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Engineering Liganded Gold Nanoclusters as Efficient
Theranostic Agents for Cancer ApplicationsVlasta Bonačić-Koutecký,^{*,[a, b]} Xavier Le Guével,^{*,[c]} and Rodolphe Antoine^{*,[d]}

Luminescent gold nanoclusters are rapidly gaining attention as efficient theranostic targets for imaging and therapeutics. Indeed, their ease of synthesis, their tunable optical properties and tumor targeting make them potential candidates for

sensitive diagnosis and efficacious therapeutic applications. This concept highlights the key components for designing gold nanoclusters as efficient theranostics focusing on application in the field of oncology.

Introduction

Cancer is one of the most leading causes of premature death worldwide.^[1] Accurate detection procedures that will allow for its early detection and diagnosis are expected to treat more efficiently numerous cancers reducing the side effects and therefore improving patient life quality.^[2] In this regard, nanotechnology offers innovative and promising opportunities for cancer diagnosis and treatment.^[3] Nanoscale based materials have been found of paramount interest as theranostic agents to fight cancer since they can integrate therapy and diagnostic in a single system.^[4] Ligand protected gold nanoclusters (Au NCs) are a new class of ultra-small nanomaterials with the potential to generate theranostic tools.^[5] Compared to others noble metal such as silver, copper, or platinum, gold metal present the advantage to have better chemical stability and biocompatibility which are crucial aspects for biomedical applications. As we will show in this concept paper, ligand protected gold nanoclusters displaying fascinating molecule-like properties possess all the components for efficient and promising

application in theranostics. Here we highlight the design strategies to turn gold nanoclusters into efficient theranostics focusing on application in the field of oncology and we invite the readers to read excellent recent reviews detailing the use of gold nanoclusters in theranostics.^[5,6] The present concept paper will focus on three aspects: (i) gold nanoclusters as efficient theranostic targets for imaging: the importance of efficient (multi)photon absorption; (ii) photo-physical processes in gold nanoclusters: the key components for efficient theranostics; (iii) surface ligand engineering: another key component for efficient cancer targeting (see Figure 1).

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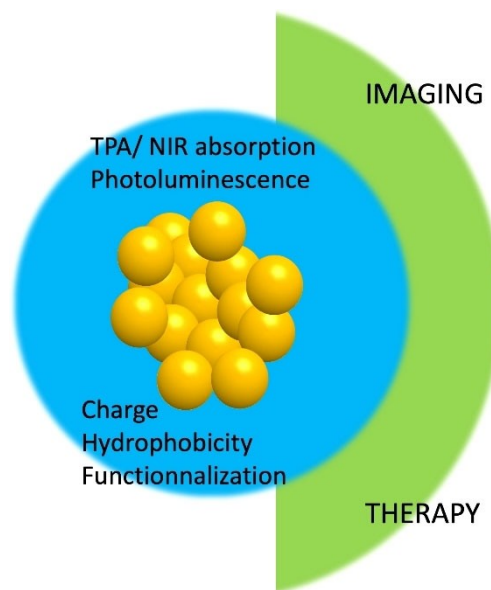


Figure 1. Schematic illustration of key components to turn liganded gold nanoclusters into efficient theranostic agents.

Gold Nanoclusters as Efficient Theranostic Targets for Imaging: The Importance of Efficient Photon Absorption

Imaging techniques using photons as exciting targets (optical imaging) provide high sensitivity and have become very popular in the field of biomedical applications. For instance, the advances in optical guided surgery enable to provide sharp resection of cancer tissues^[7] with good confidence.^[8] However, the use of optical imaging techniques for the diagnostic of deep-seated lesions remain very scarce due to limited tissue penetration of light, high scattering and auto-fluorescence of skin, tissue, blood.^[9] Therefore, it is highly desirable to develop novel optical imaging techniques combined with suitable contrast agents to overcome the above bottleneck for precise non-invasive *in vivo* imaging with high resolution.

