
# ABSTRACTS

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# Controlling hydrophobicity and self-assembly of gold nanoclusters for cellular delivery

Xavier Le Guével[1*], Estelle Porret[1], Akram Yahia-Ammar[2], Lucie Sancey[1], Maria I. Montañez[3], Jean-Baptiste Fleury[4], Niko Hildebrandt[2], Jean-Luc Coll[1]

[1]*Cancer Targets & Experimental Therapeutics, Institute for Advanced Biosciences (IAB), University of Grenoble Alpes – INSERM U1209 − CNRS UMR 5309, 38000 Grenoble, France*

[2]*NanoBioPhotonics, Institute for Integrative Biology of the Cell (I2BC), Université Paris-Saclay, Université Paris-Sud, CNRS, CEA, 91400 Orsay, France*

[3]*Andalusian Center for Nanomedicine and Biotechnology - BIONAND, 29590 Málaga, Spain*

[4]*Experimental Physics, Saarland University, D-66123 Saarbrücken, Germany*

*Corresponding author email: xavier.le-guevel@univ-grenoble-alpes.fr*

Gold nanoclusters (Au NCs) are metal particle composed of ten to hundred atoms (~1-3 nm) that exhibit molecular-like properties in this ultra-small size regime[1]. Their physico-chemical properties are highly driven by the nature of the protective ligands stabilizing the metal core in solution. Thanks to their photoluminescence in the visible and in the near-infrared region and to the ability to finely control their surface chemistry, Au NCs have found a growing interest in the field of nanomedicine. Au NCs could be seen then as potential theranostic agents combining delivery capacity and bioimaging features.

In this context, we design two different types of Au NC systems for cellular delivery: i) one based on Au NCs with a precise control of his hydrophobicity to enhance the cell internalization, and ii) a second one made of a 100 nm spherical self-assembled Au NCs to deliver biomolecules in cells.

Hydrophobicity of monodisperse Au NCs could be tuned during the synthesis using custom-made thioctic sulfobetaine molecule with an increased of aliphatic chain length. Microscopic and physico-chemical characterizations confirm the ultra-small size of the metal core and the influence of hydrophobicity to reduce protein absorption on the particle surface. Optical characterization show an enhancement photoluminescence intensity with the hydrophobicity which could either associated to better protection of the “gold(I)−thiolate binding” to water molecules or to an increase in the rigidity of the network enabling a more efficient metal−ligand energy transfer. Studies performed on artificial phospholipid membranes integrated in microfluidic device and on various cell lines stress the importance to finely tune the hydrophobicity balance of Au NCs in order to improve the penetration on cell surface without inducing cytotoxicity[2].

Self-assembly of Au NCs were produced in a fast and simple approach using cationic polymers as cross-linkers. Monodipserse spherical self-assembled Au NC with pH dependent swelling properties was then used as a model system to investigate aggregation induced fluorescence enhancement mechanism as a function of the distance between Au NCs. Our observations suggest that Au NC cross-linking has a strong effect on the ligand-to-metal charge transfer and both radiative and nonradiative recombination rates of charge carriers could be responsible of the QY enhancement. With the use multimodal imaging techniques it was also demonstrated the ability to load these self-assembled Au NCs with biomolecules (peptide, antibody) with a more efficient cell uptake than the free biomolecules[3].
These studies demonstrate then the versatility of metal nanoclusters to design smart nanosystem and open new opportunities for therapy and diagnosis.

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# Unexpected intracellular degradation of gold nanoparticles

Alice Balfourier[a], N. Luciani[a], G. Wang[b], D. Alloyeau[b], F. Gazeau[a] and F. Carn[a]

[a] *Laboratoire Matière et Systèmes Complexes (UMR 7057 / CNRS, Université Paris Diderot – Sorbonne Paris Cité), Paris, France*
[b] *Laboratoire Matériaux et Phénomènes Quantiques (UMR 7162 / CNRS, Université Paris Diderot – Sorbonne Paris Cité), Paris, France*

Despite of the great interest in biomedicine, for imaging, therapy or vectorization, gold nanoparticles (NPs) fate in biological medium, and hence in organism, is still poorly known[1]. Following cellular uptake, gold NPs are generally sequestrated in the cell lysosome, which is the subcellular organelle responsible for the degradation and recycling of extracellular material after endocytosis, where they can be stored for months. This acidic cell compartment (pH = 4-5) has been shown to been the location of metallic NPs degradation[2], however comparing to the high stability of gold, lysosome medium should be considered as chemically mild condition regarding gold NPs.
In spite of this, a precedent study has evidence gold NPs degradation *in vivo*, and a reduction of the NPs radius in mice over one year[3]. In our study, we reproduce this degradation *in vitro* to identify the underlying degradation processes.
Primary human fibroblasts have been exposed to gold NPs (citrated, D = 5 nm), cultured, and imaged over 6 months by electron microscopy. After only two weeks, specific and original degradation structures shaped like cilia have been observed (fig.1). These structures, resulting of auto-organization of very small NPs (1 to 2 nm) have been studied using high resolution scanning transmission electron microscopy (HR STEM, EDX, diffraction). At the same time, degradation pathways have been investigated though the use of DNA microarray sequencing. The results enlighten the implication of thiolated proteins that can be implicated in the degradation process.

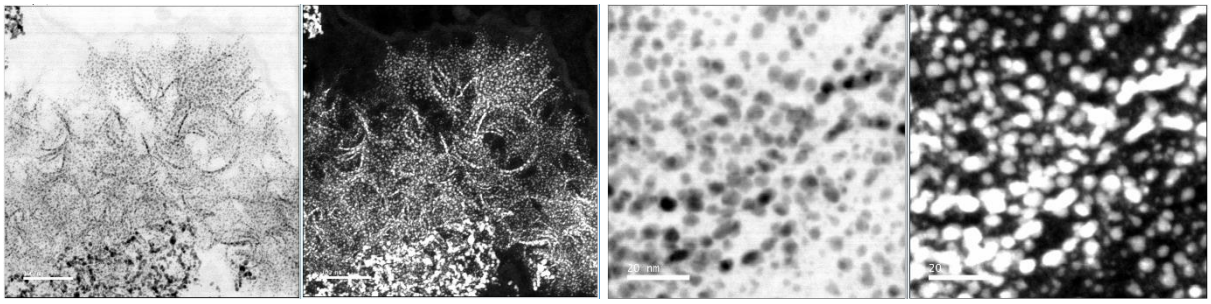

*Figure 1. STEM microscopy pictures in dark and bright field. Non degraded NPs can be seen on the bottom on the first image. Ciliary structures composed of 1 to 2 nm nanostructures auto-aligned can be observed after degradation. Scale: 100 and 20 nm respectively*

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*Corresponding author email: alice.balfourier@univ-paris-diderot.fr*

# Biological responses to encapsulating layers and cellular activities in a co-culture system of T cells encapsulated with PSS-coated gold nanorods

P. Wattanakull[1], M. C. Killingsworth, D. Pissuwan[2*]
(1) *Materials Science and Engineering Program, Multidisciplinary Unit, Faculty of Science, Mahidol University, Bangkok, Thailand*
(2) *Electron Microscopy Laboratory, Sydney South West Pathology, Sydney, Australia*
(3) *Ingham, Institute, Sydney, Australia*

Encapsulation of single cells is receiving increasing attention because there is a high possibility to use this technique in various biomedical and biological applications[1]. In the meantime, T cell-based therapy has been found to provide a high potential for cancer treatment and immunotherapeutic treatments[2,3,4]. However, it was reported that T cells could interact with cells in the immune system of recipient during cell transplantation resulting in an occurrence of some negative effects[5]. Due to this reason, T cells are one type of cells that are attractive to be combined with cell encapsulation technique for therapeutic purpose to avoid the problem of negative effect induction. Here, we demonstrate the new approach by using polystyrene sulfonate coated-gold nanorods (PSS-GNRs) to be an outer layer on the cell surface. We used Jurkat T cells as a model cell in our study. Jurkat T cells were encapsulated with poly(allyamine hydrochloride) (PAH) and/or PSS-GNRs or polystyrene sulfonate (PSS). The investigation of biological activities of T cells encapsulated with polyelectrolytes and gold nanorods was performed. The results showed that T cells encapsulated with PSS-GNRs, PAH and PSS, or PAH alone could survive and proliferate. In the case of a co-culture system, when encapsulated Jurkat T cells were co-cultured with THP-1 macrophages, the co-cultures exhibited TNF-α production enhancement. However, the TNF-α production enhancement was not found when THP-1 macrophages were co-cultured with PSS-GNR/PAH@Jurkat or PSS/PAH@Jurkat. This indicates that the encapsulating layer could help avoid the interaction between THP-1 macrophages and Jurkat T cells that related to TNF-α induction. As well, no significant inductions of IL-2, IL-1β, and IL-6 were detected in a co-culture system[6]. The layer of PSS-GNRs at the surface of cells should also provide a benefit in increasing the efficiency for diagnostic or therapeutic purposes due to the unique property of GNRs. With the positive outcome of biological activity assessment, the data here provide promising results of the possibility of using encapsulated PSS-GNR/PAH@Jurkat for immunotherapy application and other biomedical applications in the future.

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*Corresponding author email: dakrong.pis@mahidol.ac.th*

# Anti-inflammatory effect of gold nanoparticles supported on metal oxides

T. Fujita[1], T. Ishida[1], T. Murayama[1], M. Haruta[1], S. Lanone[2], J. Boczkowski[2]
(1) *Research Center for Gold Chemistry, Graduate School of Urban Environmental Sciences, Tokyo Metropolitan University, 1-1, Minami-osawa, Hachioji, Tokyo 192-0397, Japan*
(2) *INSERM U955, Équipe 4, Faculté de Médecine, Université Paris Est-Créteil, 94010 Créteil, France*

Gold nanoparticles supported on metal oxides ($Au/MO_x$) have attracted much attention due to their high catalytic performance for such as room temperature CO oxidation and selective oxidations in liquid phase. Recently, it has been revealed that $Au/MO_x$ works as a catalyst even in living organisms (at pH 7 in water). For example, Garcia and co-workers reported that $Au/CeO_2$ exhibits antioxidant activity against reactive oxygen species (ROS) in Hep3B and HeLa cell lines[1].

