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Gold Nanoclusters as a contrast agent for image-guided surgery of Head and Neck tumors

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Abstract

With the objective to evaluate the potential of ultra-small gold (Au) nanoclusters (NCs) for optical image-guided surgery, we synthesized and characterized AuNCs shelled by zwitterionic or pegylated ligands. The toxicity of the different AuNCs was evaluated on the Head and Neck Squamous Cell Carcinoma (HNSCC) CAL-33 and SQ20B cell lines *in vitro*. The safer AuNCs were administrated intravenously to mice for the determination of the pharmacokinetic properties. Biodistributions were performed on orthotopic CAL-33 HNSCC-bearing mice. Finally, the AuNCs were used for image-guided surgery, allowing the increase of the survival time *vs.* control animals, and the number of animals without any local recurrence.

Background

Head and Neck cancers are frequently observed at the late stage when the 5-years survival does not exceed 25%. At late stages, the surgery of Head and Neck Squamous Cell Carcinoma (HNSCC) remains often fairly complex due to the numerous local structures that should be sustained such as small muscles, nerves and tendons. The preservation of these healthy structures is fundamental in the perspective of restorative surgery (1). In addition, the total removal of tumor should be ensuring by the presence of healthy surrounding tissues around the lesion. This step is critical for the post-surgery treatment, which varied as function of the margin' size, *i.e.* the quantity of healthy tissue removed in addition to the pathologic area. In this context, nanotechnology might bring a strong support to overcome these issues, especially in the field image-guided surgery (2, 3). When the surgery is assisted by real-time imaging, the tumor resection might be improved with the removal of small residues in particular and sufficient margin, the healthy tissues might be spared, and the time of surgery might be reduced (4-6). NIR image-guided surgery has already been validated in small animal studies (5, 7) and is currently under investigation in clinical trials. In particular, antibody directed against Epidermal Growth Factor Receptor, *i.e.* cetuximab-IRDye800CW, is under evaluation in a clinical trial for the intraoperative assessment of tumor margins during surgical treatment of HNSCC (8). In addition, the indocyanine green (ICG) is used to identify the sentinel lymph nodes, which is associated with locoregional recurrence in the most aggressive HNSCC (9). Recently, Warram's group illustrated surgery assisted by real-time imaging. They demonstrated the valuable adjunct of imaging to the oncologic surgeon, both before, during, and after the surgery, facilitating the detection of occult lesions in particular (10). For all these reasons, image-guided surgery can be of benefit for patients in terms of post-treatment procedures, overall survival, side effects, and quality of life.

Gold nanoclusters (AuNCs) are ultra-small particles (<5 nm) that emerged as promising renal clearable nanocompounds for cancer diagnostic and therapy (11-13). Thanks to their photoluminescence properties in the NIR/SWIR region (14, 15), a spectral window favorable for *in vivo* monitoring, these probes could be tracked in real-time imaging, in various organs (16). AuNC size between 10 to 25 gold atoms was recently shown by Zheng's team to strongly affect the pharmacokinetic and the tumor accumulation in mice (17). The physico-chemical properties of the ligands protecting the metal core are another key parameters that highly influence the pharmacokinetics and the biodistribution of AuNCs in animals. For instance, short PEG chains stabilizing AuNCs have enabled to enhance the half-life from 5 to 45 min compared to AuNCs protected by glutathione (18). Different *in vivo* studies have demonstrated the potential of AuNCs in tumor's detection, including brain (15, 16, 19) or breast tumors (18, 20), but to our knowledge only Au NPs have been used for Head and Neck tumor's detection. We previously observed an increase of the blood circulation for AuNCs stabilized by a bidentate zwitterion ligand compared to the well-known NCs Au₂₅SG₁₈ from 1.5 to 6.5 min. This led to a stronger accumulation in glioblastoma mice models by passive uptake with a high tumor retention over 24 h (16).

