



SECOND YEAR REPORT

- *PhD student Name and Surname:* Veronica Schifano
- *Cycle, starting-end dates:* 38th cycle, 2022-2025
- *Tutor, co-tutor(s):* prof. Daniela Maggioni, prof. Silvia Cauteruccio

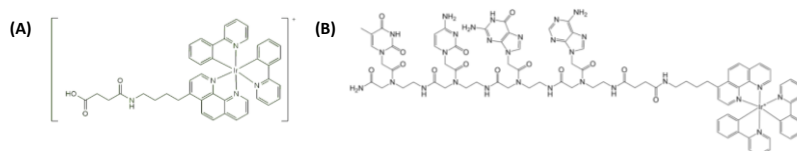
A. SCIENTIFIC REPORT

Title of the thesis

Preparation of molecular and nano-compounds triggered by light for biomedical applications

Description of the research work done during the second year (max 5 pages)

My research work during the 2nd year of my Doctorate was focused on four different projects, most of them still ongoing. The first consisted of the preparation of a conjugate of a tris-cyclometalated Ir(III) complex with a peptide nucleic acid (PNA) chain, in collaboration with Prof. Cauteruccio's group from the Department of Chemistry of the University of Milan, continuing the work started on my 1st year. The work aims to obtain a multifunctional compound with anticancer behaviour, based mainly on the Photodynamic Therapy (PDT) promoted by the Ir(III) complex, i.e. generation of singlet oxygen (¹O₂) when irradiated by a suitable light, combined with an antisense therapy given by specific sequences of PNAs, which can form a duplex with the RNA chain and interfering with RNA processing, transport or translation.



Scheme 1. Structure of the Ir-COOH complex (A) and of the Ir-PNA conjugate (B)

In particular, during the last year, we studied the interaction of the two compounds synthesized and characterized (i.e. the cyclometalated Ir(III) complex, **Ir-COOH**, and the Ir(III)-PNA conjugate, **Ir-PNA**, showed in **Scheme 1**) with HeLa cells, starting from their cellular uptake using confocal microscopy. The cell culture was carried out in collaboration with Prof. Rusmini from the Department of Pharmacological and Biomolecular Sciences of the University of Milan, while the microscopy measurements were carried out by Prof. L. D'Alfonso from the Department of Physics of the University of

Milano-Bicocca. The cells were treated and incubated for 21 h with a 25 μ M solution of both **Ir-COOH** and **Ir-PNA**, using methanol as a vehicle control. After the incubation, the compounds were removed and substituted with fresh medium. The confocal microscopy images were taken on live cells using a 720 nm pulsed laser, demonstrating the possibility to excite both compounds with a two-photon excitation. As shown in **Figure 1**, **Ir-COOH** and **Ir-PNA** have entered inside the cells, even if the behaviour of **Ir-PNA** is slightly different from the complex alone: **Ir-COOH** was homogeneously distributed inside the cytoplasm, even though it was not internalized by all the cells. On the other hand, the **Ir-PNA**'s uptake appeared to be more efficient, probably enhanced by its lipophilicity and its positive charge. Due to a limited solubility in water, inside the cells **Ir-PNA** tends to accumulate both at the cytoplasmatic and the nuclear level.

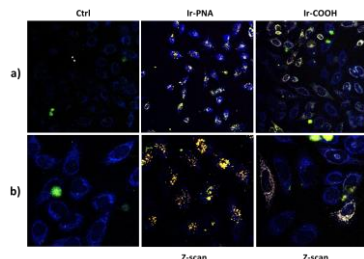


Figure 1. TPA Confocal microscopy images acquired at two different magnifications on HeLa cells treated with Ir-COOH and Ir-PNA