On the other hand, it has been reported that exposure of respiratory cells and tissues to $MO_x$ nanoparticles induces inflammation due to their toxicity. Therefore, to evaluate the effect of exposure of living cells to Au nanoparticles on $MO_x$ is also an important issue for the use and development of $Au/MO_x$ materials. In this work, we examined the cytotoxic and inflammatory response of macrophagic cells exposed to $Au/MO_x$.

$Au/TiO_2$, $Au/ZrO_2$, and $Au/CeO_2$ were prepared by deposition-precipitation (DP) followed by calcination at 573 K for 4 h or purchased from Haruta Gold Inc. The loading amount of gold of the $Au/MO_x$ were 1 wt% in preparation. Decomposition of hydrogen peroxide ($H_2O_2$) by $Au/MO_x$ was tested as a model catalytic reaction to estimate the antioxidant effect. Mice peritoneal primary macrophages were exposed for 6–48 h to 1–100 μg/mL particles of $Au/TiO_2$, $Au/ZrO_2$, $Au/CeO_2$, and $MO_x$ alone. The viability of cells was measured by MTT assay, and the quantification of the release of lactate dehydrogenase (LDH). Inflammatory response was evaluated by the quantification of cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β in cell supernatant, measured by enzyme-linked immunosorbent assay (ELISA). To assess the effect of $Au/MO_x$ on pro-inflammatory response, cells were exposed to lipopolysaccharide (LPS), which is a major component of Gram-negative bacteria wall, for 2 h and then to $MO_x$ or $Au/MO_x$ for 4 h. The concentrations of TNF-α and interleukin (IL)-1β were analyzed.

No cytotoxicity to the macrophagic cells was observed by either of MTT or LDH release assays for all the $Au/MO_x$ materials regardless of the exposure time and the Au concentration. With regard to the inflammatory response, a significant increase in TNF-α and IL-1β secretion was observed by exposure to $TiO_2$ but was much less pronounced for $Au/TiO_2$. To examine this effect of $Au/TiO_2$ in detail, LPS-induced pro-inflammatory response was measured (Figure 1). When $TiO_2$ was added, the amount of the pre-existing TNF-α and IL-1β were almost consistent with that of the LPS-induced control experiment. In contrast, the amount of TNF-α and IL-1β was significantly decreased by adding $Au/TiO_2$. This result suggests that $Au/TiO_2$ attenuates LPS-induced inflammation. The same experiments were also performed on $Au/ZrO_2$ and $Au/CeO_2$. Anti-inflammatory effect of $Au/MO_x$ was different depending on the cytokines. Namely, the orders of anti-inflammatory effect on TNF-α and IL-1β were $Au/TiO_2$ >> $Au/CeO_2$ ≒ $Au/ZrO_2$ and $Au/ZrO_2$ > $Au/TiO_2$ > $Au/CeO_2$, respectively. We hypothesized that the anti-inflammatory effect was related to their antioxidant effects. However, the catalytic activity order of $Au/MO_x$ for the decomposition of $H_2O_2$ was not consistent with the anti-inflammatory effect. Moreover, neither TNF-α nor IL-1β was adsorbed on the $Au/MO_x$ surface, excluding the decrease in the cytokines by any adsorption. Although the Au-mediated anti-inflammatory mechanism remains unclear at this stage, this study revealed that $Au/TiO_2$ and $Au/ZrO_2$ are promising candidates for anti-inflammatory agents.

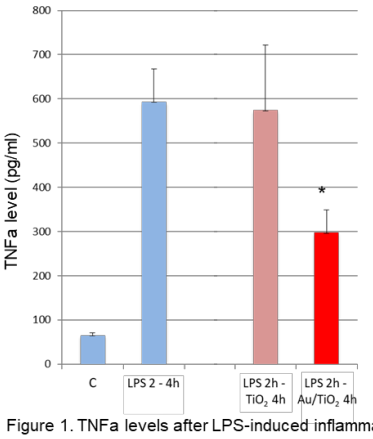


Figure 1. TNFa levels after LPS-induced inflammation and exposition to $Au/TiO_2$.

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*Corresponding author email: t-fujita@tmu.ac.jp*

# Gold nanoparticles and biology.

R. Lévy[1]
(1) *Institute of Integrative Biology, University of Liverpool, Liverpool, UK.*

Whilst nanotechnology and nanomedicine are generally seen as hot – even sometimes "revolutionary" – topics, gold nanoparticles have in fact been used in biology for therapeutic, diagnostic and biological research for over a hundred years. In this lecture, I will build from this historical context and from recent controversies on the structure (Stripy nanoparticles) and intracellular delivery of nanoparticles (SmartFlare/Spherical Nucleic Acids) to discuss some of the current challenges and opportunities in this field, illustrated by our work on the structure of peptide-capped gold nanoparticles and on the application of gold nanorods for *in vivo* cell tracking.

*Corresponding author email: Rapha@liverpool.ac.uk*

# Innovative polyvalent nano-platforms for nanomedicine: from diagnosis to therapy

F. Sciortino[1], C. Goubault[1], Jakobczyk[2], A. Burel[3], P.-A. Eliat[3], H. Y. Danger[3], F. Vérité[3], P. Rétif[4-5], S. Pinel[4], M.-B. Troadec[2],S. Chevance[1], F. Gauffre[1]

(1) *Univ Rennes, CNRS, ISCR, FR-35000 Rennes, France*
(2) *Univ Rennes, CNRS, IGDR, FR-35000 Rennes, France*
(3) *Univ Rennes, CNRS, INSERM, Biosit, FR-35000 Rennes, France*
*(4) Université de Lorraine, CRAN UMR 7039, CNRS, Vandoeuvre-lès-Nancy,France*
*(5) Unité de Physique Médicale, CHR Metz-Thionville, Ars-Laquenexy, France.*

We have recently achieved and patented[1] an innovative process for the elaboration of a new type of hybrid nanocapsules (Hybridosomes®), based on nanoparticles and polymers[2] (Figure 1). Process of aggregation, structure and porosity/elasticity of these hybrid nanocarriers were investigated by combining (cryo-)Transmission Electronic Microscopy, Static and Dynamic Light scattering, Nanoparticule Tracking Analysis and Atomic Force Microscopy[3].
The Hybridosomes® technology open up many perspectives for nanomedicine applications[2,4,5] like imaging assisted diagnosis, drug delivery or particle-based therapeutics (Figure 1). Indeed, the intrinsic properties of the inorganic nanoparticles constituting the hybridosomes®' shell can be combined and exploited i) as performant contrast agent for various type of imaging modalities (X-ray, MRI, fluorescence, …)[2] depending on the type of nanoparticles used (Au, SPIONs, QD, Ag, etc) and ii) as intrinsic therapeutic agent (radiotherapy, hyperthermia, … )[5] or tool for guided-surgery. In addition, drugs can be easily and efficiently encapsulated. Finally Hybridosomes® can be functionalized to target a specific cell or infectious organism.

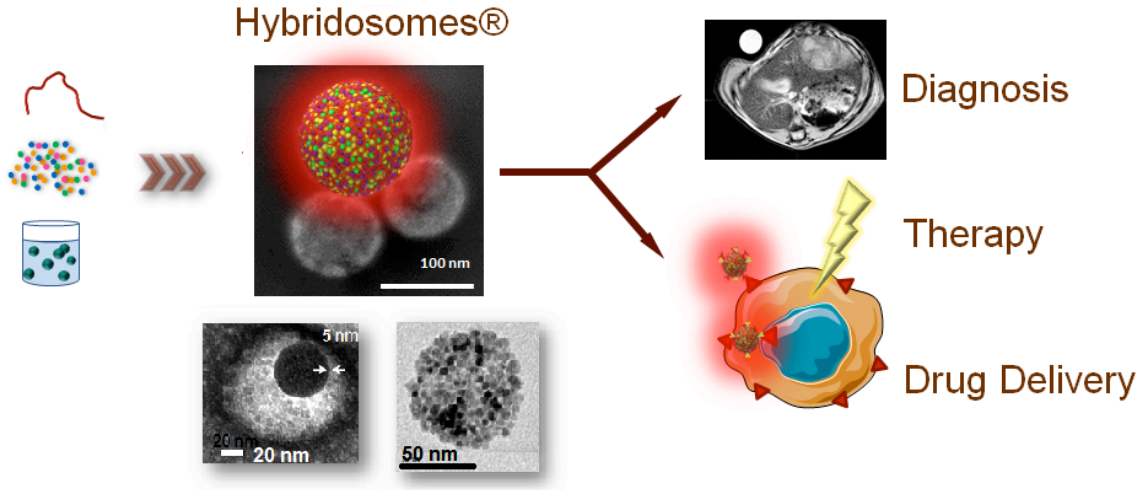


*Figure 1. Hybridosomes®: from the original assembly process to the applications in nanomedicine.*

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*Corresponding author e-mail : soizic.chevance@univ-rennes1.fr*

# A new drug vector based on ultrastable gold nanoparticles

F. Masse, M. Ouellette, E. Boisselier
*Faculty of Medicine, Laval University, CHU de Québec Research Center, Québec, Canada*

Low vision and blindness have a very important social and financial impact. Efficiency of current active molecules for ocular treatments is limited when they are administered by ophthalmic drops. Indeed, despite the high drug content of eye drops, a large proportion is eliminated by the tear film during the topical application. When optimal conditions are met, less than 0.02% of the active substance is absorbed. Therefore, it appeared necessary to design a new drug delivery system suitable for topical administration.

We have developed a new drug delivery system based on gold nanoparticles that increases the time of action of drugs administered by eye drops and thus reduces their frequency of administration (see Figure 1).[1] Ultrastable gold nanoparticles were synthesized by a new method. The mucoadhesion properties were characterized by different qualitative and quantitative techniques. Finally, the encapsulation efficiency of the gold nanoparticles for different ocular drugs was determined.