With the objective to evaluate the potential of AuNCs for image-guided surgery of orthotopic HNSCC-bearing mice, we synthesized AuNCs with different ligands, and performed their *in vitro* and *in vivo* biological investigations. First, the toxicity and the cell uptake properties of the different formulations were evaluated on 2 HNSCC cell lines, *i.e.* CAL-33 and the SQ20B cells. Then, the most favorable formulations, *i.e.* safer and well-uptaken by the cells, were administrated intravenously to mice-bearing tumors for the determination of the biodistribution and the pharmacokinetics properties of the selected NCs. Finally, two AuNCs were used for image-guided surgery, and animals were followed-up until tumor recurrence. The image-assisted removed residues were also analyzed for tumor invasion by histology.

Methods

Materials: Chemical reagents were purchased from Sigma-Aldrich (France) and deionized water was used during the synthesis.

Gold Nanoclusters: AuNCs stabilized by bidentate thiolated organic molecules were synthesized following protocols reported earlier (21). In particular, we chose a zwitterionic ligand LA-ZwEt₂ (C₁₈H₃₆N₂O₄S₃; 440.68 g.mol⁻¹) and a short pegylated ligand with a negatively charged terminal group LA-Peg_nCOOH (C₃₈H₇₄N₂O₁₆S₂; n_{PEG}=12; 879.13 g.mol⁻¹) synthesized in the lab (22, 23). Both type of AuNC were prepared with low and high ligand density by increasing the amount of the bidentate thiolated organic molecules during the synthesis. For the zwitterionic ligand, it corresponded to the molar ratio Au:ZwEt₂:NaBH₄= 1:2:2 and 1:5:2. For the PEG ligand, it corresponded to the molar ratio Au:PEG:NaBH₄= 1:3:2 and 1:5:2. In a typical synthesis for AuPegCOOH 1:3, 26.4 mg of PegCOOH was dissolved in 15 mL water at pH 10 (80 μL NaOH (2 M)) under vigorous stirring and 200 μL of HAuCl₄ (50 mM) was added. After 10 min, 200 μL NaBH₄ (100 mM) was added dropwise and the solution was kept under mild stirring for 15 hours. Freshly prepared AuPegCOOH was filtered 3 times (Amicon filters 3 kDa) to remove the excess of free ligand and diluted in PBS at the required concentration. Each stock solution was filtered (0.22 μm) before use.

The well-described AuNC made of 25 gold atoms stabilized by the peptide glutathione Au₂₅SG₁₈ was synthesized in two steps as described by Dugourd and coworkers (24), and diluted in PBS at the required concentration. Later in this manuscript, the AuNCs are cited by the name of their ligands.

NC characterization: Absorbance measurements on diluted samples were recorded on an Evolution 201 (ThermoScientific) UV-vis spectrophotometer between 300 and 950 nm. Fluorescence spectra were recorded on a Perkin Elmer LS45 fluorescence spectrometer. Hydrodynamic diameters of the AuNCs were determined by dynamic light scattering (DLS), and their surface charges by zeta potential measurements, both analyses using Malvern Instrument. For mass spectrometry measurements, a commercial quadrupole time-of-flight mass spectrometer (micro-qTOF, Bruker-Daltonics, Bremen, Germany, mass resolution 10,000) was used as previously reported (25, 26) (see also supplementary information).

Cell lines: This study was conducted using two human head and neck squamous carcinoma cell lines. The CAL-33 (Leibniz Institute, Braunschweig, Germany) cell line derived from a tongue carcinoma and was used for *in vitro* experiments, and for orthotopic models. The second cell line used was the larynx carcinoma SQ20B cell line (Institute for Advanced Biosciences, Grenoble, France). The cells were maintained in a monolayer culture in DMEM (Dulbecco's Modified Eagle's medium) supplemented with 10% of heat-inactivated FBS (Fetal Bovine Serum) (v/v) in a humidified incubator (Sanyo, Japan) at 37°C in an atmosphere containing 5% CO₂. The sub-culturing was performed twice a week.