After confirming the success in cellular uptake, the capability of the two compounds to produce $^1\text{O}_2$ *in vitro* was tested on HeLa cells, in collaboration with Prof. Rusmini from the Department of Pharmacological and Biomolecular Sciences of the University of Milan. Two multiwell plates were prepared and incubated for 24 h with **Ir-COOH** and **Ir-PNA** in a 1–50 μM concentration range. After the incubation time, one was kept in the dark, while the other was irradiated for 30 min with a commercial UV lamp emitting in the 350–400 nm range with a total power of 5.2 mW/cm². During the irradiation, the temperature was monitored and kept around 37 °C to avoid the cytotoxic effect given by the overheating of the lamp. The compounds were removed from both the plates and the cells were kept in the dark for another 6 h; hence, the absorbance was measured at 570 nm and its mean relative values were calculated for each concentration. **Figure 2** visualizes the HeLa cell viability after these treatments, showing no toxicity of the compounds in the dark (cell viability >90%), except for the two higher concentrations of **Ir-COOH** (25 μM and 50 μM). On the other hand, after the irradiation both **Ir-COOH** and **Ir-PNA** presented cytotoxicity in a dose-dependent manner, proving their ability to produce $^1\text{O}_2$ *in vitro*. By fitting those data with a sigmoidal function, the EC₅₀ values (the concentration required to induce the death of 50% of the cells) are calculated in the dark and after irradiation. Dark EC₅₀ concentrations are higher than 50 μM for both compounds, while after irradiation they decrease to 18 μM for **Ir-PNA** and 3.5 μM for **Ir-COOH**. Since during my 1st year we had established a higher quantum yield for $^1\text{O}_2$ production (Φ_Δ) for **Ir-PNA** (0.55 for **Ir-PNA** compared to 0.44 for **Ir-COOH**), we attributed the lower *in vitro* photocytotoxicity of **Ir-PNA** with its tendency to form aggregates inside the cells, which might trap the $^1\text{O}_2$ produced and limit its efficiency in reaching target biomolecules. The dark and light EC₅₀ and Φ_Δ values are shown in the following table (**Table 1**), as well as the phototherapeutic index (PI), which is an expression of the cytotoxic activity after the light treatment.

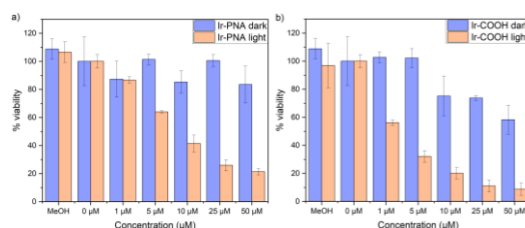


Figure 2. Cell viability calculated on HeLa cells untreated and treated with different amount of **Ir-PNA** (a) and **Ir-COOH** (b), both kept in the dark or after 30 min of irradiation

Table 1. Singlet oxygen quantum yield and cytotoxicity of **Ir-PNA** and **Ir-COOH** as calculated from a MTT test carried out on HeLa cells kept in the dark and treated with light

Species	Φ_Δ	Dark EC ₅₀	Light EC ₅₀	PI
Ir-PNA	0.55	> 50 μM	18 μM	> 2.8
Ir-COOH	0.44	> 50 μM	3.5 μM	> 14.3

Moving to the second project I've worked on, it consists of the decoration of Halloysite nanotubes (HNT) with gold nanostars (GNS). The final aim of this project is to obtain an HNT-GNS nanocomposite material with a therapeutic action based on the Photothermal Therapy (PTT) provided by GNS when irradiated with a suitable light. Moreover, we'd like to exploit HNTs as carriers for proper small molecules, through their loading inside HNT tubular structure and their release induced by the local hyperthermia generated during PTT. By choosing strategically those molecules, we'd like to exploit PDT using the same light source, whose efficiency might be enhanced by the GNS. The presence of gold could also catalyze the conversion of H_2O_2 , a species often present and produced in tumoral sites, to O_2 , to further increase PDT efficiency, even in anaerobic tumoral environments. Even though this project was started during my 1st year, we changed the synthetic approach for the preparation of the nanocomposite material, starting from the synthesis of GNS: initially, they were prepared without any capping agent because we wanted a gold surface free from anything that could interfere the linking to the HNT. Since the so-prepared nanostars presented a not well-defined shape as well as a tendency to aggregate, we decided to follow a literature procedure¹ which uses the surfactant Triton X-100 as a directing and capping agent (**GNS@TX100**).