These new ultrastable gold nanoparticles can have a major impact in nanomedicine. Indeed, the optimization of the mucoadhesion of the drugs by a new drug delivery system may significantly increase their effectiveness, reducing their frequency of administration as well as the toxicity related to their high content of active substance.

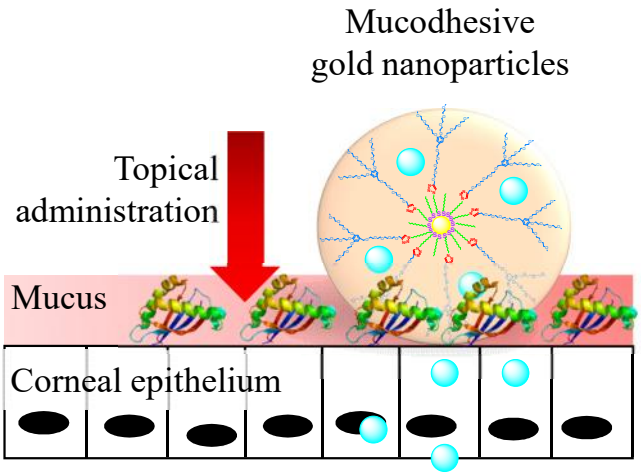


*Figure 1. Mucoadhesive gold nanoparticles*

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*Corresponding author email: Elodie.Boisselier@fmed.ulaval.ca*

# Multifunctional Gold Nanoparticles for Simultaneous PET/MR Imaging

F. Bouraleh Hoch[1], V. Thakare[2,3], V.-L. Tran[4], C. Bernhard[3], A. Deshotel[3], R. Bazzi[1], A. Oudot[5], B. Collin[3,5], F. Lux[4], O. Tillement[4], F. Brunotte[5], F. Boschetti[2], F. Denat[3], S. Roux[1]*

(1) *Institut UTINAM, UMR 6213 CNRS – Université de Bourgogne Franche-Comté, Besançon, France*

(2) *CheMatech S.A.S., Dijon, France*

(3) *Institut de Chimie Moléculaire de l'Université de Bourgogne, UMR 6302 CNRS – Université de Bourgogne Franche-Comté, Dijon, France*

(4) *Institut Lumière Matière, UMR 5306 CNRS – Université Claude Bernard Lyon 1, Villeurbanne, France*

(5) *Centre Georges-François Leclerc, Plateforme d'imagerie préclinique, Dijon, France*

Medical imaging has become a cornerstone of the fight against various diseases (cancer, cardiovascular diseases) since it allows to detect and follow up the development of disease and to guide therapy. The current trend is to combine several complementary imaging techniques to exploit the advantages of each while overcoming their limitations.[1] Among the numerous possibilities, the combination of magnetic resonance imaging (MRI) and positron emission tomography (PET) appears very attractive because it allies the high resolution of MRI to the exceptional sensitivity of PET imaging. If the development of this device is in itself a significant challenge, the design of multimodal probes also constitutes an essential step for exploiting MRI/PET fused technology.

For monitoring the biodistribution of radiosensitizing gold nanoparticles by simultaneous PET/MR imaging, the presence of two different types of chelator is required in the organic shell. Two strategies have therefore been explored for immobilizing both gadolinium ion ($T_1$-weighted MRI) and positron emitter (TEP) onto the gold core. The first one consists in the formation of a mixed shell composed of two different chelating molecules (a DOTA derivative for $Gd^{3+}$ ions and a NODA derivative for $^{64}Cu^{2+}$ ions) while the second strategy rests on the formation of the organic shell with a single molecule but functionalized by two different chelating macrocycles for a selective complexation of $Gd^{3+}$ and $^{64}Cu^{2+}$ ions.

The reduction of gold salt in presence of a mixture of two different dithiolated chelators (strategy 1) or in presence of dithiolated molecules containing two specific complexation sites (strategy 2) provides ultrasmall gold nanoparticles (core size (TEM): 2-3 nm and hydrodynamic diameter (DLS): 6-8 nm) which are able to immobilize both gadolinium ions and 64-copper(II) ions. As a result, the biodistribution of these nanoparticles can be monitored by $T_1$-weighted MRI and by PET on a same animal with the same imaging device integrating PET and MRI modalities after a single intravenous injection.

Each class of nanoparticles successfully behaves as imaging agent for integrated MRI/PET which are removed by body in large part by renal clearance. Since the ultrasmall gold nanoparticles are designed for remotely controlled therapy (radiosensitizing effect of the ultrasmall gold cores),[2,3] the data collected by combining MRI and PET will be very precious for improving the therapeutic activity of these nanoparticles.

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*Corresponding author email: stephane.roux@univ-fcomte.fr*

# Keys to enrich layer-by-layer films with gold nanoparticles for medical device coating

A. Pallotta[1], I. Clarot[1], P. Lavalle[2], A. Boudier[1]*

*(1) Université de Lorraine, EA 3452 CITHEFOR, Nancy, France*
*(2) INSERM UMR 1121, and Université de Strasbourg, France*

The design of layer-by-layer (LbL) polyelectrolyte films including nanoparticles is a growing field of innovation in a wide range of biomedical applications such as the development of coatings dedicated to medical devices. Gold nanoparticles (AuNP) are very attractive for those applications, as they can be grafted by drugs and sensitive molecules using simple synthesis protocols conferring a pharmacological activity to the developed medical devices.
In this study, AuNP were synthesized and characterized using classical physicochemical methods[1]. They were entrapped into a tripartite film based on cycles, each made with a polycation, negatively-charged AuNP and a polyanion. Nanostructured films can be deposited on various supports such as glass slides. The nanostructuration of LbL films using such metallic species was dependent on the choice of polymer and of dissolution buffer. Physicochemical parameters were evaluated to study AuNP incorporation and film stability in terms of absence of nanoparticle/film leakage to match the requirements of the future medical application. Methods such as visible spectrophotometry, capillary zone electrophoresis, quartz crystal microbalance, and high performance liquid chromatography coupled to visible detection were used. The best compromise between AuNP loading and film stability were obtained using poly(allylamine) as the polycation and Tris buffer leading to $10^{12}$ AuNP/cm² per cycle of deposition. Lastly, AuNP reactivity was modified when they were embedded into LbL films in comparison to the colloidal suspension. This reactivity was assessed in according to their interaction with biomolecules or rat whole blood[2]. The immobilized AuNP showed less interaction with biomolecules and induced less hemolysis compared to the colloidal suspension. Interestingly, to complete the obtained data on their cyto/hemocompatibility, the film stability was assessed under shear stress conditions to mimic forces applied on a medical device implanted inside an artery. We demonstrate that neither AuNP leakage from the coating nor the AuNP dissolution (resulting in gold salt) occurred using ion-pairing extraction and HPLC-visible quantification[3].
Due to the high capacity of drug grafting on gold nanoparticles, these results are promising for the development of nanostructured biomedical devices.

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*Corresponding author email: ariane.boudier@univ-lorraine.fr*

# Using bio-functionalized gold nanorods to observe the plasmonic photothermal effect on individual BaF3 cells

P. Witthöft, L. Prisner, C. Strelow, T. Kipp, A. Mews
*Institute for Physical Chemistry, University of Hamburg, Hamburg, Germany*

The exploitation of the plasmonic photothermal effect of gold structures, such as gold nanorods, for photothermal therapy in cancerous tissue is of great interest for the scientific community[1]. While a wide array of medical applications for targeted and non-targeted gold nanorods has been developed in the past, further understanding of the plasmonic photothermal effect and the parameters involved at the cellular level is of utmost importance.
We investigate the photothermal effect of gold nanorods in detail on the single-cell level. We have developed a method to selectively irradiate individual cells that were incubated with bio-functionalized gold nanorods and to monitor the induced plasmonic photothermal effect on these cells over time by measuring trypan-blue induced color changes of the cell. Figure 1 (a) exemplarily shows micrographs of a BaF3 cell before and at specific moments in time after light irradiation. In panel (b), the observed color changes, which indicate the dying of the cell, are quantified in terms of an absorbance value. We compare the efficiency of the plasmonic photothermal effect on BaF3 cells that were incubated either with non-targeted PEG-coated nanorods or with PEG-coated nanorods targeted with the aptamer AIR-3A that specifically binds to the Interleukin-6 receptor of the cells. We find that targeted gold nanorods lead to color changes twice as fast as non-targeted ones. Based on our observations we discuss the mechanism and specificity of the plasmonic photothermal effect on the single-cell level.

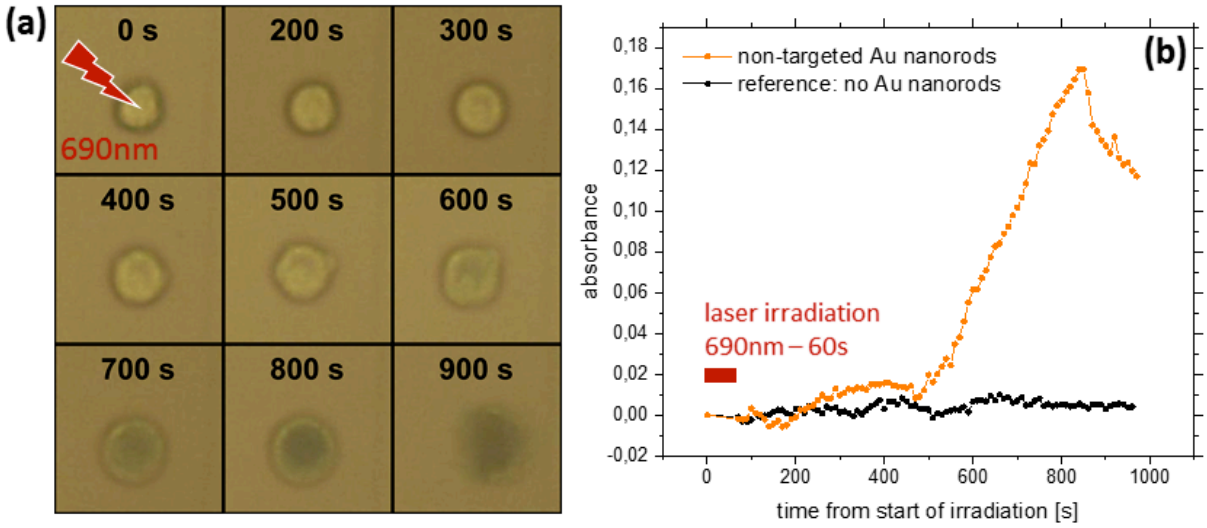


*Figure 1: (a) Micrographs of one individual cell before (0s) and at specific moments after laser irradiation. (b) Absorbance transient of the same cell.*