Viability assay: Cell viability assays were used to evaluate the cytotoxicity of AuNC *in vitro*. CAL-33 and SQ20B cells were seeded in a 96-well plate at a density of 10⁵ cells per well and cultured overnight. Then cells were incubated with 50 or 100 μL of AuNC/mL of medium for 24 h before medium renewal for the next 48 h incubation. We used staurosporine as negative control that induces massive cell death, and no AuNC as positive control for viability. After washing with PBS, the cell viability was determined using the Presto-Blue viability kit (*i.e.* 72 hours after NCs exposure). According to the manufacturer's instructions, the fluorescence was recorded on a microplate reader after 4 hours of incubation, selecting the fluorescence excitation/emission couple 560 nm/590 nm, avoiding any cross-absorption from the gold atoms.

FACS analysis: 5.10⁵ cells (CAL-33 and SQ20B) were plated in a six-well plate with DMEM with 10% FBS. After 24 h, different AuNC were added at 50 μL/mL for 4 h at 37°C and 4°C, 8 h and 24 h at 37°C.

At the end of incubation, the cells were prepared for flow cytometry analysis. The NCs' uptake was measured with LSRII (Becton Dickinson, Le Pont de Claix, France) and photon emission was selected using long-pass filters, and recorded at 730/45 and 780/60-nm. Data were analyzed with the BD FACSDiva 6.3.1 software. One should note that the fluorescence emission of the NCs, in the NIR region prevents the internalization study using conventional microscopy.

Mice and pharmacokinetic study: Animal experiments were conducted in accordance with protocols approved by the Ethical Committee of Grenoble, and the French Minister in agreement with the European directive 2010/63/UE. The pharmacokinetic (PK) study was conducted on 12 SWISS mice divided into 4 groups (n=3/group). The solutions of 200 μ L AuNC were administrated IV at 2.5 mg/mL. The PK of the Au₂₅SG₁₈ was performed previously by our group in similar condition, and was not performed again for ethical reasons (15). Approximately 20 μ L of blood were collected in heparined vials from the tail vein at the indicated times after injection. The vials were centrifuged 20 minutes to recover the plasma serum. Then, a drop of 10 μ L of serum was imaged (Optimal, Grenoble, France) and black and white pictures, acquired with a black-thinned CCD camera at -80°C (ORCAII-BT-512G, Hamamatsu, Massy, France). Image display and analysis were obtained with the Wasabi® software (Hamamatsu, Massy, France).

Head and Neck orthotopic tumor model: In total, 65 Female athymic NMRI nude mice (5-6 weeks old) were purchased from Janvier laboratories (Le Genet sur Isle, France), and used for this study. The complete tumor model can be found in the supplementary section, and in Atallah et al (7).

Biodistribution study: After tumor growth, AuNCs were injected IV (200 μ l at [Au] = 5 mg/mL, n=24 for biodistribution and n=12 for imaging). The nanoparticles' biodistribution was investigated using *in vivo* fluorescence imaging (Optimal, Grenoble, France), as previously described (16).

Surgical study: Animals were randomized 21 days after tumor implantation to perform tumor resection under visual macroscopic guidance (n=8), using near infrared (NIR) optical imaging after AuNC PegCOOH 1:3 (n=8, one mouse died from anesthesia and was removed from the study reducing to 7 the number of mice in this group) or ZwEt₂ 1:5 (n=8) administration. For all animals, the surgeon first removed the tumor masses under conventional light (macroscopic resection), and image-guided surgery was used to check the remaining fluorescence in the AuNC's groups and to assist the surgeon when residual fluorescence was observed. The animals were randomized in the different groups to minimize the differences in the means and standard deviations of the tumor volumes. Surgery was performed under general anesthesia (intraperitoneal injection of a mix of medetomidin (0.2 μ g/g) / ketamine (0.1 mg/g)) associated with a subcutaneous injection of buprenorphine (0.1mg/kg)), as described in the supplementary information section.

Histology and confocal imaging: The removed residues were embedded into OCT and sliced at 8 μ m. The sections were then H&E stained for histology. Adjacent slices were mounted with a coverglass for confocal microscopy analysis. The microscopy of the frozen remaining tissues was carried out using a confocal microscope (LSM510, Zeiss Jena, Germany) with a 10x objective. The AuNCs were excited by a 488-nm, or 405-nm emission laser and their emission was collected using a long-pass filter at 600 nm.

