorption on specific faces of the metal crystal, allowing the anisotropic growth of the NPs¹⁴. Recent studies demonstrated that the addition of AgNO_3 during the crystal growth allows a higher control on the shape of Au nano-stars¹². With this procedure pentabranch gold NPs in a size range of 70 - 350 nm were synthesized. The NPs were characterized by single-crystalline tips with [111] exposed face. Moreover, in this study is underlined the role of bromide ions, playing a synergistic effect with Ag^+ as templating agent. Also the choice of the reducing agent has a strong influence on the synthesis of star shaped Au NPs. Some of the most employed once are hydroxylamine sulfate¹⁵, N,N-dimethylformamide (DMF)¹⁶, AA^{17,18} and hydroquinone¹⁹.

One-pot strategies are widely applied for the production of multi-branched Au NPs because they are highly versatile and easily approachable procedures. The main drawback of this strategies is the high sensitivity to small changes in the reaction parameters and surrounding environment¹. Several one-pot synthetic procedures for the synthesis of gold nano-stars, were developed in the last years. Recently, multiple-twinned crystals has been synthesized by mean of gold reduction with ascorbic acid, registering a preferential growth of branches on the {111} facets¹⁴. Another recent synthetic protocol is based on the employment of HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid) as reducing and stabilizing agent. With this procedure branched and flower-like Au NPs were obtained in high yields²⁰.

2. Results and discussion

2.1 Aim of the work

The synthesis of Au NPs in continuous-flow, is a research field that is attracting ever growing interest.²¹ In fact, the application of the fluidic chemistry to the production of nano-materials can bring numerous advantages. Among them the most important are the higher reproducibility, due to an improved control of the reaction condition, and the possibility to scale up the production, employing several in parallel reactors. Notwithstanding the implemented efforts in the field, many

challenges in the optimization of the micro and meso-fluidic production of this types of nano-materials are still to deal with.^{2,22} In particular, the synthesis of nanoparticles with controlled structural features, including anisotropic and hybrid materials, has not been extensively investigated.²³ Among anisotropic nanoparticles, gold nano-stars cover a prominent role thanks to their interesting optical, chemical and physical properties.²⁴ In this chapter is reported a novel manufacturing method for the preparation of gold nano-stars in fluidic conditions, by employing a designed bench-top fluidic reactor (**Figure 1. a**).

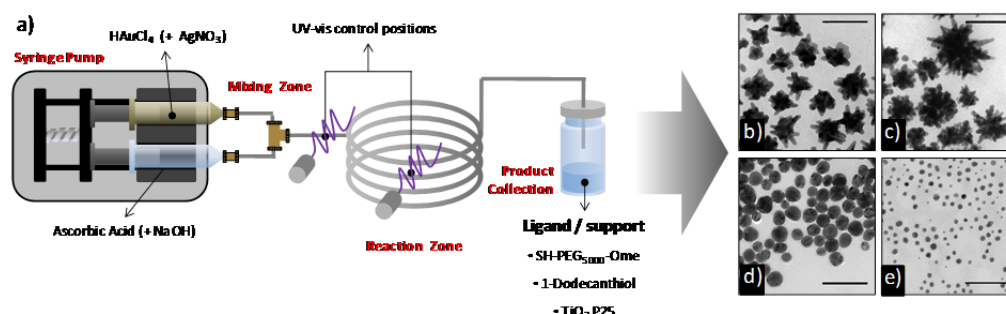


Figure 1. a) Schematic representation of the bench-top reactor designed for the synthesis of AuNPs. TEM micrographs of **b)** star-shaped Au NPs **c)** hyper-branched Au NPs **d)** 30 nm spherical Au NPs **e)** 10 nm spherical Au NPs. The scale bars correspond to 100 nm.

This synthetic procedure is extremely versatile and allows to obtain a continuous production of high quality gold nano-stars. In fact, four morphologies of gold nanoparticles (star-shaped Au NPs, hyper-branched Au NPs, 30 and 10 nm spherical Au NPs) can be obtained using the same equipment, by tuning reagent ratios and reaction conditions (**Figure 1. b-e**). Moreover, by applying a convenient one-pot strategy, the production of different functionalized nano-materials (dodecanthiol-functionalized Au NPs or PEG-functionalized Au NPs) as well as Au NPs deposited onto solid supports (i.e. TiO_2), has been achieved. Finally, by taking advantages from the spatial resolution of the meso-fluidic channel, we investigated the temporal evolution of the gold nano-stars during the formation process. The data obtained by on-line UV-visible spectroscopy were supported by TEM analysis of samples taken at three different check points, in order to have a full overview of the process. Moreover, as proof of concept the complete in-flow synthesis of a nano-structured functional material has been performed. Exploiting the properties of gold nano-stars to enhance the Raman signal of molecules close to their surface, a Raman probe for cellular imaging has been synthesized and tested on A549 cell line.

2.2 Fluidic production of morphologically controlled Au NPs

The bench-top fluidic reactor employed in this work was designed using commercially available components that can be easily purchased and found in most any laboratory (**Figure 2**). A programmable syringe pump (Legato 200, KD Scientific) was used to generate the flow and the syringe containing the metal precursors (HAuCl_4 and AgNO_3) was shielded from the light, to avoid a premature nucleation of the metals. The mixing zone was constituted by PEEK T-mixer with a swept volume of $0.57 \mu\text{l}$ (P-727 - PEEK Tee .020 thru hole Hi Pressure,). The reaction zone consisted in a PTFE tube with a length of 160 cm and an ID (internal diameter) of 1 mm.

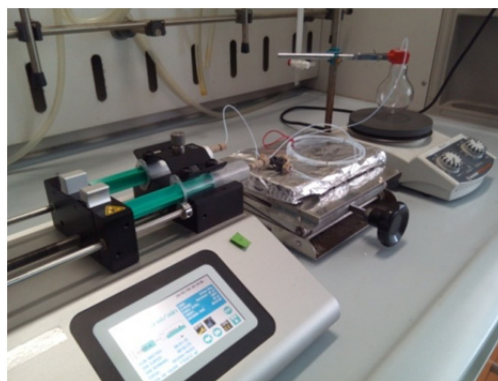


Figure 2. Picture of the bench-top fluidic reactor

PTFE is inert to aqua regia ($\text{HCl}:\text{HNO}_3 = 3:1$), employed to remove possible residues of metallic gold from the walls of the tubes at the end of the reaction. This makes the polymer as the ideal material for the reactors tubes. The employment of 1 mm ID can avoid the blockage of the reactor due to the fouling of the walls. Moreover, can lead as well to a higher flow rate and a consequently increased production of nano-materials. During the design of the reactor we considered to insert in the reaction zone two different positions to perform on-line UV visible spectroscopy (at 5 and 80 cm). The measurements were registered in real-time, employing an optical window characterized by an internal volume of 200 μl and an optical pathway of 1mm. A flask equipped with magnetic stirrer has been disposed at the end of the reactor, for the collection of the formed nanoparticles. In this way is possible to drop NPs directly inside a solution or suspension of the stabilizing material, obtaining a one pot functionalization of the nano-system.

The chosen ID, within the applied flow rate ranges, allowed to obtain a strictly laminar flow, maintaining a micro-fluidic regime, ideal for the application. In this way the employed apparatus can be defined borderline between a meso and micro fluidic reactor. In fact, a micro-fluidic reactor is generally defined basing on its internal diameter (ID). It is commonly accepted that micro-fluidic devices should have an ID smaller than 500 μm . Instead the devices possessing a internal diameter in the range of 500 and 1000 μm are called meso-fluidic. However, this is a restrictive definition and does not take in consideration that the passage from a micrometer to a millimeter scale does not necessary imply a change in the fluid performances. A more rational definition considers the Reynolds (Re) and Péclet (Pe) numbers as discerning parameter for the fluid behaviour. Following this definition is possible to define micro-fluidic reactor, a device operating at Reynolds and Péclet numbers respectively lower than 250 and 1000. The Reynolds number can be expressed using the following equation:

$$Re_d = \frac{\rho v D}{\mu} \quad \text{eq. 1}$$

It is a dimensionless value that describes the proportion between inertial and viscous forces in a fluid. D is the internal diameter of the pipe (expressed in m), ρ is the density (Kg/m^3), v the velocity (m/sec), and μ represents the dynamic viscosity of the media ($\text{Pas}\cdot\text{sec}$). When the Re is lower than 2300 the flow regime can be considered laminar, and no turbulences and back-mixing events take place. When Re has a value below 250 the flow regime is characterized by a strictly laminar flow, and the device can be considered micro-fluidic. At our operating conditions (**Table 1**) the Re number is always below the limit value and the system can be considered a micro-fluidic device.