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*Corresponding author email: phillip.witthoeft@chemie.uni-hamburg.de*

# Golden Nanoflowers for Combining Hyperthermia and Radiotherapy

N. Mohamed Saïd[1], J. Volatron[2], G. Jimenez Sanchez[1], I. Marangon[2], S. Dufort[3], F. Denat[4], F. Boschetti[5], G. Le Duc[6], F. Gazeau[2], R. Bazzi[1], S. Roux[1]*

(1) *Institut UTINAM, UMR 6213 CNRS – Université de Bourgogne Franche-Comté, Besançon, France*

(2) *Laboratoire Matière Systèmes Complexes, UMR 7057 CNRS – Université Paris Diderot, Paris, France*

(3) *Nano-H SAS, Saint-Quentin Fallavier, France*

(4) *Institut de Chimie Moléculaire de l'Université de Bourgogne, UMR 6302 CNRS – Université de Bourgogne Franche-Comté, Dijon, France*

(5) *CheMatech S.A.S., Dijon, France*

(6) *European Synchrotron Radiation Facility, Grenoble, France*

Owing to their large range of properties which can be accurately tuned by the chemical composition, the shape and the dimensions, multifunctional nanoparticles appear as promising candidates for image-guided therapy.[1]

In this context, we developed the synthesis of gadolinium chelate coated gold nanoparticles (Au@L-Gd with L a linear or macrocyclic polyaminocarboxylate chelator) which are designed for image-guided radiotherapy.[2] Despite promising results, the radiosensitizing effect appears to be underexploited owing to a too rapid renal clearance which limits their accumulation in solid tumor.[3] Hence the exploitation of the potential of these gold nanoparticles for image-guided radiotherapy requires the increase of the circulation time for reaching efficiently their specific target while preserving the ability for renal clearance.

For achieving an enhanced circulation time and therefore a greater accumulation in solid tumor, Au@L-Gd nanoparticles were immobilized onto large bioresorbable carriers (maghemite nanoflowers). The resulting golden nanoflowers are constituted of monocrystalline grains (11 nm) which are assembled in a flower-shaped structure and gold nanoparticles (Au@L-Gd).[4,5] Such an association allows combining the imaging modalities and therapeutic activities of each part of the golden nanoflowers ($T_1$-weighted MRI, radiosensitization from Au@L-Gd nanoparticles and $T_2$-weighted MRI, magnetic hyperthermia from nanoflowers). The circulation of golden nanoflowers after intravenous injection is longer-lasting: the golden nanoflowers are still observed in the tumor 1 h after the injection in contrast to Au@L-Gd nanoparticles. This was mainly attributed to the larger size of the golden nanoflowers (30 nm vs 2.5 nm). As a consequence, the radiosensitizing effect of the golden nanoflowers provides for a same gold content in the injected suspension a higher increase in life span than in the case of Au@L-Gd. Moreover, the combination of magnetic hyperthermia and radiosensitization which is rendered possible by the immobilization of gold nanoparticles onto maghemite nanoflowers permits to control the tumor growth.

The immobilization of the gold nanoparticles Au@L-Gd onto nanoflowers allows therefore to better exploit the radiosensitizing effect of the gold nanoparticles.

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*Corresponding author email: stephane.roux@univ-fcomte.fr*

# Multi-core vs single-core Gold Nanoparticles: intracellular confinement effect on NIR Photothermia

A. Plan[1,2*], R. Aufaure[1], L. Motte[1], E. Guenin[3], C. Wilhelm[2], Y. Lalatonne[1]
(1) *LVTS, Inserm U1148,University Paris 13, Bobigny, France*
(2) *MSC, CNRS 7057, University Paris 7, Paris, France*
*(3) TIMR, EA4297,Université de Technologie de Compiègne, Compiègne, France*

Gold nanoparticles (GNPs) provide multi-functionalities for biomedical applications due to their suitable properties for drug delivery, cancer treatment, and imaging and in vitro diagnosis. One major input for nanomedicine is their optical properties, being able to absorb light within the Near Infrared (NIR) window where light has its maximum depth of penetration in tissue[1]. Their properties can be tuned via chemical composition, size, shape, ligands and colloidal stability.

Within the present study we decided to control GNPs confinement to tune their optical absorption towards NIR wavelengths in cellular environment. For this single[2] and multi-core (not published) pegylated GNPs have been synthesized using phosphonate ligands. Besides we obtained 10 nm single-core GNPs and tunable multi-core GNPs from 30 to 70 nm (Figure 1). The multi-core GNPs confinement is leading to a red shift absorption making them at potential photothemia agent. Then in situ cellular measurement has been performed by incubating single- and multi-core GNPs with PC3 prostatic cancer cells.

We then compared their efficiency of photothermia in solution and cells. Multi-core GNPs photothermia competed well with most efficient photothermia agent, such as nanostars[3], for thermal efficiency in water. Unexpectedly, in cellular conditions, single-core GNPs NIR photothermia ensured a temperature rise, due to GNPs confinement in lysosomes. Single- and multi-core structure and concentration effect have been studied leading to high heating amplification and subsequent cell apoptosis and necrosis.

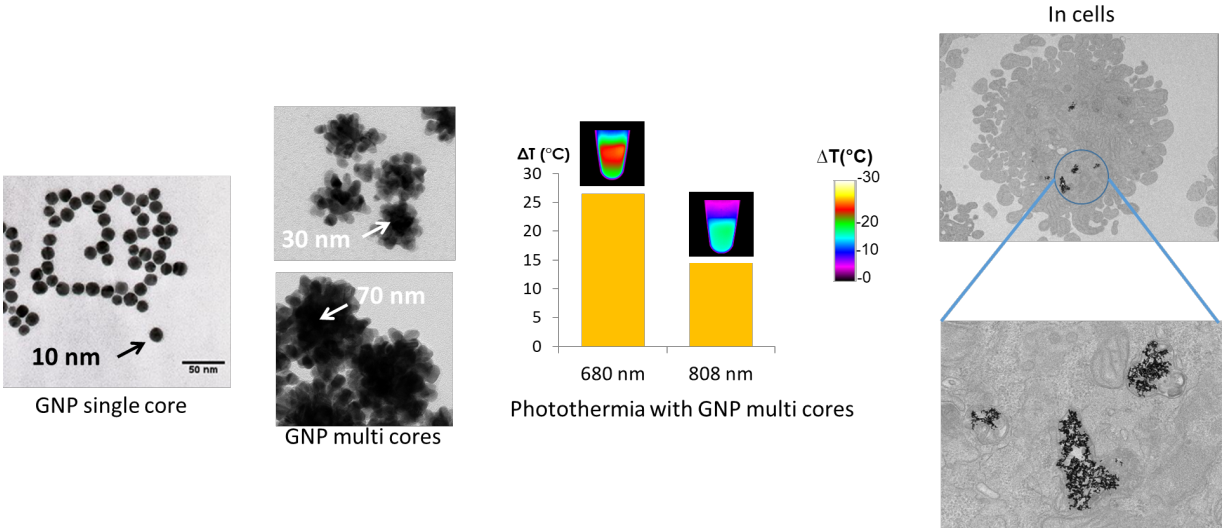


*Figure 1. From single- to multi-core Gold nanoparticles and their control intracellular optical properties for NIR photothermia cancer treatment*

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*Corresponding author email: anouchka.plan@gmail.com*

# Photothermal Therapy: optimal nanogold morphology for efficient heat generation

Andreea Campu[1], Laurentiu Susu[1], Frederic Lerouge[2], Stephane Parola[2], Monica Focsan[1], Simion Astilean[1]

(1) *Nanobiophotonics and Laser Microspectroscopy Center, Interdisciplinary Research Institute on Bio-Nano-Sciences and Biomolecular Physics Department, Faculty of Physics, Babes-Bolyai University, Cluj-Napoca 400084, Romania*
(2) *Ecole Normale Superiéure de Lyon, CNRS, Université Lyon 1, Laboratoire de Chimie UMR 5182, 46, allée d'Italie, F-69364, Lyon Cedex 07, France*

Nowadays, cancer represents worldwide one of the main leading causes of death also becoming one of the most investigated diseases. The scientific interest is not only to detect its presence at an early stage and get an insight on its progress, but also to treat it without harming healthy tissue. A minimally-invasive therapy that has attracted a lot of attention and holds a great promise of success is the Photothermal Therapy (PTT), where the ill tissue is destroyed by locally generated heat. [1] The unique optical properties such as well-controlled size, shape and surface chemistry designate the anisotropic gold nanoparticles as potential candidates of choice for PTT heat sources. Moreover, the enhanced absorption induced by Localized Surface Plasmon Resonance (LSPR) is even higher when the resonance peak is located in the near-infrared (NIR) region where the gold nanoparticles possess high extinction coefficients – translating in high depth PPT due to high penetration of infrared light. [2]

In this work, we present a meticulous study of the photothermal properties of two similarly-shaped gold nanostructures, nanobipyramids (AuBP) and nanorods (AuNR), in almost identical experimental conditions. AuNR have been already investigated how their morphology affects the photothermal effects [3], but to our knowledge such a characterization is missing in the case of AuBP as well as a comparison between them. Firstly, we synthesized both AuBP and AuNR with longitudinal LSPR responses spanning from the visible to the NIR region of the spectrum. The extinction spectra show intense narrow bands which reflect a high yield and monodispersity, Transmission Electron Microscopy (TEM) images confirm the homogeneity of the nanoparticle shapes, while along with Dynamic Light Scattering (DLS) measurements the AuBP and AuNR dimensions are determined. The as-prepared colloidal nanostructures are covered in a double-layer of surfactant which is removed by performing two washing steps according to nanoparticle characteristics without affecting their stability in solution. The samples were irradiated using two different laser excitation wavelengths, 785 and 808 nm, respectively, in continuous mode for 30 minutes. In order to determine the photothermal properties of each sample, the experimental parameters such as sample volume, optical density, measure area and laser power have been varied one at a time. The aim of the study is to determine which is the optimal morphology for efficient PTT and what are the irradiation conditions to achieve maximum energy conversion in the shortest time possible. The most efficient system will be further used for *in vitro* investigations in view of implementing PTT alone or in combination with other plasmon-based therapies.