Results

Library of nanoclusters

AuNCs are ultra-small gold particles with core size between 1 and 3 nm. To protect the body from the reactive gold species, the core of the NCs can be shelled by various ligands. In this study, 5 NCs were

synthesized: 2 with a lipionic zwitterionic ligand (*i.e.* ZwEt₂), 2 with the pegylated ligand PegCOOH and the last one with glutathione (SG). Thickness of the zwitterionic and pegylated layers covalently bonded to the gold core could be tuned during the synthesis while keeping a similar core size as represented in the **Figure S1**. The increase of the organic shell leads to an obvious increase of the molecular weight of AuNCs, which is confirmed by electron-spray ionization mass spectrometry (ESI-MS). For instance AuZwEt₂ 1:5 has an average molecular weight around 17.5 kDa, which is 22% bigger than AuZwEt₂ 1:2 with a molecular weight around 14.5 kDa (see **Figure S2**). Surprisingly, AuPegCOOH 1:3 is smaller than AuZwEt₂ 1:2 with a molecular weight estimated at ~9 kDa but an increase of the pegylated ligand during the synthesis for AuPegCOOH 1:5 leads to a molecular weight (MW) around ~22 kDa heavier than AuZwEt₂ 1:5. Despite a similar lipionic acid moiety to anchor the gold core, the difference in size between zwitterionic and pegylated ligands might be related to the electrostatic interaction between the ligands during the AuNC's formation in solution. This could influence the number of ligand per AuNCs considering no significant change of the metal core size determined by microscopy measurements (data not shown). However, this observation needs to be considered carefully because of the quite broad MW distribution obtained by ESI-MS for these AuNCs. In addition and as previously reported (27), ESI-MS analysis also demonstrated that the effective charges (Z_{eff}) extracted from the measured Zeta-potential (ζ) of NPs in solution were partially correlated with the average values of charge Z_{average} of NPs in gas phase. Interestingly, this correlation seems also valid for AuNCs investigated in this study, where a nice correlation between the Zeta-potential and the average charge state of Z_{average} is observed (see Table 1). The atomically precise AuNCs stabilized by glutathione Au₂₅SG₁₈ have a molecular weight of 10.4 kDa.

Hydrodynamic diameters measured by dynamic light scattering (DLS) show a size below 10 nm for all types of AuNCs with an increase of diameter for thicker organic shell. From zeta potential measurements, all AuNCs were negatively charged at pH 7, and less charged for the zwitterionic species AuZwEt₂. The main physico-chemical characteristics and properties of the different NCs are summarized in the **Table 1**. Absorbance profiles for AuZwEt₂, AuPegCOOH, and Au₂₅SG₁₈ present similar pattern with a decrease of signal at longer wavelength (**Figure S3**). All types of AuNCs emit in the red/near-infrared region with a blue-shift and an increase of their brightness for thick organic shell (**Figure S3, Table 1**). This behavior has been extensively reported and is correlated to the nature, the density, and the rigidity of the ligands stabilizing the metal core of AuNCs (28-30).

Toxicity profiles and cell internalization of the different NCs

The toxicity of the different NCs was determined at 50 and 100 µg gold/mL on two different human HNSCC cell lines, namely CAL-33 and SQ30B cells (**Figure 1**). The viability assay was performed after a 24-hour exposure followed by a further 48-hour incubation, *i.e.* 72 hours in total. Due to the natural blue-color of the AuNCs, the presto-blue assay was performed in fluorescence mode, to avoid any overlapping due to the solutions themselves.

If most of the tested NCs were not or weakly toxic, the main toxicities were measured for the AuPegCOOH, with a particular susceptibility of the SQ20B cells for AuPegCOOH (1:3) at high concentration (*i.e.* 100 µg/mL). For these AuNCs, the density of the ligands in the NCs' shell did not affect the safety profile of the NCs: both ligand densities were equally safe, except for the SQ20B cells exposed to AuPegCOOH (1:3), as described in the **Figure 1B**.