<i>Reynolds numbers in the operating conditions</i>			
<i>ID (m)</i>	<i>Flow rate (mL/min)</i>	<i>Flow rate (m/sec)</i>	<i>Reynolds number</i>
1x10 ⁻³	2,5	0.027	27
1x10 ⁻³	5	0.053	53
1x10 ⁻³	10	0.106	106
1x10 ⁻³	15	0.159	159

Table 1. Reynolds numbers obtained for the applied operating condition

Instead the Péclet number describes the proportion existing between the heat transport related to advective and diffusive phenomena. The thermal Pe number describes the heat transfer rate from the tube walls to the middle of the fluid and is a peculiar parameter of the micro-fluidic condition. It can be derived employing following equation:

$$Pe = \frac{dv\rho C_p}{k} \quad \text{eq.2}$$

In this equation d and v represents respectively the internal tube diameter (expressed in m) and the velocity of the flow (m/sec). The remaining parameters involved in the equation are relative to the physical characteristics of the media: C_p the specific heat capacity (J/mK), ρ the density of the media (Kg/m³) and k the thermal conductivity (W/gK). Micro-fluidic devices are characterized by high thermal transfer and that correspond to a Pe number below 1000. As shown in **Table 2** at flow rates below 15 mL/min, the calculated Pe numbers are comprised between 185 and 740. At a flow rate of 15 mL/min we are in a border-line situation having a Pe of 1110.

<i>Péclet numbers in the operating conditions</i>			
<i>ID (m)</i>	<i>Flow rate (mL/min)</i>	<i>Flow rate (m/sec)</i>	<i>Péclet number</i>
1x10 ⁻³	2,5	0.027	185
1x10 ⁻³	5	0.053	370
1x10 ⁻³	10	0.106	740
1x10 ⁻³	15	0.159	1110

Table 2. Péclet numbers obtained for the applied operating condition

In conclusion, if we consider only the pipe ID, the described device should be considered a meso-fluidic reactor. Instead, evaluating the flow behaviour, the device effectively operates in micro-fluidic regime, exploiting all the advantages typical of a micro-fluidic reactor.

Employing the described apparatus, a synthetic procedure for the production of morphologically controlled Au NPs was developed. By means of a careful modulation of the reaction conditions, it was possible to obtain 4 types of Au NPs characterized by different morphologies, dimensions and physico-chemical properties. The reaction at the base of the process is the reduction of tetrachloroauric acid (HAuCl_4) by mean of ascorbic acid (AA) that plays both the role of reductive and temporary stabilizing agent leading to the formation of Au^0 crystals. Instead AgNO_3 is used as templating agent in order to control the nanoparticles morphology. In literature is reported that silver ions can be preferentially reduced on [110] faces of the gold crystal, by mean of under potential deposition (UDP). Consequently, silver can shield preferentially these facets of the NPs, performing a similar role of CTAB (cetyl trimethylammonium bromide) and inhibiting their grown. In this way, only the other faces of the crystal are allowed to grow, affording nanoparticles characterized by multi-branched shape. There are several factors that can play a role in this process, strongly affecting the morphological outcome of the synthesis. Among these factors, we performed a complete screening on the $\text{AgNO}_3/\text{HAuCl}_4$ ratio, the pH of AA solution and the applied flow rate in order to understand their effect on the synthesis outputs. A careful modulation of these factors allowed us to implement a fine control over the morphology of the nanoparticles (**Figure 3**).

First of all, we deeper analysed the effect of the $\text{AgNO}_3/\text{HAuCl}_4$ ratio on the anisotropy of the Au NPs (**Figure 4**). Maintaining fixed the parameters of flow rate (5 ml/min), Au/AA ratio (4) and pH (3,93) we tuned the $\text{AgNO}_3/\text{HAuCl}_4$ ratio from 0 to 10%. The employment of 10% of silver nitrate can lead to obtain uniform star-shaped nanoparticles characterized by a diameter centred around 70 nm with a distribution of 25 nm. Gradually decreasing the amount of AgNO_3 from 10% to 0%, an increasing fraction of Au NPs assumes spherical morphology and different ratio mixtures of anisotropic and isotropic nanoparticles were achieved (**Figure 4**). When the lower limit is reached (no addition of silver nitrate to the metal precursor) quasi-spherical Au NPs with an average diameter of 35.63 ± 8.27 nm were synthesized (**Figure 4**). From the UV-vis spectra it is possible to appreciate that star-shaped and spherical nanoparticles owns different absorption profiles, corresponding to completely different optical and plasmonic properties

The pH of the AA solution is as well an essential factor to implement a control on the shape of the NPs. In fact, the reductive potential of AgNO_3 and the AA reactivity can be strongly affected by the protons concentration in the solution. The under-potential deposition process of silver on gold surface can be favoured or hindered by a combination of this two factors.^{18,25} In particular the experimental data show that this process can be promoted in acidic conditions. The effect of the pH_{AA} on the synthesis of star shaped Au NPs has been evaluated keeping constant the flow rate (5ml/min) and $\text{AgNO}_3/\text{HAuCl}_4$ ratio (10%). AA equivalents were tuned from 8 to 2 and, consequently, the pH of the reaction media between 3.52 and 4.29. In particular, the experimental data show that this process can be promoted in acidic conditions. In fact, an enhanced degree of ramification in the branched NPs can be observed increasing the quantity of AA from 2 to 8 eq. The employment of 8 equivalents of AA (flow rate = 5 ml/min; $\text{pH}_{\text{AA}} = 3.52$; 10% of AgNO_3) can enhance the UDP efficiency leading to the obtainment of hyper-branched Au NPs (**Figure 5**). Instead, increasing the pH value ($\text{pH}_{\text{AA}} = 4.29$) fewer tips and un-homogeneous NPs are formed. In this case from the UV-vis spectra possible to appreciate that, increasing number of branches and the anisotropy of the NPs, the plasmonic peaks of the Au NPs are broader and shifted to the near

infrared region. The effects of the pH_{AA} on the morphology were evaluated as well for the spherical NPs. The flow rate conditions (5ml/min) and $AgNO_3/HAuCl_4$ ratio (0%) were maintained constant, and the pH values were varied between 3.93 and 10.28. To reach a pH value of 10.28 a proper amount of NaOH was added to the AA solution. In strongly basic conditions small Au NPs with a mean diameter of 10.13 ± 2.52 nm were obtained.

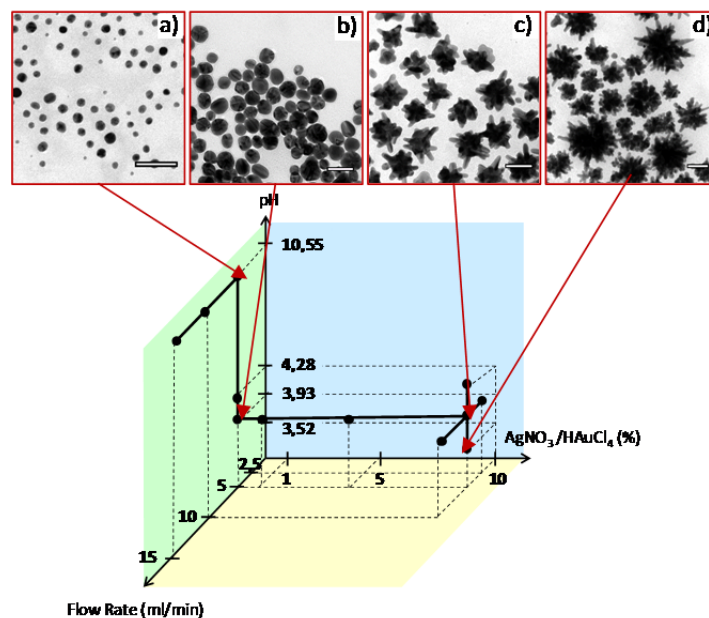


Figure 4. Graphical representation of the experimental tests performed varying the parameters of: pH, flow rate and $AgNO_3/HAuCl_4$ ratio. The TEM micrographs are referred to: **a)** PEGylated 10 nm spherical Au NPs **b)** PEGylated 30 nm spherical Au NPs **c)** PEGylated star-shaped Au NPs **d)** PEGylated hyper-branched Au NPs. (the scale bars are correspondent to 50 nm). The red arrows indicate the reaction parameters employed for their synthesis.