**Acknowledgments** Funding by the CNCS-UEFISCDI Romania, under the project number PN-III-P4-ID-PCE-2016-0837 is gratefully acknowledged.

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*Corresponding author email: andreea.campu@gmail.com*

# Single-step and sensitive detection system-based on dual-color light scattering of metal nanoparticles

J. Wenger[1], S. Vial[1]
(1) *CNRS, Aix-Marseille Université, Centrale Marseille, Institut Fresnel, UMR 7249, 13013 Marseille, France*

DNA and protein-based detection methods have many applications in clinical diagnosis, food science, and environmental control. However, most of the analytical techniques currently in use, such as ELISA and PCR, are expensive, cumbersome and time consuming. Today, there is a clear need for a disruptive detection technique meeting the following key criteria: small sampling volumes, single step analysis and accuracy. To tackle these challenges, we have recently introduced a novel analytical technique based on Photon Cross Correlation Spectroscopy (PhoCCS) of metallic nanoparticles (NPs).[1,2] It opens new and breakthrough features for diagnosis, by providing increased sensitivity, rapid detection and reduced costs. PhoCCs is a dual-color technique, enabling to distinguish the scattering properties of two spectrally distinct nanoparticles (e.g. silver NPs and gold nanorods (AuNRs)) in solution phase (≥ 10 µl drop) (Fig 1). Upon illumination using two laser beams, the NPs passing through the volume of analysis are excited and their resulting scattered lights are collected and analyzed together. In the absence of target, both NPs move independently and their signals are uncorrelated. Upon target addition, the probe-anchored NPs (probes A and B) assemble via molecular recognition with the target, giving rise to temporal coincidences.
Here, we will demonstrate its effectiveness for the detection of a specific DNA fragment of sesame, an allergenic food ingredient, as well as for the detection of human free prostate-specific antigen (f-PSA), a prostate cancer biomarker. The technique implies a single mixing step, no washing, and allows sensitive detection within less than one hour for the total assay duration.

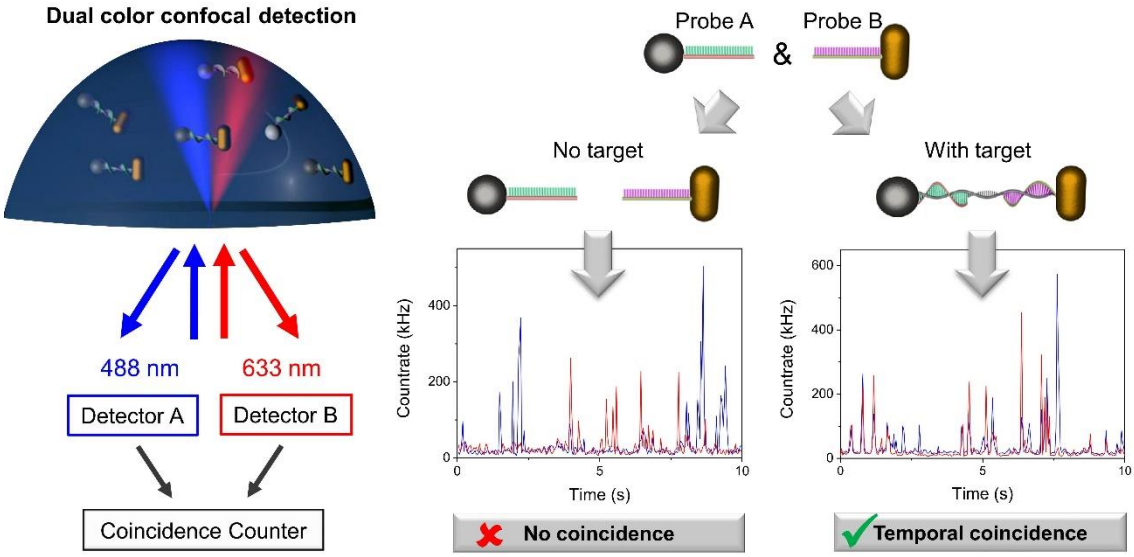


*Figure 1.* Schema representing the dual-color light scattering detection system. In the presence of the target DNA, nanoparticle probes A and B associate, yielding temporal coincidence between the detection channels

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*Corresponding author email: stephanie.vial@satt-paris-saclay.fr*

# Naked Eye Readout Detection of Staphylococcal Enterotoxin A (SEA) in Milk by Gold Nanoparticle-Based Colorimetric Biosensor

L. Zhang[1,2,3], P. Chen[2], B. Liedberg[2], M. Salmain[3], S. Boujday[1]
(1) *Laboratoire de réactivité de surface, Sorbonne Université, Paris, France*
(2) *Centre for biomimetic sensor science, Nanyang Technological University, Singapore, Singapore*
(3) *Institut parisien de chimie moléculaire, Sorbonne Université, Paris, France*

Colorimetric detection methods are convenient and effective in many applications as their major advantage is that readout requires only human eye.[1] In many routine clinical diagnostics, the concentration of analyte is often not high enough to generate a visible signal readable by naked eye.

Colloidal gold nanoparticles (AuNPs) display an intense absorption band in the visible range with extremely large extinction coefficient, which is due to the localized surface plasmon resonance (LSPR) phenomenon. This enables its high potential use for colorimetric reporting of molecular recognition events through readout by naked eye or for a quantitative detection by standard absorbance measurements.[2]

Some strains of *Staphylococcus aureus* produce staphylococcal enterotoxins (SEs) and it is generally admitted that ingestion of 100 ng of toxin may be sufficient to cause intoxication symptoms. Twenty-one different SEs have been identified to date with staphylococcal enterotoxin A (SEA) being the most frequent toxin involved in food poisoning outbreaks by *S. aureus*.[3]

Here we established a sandwich-format colorimetric biosensor on glass slides based on AuNPs for SEA detection. The sensing layer was built up by treatment of glass with either epoxide- or amine-terminated alkoxysilane, with or without adding protein A to immobilize the antibody, which was applied to capture target SEA. AuNP-labeled Ab served as a revelation reagent by forming a sandwich immune complex. The optimized sensor was applied for SEA detection in model buffer medium and in milk.

In the absence of SEA, no immunogold was bound to the capture Ab. In the presence of SEA in samples, a red color was produced within the detection zone on glass slide and could be easily distinguished by the naked eye (see figure 1), down to SEA quantity of 1 ng. The signal was further quantified using a benchtop UV-Vis spectrometer. The limit of detection (LOD) of SEA spiked in real milk samples reached 6 ng/mL from the dose-response curve.

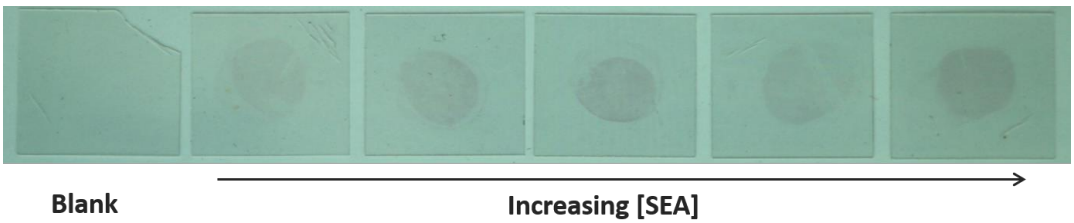


*Figure 1. Readout of colorimetric assay of detection of SEA at different concentration by naked eye*

A specific, sensitive colorimetric biosensor for detection of SEA was well established, which requires simple optical equipment or even visual detection. This simple method gives an even better LOD compared with the well-established methods, for instance quartz crystal microbalance[4].

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*Corresponding author email: lu.zhang@upmc.fr*

# Nanobiosensor coupling SERS and QCM: optimization of gold nanocylinder arrays on gold

Médéric Lequeux[1,2], Nestor Gisbert Quilis[4], Jakub Dostalek[4], Nathalie Lidgi Guigui[1], Michèle Salmain[5], Wolfgang Knoll[4], Souhir Boujday[2], Marc Lamy de la Chapelle[1,3]

[1] *Laboratoire CSPBAT, CNRS UMR 7244, UFR-SMBH, Université Paris 13, 74 rue Marcel Cachin, 93017 Bobigny, France*

[2] *Sorbonne Université, CNRS, UMR 7197, Laboratoire de Réactivité de Surface, F-75005 Paris, France.*

[3] *Institut des Molécules et Matériaux du Mans (IMMM - UMR CNRS 6283), Le Mans Université, Avenue Olivier Messiaen, 72085 Le Mans cedex 9, France*

[4] *Biosensor Technologies, AIT-Austrian Institute of Technology GmbH, Muthgasse11, 1190 Vienna, Austria*

[5] *Sorbonne Université, CNRS, Institut Parisien de Chimie Moléculaire (IPCM), F-75005 Paris, France*

Streptomycin is an antibiotic used to treat some bacterial infections like tuberculosis and *Mycobacterium avium* complex. Nowadays, owing to its large use in agriculture, livestock and aviculture, there is a great need to quantify the concentration of streptomycin to prevent antibiotic resistance. Previous study[1] on this molecule showed that there is an aptamer which could recognize streptomycin with good affinity and selectivity.

We propose to detect this molecule by coupling SERS and QCM techniques with streptomycin aptamer.