Near-Infrared (NIR) light (emission in 700–950 nm NIR-I window and emission in the 1000–1700 nm NIR-II window) can pass through biological tissues more efficiently than visible which has led to the rapid emergence of NIR optical imaging for deep tissues imaging.^[10] However, in the regime of ballistic light, the spatial resolution is in the micron range, the imaging depth still remains very modest (lower or in the order of millimeter). To overcome this limitation, an elegant approach relies on the combination optical resolution and acoustic depth penetration by photoacoustic microscopy.^[11] Photoacoustic microscopy is clearly becoming a promising biomedical imaging modality for deep-tissue applications with already clinical applications to monitor endogenous contrast agents for breast cancer diagnosis, and tissue transplants.^[11]

Since most imaging techniques use photons as exciting targets, nanoscale-based materials developed as contrast agents should display a high photo-absorption in the NIR to an improved imaging sensitivity at high depth in cellular and *in vivo* environments. Understandably, a first strategy consists in developing nanomaterials with small optical gaps (e.g. in the NIR-I or even NIR-II absorption). Luminescent ligand protected noble metal nanoclusters, especially gold nanoclusters composed of a few dozen of Au atoms and protected by ligands, exhibit quantum confinement effects and molecule-like

properties.^[12] For instance, lot of efforts have been dedicated to correlate the structure and the optical properties of ligated gold nanoclusters using mainly thiolated (bio)molecules. A fine selection of ligand and co-ligand enable to produce gold nanoclusters with strong absorption bands from the visible (400–700 nm) to the near infrared region (700–1000 nm).^[13] Interestingly, recent studies report Au NCs with emission in the NIR-II region^[14] using visible and NIR excitation for *in vivo* imaging applications.^[6b,14a,15]

A second strategy consists in decreasing the photon energy of the excitation light (to reach the IR windows) by multiphoton absorption processes. Multiphoton absorption is a non-linear process based on two or more photons simultaneously absorbed in one event. Two-photon absorption (TPA) is one of the most popular process used in multiphotonic techniques which allows to reach high depth in cellular environment with high resolution (organoids, tissues, organs for example).^[16] The understanding of the structure-property relationship of molecular TPA is of great importance for the rational design of optimized two-photon chromophores. The analogy with push-pull molecules can be established in order to describe how theoretical data can be used to get an extensive comprehension of the physics underlying the two-photon process and its amplitude. Simultaneously this case suggests an exploratory root for novel chemical engineering for further enhancement of TPA in atomically precise clusters of gold. Push-pull dipolar molecules are characterized by a low-lying, high-intensity absorption band, related to the intramolecular charge transfer between the electron donor and acceptor groups.^[16]

The TPA cross section of such molecules is considered to be governed by two factors: transition dipole moments and transition energies of the molecule. In molecular design, this can be realized by increasing the π -conjugation length, or by introducing electron donor/acceptor groups. Amazingly, liganded metal nanoclusters display large transition dipole moments (due to ligand-to-core or inversely core-to-ligand excitations and reinforced by a non-uniform electronic distribution in the metal core). We coined this new class of NLO materials as “Ligand-Core” NLO-phores (see Figure 2).^[17] In addition, decreasing the energy between intermediate and ground states can significantly enhance TPA cross sections. If

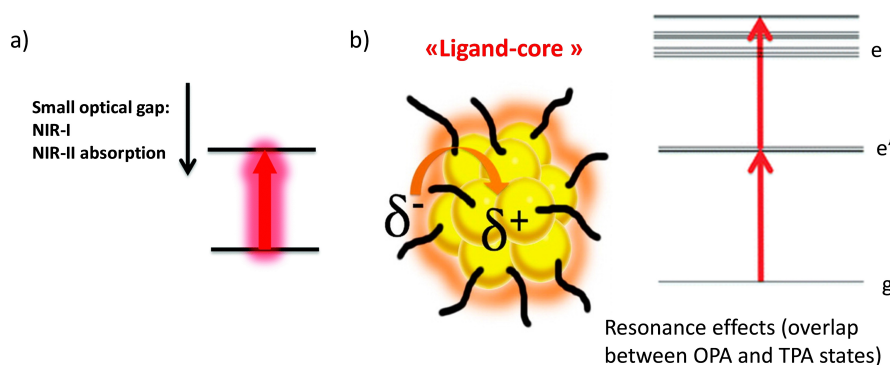


Figure 2. Schematic illustration of strategies consisting in developing nanoclusters (a) with small optical gaps and (b) using multiphoton absorption processes thanks to “ligand-core” NLO-phore with ligand-protected gold clusters. OPA and TPA are one- and two-photon absorption.