The cell uptake of the NCs was evaluated using flow cytometry on CAL-33 cells. As summarized in the **Figure 2**, after 8 hours of incubation, AuNCs were moderately internalized. Only the fluorescence signal of the AuZwEt₂ (1:5), and AuPegCOOH (1:3) were recorded inside the CAL-33 cells. Percentages of cells positively stained by the AuNCs reach 17% and 38% for the AuPegCOOH (1:3) and the AuZwEt₂ (1:5), respectively (see **Figure 1D**). We hypothesized that the weak AuPegCOOH (1:5) uptake may be

in part attributed to a higher contribution of the Peg coating as compared to AuPegCOOH (1:3), which exhibit anti-fouling effect (31).

In vivo distribution and behavior of the NCs

The distribution of the NCs was evaluated in mice-bearing sub-cutaneous CAL-33 tumors, using optical imaging. Organs were sampled 5 hours post-intravenous (IV) administration and analyzed. The results are summarized in the **Figure 3**, and the **Table S1**. As previously reported, most of the AuNCs were rapidly eliminated through the urine, *i.e.* after renal filtration, due to their small size (15, 16). The remained NCs were mainly distributed in the hepato-biliary system (liver and spleen), and the kidneys. We noticed some uptake in the stomach for AuZwEt₂ (1:2) while the others AuNCs were not specifically accumulated in this organ. This accumulation might be due to its hepato-biliary elimination. The AuNCs were then mostly found in the skin and the tumor, 5 hours post injection. Interestingly, the AuZwEt₂ 1:5, and the Pegylated AuNCs were rapidly eliminated from the body, with very moderate to low liver and kidney accumulations. Therefore, the most promising AuNCs were selected for next PK and imaging investigations. Due to its long liver retention, the AuZwEt₂ (1:2) was not selected for the *in vivo* studies. AuZwEt₂ (1:5), and the pegylated AuNCs were injected to healthy mice to determine their plasma half-lives, and compared to Au₂₅SG₁₈. The blood samples were collected directly after the intravenous administration, and over the next 2 hours. As indicated in the **Figure 4**, the plasma half-lives are longer than that of Au₂₅SG₁₈ (see also Suppl. **Table S2**), with prolonged exposure. The nature of the ligand and the thickness of this organic layer stabilizing the gold core have a significant effect on the pharmacokinetics. All the AuNCs presented plasma half-life fitting with two-phase decay equation, which is a combination of 2 plasma half-lives, *i.e.* a fast and a slow half-life. Indeed, zwitterionic ligand Et₂ (1:5) prolonged the biopersistence of the AuNCs in the plasma, reaching almost 4 min and 11.5 minutes for fast and slow half-lives respectively. For pegylated AuNCs, the fast half-lives were very short with 0.45 and 0.32 min for AuPegCOOH (1:3), and AuPegCOOH (1:5), respectively. The slow half-lives were longer, reaching almost 10 minutes for AuPegCOOH (1:3), and more than 13 minutes for AuPegCOOH (1:5). Such compounds with both fast and slow half-lives are partially absorbed in organs, such as the liver, before being released from this one into the plasma, and available for clearance. Such two-phase decay equation was expected, and previously reported by Zheng and collaborators with pegylated AuNCs (18). One should note that the Pegylated AuNC from this group was ≈ 5.5 nm size, but possesses very long half-lives of 56 minutes and 9 hours. Their pegylated AuNCs were also retained in the kidneys around 10% of ID/g until 48 h post injection. In our case, the density of the peg should be higher, leading to more extended structure of the peg around the gold core, leading to the increase of the final size of the AuNC. However, in our case, the AuPegCOOH are well eliminated from the kidneys, and the blood. The dose-normalized AUC indicated a strong enhancement of the bio-availability of the AuNCs stabilized by organic ligands, with an increased by a factor 4 to 6.7 for the pegylated, and 10.8 for the zwitterionic ligand, respectively, as compared to Au₂₅SG₁₈. Such increased availability should favor the amount of AuNCs reaching the tumor region.