QCM is a technique that measures the change in resonance frequency of quartz crystal resonator sandwiched between two gold electrodes. We propose to add nanostructures onto the gold surface to perform SERS as well.

We present here our work on the construction of arrays of gold nanocylinders on a gold surface so that the SERS and QCM signals are optimal. Gold nanostructures deposited on gold thin film exhibit specific optical properties with the observation of localised surface plasmon (LSP) as well as Bragg modes where the propagating and localized modes are resonantly coupled through the array periodicity.[2] It has already been demonstrated that Surface Enhanced Raman Scattering (SERS) is higher in this configuration compared to the one recorded for gold nanostructures on insulating media.

The nanostructures were made by e-beam lithography in the shape of nanocylinders with a diameter between 80 and 250 nm and a periodicity of 400nm on a gold subfilm with a thickness between 20 and 50 nm. Thanks to this study we defined the optimal nanostructures to couple QCM and SERS.

In a second step, this biosensor was validated for the detection and the quantification of the streptomycin. Thanks to its capture by its associated aptamer chemisorbed on the nanostructured QCM sensor, we tested this biosensor with different concentrations of streptomycin.

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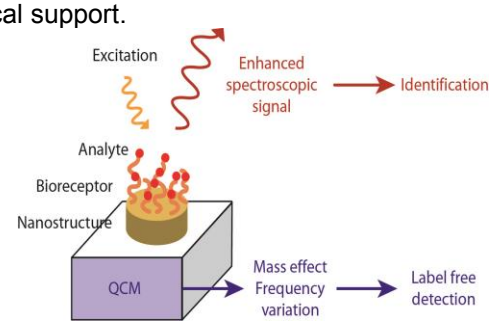


*Figure 1: Biosensor scheme*

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*Corresponding author email: medlequeux@gmail.com*

# Gold nanoparticles SPRi enhanced signal for small molecules detection with split aptamers

A. Buhot*, F. Melaine, C. Coihlac, Y. Roupioz
*Univ. Grenoble Alpes, CEA, CNRS, INAC-SyMMES, 38000 Grenoble, France*

**Aptamers** are single-stranded DNA or RNA molecules capable of binding to target molecules like proteins, metal ions or drugs. Due to their specific binding affinities and other advantages compared to antibodies (higher stability, lower cost, easy chemical modification…), they provide a great opportunity to produce sensing surfaces for effective and selective detection of small molecules.

**Surface Plasmon Resonance imaging (SPRi)** has become one of the most widely used label-free method for the study of bio-recognition events on surfaces. This technique provides a rapid approach, however, limited in sensitivity by low refractive index changes occurring when small molecules (<500 Da) are captured on the biosensor. Whereas significant reflectivity variation are observed upon the interaction of large molecules like proteins to the sensing interface, for small targets such as **adenosine**, the reflectivity variation is often too small to be detected by SPRi. Thereby, only few studies have been reported on developing SPRi-based biosensor for small molecules detection using aptamers.

We developed a bioassay based on three different but compatible and complementary strategies[1,2]:
1/ **the engineering of split aptamer sequences** adapted from the adenosine model aptamer,
2/ **the use of gold nanoparticles** for Surface Plasmon Resonance amplification signal and
3/ **the thermodynamic stability** of the complex formed to quantify the small molecule adenosine.
The experimental results have demonstrated that the combined strategies allow us to obtain state-of-the-art detection limit below 50nM. Furthermore, the determination of **the melting temperatures** of the complex formed by the split aptamers and the adenosine targets open the door for an access to the thermodynamical parameters.

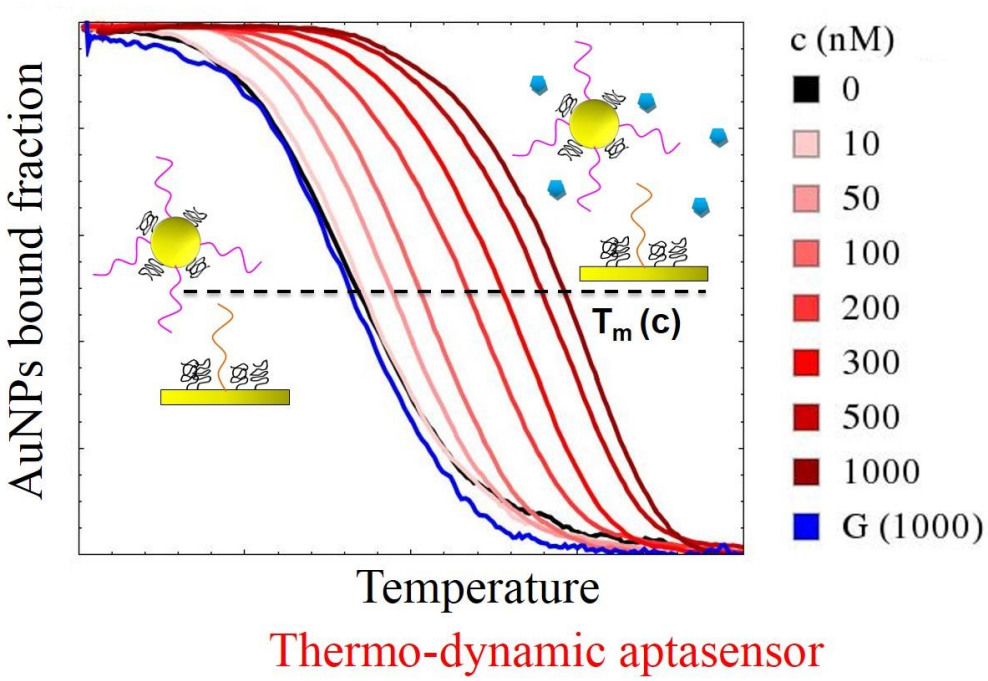


*Figure 1. Adenosine detection from melting temperature shift of split-aptamer functionalized AuNP*

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*Corresponding author email: arnaud.buhot@cea.fr*

# Optical Losses in Gold-Based Plasmonic Biosensors: Influence of Crystalline Structure

Y. Stebunov[1,2*], D. Yakubovsky[1], A. Arsenin[1,2], V. Volkov[1,3]
(1) *Moscow Institute of Physics and Technology, Dolgoprudny, Russia*
(2) *GrapheneTek, Skolkovo Innovation Center, Moscow, Russia*
(3) *SDU Nano Optics, Mads Clausen Institute, University of Southern Denmark, Odense, Denmark*

Thin gold films are a key component of most commercial and laboratory-built plasmonic biosensors, which have found their applications in various fields, ranging from biochemical and pharmaceutical research to medical diagnostics. The biosensing principle of such devices is based on the excitation of surface plasmon resonance (SPR) in gold films and monitoring of excitation conditions during biosensing assay. The performance of plasmonic biosensors depends on optical properties of metal films and, in particular, on optical losses in metal, which in turn are partially determined by electron scattering on crystallite boundaries[1]. Here, we investigate how crystalline structure of gold films influences sensitivity and resolution of plasmonic biosensing. The SPR excitation was considered according to the Kretschmann's configuration, which composes 1) the glass prism with refractive index (RI) 1.523; 2) 47-nm-thick gold films; and 3) the top aqueous layer with RI of 1.33[2]. Figure 1(a) shows the SPR angular reflectivity curves for structures comprising polycrystalline gold films with different crystallinity. The full-width at half-maximum (FWHM) $\alpha_{FWHM}$ of SPR angular curve is a characteristic of optical losses and, therefore, depends on the crystallite size *D* (Figure 1(b)). The resolution of SPR biosensing can be described by the figure of merit (FOM), defined as a ratio of biosensing sensitivity to FWHM. The dependence of FOM on the crystallite size is also shown in Figure 1(b), which demonstrates the increase of FOM up to 60% for different crystallites sizes. So, the performance of gold-based plasmonic biosensors strongly depends on the crystalline structure of metal films used for the excitation of SPR. Due to this, the development of efficient plasmonic biosensors should carefully address various aspects of gold film deposition, including substrate preparation as well as the choice of a fabrication method and corresponding deposition regimes.

This work was supported by the Russian Foundation for Basic Research, grants no. 18-07-01339-a and 18-07-01379-a.

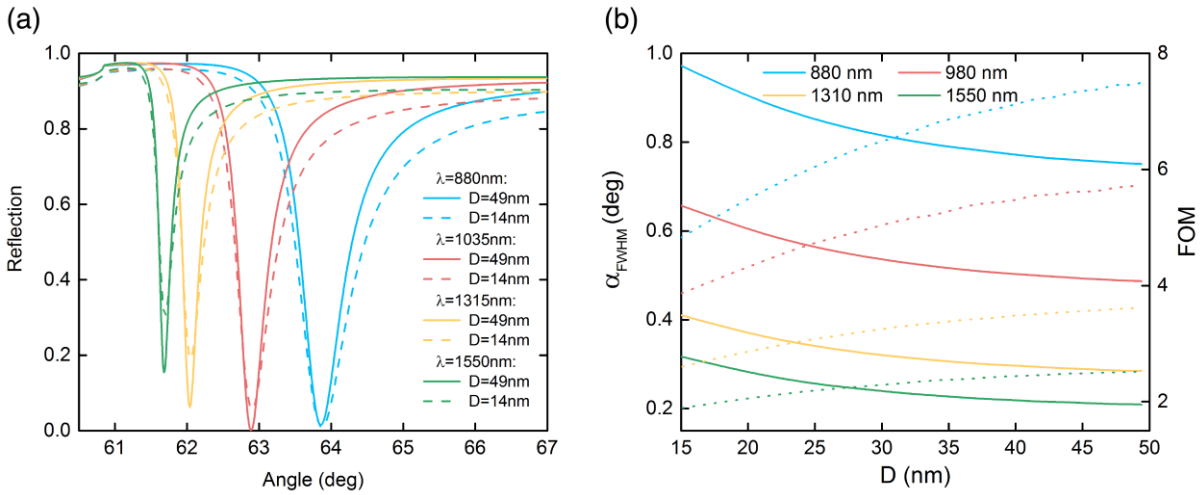


*Figure 1. (a) SPR angular reflectivity curves for polycrystalline gold films with crystal sizes of 14 and 49 nm. (b) Full-width at half-maximum and figure of merit for SPR biosensing based on thin gold films with different crystallinity.*