the intermediate state (e') is located halfway between ground state (g) and final state (e), a "double resonance" condition can be achieved, which can lead to a dramatic enhancement of TPA cross sections (TPA reaching thousands of GM).^[17] Ligand protected gold nanoclusters can be viewed as a "multi-shell system" composed by (1) a metallic core, (2) a metal-ligand interface, in particular with staple motifs leading to metal-sulfur bonds, and (3) the surface ligand molecules. These three shells may communicate in two different ways: charge transfer from ligand to metal core (analogy with ligand-to-metal charge transfer or ligand-to-metal-metal charge transfer observed in metal complexes) and through direct bonding or direct donation of delocalized electrons of electron-rich groups of the ligands. Such "communications" between ligands and metal core may increase the transition dipole moments leading to enhanced TPA cross sections.^[17,18]

Photo-Physical Processes in Gold Nanoclusters: The Key Components for Efficient Theranostics

Even if the detailed photo-physical processes in gold nanoclusters are still far from being complete, relaxation pathways following (multi)photon absorption in metal nanoclusters are originating from a subtle interplay between excitations arising within the metal core and from charge transfer between the metal core and surface ligands. The lowest excited states in absorption spectra usually belong to "core" in nature. When highest excited states are involved, the nature of excited states are both of "interface-like" and "core-like" (or combination of both). The characteristic of such excited states is more "interface-like" (or also called "oligomer band" or "Au-S band") for which contribution from the Au-S interface in molecular orbitals is more pronounced. Also, electron-rich donor groups from surface ligands may add some contribution to "surface-like" excited states. In addition, multiple energy transfers associated with intersystem crossings (inter-system crossing reinforced) which opens for overall boost in PL emission and longer PL lifetimes. The following energy diagram derived from experimental and theoretical findings is given in Figure 3.^[19] For liganded nanoclusters, there are many parameters that affect

the way the energy flows following photoexcitation. The main parameters are molecular floppiness, solvent accessibility and metallophilic interactions. Loose and floppy ligand molecules exhibit several rotational and vibrational degrees of freedom and will seriously lower fluorescence intensity. Solvent, in particular solvent accessibility to metal core or metal-sulfur interface may play a role in the de-excitation pathway of nanoclusters. If metal NCs are in a high vibrational level of the triplet state, energy can be lost through collisions with solvent molecules (vibrational relaxation), leaving it at the lowest vibrational level of the triplet state. It can then again undergo intersystem crossing to a high vibrational level of the electronic singlet ground state, where it returns to the lowest vibrational level through vibrational relaxation.

Figure 3 summarizes the different photophysical process occurring in ligand protected gold nanoclusters. This figure serves as a snapshot of the huge potential of such ultra-small nanomaterial as efficient theranostic agents using light as trigger to induce PL for optical detection and thermal and/or photodynamic processes for cancer therapy. On one hand, radiative relaxation pathways (occurring from surface, core and triplet states) lead to a rich and color-tunable photoluminescence (PL), in particular for NIR imaging. On the other hand, non-radiative pathways promote interaction of nanoclusters with their surrounding media (liquid) and thus inducing heating, leading to nanobubbles formations enhancing acoustic waves, particularly appealing for photoacoustic imaging. Beyond imaging capabilities (both by photoacoustic and NIR fluorescence techniques), such photophysical process may promote interesting processes for therapy. Indeed, inter-system crossing opening for triplet states populations can be the basis for reactive oxygen species generation (key ingredient for photodynamic therapy).^[20] Of course the non-radiative pathway inducing heating can be used also for therapy actions, known as photothermal therapy.^[21]

Surface ligand engineering^[22] can allow for a fine tuning of such photophysical process, leading to nanoclusters with enhanced luminescence, photoacoustic response or both. Increasing the PL can be achieved by modifying the local rigidity at the vicinal level of the gold surface by introducing small organic molecules,^[23] or by increasing the rigidity of the organic shell surrounding the gold core.^[24] We developed such