In the perspective of image-guided surgery, the imaging properties of these AuNCs were determined, in terms of Tumor/Skin ratios. This investigation was necessary for the selection of the best AuNCs, able to reach the highest Tumor/Skin imaging ratios in the context of orthotopic tumors, and for the adjustment of the surgery protocol, *i.e.* the time between the injection of the AuNC and the surgery itself, for optimized distribution. As represented in the **Figure 5**, the pegylated AuNCs presented similar behaviors, *i.e.* a rapid tumor uptake reaching a Tumor/Skin ratio value of 1.5 to 2 since the first 30 minutes post-injection (see **Figure 5B**). The zwitterionic AuZwEt₂ exhibited a stable Tumor/Skin ratio of 1.5 from 1 to 5 hours post-injection, and favorable Tumor/Muscle ratio close to 3, similarly to the one measured for the pegylated AuNCs (data not shown). In view of these data, we decided to use the NCs AuPegCOOH 1:3 and the AuZwEt₂ 1:5 for the following image-guided surgery investigations; however, as both compounds presented similar results, the data were merged for these two AuNCs.

After orthotopic CAL-33 tumor engraftment, the tumors were measured and the mice were split into 3 groups: (i) control mice whose tumors were removed without any contrast agent ($n = 8$), mice treated with the AuNC, either (ii) AuPegCOOH (1:3) ($n = 7$), or (iii) AuZwEt₂ (1:5) ($n = 8$), before surgery. The surgeon first removed the main tumor mass using the white light (macroscopic tumor resection) in all groups, and for the groups (ii) and (iii), used the optical imaging as a quality control. If any fluorescent mass persisted in the cheek of the animal, the surgeon used the optical live imaging system to localize the tumor residue and to remove it (**Figure 6**). The residues removed by the optical imaging were counted, and further analyzed by histology to ensure the presence of tumor cells, and by confocal imaging to localize the AuNCs within the tissues (**Figure 6F-G**).

As shown in the **Figure 7A**, the main tumor masses macroscopically removed were almost similar in the different groups, reaching a median value of 180 mg for the control group, *versus* 170 mg and 167 mg for the AuNC groups, respectively. Such information confirmed that before surgery, the tumor masses were statistically similar. Following the surgery, the animals were monitored for tumor relapse. All the animals of the control group relapsed rapidly and were euthanized for ethical reasons within 40 days, reaching a mean survival time of 34.8 ± 5.95 . The image-guided surgery allowed the increase of the mean survival time to 45.1 ± 21.8 days and 47 ± 27.2 days, corresponding to 29.9 % and 35.3 % increase in life span (ILS) *vs.* control animals, respectively for AuPegCOOH (1:3) and AuZwEt₂ (1:5). The quality of the image-guided surgery facilitated the removal of the tumor residues for 8 mice, including 3 mice with prolonged survival (1 for the group AuPegCOOH (1:3) and 2 for the group AuZwEt₂ (1:5)) without any local tumor recurrence until the end of the experiment on day 90. All the removed residues were analyzed by histology and revealed the presence of tumor cells (**Figure 6E**, see also **Table S3**). In addition, the presence of AuNCs was observed by confocal imaging on selected residues. The AuNCs were heterogeneously distributed in the tumor mass, with a particular accumulation in some cells into small and cytoplasmic vesicles (**Figure 6F-G**).

Discussion

AuNCs with different surface chemistries were synthesized, characterized and biologically evaluated in this study. The nature of the biocompatible ligands protecting the gold metal core and their density have been tuned to construct 4 different structures that were compared to the well-defined Au₂₅SG₁₈ NCs. An increase of zwitterionic or pegylated coating on the gold core leads a photoluminescence enhancement of the AuNCs due to a more efficient energy transfer of the gold shell, and a better protection from the environment (28, 29). As expected, a thicker ligand coating on AuNCs that still remains quite small (<22 kDa) seems to enhance the interaction with cell surface, through stronger cell internalization with zwitterionic ligand and antifouling effect for charged pegylated ligand.