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*Corresponding author email: stebunov@phystech.edu*

# Gold Nanocrystals as 3D High-precision Motion Tracker

Yuji C. Sasaki [1, 2, 3*], M. Kuramochi [1, 2], H. Sekiguchi [3], K. Mio [2]
(1) *Graduate School of Frontier Sciences, The University of Tokyo, Chiba, Japan*
(2) *JapanAIST-UTokyo Advanced Operand-Measurement Technology OIL, Chiba, Japan*
(3)*SPring-8/JASRI, Hyogo, Japan*

*in vivo* observations have greatly progressed due to the remarkable development of fluorescence single-molecule detection techniques using visible lights. These single-molecular techniques have provided positional information at an accuracy of about wavelength/100, far below the optical diffraction limit (wavelength/2). In 1998, we achieved time-resolved x-ray (wavelength~0.1nm) observations of 3-dimentional (3D) picometer-scale (wavelength/100) Brownian motions in individual DNA molecules[1]. We proposed a method to observe intramolecular motions by labeling gold nanocrystals with individual single protein molecule and observing the motions of diffracted X-ray spots from labeled individual gold nanocrystals. This DXT (=Diffracted X-ray Tracking) can trace all rotational 3D motions within single protein molecule using white X-rays. The cysteine and methionine site in the protein molecules can have a covalent bond to the surface of gold nanocrystals. Therefore, we succeeded time-resolved (to nano-seconds from micro-seconds) x-ray observations of dynamical Brownian motions of individual single channel in aqueous solutions through the labeled gold nanocrystals for the first time in the world[2]. Until now, we are trying to observe Brownian motions of actin-myosin interactions, denatured proteins[3,4], functional protein membranes[2,5] (bacteriorhodopsin, AChBP, AChR, and KvAP), antigen- antibody interactions[6,7], peptide/MHC complex for T cell activation[8], and monitoring super-weak force (pN).

Additionally, we successfully observed the nano-scale dynamics of supersaturated protein (lysozyme) solutions with time-resolved X-ray observations[9]. We demonstrated that supersaturated protein solutions have femto newton-scale force fields. This observed force field by manipulated nanocrystal is originated from asymmetric Brownian motions, we call as nano-flow field.

As described above, normal DXT must use white x-rays. Therefore, when using monochromatic X-rays, it is impossible to track all motions of diffraction spots. However, we detected a clear blinking in diffracted X-ray intensity. Now, we call Diffracted X-ray Blinking (DXB). The observed X-ray blinking intensity from the labeled and moving gold nanocrystals correlated with the velocity of the diffraction spots by autocorrelation function (ACF). Recently, we developed this technique to observe the molecular dynamics using laboratory X-ray source; Rigaku FR-D (Cu anode, 50kV, 60mA) and a high sensitive detector; PILATUS-100K. We try to distinguish molecular dynamics of AChBP between with or without toxin using new our laboratory analytical instrumentation. We have recently succeeded in being able to measure *in vivo* single molecular observations in living cells with DXB even with a laboratory x-ray source for the first time in the world.

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*Corresponding author email: ycsasaki@edu.k.u-tokyo.ac.jp*

# Additive-free functional gold nanoparticle conjugates for biomedical applications

**C. Rehbock***, C. Streich, L. Gamrad, S. Barcikowski

*Technical Chemistry I and Center for Nanointegration Duisburg-Essen (CENIDE), University of Duisburg-Essen, Universitaetsstrasse 7, 45141 Essen, Germany*

Gold nanoparticle (AuNP) are interesting for biomedical applications due to their optical properties as well as high biocompatibility. Furthermore, they can be easily functionalized with biomolecules via thiol chemistry, forming hybrid materials termed nano-bio-conjugates (NBC). Laser ablation in liquids (LAL) is an emerging technique[1] for the synthesis of AuNP-NBC suitable for biomedicine, in particular because no organic additives are required and toxic cross effects can be avoided. Size control of these AuNP can be achieved by the addition of electrolytes at low salinity during synthesis, resulting in monodisperse particles with average diameters <10 nm [2]. Furthermore, the totally ligand-free surface of the LAL-generated AuNP eases bioconjugation with functional molecules, yielding surface coverages up to five times higher than for AuNP synthesized by ligand exchange.[3] In this work, two recent applications of LAL-fabricated AuNP-NBC are presented. In one approach, monodisperse AuNP (< 10 nm) were utilized as a platform for functional peptide ligands, aiming at the dissociation of protein aggregates, believed to be involved in the pathology of neurodegenerative diseases like Alzheimer´s disease using the model protein Aβ. With a set of complementary biophysical methods we could conclusively show that the conjugates performed similar, in some cases even better than the free ligands in dissolving Aβ fibrils and oligomers (Figure 1a)[4]. A second approach entailed the use of deliberately agglomerated AuNP-peptide-conjugates as platforms for photo-induced intracellular release The AuNP agglomerates were internalized by model cell lines and pulsed laser irradiation induced a controlled endosomal rupture and desagglomeration of the particles. This went along with the release of cargo molecules (dyes) into the cytoplasm (Figure 1b), while overall cell viability was unaffected.[5] This work could offer novel strategies in laser-induced drug delivery.

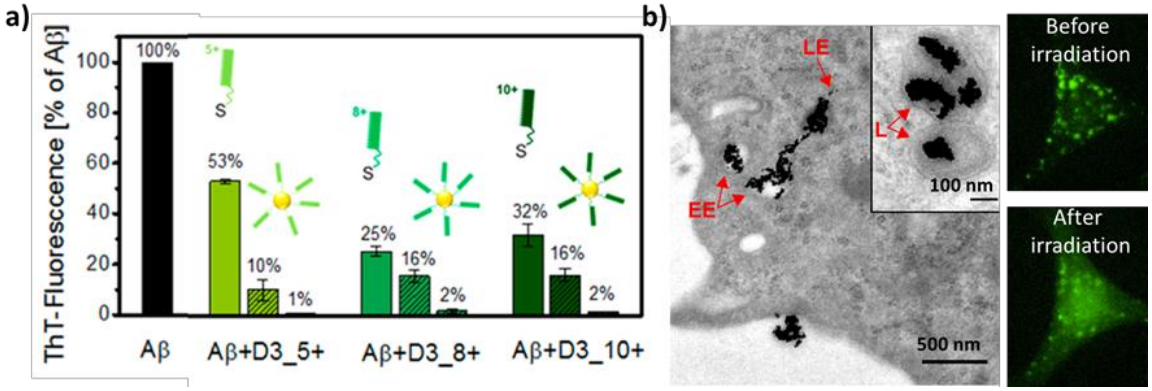


*Figure 1 a) ThT fluorescence assay, indicating β-sheet aggregate content in Aβ, with free D3 peptide ligands as well as D3-gold-nanoconjugates. Ligands with different positive net charges (5+ - 8+) were tested. [4] b) TEM images of AuNP agglomerates localized within endosomes (EE=early endosomes, LE=late endosomes) as well as confocal images of cells prior to and after laser irradiation, indicating endosomal rupture and even distribution of a cargo dye in the cytoplasm.[5]*

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*Christoph.rehbock@uni-due.de*

# Gold-coated harmonic nanoparticles for multi-modal targeted imaging and treatment of cancer

R. Taitt[1], E. Millet[1], M. Urbain[2], V. Kilin[3], Y. Mugnier[2], L. Bonacina[3], A. Géloën[4], R. Le Dantec[2], Y. Chevolot[1], V. Monnier[1]

*(1) Université de Lyon, Institut des Nanotechnologies de Lyon UMR CNRS 5270, Ecole Centrale de Lyon, 36 Avenue Guy de Collongue, F-69134 Ecully, France.*
*(2) Université Savoie Mont Blanc, SYMME, F-74000 Annecy, France.*
*(3) Université de Genève, GAP-Biophotonics, 22 chemin de Pinchat, Carouge, 1211 Geneva 4, Switzerland.*
*(4) Université de Lyon, CARMEN INSERM U1060, INSA Lyon, F-69621 Villeurbanne, France.*

In moving towards more efficient and targeted treatment of cancer, the design of systems capable of simultaneously imaging and providing treatment is now imperative. Nanostructures are proposed as ideal platforms for such multi-modal systems as the nanostructure itself can have enhanced optical or therapeutic capabilities, but additionally, the main property gained at this scale, increased surface area to volume ratio, allow for the incorporation and grafting of different compounds and bio-molecules onto a single particle. This allows for some degree of creativity in conceptualizing what combination of materials will give the greatest advantage. With respect to advances in cancer treatment, gold nanostructures have been demonstrated as photothermal agents in laser-mediated localized hyperthermic treatment of cancer[1], due to its ability to convert incident light energy corresponding to its plasmon resonance band into thermal energy, and by careful manipulation of the nanostructure design, the characteristic plasmon band can be tuned. Additionally, inorganic harmonic nanoparticles have recently emerged as viable tissue imaging probes for multi-photon microscopies due to their inherent non-linear optic activity that allows the second harmonic generation (SHG) of an incident light beam[2]. Their attractiveness when compared to traditional fluorescent probes lies in the absence of photo bleaching, the tuneability of the excitation and emission wavelengths and the inherent high resolution of multi-photon microscopy.

The aim of this work is to encapsulate harmonic nanoparticles of lithium niobate ($LiNbO_3$) with a gold shell, where both core and shell are activated under near infrared (NIR) light, to allow for the imaging and photothermal treatment of cancer cells. The controlled thickness of this gold shell is a crucial step in this work because it directly influences the plasmon band position in the NIR spectral region. Therefore, we will detail our synthesis protocol for growing a homogenous gold shell onto $LiNbO_3$ nanoparticles using an ion-reducible layer-by-layer approach to precisely control shell thickness and consequently the plasmon band position. SHG emission of these core-shell nanoparticles will then be investigated.