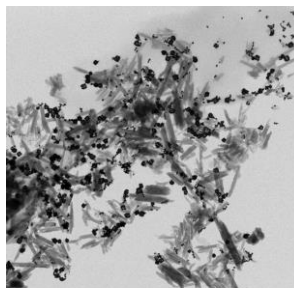


Figure 3. TEM image of **HNT-APTES-GNS@TX100** (scale bar: 1 μm)

¹ G. Dacarro et al. *Jour. Coll. Int. Sc.* 2017, 505, 1055-1064



We then changed the linker molecule between HNT and GNS, moving from the 3-aminopropyl ethoxy silane (APTES), at first used with “naked” GNS, to the PVP10 polymer, since APTES provided a too-weak interaction with **GNS@TX100**, as it is visible from the TEM image in **Figure 3**. The final synthetic path followed for the preparation of **HNT-PVP10-GNS** nanocomposite is schematized in **Scheme 2**. Non-commercial Australian HNTs have been used for the preparation of the nanocomposite, because of their high suspensibility properties and their little polydisperse length distribution. To functionalize the external surface of those HNTs with PVP, they were first treated with NaOH for 24 h at RT, washed with H₂O milliQ through centrifugation until pH 7 and dried under vacuum. Then, the HNTs exposing -OH groups were treated for 24 h at RT with the polymer dissolved in H₂O, again washed through centrifugation and dried under vacuum. The success of this functionalization step was ascertained with FTIR spectroscopy which showed the presence of the C=O stretching band.

Moving to the decoration of the so-functionalized HNTs with **GNS@TX100**, it was performed through their overnight interaction (about 17 h) at RT. The excess of TX-100, already present in solution and further released during the interaction, was washed with repeated centrifugation cycles. Two different HNT:Au mass ratios were tested, obtaining two final samples (10:1 and 5:1) which were characterized with TEM and photothermal measurements.

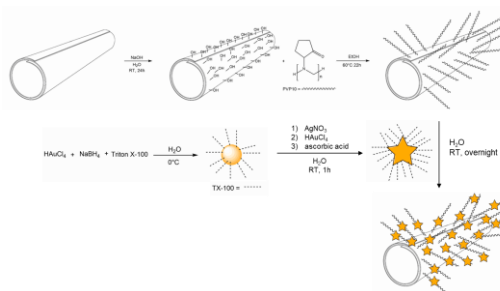
The TEM images (**Figure 4**) show an overall good homogeneity in HNT decoration, with several nanostars attached on their external surface. In particular, in the 5:1 HNT:Au mass ratio sample HNTs appear decorated with more GNS, as expected from the higher Au content, but less homogeneously distributed. Moreover, in both samples, GNS possess a well-defined star shape with a very minor number of aggregated nanostars. Furthermore, these nanocomposites were found to be stable after multiple centrifugations, overcoming the stability issues observed in the nanocomposite synthesized with the previous approach.

Another consideration that we made on **HNT-PVP10-GNS** prepared with this method concerns

the efficiency of the HNT-Au interaction. We calculated the expected amount of gold in the nanocomposite material starting from the literature yield for the synthesis of GNS (also experimentally verified) considering quantitative the interaction with HNT. Then, we compared this value with the amount of gold determined with ICP-OES in the samples.

The yield calculated for the HNT-GNS interaction showed to be very high, and equal to 75.5% for the 10:1 ratio sample and to 99.5% for the 5:1 ratio one. This finding states that the best HNT:Au mass ratio to perform this interaction is precisely 5:1. For the future, due to the impossibility of increasing the concentration of GNS colloidal suspensions, we could think to improve the HNT coverage by repeating the interaction using the pellet recovered after the first cycle and employing it in a new interaction cycle by using a new freshly prepared suspension of GNS.

Finally, the photothermal properties of the two samples were studied in collaboration with Prof. L. D'Alfonso from the Department of Physics at the University of Milano-Bicocca. The samples were deposited on a glass slide, dried in the air and irradiated for about 3 minutes with a monochromatic laser with 720 nm wavelength, near the maximum absorption of the nanocomposites determined with DRUV spectroscopy, and tunable power. **Figure 5** reports only the heating curves over time



Scheme 2. Synthetic path followed for the preparation of **HNT-PVP10-GNS** nanocomposite.