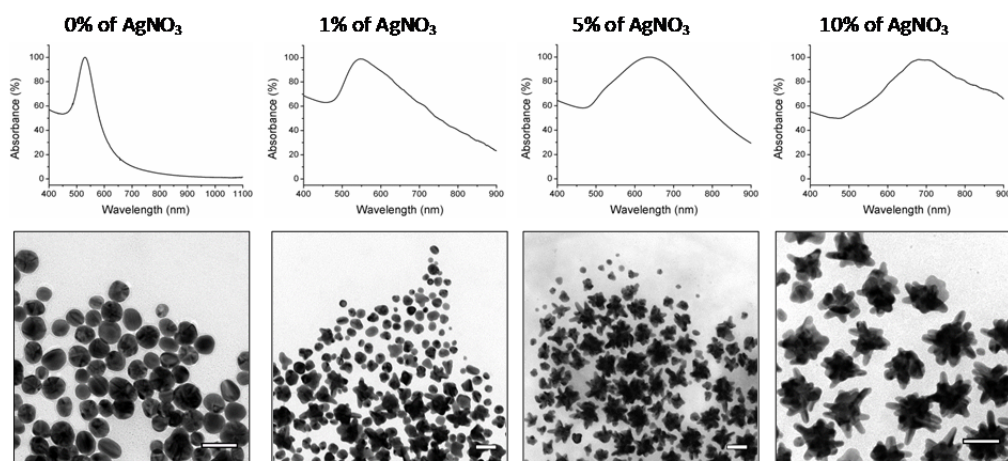


Figure 5. UV-vis spectra and TEM images of Au NPs achieved varying the $AgNO_3/HAuCl_4$ ratio. The scale bar corresponds to 50 nm.

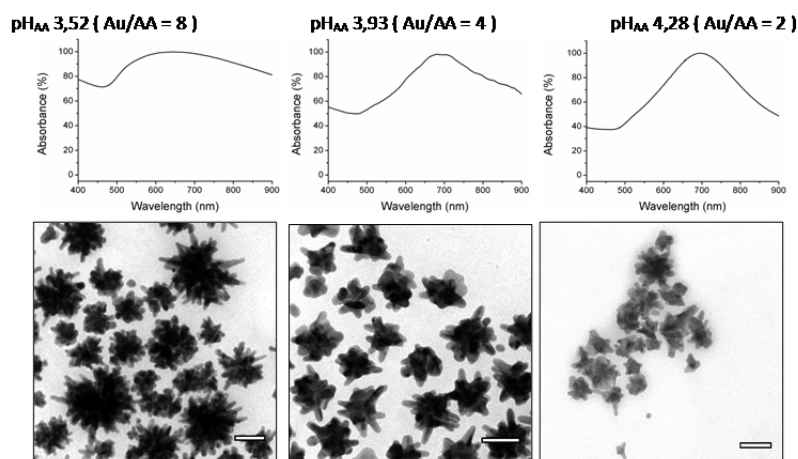


Figure 6. UV-vis spectra and TEM images of the Au NPs achieved varying the pH of the ascorbic acid solution. The scale bar corresponds to 50 nm.

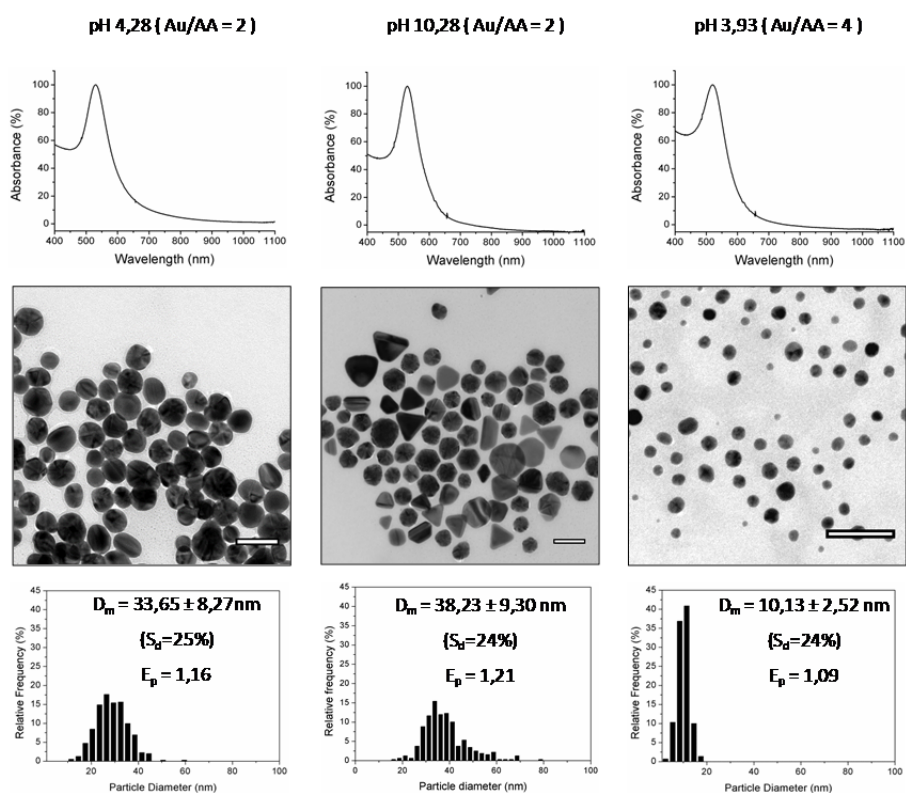


Figure 6. UV-vis spectra, TEM images and statistical distributions of the isotropic Au NPs achieved varying the pH of the AA solution. The scale bar corresponds to 50 nm.

This can be explained by the fact that AA is a weak acid, with a pKa at 4.1. At pH values higher than the pKa, the main reductive specie present in solution is the ascorbate ion. This specie is characterized by lower chemical stability and higher reactivity with respect to AA. As well the presence of free charges on the ascorbate ion can better stabilize the Au NPs in solution favouring a rapid nucleation process instead of the growing phase of the NPs. The result is the preferential formation of Au clusters with an average diameter of 10.13 ± 2.52 nm (**Figure 6**). At a pH_{AA} of 3.93 the situation results completely different, with the presence of ascorbic acid as main reducing specie. In these conditions bigger nanoparticles are obtained, with a mean diameter of 33.65 ± 8.27 nm.

As we are operating in fluidic conditions we need to consider also the mixing efficiency of the system and the flow rate as fundamental discerning parameters for the outcome of the reaction. Star shaped Au NPs demonstrated to be highly sensitive to variations in flux speed. Maintaining fixed the AgNO₃ concentration at 10% and the pH of the ascorbic acid solution at 3.93, three different flow rates were investigated: 5 mL/min resulted the best rate to obtain regular high aspect-ratio nano-stars (**Figure 7**). At slower flux speed (2.5 mL/min) un-homogeneous and poly-dispersed branched NPs are obtained. Instead a fast and uncontrolled mixing process seems to occur at faster flow rate (10 mL/min), leading to the undesired formation of quasi-spherical NPs. To analyse the effect of the flow rate on spherical nanoparticles we operated in absence AgNO₃ and at pH_{AA} of 10.55. In general, the isotropic nanoparticles demonstrated to be less sensitive to variations in the flow rate. Anyway, with an excessive flow rates (15 mL/min) the formation of undesired large nanoparticles was observed, probably due to a poor efficiency during the mixing phase.