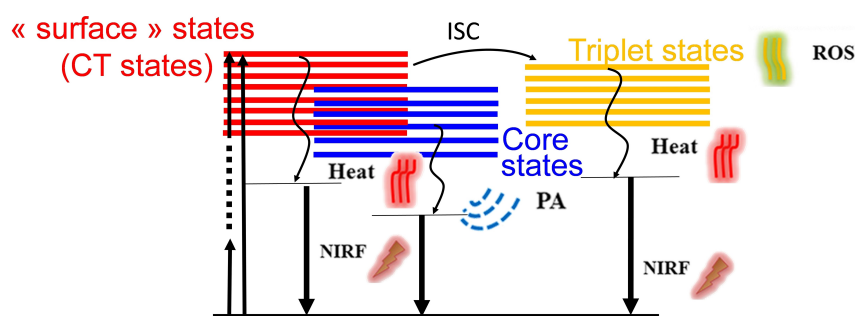


Figure 3. Proposed mechanism for relaxation pathways in protected gold nanoclusters following (multi)photon absorption. NIRF : NIR fluorescence, PA : Photoacoustic, ROS : Reactive oxygen species, ISC : Inter system crossing.

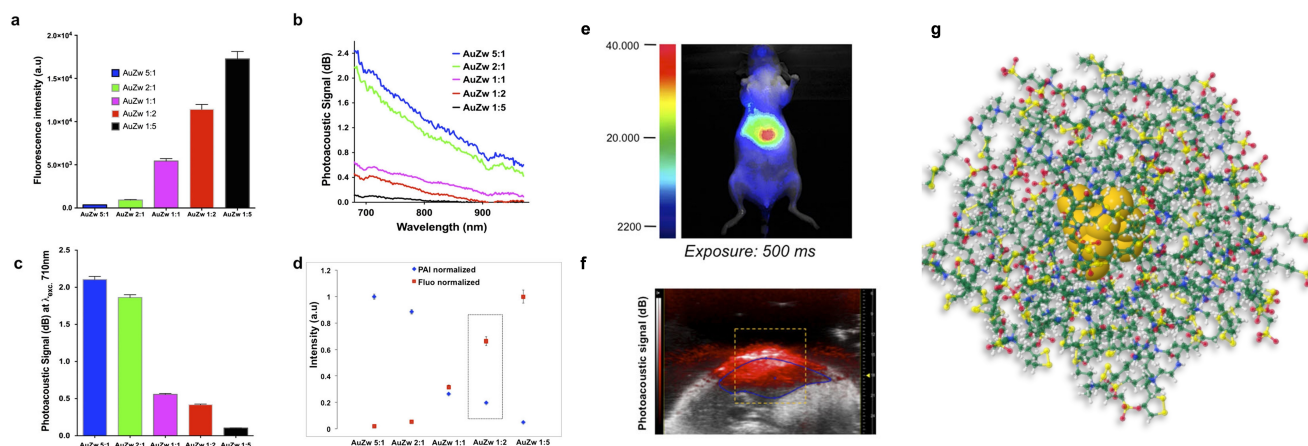


Figure 4. (a) Fluorescence intensity of zwitterion protected Au NCs measured with a NIR 2D-Fluorescence Reflectance Imaging device (λ_{exc} 780 nm; LP 830 nm). Photoacoustic signal of zwitterion protected Au NCs (b) between 690 and 970 nm and (c) at λ_{exc} 710 nm. (d) Normalized fluorescence and photoacoustic signals for zwitterion protected Au NCs. (e) Non-invasive in vivo fluorescence and (f) photoacoustic imaging 3 hours after nanoclusters intravenous injection. (g) Zwitterion functionalized gold nanoclusters investigated by performing QM/MM computation simulations. Adapted from ref. [25b] with permission; Copyright© 2017 AIP Publishing.

second strategy using zwitterion ligands for stabilizing gold core.^[25] In addition we managed to change both the gold core size and the ligand coverage by wet chemistry synthesis. Intense fluorescence signal is reported for the highest ligand coverage whereas photoacoustic signal is stronger for the largest metal core. A trade-off is obtained with a moderate ligand coverage allowing for conducting both NIR fluorescence and photoacoustic imaging with the same Au NCs in vitro and in vivo experiments. They demonstrate an efficient cell uptake in cell lines, a fast renal clearance and a good correlation between near infrared fluorescence and photoacoustic measurements of Au NCs in liver (see Figure 4).