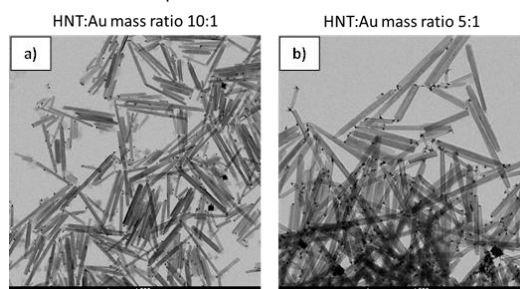


Figure 4. TEM images of HNT-PVP10-GNS samples prepared with 10:1 (a) and 5:1 (b) HNT: Au mass ratio (scale bar: 500 nm)

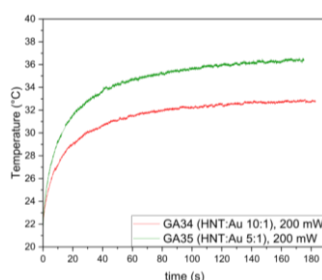


Figure 5. Heating curve over time of the 10:1 and 5:1 samples, irradiated with a 720 nm laser with 200 mW incident power

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recorded on the two samples using a laser power of 200 mW, as for the two employed higher power levels (300 mW and 400 mW), the behaviour of the two nanocomposites was analogous.

From these curves, the thermal capacity was calculated as the ratio between the total heat exchanged by the system and its total temperature variation: $C = \frac{5\tau P}{\Delta T}$

The total heat exchanged in this formula is given by the product between the incident power and the time required by the sample to reach the equilibrium temperature (5τ , where τ is a parameter determined from the heating curve and is defined as the characteristic heating time for the material)².

Moreover, to compare quantitatively the two thermal capacity values, they were normalized with the exact amount of gold contained by the two samples, which was determined using ICP-OES. This way, the specific heat capacity was obtained, and it is described by the following formula: $C_s = \frac{C}{m}$

In the following table (Table 2), the ΔT and τ values for the two samples at 200 mW power are reported, as well as the calculated thermal capacity, the gold amount in the samples and the specific heat capacity values.

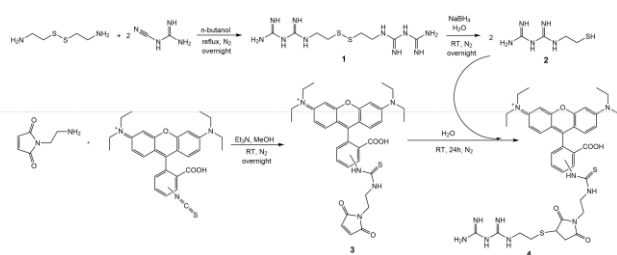
Table 2. Temperature variation, characteristic heating time and thermal capacity values for HNT: Au nanocomposites with a mass ratio of 10:1 and 5:1 determined and calculated from the heating curves

HNT: Au ratio w/w	ΔT at 200 mW	τ	Thermal Capacity	Au content	Specific Heat Capacity
10:1	11.1 °C	21.5 s	119 mJ/K	$9.81 \cdot 10^{-3}$ mg	$12.16 \cdot 10^3$ mJ/mg·K
5:1	14.8 °C	23.0 s	118 mJ/K	$2.09 \cdot 10^{-2}$ mg	$5.67 \cdot 10^3$ mJ/mg·K

The comparison between the two specific heat capacities gives an unexpected result hence we expected a direct correlation between this value and the gold content in the sample, while the specific heat capacity calculated for the 10:1 mass ratio sample was about 2 times higher than the 5:1 mass ratio. We tentatively explained this result considering the more homogeneous decoration of HNT for the 10:1 mass ratio sample as shown by TEM images and especially the more pronounced presence of small GNS aggregates, which make the adducts thermally less active at the same amount of gold content.

The next step will be the loading of a proper photosensitizing compound inside the inner lumen of HNTs to test if and how the efficiency in $^1\text{O}_2$ production is increased by the presence of GNS.

A third project I've worked on during this 2nd year consists of the preparation of a luminescent metformin derivative, to study how metformin interacts and inhibits tumoral cells. Metformin belongs to the family of biguanide molecules and is a widely used drug to treat type II diabetes since it can suppress hepatic glucose production and improve insulin sensitivity and glucose uptake by skeletal muscles. Recently, in patients treated with this drug, a lower incidence of various kinds of tumours has been recorded, suggesting a correlation between their growth and their proliferation and assumption of metformin. Anyhow, the mechanism in which it exerts this action is still uncertain and subjected to study, even if some hypotheses have been formulated. For example, the inhibition of mitochondrial complex I and the activation of an AMP-dependent kinase are two mechanisms suggested³. In this panorama, the aim of our work, which starts as a collaboration with Prof. Mazzanti from the Department of Biosciences of the University of Milan, is to link a biguanide moiety to a luminescent compound, to visualize and study how the molecule is internalized and interacts with various kinds of tumoral cells, exploiting the confocal microscopy technique.