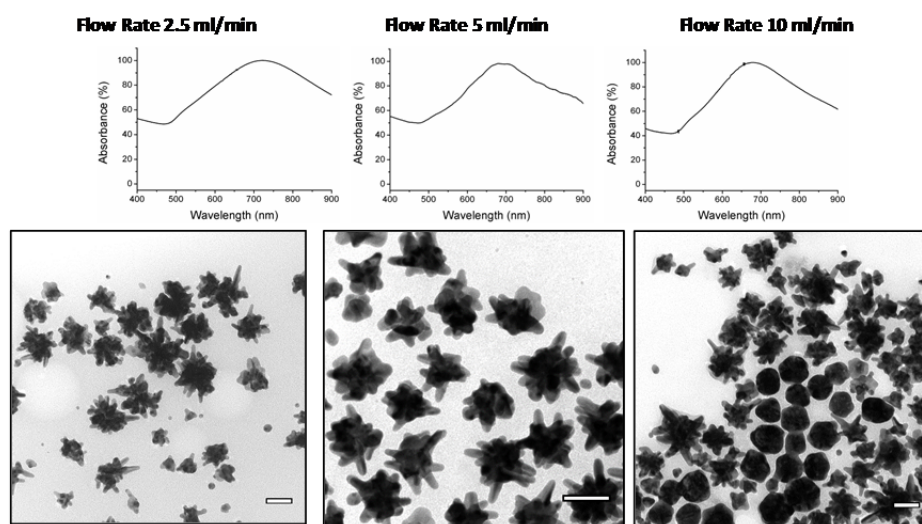


Figure 7. UV-vis spectra and TEM images of anisotropic Au NPs achieved varying the flow rate conditions. The scale bar corresponds to 50 nm.

In the end, from the careful modulation of the described parameters we were able to obtain 4 different types of gold nanoparticles, employing the same fluidic apparatus (star-shaped Au NPs, hyperbranched Au NPs, 30 nm spherical Au NPs, 10 nm spherical Au NPs). The nanoparticles obtained, summarized in **Figure 9**, own different physical, chemical and optical properties and are suitable for a vast pool of applications.

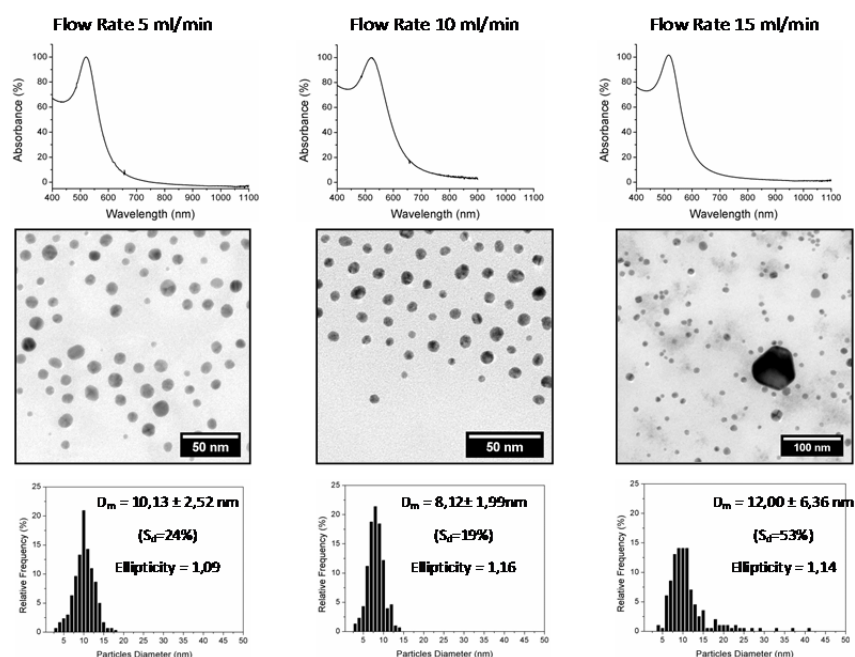


Figure 8. UV-vis spectra, TEM images and statistical distributions of the isotropic Au NPs achieved varying the Flow rate of the AA solution. The scale bar corresponds to 50 nm.

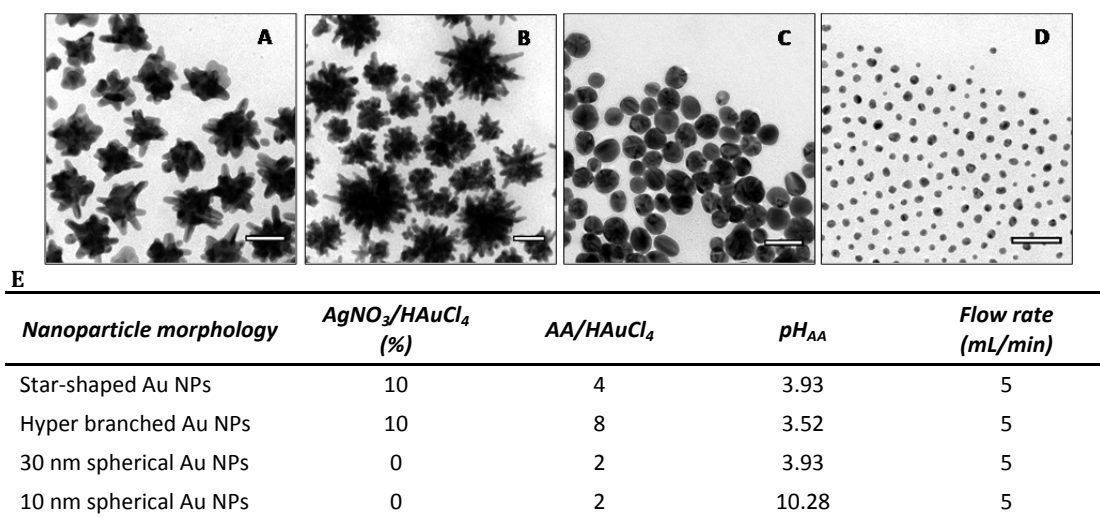


Figure 9. Representative TEM micrographs of **a)** PEGylated star-shaped Au NPs **b)** PEGylated hyperbranched Au NPs **c)** PEGylated 30 nm spherical Au NPs **d)** PEGylated 10 nm spherical Au NPs. The scale bar corresponds to 50 nm. **e)** Summary table of the synthesized nanoparticles and of the experimental condition applied.

An essential part of a functional engineered nano-material is the superficial chemistry or the supporting materials, which plays a fundamental role in the identity of the system. In fact, each

application requires the use of a peculiar coating material that confers to the NPs the desired properties like: dispersability in a specific media, chemico-physical activity or active targeting. One of the advantages of our fluidic reactor is the chance to collect the formed nanoparticles in straight-line, directly into a suspension or solution of different supporting and coating agents. The first screened surfactant is HS-PEG₅₀₀₀-OMe. This type of polymer is diffusely employed in nano-medicine to cover NPs, giving outstanding properties of biocompatibility, water stability and stealth ability. The ascorbate stabilized Au NPs were directly dropped inside an HS-PEG₅₀₀₀-OMe aqueous solution at RT. In these conditions the PEG chains easily exchange the ascorbate present on the surface, thanks to the high affinity between thiol moieties and gold atoms. Another surfactant screened was dodecyl thiol. We choose this type of molecule with the aim to obtain NPs soluble in apolar organic solvents (like hexane or toluene). Experimentally, we directly collected the NPs solution into a flask containing an excess of pure hydrophobic ligand. The affinity between gold and sulphur permits to sequester the Au NPs from the water suspension, precipitating them on the bottom of the flask. This easily accessible ligand exchange procedure allowed to obtain Au NPs stable in toluene without affecting the shape of the nanoparticles. From TEM micrographs is visible a grey shadow surrounding the metal core, proving the actual coating with the dodecyl thiol (**Figure 10 b**). In the end, we supported the star-shaped gold nanoparticles on P25 titania powder (suspended in water). This type of TiO₂ is widely used for photo-catalytic applications. In **Figure 10 a** HR-TEM image of TiO₂ supported gold nano-stars shows that the nanoparticle shape is not affected by the supporting material. It is interesting to notice that the interaction between gold and titania is so strong that can cause, in the contact region, slightly deformation of the nano-stars tips (**Figure 11**).