Another appealing and promising surface ligand engineering is to use proteins a scaffold for entrapping and anchoring gold nanoclusters. The close environment of the protein template can allow for tailoring the photophysical process of entrapped gold nanoclusters. This strategy was nicely used by our groups with a single Au₂₅ anchored to bovine serum albumin protein (BSA).^[26] Joint experimental (small-angle X-ray scattering) and molecular modeling approach allowed to localize a single Au₂₅ within protein to cysteine residue at the Au NC surface (see Figure 5). PL properties have been strongly modified by enhancement and red shift in NIR-II due to attachment of Au₂₅ to BSA, but the structure of Au₂₅ remains unchanged. Cysteine ligand exchange during the attachment of Au₂₅ to BSA increased contribution of core-interface charge transfer states. It is of interest to use 700 nm excitation for in vivo optical imaging and photo activation both in linear and nonlinear optical regimes. This study opened the way to design new photo emitters with tunable NIR-I/NIR-II emission by visible or NIR excitation. Engineering mutations in proteins by cysteines should allow for more selective control of the position of atomically precise metal NCs in proteins and of the number ligands exchanged. This will open new routes for extending use in diagnostics and therapeutic applications.^[26]

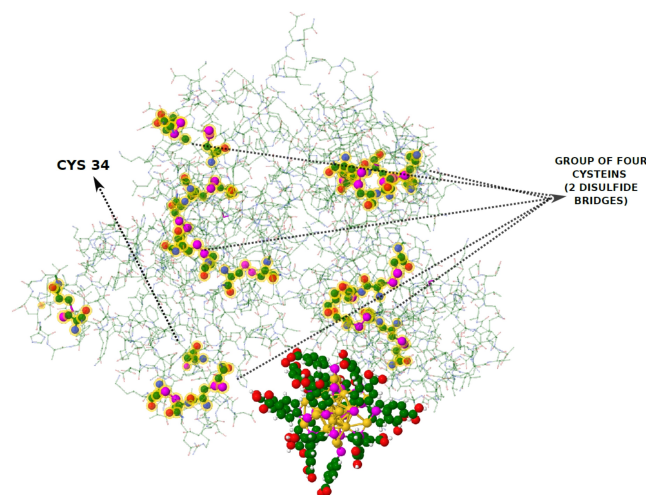


Figure 5. Crystal structure of BSA and DFT structure of Au₂₅pMBA₁₈. Cysteine residues in BSA are highlighted.

Surface Ligand Engineering: Another Key Component for Efficient Cancer Targeting

Colloidal stability, biocompatibility, drug loading and targeting capacity are key aspects to design efficient theranostic agents for cancer therapy.^[5,6b] For this purpose, the surface ligand engineering needs to be correctly balanced to prevent particle aggregation and to provide access to functional groups for targeting and/or drug delivery. Major steps in the synthesis of ligands protected gold nanoclusters with high purity and controlled structure have been achieved to reach this goal.^[27] For instance, numerous of small thiolated organic molecules have been employed to produce stable atomically precise gold nanoclusters with a fine control of their surface chemistry such as their charge^[28] and their hydrophobicity which directly impact on their cellular interaction.^[29] This strategy has been

employed to enhance cellular uptake with promising results in mice models leading to high tumor uptake and retention by passive targeting.^[30] The choice of the ligand (and possible co-ligands) protecting the metal core requires a careful attention as labile ligand coordination might lead to ligand removal. This will lead to possible modification of the metal nanocluster structure, aggregation or ligand exchange. This will strongly influence the biocompatibility and the photophysical properties of the metal nanoclusters.^[31]

In order to improve the selectivity and reduce the non-specific accumulation of Au NCs in reticuloendothelial system, modification of Au NC surface has been explored to bring active targeting. The high diffusion of such nanoobjects combined with the capacity to target receptors overexpressed in cancer cells (integrin, folate, etc.) is reckoned as an elegant approach to not only reach the periphery of the tumor microenvironment but also deeper in the tumor and being internalized in cancer cells. This aspect is essential to address in order to reach an efficient treatment of cancer by radio/phototherapy and/or chemotherapy.