In this study, we confirmed the efficient renal clearance of these AuNCs as already reported for such small sized nano-objects (16, 32). In addition, their rapid renal clearance should contribute to limit their toxicity, if any. However, a small part of the AuNCs was also trapped by the liver and the spleen, as already reported by us and other teams (15, 16, 33-35). As expected, the pharmacokinetics, *i.e.* AUC, indicated that smaller AuNCs as Au₂₅SG₁₈ were rapidly eliminated from the blood (half-life < 2 min) while the 17.5 kDa zwitterionic AuNCs could reach a half-life around 10-15 min, which is 2 times longer than other zwitterionic AuNCs recently reported (12, 15). One should note the pegylated AuNCs were cleared from the blood stream by a two-phase decay kinetics, with a first fast clearance (<1 min). In absence of specific targeting ligand at the surface of the AuNC, the NCs are weakly internalized in monolayer cell cultures. *In vivo*, they are able to reach the tumor site after intravenous administration, and furthermore are internalized inside the tumor cells, into small vesicles as demonstrated by fluorescent confocal imaging (**Figure 6**). From the preliminary experiments, 2 AuNCs appears

promising for image-guided surgery investigations: the zwitterionic ZwEt₂ (1:5) and the PegCOOH (1:3). Coating nanoparticles with pegylated molecules have shown to improve the anti-fouling effect, leading to the significant increase in blood circulation (36, 37), and sometimes the tumor accumulation by EPR effect (38, 39). However, there are still some debates about the tumor cell uptake and the strong liver accumulation of pegylated NPs. In addition, zwitterionic AuNCs may have better Tumor-to-Kidney ratio than pegylated AuNCs (18), which question about the most suitable surface chemistry on ultra-small particle such as AuNCs. In our case, both AuNCs presented similar Tumor/Muscle ratio justifying their use for image-guided surgery.

The optical properties of the AuNCs are due to their ultra-small size, and the nature of the coating ligand (23, 40, 41). Such properties might be tuned to be helpful for medical applications, in particular for image-guided surgery. In HNSCC, margin delineation impacts tumor recurrence and patients' quality of life (preservation of vital structures as nerves, swallowing and speech functions) (42). In this study, the AuNCs allowed to identify small remaining tumor lesions, after surgical tumor removal. However, in the NIR optical window, some parts of the cheek as the tendons are autofluorescent. Therefore, the optimization of the fluorescence quantum yield would facilitate the detection of the residues *versus* the nerves and tendons. In this study, both the two types of AuNCs selected showed a tumor-to-healthy tissue ratio >1.5 within the first hour after administration allowing a rapid, and prolonged tumor detection. The image-guided surgery was helpful for half of the mice (53% of the animals), allowing the detection and removal of undetected tumor residues. Such strategy increased the mean survival time of the animals of \approx 30/35% as compared to the control group. However, improvement of the AuNCs formulation might increase such favorable results (7), in particular with augmentation of the fluorescent quantum yield leading to higher sensitivity of detection, and tumor accumulation. Most of the AuNCs studied *in vivo* are stabilized by the peptide glutathione (11, 13, 17). These AuNCs exhibit an efficient renal clearance, but possess a short blood half-life (<6 min), preventing a strong and prolonged tumor uptake. We recently demonstrated how the selection of thioctic sulfobetaine ligand as stabilizers could slightly enhance AuNCs blood circulation time to 6.5 min, and increase tumor retention in glioblastoma-bearing mice (15, 16). Here, we moved to other constructs based on bidentate thiol ligand with charged pegylated moiety and hydrophobic zwitterionic moiety to prolong the AuNC's half-life, while keeping a high renal clearance accompanied by a favorable tumor accumulation and retention. From the 2 tested AuNCs, both presented similar properties in terms of absence of toxicity, cell uptake, and quantum yield. However the AuZwEt₂ presents a better circulation time, with a 2.6 extended AUC *versus* AuPegCOOH (1:3), leading to a favorable Tumor/Healthy tissue ratio.

To conclude, the protection of the gold core by small biocompatible ligands as zwitterionic or pegylated moieties helped to improve the optical properties of the AuNCs, and to prolong their plasma half-life while conserving their renal elimination. One of the major interests of AuNCs is their multiple uses for both optical imaging, enhanced radiation therapy (13, 35), similarly to larger Au nanoparticles, or photothermal therapy (43, 44). The combination of image-guided surgery with a curative modality could reinforce the treatment efficacy. Such combining modalities would be of great interest in the next development of the AuNCs for the therapeutic care of Head and Neck pathologies. A particular interest would be the development of SWIR emitted AuNCs with good quantum yield for better tumor to healthy tissue ratios.