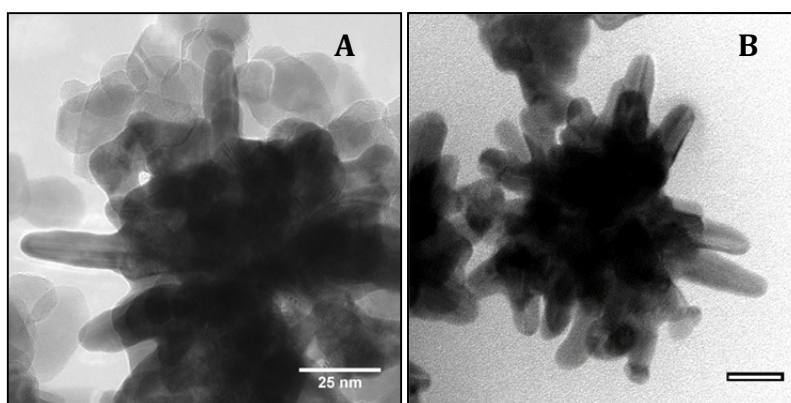


Figure 10. HR-TEM images of **a)** TiO₂ supported star-shaped Au NPs **b)** Star-shaped Au NPs stabilized with dodecylthiol. The scale bars are correspondent to 25 nm

The HR-TEM micrograph on a single branch of star-shaped Au NP (**Figure 11**) shows a complex polycrystalline phase due to the presence of twin defect along the longer axis. The d-spacing was separated by 0.235 nm, which is ascribed to the [111] reflection for the face-centred cubic lattice of the Au/Ag NPs. These results are consistent with previously reported studies on the formation of Au nano-star where the branches growth from twin sites of polycrystalline core particles in the [100] direction.^{26,27}

Exploiting the spatial resolution obtained inside the fluidic reactor we collected interesting information on the time evolution of the formation of gold nano-stars. The fluidic reactor was equipped with two optical windows to perform on-line and in real time UV-vis spectroscopy. The obtained data were completed with the UV-vis spectra of the recovered material and the TEM

images of the NPs at different tube length. It is interesting to notice that after the mixing phase the nucleation of the nanoparticles takes place instantaneously; at the first check-point (5 cm after the T-joint mixer) it is possible to appreciate the presence of the formed nucleus (**Figure 11.b**).

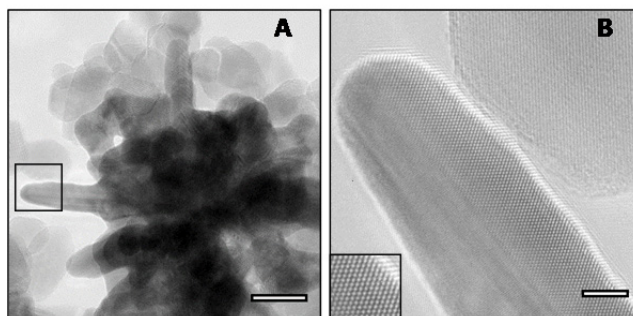


Figure 11. a) TEM micrograph of an Au nano-star immobilized on TiO₂; b) The HR-TEM image of the branch of the region contained in the black square (a). The [111] crystal planes are indicated by the white lines. The scale bars are respectively 20 and 2 nm.

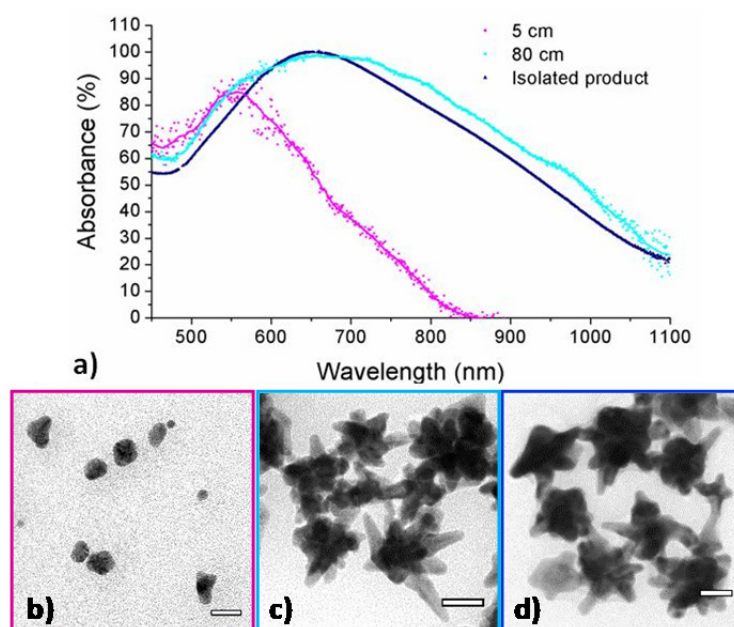


Figure 12. a) UV-visible spectra registered in real time 5 and 80 cm at the T-joint mixer and on the recovered nanoparticles. Representative TEM micrographs of the nanoparticles at b) 5 cm after the mixing c) 80 cm after the mixing d) the collecting point. The scale bar is correspondent to 20 nm.

At this check-point is already possible to appreciate the formation of the first tips in the crystal structure. The UV-visible spectrum registered on-line and in real time is coherent with the nanoparticles seen by TEM analysis, presenting a broad plasmonic peak centred at 550 nm (**Figure 12 b**). At the second check-point (80 cm after the T-joint mixer) the gold nano-stars were already well formed (**Figure 12 c**) and analogous to the nanoparticles collected at the end of the reactor

(Figure 12 d). This proof give use the confidence that the formation of the nano-stars takes place entirely inside the channel and the second half of the tube is useful to guarantee the uniformity of the sample, allowing all the nanoparticles to reach the same growing stage.

2.3 Fluidic production of Raman probe for cell imaging

Having in our hands a reliable and reproducible procedure for the fluidic production of star-shaped AuNPs, we decided to exploit the acquired knowledge to produce an effective functional material, completely in fluidic conditions. In particular, we focused our efforts on the production of a Raman nano-probe for cellular imaging. To obtain this probe, we exploited the peculiar properties of star-shaped Au NPs, that are able to enhance the Raman signal of molecules that are close to their surface.²⁸ In fact local electromagnetic (EM) fields, near to noble metal NPs, result strongly enhanced due to the optical excitation of plasmon resonances bands (LSPRs) typical of these NPs. This phenomena is the main mechanism at the base of the surface-enhanced Raman scattering (SERS).^{29,30} The morphology of the nanoparticles can play a synergistic effect to the noble metal composition on the increasing SERS effect intensity. In particular, star-shaped NPs demonstrates a stronger SERS enhancement than isotropical ones. This is due to the anisotropic distribution of the electromagnetic field that create “hot spots” close to the tips and edges of the multi-branched nanoparticles.³¹ In fact the small curvature radius present at the ends of the tips can enhance the electromagnetic field generating the majority of the SERS signal.

In order to afford the continuous production of the Raman probe we slightly modified the previously proposed fluidic set up. In particular a second collection chamber, equipped with a magnetic stirrer, was included to allow the absorption of the Raman code³² (methylene blue, MB) on the gold surface (Figure 13). Then the NPs, after 10 minutes of incubation were transferred, by mean of a peristaltic pump, inside a second collection chamber. Here the nanoparticles were coated and stabilized with a HS-PEG₅₀₀₀-OMe. The system was left to react with PEG for 1 hour, then recovered, concentrated on Amicon centrifuge filter units (30 kDa) and purified with dialysis membrane (10 kDa). The nanoparticles were stocked as a 1 mg/ml solution.

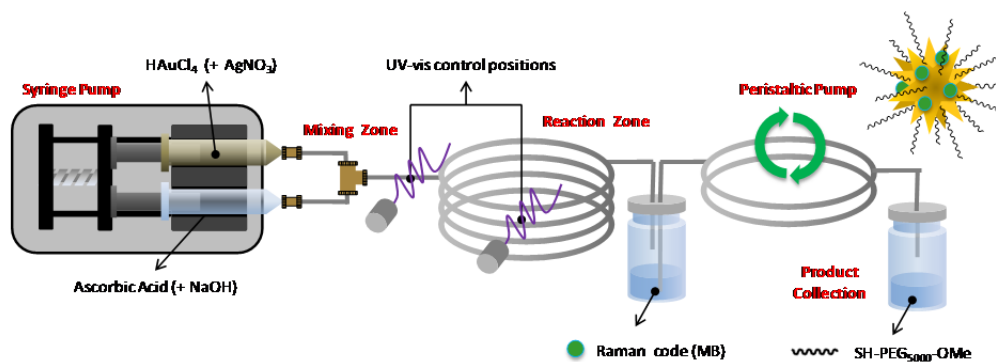


Figure 13. Schematic representation of the fluidic reactor employed for the synthesis of the Raman nano-probe

HR-TEM micrographs of the nano-material confirmed that the process of surface functionalization did not affect the shape and morphology of the nano-stars (Figure 13 c). Moreover a N-EFTEM map has been performed in order to visualize the nitrogen of the MB on the surface of the NPs

(Figure 14 a,b). The mapping established the successful absorption of the Raman code on the nanoparticles.