Post functionalization of the ligands protecting the gold core via standard chemical reaction (click reaction, imine reaction, esterification, Michael reaction) are nowadays well-established and enable to control the number of molecules of interest grafted per NCs.^[32] This has been achieved to conjugate mono and oligomeric peptides, carbohydrates, oligonucleotide which drastically enhance the sensitivity and selectivity of cellular uptake.^[33] Another strategy to introduce the molecule (drugs, targeting agent) to Au NC surface relies on a ligand exchange process exploiting the high surface reactivity of Au NCs. As a tiny modification of the structure of Au NCs strongly influences their optical properties, very careful characterizations are required after each step of post-functionalization.

Since the seminal work of J. Xie in 2009 leading to the sequestration of gold nanoclusters in bovine serum albumin by a process similar to biomineralization,^[34] it opened a large avenue to the synthesis of metal NCs (gold, silver, copper, platinum) using natural and custom-made biomolecules as stabilizing agents such as peptides, enzymes, proteins, antibodies, nanobodies, oligonucleotides or aptamers.^[35] A strong effort has been dedicated to form monodisperse NCs keeping the structural conformation and the activity of the biomolecules.^[36] Many studies demonstrated the active targeting of peptide-Au NCs to cancer cells and their specific accumulation in tumor bearing mice with an efficient renal clearance preventing toxicity risks.^[37]

Concluding Remarks

Ligand protected gold nanoclusters possess all the key components in their photophysical process following (multi)photon absorption to produce both therapy and diagnosis into a single system. Ligands play a central and pivotal role in ligand protected gold nanoclusters for efficient "ligand-core" NLO-phores and also for efficient cancer targeting thanks to their easy post-synthesis functionalization.

However, there is still a room for improvement for tailoring photophysical process, through ligand engineering, on demand depending on the desired theranostics applications. Thanks to the progress of surface engineering of metal nanoclusters, next generation of metal nanoclusters including Au NCs are now developed based on hybrid assembly of NCs with organic and inorganic nanoparticles. Moreover, the gold nanostructures combination with other materials (in particular 2D platforms)^[38] or small molecules (in particular dyes) to improve the nano-materials photothermal capacity is also largely developed.^[39] This led to a vast library of stimuli responsive nanosystems with multimodal imaging capability enabling control release by endogenous or exogenous triggering.^[40] For instance, a recent work reported the conjugation of NIR-II emitting Au NCs ($\lambda_{em} = 1000$ nm) to NIR-IIb ($\lambda_{em} = 1500$ – 1600 nm) emitting rare earth nanoparticles by a thiolated linker. This linker could be cleaved in cancer cells and led to a modification of the optical properties which could be monitored non-invasively in tumor in mice models by NIR-II imaging.^[41]

Another recent promising strategy to develop metal nanoclusters for theranostic application relies on the self-assembly of atomically precise NCs.^[19,42] Indeed nanoclusters produced with atomic precision with enhanced stability, and diverse surface functionalities, render them attractive as platform for developing superstructures via the self-assembly process. The amplification of the photophysical properties in such hierarchical structures opens up a new route to design nanosystems with high molecular sensitivity and can find applications from multimodal imaging and biosensing to therapies.

Finally, to envision a clinical transfer of these renal clearance particles, an important aspect of NCs that needs to be properly addressed is the long-term toxicity of such ultra-small particles. For example, gold NCs detected within the nucleus^[22] or at the physiological level can be excreted after few weeks from tissues to the blood stream.^[37b,43]

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Conflict of Interest

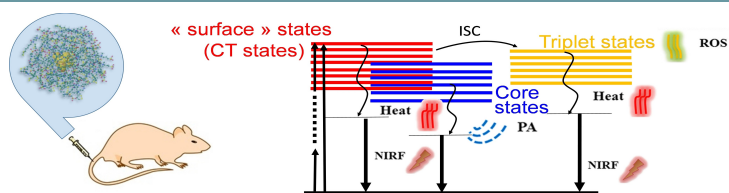
The authors declare no conflict of interest.

Keywords: bioimaging · cancer · gold nanoclusters · multiphotonic process · theranostic agents · photoluminescence

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CONCEPT



The key components for designing gold nanoclusters as efficient theranostics are highlighted in this

Concept article with a focus on applications in oncology.

Prof. V. Bonačić-Koutecký*, Dr. X. Le Guével*, Dr. R. Antoine*

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Engineering Liganded Gold Nanoclusters as Efficient Theranostic Agents for Cancer Applications