Scheme 3. The comprehensive synthetic path followed to obtain the oxidized (1) and reduced (2) biguanide molecule, the rhodamine-maleimide adduct (3) and the final rhodamine-biguamide product (4)

² A. Pàsciak et al. *ACS Appl. Mater. Interfaces* 2021, 13, 44556-44567

³ Chen et al. *J. Pharm. Investig.* 2020, 50, 219

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In **Scheme 3** the synthetic path that we have followed to prepare this luminescent molecule is schematized. The strategy involved exploits the high reactivity of the SH group toward the double bond of a maleimide to link the luminescent fragment (rhodamine) with the metformin fragment. For this reason, a strategy to obtain a metformin derivative bearing a terminal SH group and a rhodamine fragment containing a maleimide group was designed. First of all, it should be noted that the reactivity of cyanoguanidine towards primary amines at high temperatures and in the presence of acid catalysis is high but, unfortunately, it is not limited to the $C\equiv N$ moiety and often byproducts deriving from the concomitant reactivity of an amine group of the guanide fragment is occurring. Hence, in principle, the right reaction temperature as well as the amount of H^+ employed should be carefully investigated. Our first goal was the preparation of the biguanide, through a reaction between the cystamine dihydrochloride and the 1-cyanoguanidine, followed by the reduction of the disulfuric bond with $NaBH_4$. Once this intermediate was obtained, the following step was its linkage to the luminescent rhodamine, appropriately functionalized with a maleimide group. All the synthetic steps have been carried out under an N_2 atmosphere and followed with NMR spectroscopy.

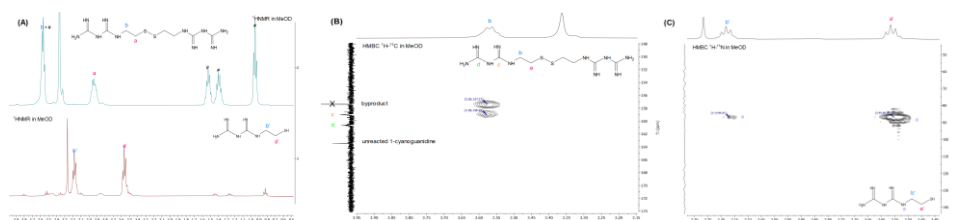


Figure 6. 1H NMR (A, blue trace) and HMBC 1H - ^{13}C (B) spectra of the oxidized biguanide (**product 1**), and 1H NMR (A, red trace) and HMBC 1H - ^{15}N (C) spectra of the reduced biguanide (**product 2**), registered in MeOD solution at RT

The reaction to prepare the oxidized biguanide was performed in a n-butanol solution at reflux. From the 1H and 1H - ^{13}C HMBC experiments (**Figure 6A**, blue trace, and **6B** respectively), we confirmed the success in the preparation of this molecule, from the correlation between one of the two protons assigned to the aliphatic CH_2 (placed at 3.58 ppm) and the carbon signal at 159.0 ppm, compatible with one of the two $C=NH$ groups of the biguanide. The reduction of the disulfuric bridge in compound **1** was carried out overnight at RT in water solution, through the addition of an excess of $NaBH_4$. The shift of the two aliphatic protons at higher fields confirmed the success of the reduction (**Figure 6A**, red trace), while the persistence of the correlation in the 1H - ^{13}C HMBC spectrum (**Figure 6B**), as well as the 1H - ^{15}N correlation between the two protons and a ^{15}N signal at 86.9 ppm, assigned to the biguanide, confirmed that the group remained unchanged after the reduction step (**Figure 6C**).

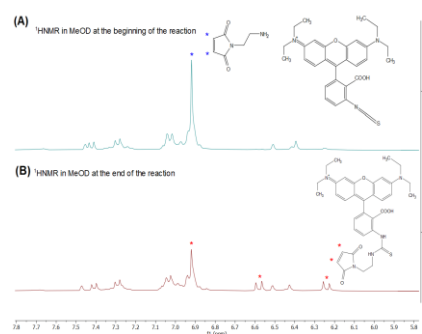


Figure 7. 1H NMR spectra of the functionalization of rhodamine isothiocyanate to obtain **product 3**, recorded at the beginning of the reaction (A) and after 28h (B) in MeOD solution at RT