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Disclosure

The authors declare no conflicts of interest.

Captions

Table 1: Main physicochemical characteristics of the AuNCs. The Zeta-potential was measured at pH 7, and the maximum emission was obtained for any excitation from 350 to 600 nm. *: The quantum yield as determined at the maximum emission wavelength using QD705 as reference. ^{a)} The average charge state Z_{average} , is calculated from the observed charge state distribution in ESI-MS spectra (plotted in m/z) using the formula: $Z_{\text{average}} = \frac{\sum N_i z_i}{\sum N_i}$ where N_i corresponds to the number of ions of charge state z_i (27).

Figure 1: Cell viability assay performed 72-hours after incubation with the NCs at 50 and 100 $\mu\text{g/mL}$ on HNSCC CAL-33 (A), and SQ20B (B). Staurosporine was used as a cell-death inducer (red control diagram). Results are expressed as the mean of cell viability (% vs. control) \pm standard deviation (SD).

Figure 2: NCs' uptake in CAL-33 cells after 8 hours of NCs exposure at 50 $\mu\text{g/mL}$. (A-C): Smooth histograms of the cells normalized to the control condition. The positive cells were gated with the marker M1, assuming that less than 1% of the control cells induce autofluorescence signal. The percentage of isolated cells positively stained for the NCs is presented in the table (D).

Figure 3: Distribution of the different AuNCs, 5 hours after intravenous administration in mice-bearing sub-cutaneous CAL-33 tumor. The results are expressed as the mean of the fluorescence intensity/pixel/100 ms of 3 mice/group, and the error bar represents the SD.

Figure 4: Blood PK of selected NCs after intravenous injection. The NCs concentrations were determined from the fluorescence intensity values of the plasmas collected just after the injection, and at 5, 10, 15, 30, 60, and 120 min, for AuZwEt₂ (1:5) (A), AuPegCOOH (1:3) (B), AuPegCOOH (1:5) (C), and Au₂₅SG₁₈ (D). Two PKs parameters are summarized in the table (E). DN AUC_{0-end}* corresponds to the area under the curve taken from the injection time to the end of blood plasma sampling, normalized to the dose of 1 mg of gold. DN AUC_{0-end}* is expressed as min*mg/mL. T_{1/2} is the blood plasma half-life expressed in minutes. The AuNCs were best fitted with two-phase decay equation, generating a fast and a slow half-lives (see also Table S2 with goodness of fit).

Figure 5: Kinetic of the tumor-to-skin ratios obtained from the optical analysis. (A): The Tumor/Skin ratios were measured at 0.5, 1, 2.5, and 5 hours post injection in mice-bearing orthotopic CAL-33 tumors. The Tumor/Skin ratios are expressed as the mean of the fluorescent ratios normalized to T=0 \pm SD (n=3/group). (B): Example of pictures obtained after administration of AuPegCOOH (1:3).

Figure 6: Illustration of the image-guided surgery principle. The mouse was injected with AuPegCOOH (1:3) 30 minutes before the fluorescence imaging (A). Then, the main tumor mass was removed (B), and the animal was imaged to check the presence of any tumor residue. In (C), the naturally fluorescent tendon is clearly seen (white arrow), and a tumor residue is also observed (yellow arrow, and (D). This residue presents tumor cells (yellow arrows). (E) Histology image of a representative removed residue.

The yellow arrows indicate the tumor areas. Confocal microscopy confirmed the presence of AuNCs in the outer (F) and in the center (G) of the tumor residue. Scale bar: 10 μm .

Figure 7: Image-guided surgery and animal survival. (A): The animals were split in 3 groups: without (control) and with image-guided surgery using AuPegCOOH and AuZwEt₂. The tumors were macroscopically removed and weighted. The results are presented as a box-plot with the median value in red. (B): Animal survival for control and AuNCs groups. (C): The survival time of the animals is expressed as the mean \pm the standard deviation in days, but also as the median survival time (MeST). The increase in life-span (ILS) is expressed as the percentage *versus* the life-span of control group. The number of mice with image-guided tumor residue removal is indicated, as the percentage of tumor positive residues.

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