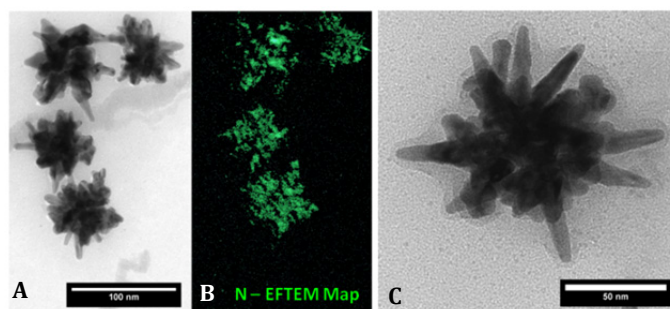


Figure 14. Reference TEM image (a) and N-EFTEM map (b) of the obtained gold nano-stars, proving the actual absorption of MB on the metallic surface. c) HR-TEM image of a single nanoparticle where is possible to confirm that the procedure does not affect the morphology of the NPs. Moreover, a clear uniform organic shell due to the presence of PEG is visible from the image.

Then we measured the Raman signal deriving from the aqueous solution of the Raman probe using a Raman-IR microscope (Renishaw in Via Raman) in the following conditions: $\lambda = 756 \text{ nm}$; LP = 10%; exposure time = 10 s; 1 accumulation. The spectrum corresponds to the typical spectra of MB with intense and sharp peaks. (Figure 14)

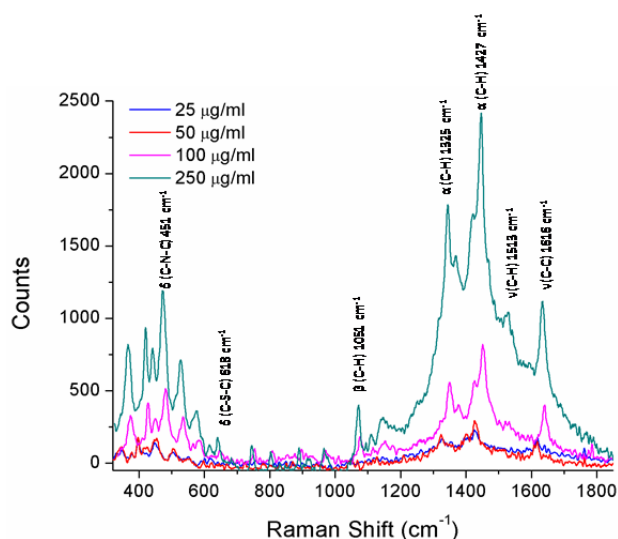


Figure 15. Raman spectra of the nano-probe registered at different probe concentrations. The attributions of the main peaks are reported in the spectra.

In accordance with previous works the main peaks has been assigned as following: 451 cm^{-1} corresponds to $\delta(\text{C-N-C})$ skeleton bending, 618 cm^{-1} to $\delta(\text{C-S-C})$, 1051 cm^{-1} to the $\beta(\text{C-H})$, 1325 cm^{-1} and 1427 cm^{-1} to $\alpha(\text{C-H})$, 1513 cm^{-1} to the asymmetrical stretching of $\nu(\text{C-H})$, 1616 cm^{-1} is referred to the $\nu(\text{C-C})$ stretching of the aromatic ring.³³

In order to apply the produced Raman probe with cells we had to ensure that the signal was still detectable at concentrations applicable in cell culture. For this reason, we measured the signal deriving from a series of diluted solution of the Raman probe (**Figure 15**). The principal peaks resulted detectable until 25 $\mu\text{g/ml}$. On the base of these observations we decided to incubate the Raman probe with A549 cells for 24 h with a concentration of 200 $\mu\text{g/ml}$ (at 37 °C in humidified atmosphere with 5% of CO_2). After the incubation times the cells were washed and fixed with formaldehyde and the map registered. The spectra were collected point-by-point and the intensity at the baseline of the $\delta(\text{C-N-C})$ signal at 451 cm^{-1} was employed to create the map (**Figure 16**).

From the resulting image, it is possible to appreciate the actual internalization of the probe, with a quite uniform distribution inside the cell. Moreover, the back ground resulted clean, confirming that the signal is detectable only in concomitance with cells structures. This experiment is a proof of principle that is possible to produce in continuous flow condition a functional Raman probe, employing gold nanoparticles as amplifiers of the Raman signal. We are actually performing further studies on this nano-system to improve the resolution of the Raman mapping and to evaluate the cytotoxicity of the nano-probe. Once clarified these aspects the nanoparticles will be functionalized with peptides for the specific targeting of mitochondria, in order to obtain a nano-probe with selectivity toward these organelles.

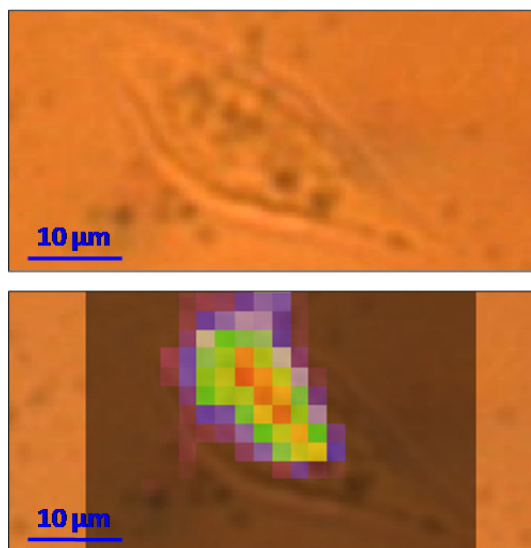


Figure 16. Raman mapping of a single A 549 cell treated with the nano-probe (200 $\mu\text{g/ml}$; 24h). The spectra were registered employing a 756 nm at the 10% of laser power with an exposure time 10 s for each point. The map was created employing the intensity value of the $\delta(\text{C-N-C})$ signal at 451 cm^{-1} .

3. Conclusions

In this chapter, has been reported the fluidic continuous production of high aspect-ratio Au nano-stars. The proposed synthetic method is very versatile. It allows to produce 4 different types of gold nanoparticles (star-shaped Au NPs, hyper-branched Au NPs, 30 and 10 nm spherical Au NPs) using the same fluidic set-up and finely modulating the reaction conditions and reagent amounts. The best reaction parameters for the process were identified screening the pH, AgNO_3 amounts and flow rate. With the described manufacturing method was also possible to afford the one-pot

production of functionalized nano-materials, obtaining nanoparticles supported on solid state or dispersible both in organic as in water media. Moreover, this reliable and reproducible synthetic procedure can allow an easy scale up to higher nanoparticles productions. Furthermore the nano-stars formation process has been monitored by mean on-line and in real time UV-visible spectroscopy and TEM microscopy. The nanoparticles were monitored at different check points taking advantage from the spatial resolution of the reactor and commuting it in temporal information. In the end, with a slightly modification of the fluidic apparatus, an effective Raman nano-probe has been produced in continuous fluidic conditions. The Raman signal deriving from the nano-probe has been measured at different probe concentrations and the NPs cellular internalization evaluated over A549 cancer cell line.