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*Corresponding author email: rachael.taitt@ec-lyon.fr*

# Radioactive gold nanoparticles: therapeutic impact in a prostate cancer model studied by electron microscopy and microdosimetry

M. Laprise-Pelletier[1], Y. Ma[2], J. Lagueux[1], M.-F. Côté[1], L. Beaulieu[2], M.-A. Fortin[1*]

*(1) Centre de recherche du Centre hospitalier universitaire de Québec – Université Laval (CR CHU de Québec), axe Médecine Régénératrice, Québec, G1V 4G2, Qc, Canada AND Centre de recherche sur les matériaux avancés (CERMA) AND Département de Génie des Mines, de la Métallurgie et des matériaux, Université Laval, Québec, G1V 0A6, Qc, Canada*

*(2) CR-CHU de Québec, Axe Oncology, Département de radio-oncologie AND Centre de recherche sur le cancer (CRC) AND Département de physique, de génie physique et d'optique, Université Laval, Québec, G1V 0A6, Qc, Canada*

Several theoretical and experimental studies have confirmed the therapeutic potential of gold nanoparticles (Au NPs) as sensitizers for radiotherapy. In particular, the irradiation of Au NPs with low-energy photons such in low-dose rate brachytherapy (e.g. with the radioisotope $^{103}$Pd, ~ 20 keV photons; Figure 1.A), is particularly efficient for controlling the volume of prostate cancer tumors over months[1]. In fact, the probability of photons to interact with Au atoms through the photoelectric effect, is much higher at low photon energies and this has been considered until now as the driving mechanism for tumour control with Au NPs coupled to low dose rate brachytherapy. However, the secondary emissions produced by the photon-Au interaction (e.g. secondary electrons) are not very energetic and it is not clear at this step if their trajectory could reach the nucleuses and impact on the DNA. To improve our understanding of the radiosensiting effect provided by Au NPs irradiated by low energy photons, microdosimetry studies are needed. In the present study, radioactive $^{103}$Pd:Pd:Au core-shell nanoparticles were synthesized according to a recently published methodology (Figure 1.B).[2] The particles were injected in prostate tumours grown in the mouse model (PC3 cells injected in the flank of nude mice). The tumours were harvested at time points, sliced, and observed in transmission electron microscopy (TEM), thereby revealing biodistribution maps of Au NPs at the micrometric scale (Figure 1, C). A Monte Carlo-based dosimetric model was developed to evaluate at the sub-cellular scale (100 x 100 x 100 nm$^3$ voxels) and based on the biological TEM images, energy deposition in the tissues containing $^{103}$Pd:Pd:Au NPs. The simulation results confirmed high-intensity dose deposition in the immediate vicinity of Au NPs, and not close to the nucleuses. This suggests that the strong tumour volume control observed experimentally in previous studies[1], is most likely attributed to indirect damage (e.g. production of reactive oxygen species). Such mechanisms should be integrated in the model in the perspective of transferring Au NPs as radiosensitizers for low dose rate brachytherapy.

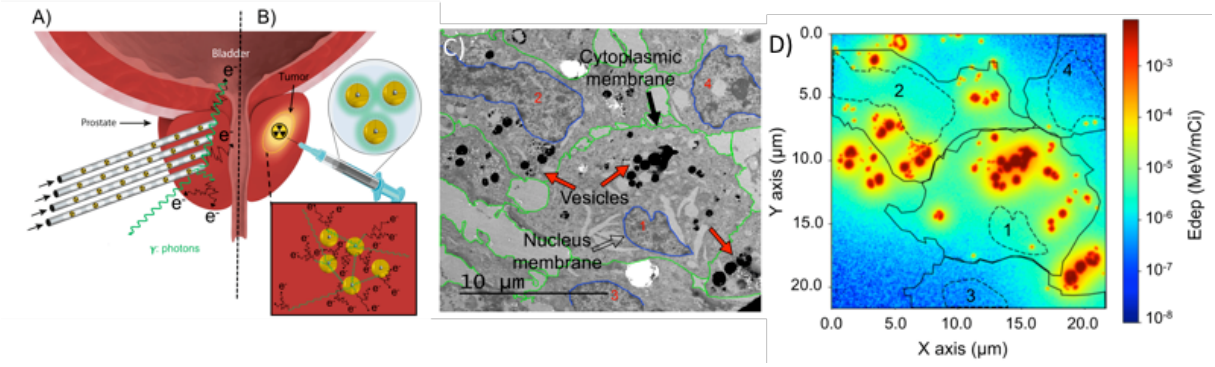


*Figure 1. A) Conventional low dose-rate brachytherapy procedure for prostate cancer and B) new procedure involving injections of radioactive particles ($^{103}$Pd:Pd-Au NPs). C) The intratumoral distribution of Au NPs was studied at the cell level with TEM (here: 24 h after injection). D) A microdosimetry study based these images and on a Monte-Carlo calculation approach, revealed energy deposition maps (Edep) at the cellular level (expressed in units of MeV/mCi).*

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*Corresponding author email: marc-andre.fortin@gmn.ulaval.ca*

# Could quantification of radicals help us in understanding gold nanoparticles radiosensitization mechanism?

E. Brun[1], C. Sicard-Roselli[1]
(1) *Laboratoire de Chimie Physique, UMR CNRS 8000, Université Paris-Sud, Orsay, France*

In 2004 Hainfeld et al. highlighted the important role gold nanoparticles (GNP) could play for therapy[1]. They demonstrated in mice that the adjunction of GNP to X-ray treatment could lead to complete tumor regression. Surprisingly, this promising effect is not yet transposed to clinical phases. One main reason could be the absence of consensus about the benefit obtained by coupling GNP and irradiation. Not only the adding value of GNP can be quite different from a publication to another, but the GNP efficiency highly varies from a cell type to another[2]. With this in mind, we decided to study the GNP-radiation interaction in order to get a good knowledge of the mechanisms involved and developed a protocol to quantify the electrons and hydroxyl radicals emitted by irradiated nanoparticles[3]. For uncoated GNP, massive quantities of both species were quantified and unexpectedly, gamma rays induce more radicals than X-rays. We propose a key role of interfacial water around nanoparticles, with a specific organization responsible for high radical production. As nanoparticles need to be coated for biological applications, we also studied the impact of functionalization on radical production. This work highlights the fact that a strategic coating choice appears necessary to design the most efficient radiosensitizing nanoparticle.

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*Corresponding author email: emilie.brun@u-psud.fr*

# Investigation of the role of gold nanoparticles in radiation induced oxidation of amino acids and proteins.

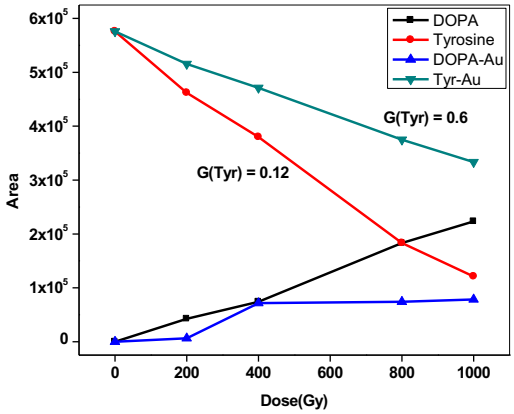
**Yogitabali M. Narode[1], Geeta K. Sharm[1]*, Kirankumar K. Sharma[2].**

***1. National Centre For Free Radical Research, Department of Chemistry, Savitribai Phule Pune University, Pune, India.***
***2. School of Nanoscience and Technology, Shivaji University, Kolhapur, India.***
***E-mail*: geetas@chem.unipune.ac.in**

Proteins are one of the most important building blocks of life and are also one of most accessible target for oxidative damage by reactive oxygen species (ROS). Under normal circumstances, there is a well-managed balance between formation and neutralization of ROS so that there is minimal modification of biomolecules. Under oxidative stress conditions, biomolecules become subjected to attack by excess ROS and significant molecular and physiological damage can occur[1]. Nanoparticles are being increasingly used in the field of biomedicine as drug carrier and also for imaging purpose. Detailed investigation of the interaction of NPs with biomolecules has there fore gained momentum.

We synthesized gold nanoparticles by using Creighton's chemical reduction method[2]. The effect of free radical induced oxidation in bovine serum albumin and the role of gold nanoparticle in the oxidation mechanism was investigated using UV–visible spectroscopy, fluorescence, Fourier transform infrared spectroscopy (FTIR), circular dichriosm(CD), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and DTNB assay.

The γ-irradiation of the protein causes the disruption of the ordered structure of protein. The CD study shows that % α helix after irradiation in presence of AuNPs is more (61%) as compared to when irradiated in absence of AuNPs (53%). SDS-PAGE study shows that the degradation of protein on irradiation was prevented partially in the presence of gold nanoparticles. Estimation of thiol content after irradiation suggest significant protection of the thiol groups against radiation induced oxidation in presence of AuNPs. To understand in detail, the role AuNPs in these oxidation reaction, tyrosine, one of the two fluorescent amino acid in BSA was selected. $^{\bullet}$OH induced oxidation of tyrosine leads to the $^{\bullet}$OH adduct, 3,4-dihydroxy phenylalanine (DOPA) or dityrosine (via one $e^-$ oxidation pathway).[3] In our study it was observed that the reaction leading to formation of DOPA is less in the presence of AuNPs, as is evident from $G_{(Tyr)}$ calculated from our steady state experiments




Interestingly our product analysis studies (LC-MS, UV-Visible) have revealed that the pathway leading to the formation of DOPA is influenced by the presence of AuNPs. The concentration of DOPA formed in presence of AuNPs was found to be lower than that irradiated in absence of AuNPs. The mechanism of the $^{\bullet}$OH induced oxidation of Tyr and the effect of AuNPs on this mechanism will be discussed in detail.

Figure 1: a) linear plot area Vs dose (Gy) of tyrosine irradiated at different dose in presence and absence of AuNPs from HPLC.

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*Corresponding author email:* ***E-mail*: yoginarode22@gmail.com**