The two last reactions of the synthetic path were the functionalization of the rhodamine isothiocyanate with a maleimide linker and, finally, the linking of the so-functionalized fluorophore with the reduced biguanide. The first step was carried out in methanol solution, for 28 h at RT, and followed through the decrease of the singlet at 6.92 ppm, assigned to the protons of the double $C=C$ bond of the starting aminobutyl maleimide molecule (**Figure 8A**), and the simultaneous appearance of two doublets at 6.58 and 6.24 ppm (**Figure 7B**), assigned to the same protons, which possibly, for steric hindrance that hamper the free rotations of the C-C and N-C bonds of the maleimide pendant, are no more magnetically equivalent.

Finally, an attempt to link the so-functionalized rhodamine the reduced biguanide was carried out. Despite the hints suggested by the NMR spectra on a positive outcome for this reaction, they were not confirmed by MS-ESI, that did not show the molecular peak of the desired product.

In the next future we will try again this reaction, changing some parameters as well as the solvent, trying to obtain a clear enough mass spectrum and, if needed, we will move to another luminescent compound in substitution of the rhodamine, such as a $Ru(II)$ complex. Then, we will purify the



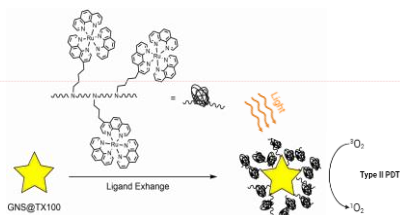
product and prof. Mazzanti's group will carry out the biological studies to investigate the antitumoral mechanisms of Metformin.

To conclude, the fourth and last project I've worked on consisted of the functionalization of GNS with a Ru(II)-poly(amidoamine) complex, namely **phenAN**⁴, to obtain a hybrid compound with an antitumoral action based on PDT, as schematized in **Scheme 4**. Studies on these types of complexes have already been conducted by our research group, showing a good uptake efficiency and intracellular organization in vesicles localized in the nucleus, together with a good efficiency in ¹O₂ production. As it has already been said, the presence of GNS might increase the capability of **Ru(II)-phenAN** to produce ¹O₂ through an energy transfer to the complex or directly to molecular oxygen, due to the Localized Surface Plasmonic Resonance (LSPR) typical of such nanostructures. Also, Au is involved in the photo-oxidation of H₂O₂ to O₂, providing a higher efficiency of the treatment also in anaerobic tumoral environments. In addition, we aim to exploit the plasmonic absorption band of GNS to excite these compounds in the biological transparent window by exploiting a deeper tissue penetration, and to combine PDT with PTT, giving rise to the formation of multifunctional therapeutic compounds.

During the past months I focused mainly on the preparation of the hybrid compound: starting from the GNS@TX-100, prepared following the same literature synthesis already described above, and the Ru(II)-phenAN polymer (**Figure 8A**), synthesized in collaboration with Prof. Ranucci from the Department of Chemistry of the University of Milan, different approaches have been attempted.

The first consisted of a ligand exchange between the Triton X-100 and the complex, exploiting the interaction between the gold surface and the carbonyl groups of the polymer. The 50:1 Au:polymer molar ratio was tested at first, through a 5 h interaction at RT and washing the excess of TX-100 by successive centrifugations. Neither the pellet nor the supernatant showed any luminescence, suggesting that the polymer was linked to the gold surface and not dissolved in the supernatant, but since the GNS did not resuspend easily in water, we hypothesized that a larger amount of polymer was needed to stabilize the GNS.

To obtain a better stabilization, two higher quantities of polymer were then tested, following the same procedure but with a longer interaction time (17 h): 5:1 and 1:2 Au:polymer molar ratios, corresponding to a 3.5 and 34.7 polymer mass excess, respectively. This time the GNS resulted stabilized enough in both samples, while only in the supernatant of the 1:2 ratio sample an excess of the complex was detected. Moreover, we carried out photophysical measurements on these two last samples, in which no fluorescence has been detected, suggesting the quenching of the complex luminescence by GNS. Because of the dependence of this quenching both on the superposition of the plasmonic band of GNS and the emission wavelength of the complex and the distance between the two components, we tried to increase their distance by functionalizing the double bonds at the ends of the polymer with cystamine, to expose amino groups and set a stronger interaction with gold (**Figure 8B**). Lastly, the so-functionalized complex was used to decorate the GNS, in a 30-times excess compared to gold mass, following again the same interaction procedure as before. The obtained **GNS-Ru(II)-phenAN** again did not show any luminescence under the UV lamp. Nevertheless, in the next future, we will perform some tests to see if the complex luminescence can be restored once its interaction with GNS is disrupted, potentially exploiting the local hyperthermia produced by GNS when irradiated. Alternatively, we will test the efficacy in the cleavage of the Au-polymer interaction based on a redox reaction exploiting a mild reducing agent such as dithiothreitol (DTT) as a mimic of glutathione GSH, by following a possible recovery of the emission of Ru moiety.