4. Experimental

4.1 Materials and methods

HAuCl₄·3H₂O, AgNO₃, ascorbic acid, dodecyl thiol and NaOH were purchased from Sigma-Aldrich, and used without further purification. HAuCl₄·3H₂O was stored at 4°C, shielded from light, as 10 mM solution and NaOH was stored as 1M water solution. AgNO₃ 10 mM and ascorbic acid 100 mM solutions were freshly prepared before each synthesis (avoiding the exposition to the light). HS-PEG₅₀₀₀-O CH₃, purchased from Rapp Polymer GmbH, was used as received and stored under dry argon atmosphere at -20 °C. TiO₂ P25 was purchased by Degussa. The fluidic reactor, between the different synthesis, was washed in the order with aqua regia (HCl (37%):HNO₃ (65%) 3:1) and copious deionized water. Ultrapure deionized water, MilliQ water, was used for the preparation of aqueous solutions. Purification of water dispersible colloidal Au NPs was performed by ultrafiltration using Millipore Amicon Ultra-4 Centrifugal Filter Units with a cut-off of 300 kDa. TiO₂ Au NPs were purified by repeated precipitation in spin centrifuge. Au NPs were characterized by transmission electron microscopy (Zeiss LIBRA 200FE, equipped with: 200 kV FEG, in column second-generation omega filter for energy selective spectroscopy (EELS) and imaging (ESI), HAADF STEM facility, EDS probe for chemical analysis, integrated tomographic HW and SW system) and UV-vis spectroscopy (Agilent 8453 instrument). A disposable cuvette with 1 cm optical path length was used for the out-line measurements. A optical window with a path length of 1mm and an internal volume of 200 µl was used for in-line real time measurements. The samples for TEM analysis, in the case of colloidal NPs, were prepared depositing a drop of nanoparticle solution on a carbon-coated copper grid (300 mesh) and evaporating the solvent. For TiO₂ supported NPs, the samples were dried to powder, smashed and re-suspended in 2-propanol. A drop of this suspension was deposited on holey carbon copper grids. The particle size distribution was obtained by measuring at least 300 nanoparticles. For star-shaped nanoparticles are provided two different dimensions corresponding to the major axis (comprising the tips) and the minor axis (excluding the tips). For spherical nanoparticles, have been evaluated the minor and major diameter. From their ratio can be calculated the ellipticity parameter describing the isotropy of the nanoparticles:

$$E_p = \frac{d_{max}}{d_{min}}$$

closer to one is the E_p , more spherical are the nanoparticles.

4.2 Au NPs synthetic procedure

All the presented Au NPs were synthesized using the apparatus described in the section 2.2. Prior to proceed with the synthesis, the fluidic reactor was washed with deionized water and dried with air. In the first syringe were loaded 20 mL of a solution of ascorbic acid and eventually NaOH. In the second syringe (shielded from light) was loaded the same volume of a solution of HAuCl₄ and AgNO₃, prepared suddenly before the beginning of the reaction. The reaction conditions and amounts of reagents were modulated in order to obtain the desired products (**Table 3**). To functionalize the nanoparticles with different ligands/supports, in the collecting flask were prepared: 1) 3 ml of an aqueous solution of HS-PEG₅₀₀₀-OMe (1 mg/mL), 2) 20 µl of pure dodecyl thiol and 3) 5 ml of a suspension of TiO₂ P25 (7 mg/mL). The just formed nanoparticles were straight dropped into the collection flask and stirred for 1h allowing the direct surface functionalization.

Nanoparticle morphology	[HAuCl ₄] (mM)	[AgNO ₃] (mM)	[AA] (mM)	[NaOH] (mM)	Final Volume* (mL)	pH _{AA}	Flow rate (mL/min)
Star-shaped Au NPs	0.2	0.02	0.8	0	20	3.93	5
Hyperbranched Au NPs	0.2	0.02	1.6	0	20	3.52	5
30 nm spherical Au NPs	0.2	0	0.4	0	20	3.93	5
10 nm spherical Au NPs	0.2	0	0.4	0.0015	20	10.28	5

Table 3. Reaction condition and amounts of reagents employed for the synthesis of the four main types of nanoparticles described in the chapter. *The volume is referred to the solution charged in each syringe.

Different purification procedures were used to isolate the nanoparticles according to the employed ligands/support. HS-PEG₅₀₀₀-OMe functionalized nanoparticles were washed at least 5 times by ultra-filtration, using Millipore Amicon Ultra-4 Centrifugal Filter Units with a cut-off of 300 kDa. The precipitation of dodecyl thiol functionalized Au NPs was exploited to isolate them. The aqueous supernatant was removed and nanoparticles were recovered using toluene as solvent. Then the nano-material was washed at least 5 times with a 1:1 mixture of ethanol and acetone. At the end the Au NPs were stored in toluene as colloidal solution. TiO₂ P25 supported Au NPs were isolated by centrifugation and washed at least five time with fresh milliQ water.

At the end of the reaction the PTFE tubes and the flasks were washed, in the order, with aqua regia and copious distilled water. The PEEK mixer was sonicated in distilled water.

4.3 *Characterization of star-shaped Au NPs*

Dimensions from TEM images

Major axis= 69.84 ± 25.42 nm ($S_d=36\%$); **Minor axis** = 37.00 ± 11.25 nm ($S_d=30\%$)

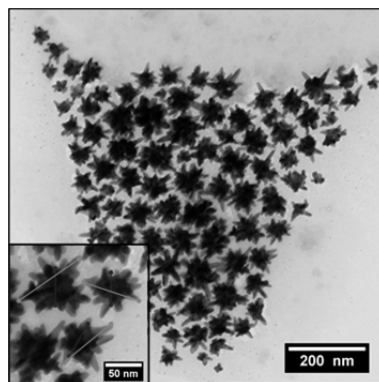


Figure 17. TEM image representing the star-shaped PEGylated Au NPs. In the inset are represented three examples of measurements of major and minor axis as performed.

4.4 *Characterization of hyper-branched Au NPs*

Dimensions from TEM images

Major axis= 53.47 ± 29.33 nm ($S_d=54\%$); **Minor axis** = 29.88 ± 14.21 nm ($S_d=48\%$)

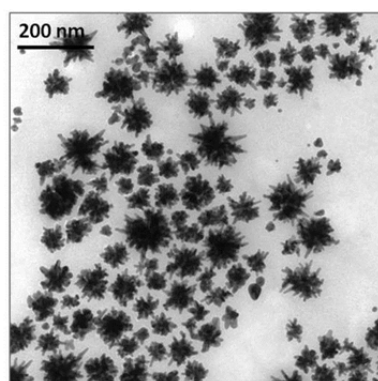


Figure 18. TEM image representing the hyper-branched PEGylated Au NPs.

4.5 Characterization of 30 nm spherical Au NPs

Dimensions from TEM images

Major axis= 33.65 ± 8.27 nm ($S_d=25\%$); **Minor axis** = 28.84 ± 6.71 nm ($S_d=23\%$)

Ellipticity parameter= 1.16

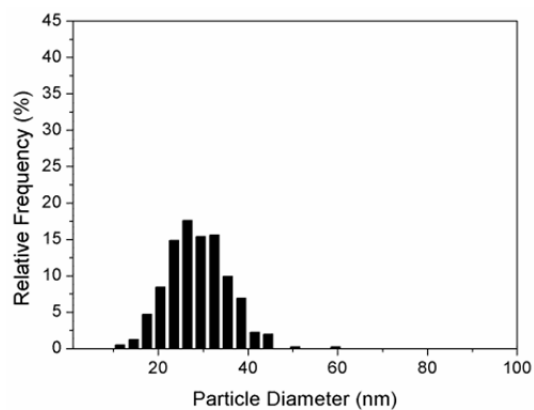


Figure 19. Dimensional distributions related to the measurements performed on the minor diameters of 30 nm spherical PEGylated Au NPs.

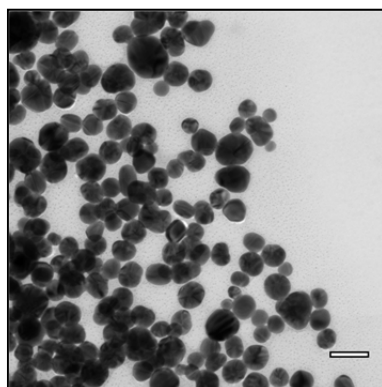


Figure 20. TEM image representing the of 30 nm spherical PEGylated Au NPs

4.6 . Characterization of 10 nm spherical Au NPs

Dimensions from TEM images

Major axis= 10.13 ± 2.52 ($S_d=24\%$); **Minor axis** = 9.29 ± 2.18 ($S_d=23\%$)

Ellipticity parameter= 1.09

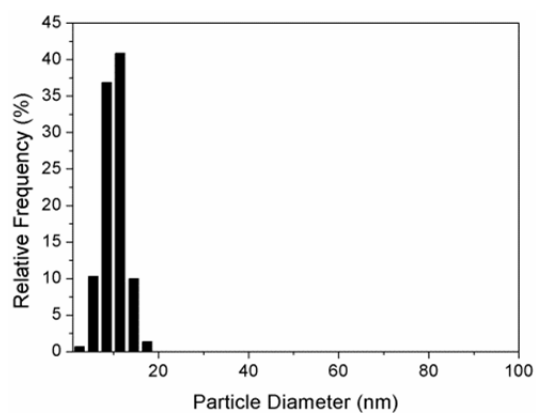


Figure 21. Dimensional distributions related to the measurements performed on minor diameters of 10 nm spherical PEGylated Au NPs.

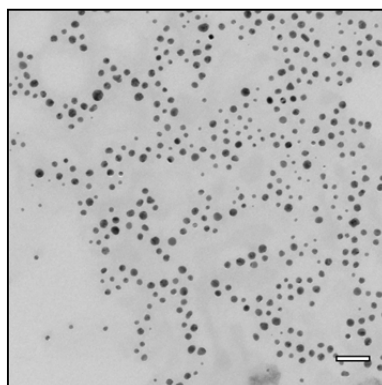


Figure 22. TEM image representing the of 10 nm spherical PEGylated Au NPs

4.7 . *P-25 TiO₂ supported star-shaped Au NPs*

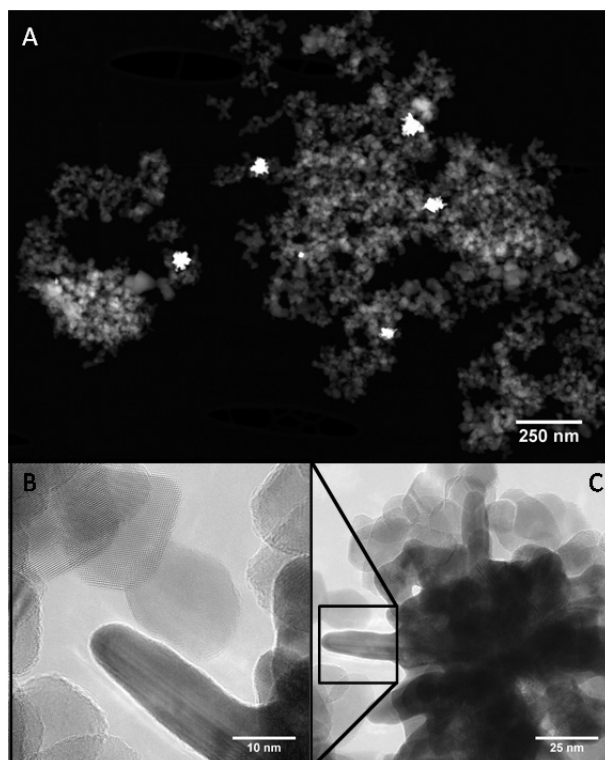


Figure 23. a) STEM image of P-25 TiO₂ supported star-shaped Au NPs. b) Higher magnification of a single point showing a slightly deformation in the contact region, due to the strong interaction between gold and TiO₂. c) TEM image of a single nanostar.

4.8 . *Dodecyl thiol stabilized star-shaped AuNPs*

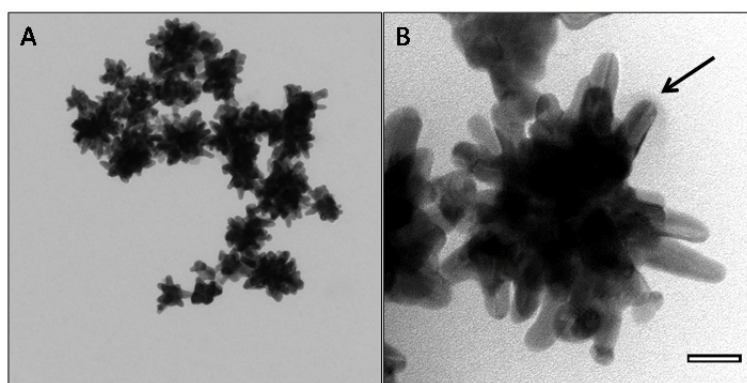


Figure 24. a) TEM image of the dodecyl thiol stabilized Au NPs. b) Higher magnification showing the details of a single nanostar. The shadow, pointed by the red arrow, is ascribable to the hydrophobic organic shell.

4.9 . Fluidic synthesis of the nano-structured Raman probe

The nano-structured Raman probe was synthesized using a slightly modified version of the apparatus described in **Section 2.2**. In particular a second collection chamber, equipped with a magnetic stirrer, was included to allow the absorption of the Raman code³² (methylene blue, MB) on the gold surface (**Figure 12**). Prior to proceed with the synthesis, the fluidic reactor was washed with deionized water and dried with air. In the first syringe were loaded 20 mL of a solution of ascorbic acid (0.8 mM). In the second syringe (shielded from light) was loaded the same volume of a solution of HAuCl₄ (0.2 mM) and AgNO₃ (0.02 mM) prepared suddenly before the beginning of the reaction. The mixing was performed with a flow rate of 5 ml/min then the just formed nanoparticles were straight dropped into a first collection flask containing 3 ml of an aqueous MB solution (0.25 mM) and stirred for 10 min at RT. The nanoparticles were then transfer to a second collection flask containing 3 ml of an aqueous solution of HS-PEG₅₀₀₀-OMe (0.2 mM). At the end of the reaction the PTFE tubes and the flasks were washed, in the order, with aqua regia and copious distilled water. The PEEK mixer was sonicated in distilled water.

The functionalized nanoparticles were washed at least 5 times by ultra-filtration, using Millipore Amicon Ultra-4 Centrifugal Filter Units with a cut-off of 30 kDa. Then the purification was completed by dialysis with 10 kDa cut-off membranes. The nanoparticles were concentrated and stored at 1mg/ml solution (concentration determined by weight of the lyophilized nanomaterial)

4.10 Raman spectra

Raman spectra were registered employing a confocal Raman Renishaw inVia microscope equipped with 785 nm laser (maximum laser power 270 mW), 1024 × 512 CCD detectors and 1200 L/mm diffraction grating. The spectra were performed in employing 1 mL glass vials (purchased by ThermoFisher) containing 500 µL of Raman probe aqueous solution with 50 × long distance objective, 10 s integration time and a laser power (LP) of 27 mW in a single accumulation. The spectra were analysed and elaborated employing Wire 3.4 software (provided by Renishaw) .

4.11 Raman mapping on fixed A549 cells

A549 cells were grown in RPMI media enriched with 10% of fetal bovine serum (FBS) and 1% of penicillin-streptomycin (PS) in a humidified atmosphere at 37 °C with 5% CO₂ for 24 hours. To perform a Raman mapping experiment 100'000 A549 cells were seeded on 27 mm (inside bottom diameter) Nunc™ Glass Bottom Dishes (purchased from Thermo Fisher Scientific) and grown in a humidified atmosphere at 37 °C with 5% CO₂ for 24 hours. Then the cells were incubated with 1 ml of 200 µg/ml solution of Raman nano-probe (dissolved in Full RPMI media) for 12 hours. The nanoparticle was removed and the cells washed twice with warm PBS. At the end the cells was with a 3.7% formaldehyde solution (1ml for 10 min at R.T.) and stored with a thin PBS layer on the top. The Mapping was performed within 1h from the fixing procedure. The Raman mapping on cells was registered employing a confocal Raman Renishaw inVia microscope equipped with 785 nm laser (maximum laser power 270 mW), 1024 × 512 CCD detectors and 1200 L/mm diffraction grating. The map was created from spectra performed in static mode, centring the scattering at 400 cm⁻¹. 50 × long distance objective was employed with 10 s integration time and a LP of 27 mW in a single accumulation. The imaging was performed measuring consecutive spectra on an area of 40x30 µm with a step on 2µm in x and y directions. The map was created employing the intensity value of the δ(C-N-C) signal at 451 cm⁻¹

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