Scheme 4. Schematic representation of the GNS-Ru-phenAN hybrid compound, and of its use in PDT for anticancer treatment

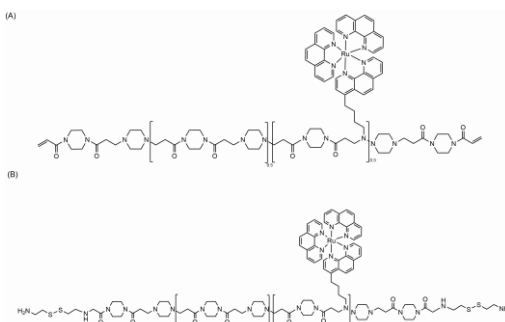


Figure 8. Structure of the Ru-phenAN complex (A) and of Ru-phenAN functionalized with cystamine (B)

⁴ L. Mascheroni et al. *Inorg. Chem.* 2019, 58, 21, 14586-14599



Publications

- Miriam Parmigiani, Veronica Schifano, Angelo Taglietti, Pietro Galinetto, Benedetta Albini. Increasing gold nanostars SERS response with silver shells: a surface-based seed-growth approach. *Nanotechnology*, 2024, 35, 195603
- Hady Hamza, Veronica Schifano, Marco Aldo Ortenzi, Gabriele Di Carlo, Francesca Tessore, Daniela Maggioni. Halloysite nanotubes as a vector for apolar perfluorinated porphyrin-based photosensitizers for singlet oxygen generation. *Materials Today Chemistry* (*submitted*)
- Veronica Schifano, Rosa Maria Dell'Acqua, Maria Vittoria Dozzi, Monica Panigati, Paola Rusmini, Angelo Poletti, Silvia Cauteruccio, Daniela Maggioni. Synthesis and Characterization of an Ir(III)-Peptide Nucleic Acid (PNA) conjugate as a Singlet Oxygen Photosensitizer for Photodynamic Therapy (*draft in preparation*)

Communications (poster and oral)

- Poster: "Preparation and characterization of a PNA-Ir(III) conjugate for $^1\text{O}_2$ production in PDT", 17th European Biological Inorganic Chemistry Conference (EuroBIC-17)

B. OTHER ACTIVITIES DONE DURING THE SECOND YEAR

List of attended courses (title of the course and name of the teacher)

- "Youth and Enterprise" prof. Dominique Roberto
- "Instrumental methods for the analysis of materials and of surfaces: molecular spectroscopy, optical microscopy, X-ray fluorescence and scanning electron microscopy" prof. Vittoria Guglielmi

Attended Schools, Congresses and Seminars

- 17th European Biological Inorganic Chemistry Conference (EuroBIC-17), Münster (Germany), 25th-29th August 2024
- "Chemist's Interactions" seminars as requested and other seminars offered by the Department of Chemistry

Assistance, tutoring

- Tutoring activity for the "Chimica generale e inorganica" course, Bachelor course in Biotechnology, Department of Pharmaceutical and Biomolecular Sciences (32 hours)
- Tutoring activity for the "Metodi chimici per le biotecnologie" course, Bachelor course in Biotechnology, Department of Pharmaceutical and Biomolecular Sciences (24 hours)

"Orientamento" PLS activity: Diffusion and promotion of chemistry and industrial chemistry activities within non-academic communities

- "Laboratorio di orientamento alla chimica – analizziamo l'acqua" prof. Daniela Maggioni and prof. Francesca Tessore (2 hours)

Period in other university, research center or industry

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UNIVERSITÀ DEGLI STUDI DI MILANO

Ph.D. COURSE IN
INDUSTRIAL CHEMISTRY

Date 13/09/2024

Signature Veronica Schifano