

Universidade do Minho
Escola de Ciências

Ânia Barata Micaelo

**Bionanostructures for intracellular
temperature sensing during
photothermal cancer treatment**

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photothermal cancer treatment**

Master thesis

Master in Biophysics and Bionanosystems

Work developed under the supervision of

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Universidade do Minho

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration. I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Bionanoestruturas para medição de temperatura intracelular durante terapia fototermal contra o cancro

Resumo

O cancro é uma das causas de morte mais comuns em países desenvolvidos, com tratamentos agressivos e de fraca eficácia, agravando-se pela inexistência de uma cura universal para todos os tipos de tumores. A hipertermia surge como um potencial tratamento complementar, ao debilitar as células cancerígenas e aumentando a sua suscetibilidade a outros tratamentos como quimioterapia e radioterapia, permitindo uma maior eficácia na sua aplicação, reduzindo a exposição a tais tratamentos, causadores de inúmeros indesejados e danosos efeitos secundários.

O objetivo deste estudo foi desenvolver e caracterizar nanohíbridos multifuncionais para terapias fototermiais, com capacidades simultâneas de aquecimento e medição de temperatura celular. A geração de calor é efetuada pela excitação de plasmões de superfície localizados de nanobastões de ouro integrados numa capa mesoporosa de sílica, enquanto a leitura da temperatura é executada pelas moléculas fluorescentes conjugadas de Rodamina B, que apresentam um tempo de vida de fluorescência dependente da temperatura, que decresce à medida que a temperatura sobe. Ao aplicar estes nanohíbridos a células tumorais e irradiar os mesmos com um laser de 808 nm (capaz de atravessar tecidos), o intuito será gerar calor e debilitar as células, enquanto a leitura da temperatura atingida dentro das células nas localizações dos nanohíbridos marcados é feita através de microscopia de imagens de tempo de vida de fluorescência. A leitura da temperatura permitirá a otimização das condições para atingir a temperatura intracelular ideal sem causar danos nos tecidos circundantes.

Neste trabalho, os nanohíbridos foram sintetizados e caracterizados com êxito, enquanto a sua biocompatibilidade foi estudada em duas linhas celulares (células A431 e HeLa). Além disso, o aquecimento fototermal com nanotermómetro integrado foi estabelecido, ao medir a eficácia de aquecimento e a curva de calibração do tempo de vida de fluorescência dependente da temperatura. Adicionalmente, o nanohíbrido foi aplicado no estudo de mapas de temperatura resolvidos no espaço, em células vivas sujeitas a tratamento fototermal, exibindo uma sensibilidade de cerca de $0.23\% \text{ }^{\circ}\text{C}^{-1}$. Porém, a robustez dos nanohíbridos sob mudanças noutros fatores ambientais (ex.: pH) é desconhecida, precisando de estudos e aperfeiçoamentos.

Palavras-chave: Nanohíbrido; Ouro; Rodamina B; Temperatura; Tempo de vida de fluorescência.

Bionanostructures for intracellular temperature sensing during photothermal cancer treatment

Abstract

Cancer is one of the most common causes of death in developed countries, with aggressive and often poor treatment efficacy, while also lacking a universal cure suited for every type of tumor. Hyperthermia is presented as a potential complementary treatment option, by debilitating the cancer cells and increasing their susceptibility to other treatments, such as chemotherapy and radiotherapy, allowing for improved effectiveness on their application, by reducing exposure to such treatments that cause numerous undesired and harmful side-effects.

The aim of this study was to develop and characterize multifunctional nanohybrids for photothermal therapies, with simultaneous heating and intracellular temperature-sensing capabilities. The heat generation shall be achieved by excitation of localized surface plasmons of gold nanorods embedded in a mesoporous silica shell, while the temperature readout shall be attained by conjugated fluorescent Rhodamine B molecules, which present a temperature-dependent fluorescence lifetime that decreases as the temperature increases. By applying these nanohybrids to tumor cells and irradiating them with a 808 nm laser (capable of tissue penetration), it is intended to generate heat and weaken cells, while fluorescence lifetime imaging microscopy is used to assess the temperature achieved inside of the cells in the locations of the labeled nanohybrids. The temperature reading shall allow optimization of the conditions to achieve the desired level of intracellular temperature without damaging surrounding tissues.

In this work, these nanohybrids were successfully synthesized and characterized, as well as assessing their biocompatibility for two cell lines (A431 and HeLa cells). Furthermore, the photothermal heating with integrated nanothermometer was established by recording heating efficacy and the temperature dependent fluorescence lifetime calibration curve. Additionally, the nanohybrid was applied to study the spatially resolved temperature maps in photothermally treated live cells, with a sensitivity shown to be around $0.23\% \text{ }^{\circ}\text{C}^{-1}$. However, the nanohybrids' robustness when facing changes in other environmental factors (e.g. pH) is unknown, requiring further studies and improvements.

Keywords: Fluorescence Lifetime; Gold; Nanohybrid; Rhodamine B; Temperature.

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A – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected. . 111

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A – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected. . 111

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A – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected. . 112

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A – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected. . 112

Abbreviations

APD – Avalanche Photodiode

C12EDMAB – Dodecylethyldimethylammonium Bromide

CTAB – Cetyltrimethylammonium Bromide

DLS – Dynamic Light Scattering

DMEM – Dulbecco's Modified Eagle Medium

DNA – Deoxyribonucleic Acid

EGFR – Epithelial Growth Factor Receptor

EPR – Enhanced Permeability and Retention

FBS – Fetal Bovine Serum

FLIM – Fluorescence-Lifetime Imaging Microscopy

FPA – Fluorescence Polarization Anisotropy

FWHM – Full Width Half Maximum

GFPs – Green Fluorescent Proteins

HPVs – Human Papillomaviruses

HTLV-1 – Human T-lymphotropic virus 1

INL – International Iberian Nanotechnology Laboratory

IRF – Instrument Responsese Function

KSHV – Kaposi Sarcoma-Associated Herpesvirus

LSPR – Localized Surface Plasmon Resonance

MCPyV – Merkel Cell Polyomavirus

MEM – Minimum Essential Medium

MSN – Mesoporous Silica Nanoshell

N. A. – Numerical Aperture

NBD – Nitrobenzoxadiazole

NIR – Near infra-red

PBS – Phosphate Buffer Solution

PEG – Polyethylene Glycol

PTAs – Photothermal Conversion Agents

PTT – Photothermal therapy

RhB – Rhodamine B

RhBITC – Rhodamine B isothiocyanate

RNA – Ribonucleic Acid

SPC – Single Photon Counting

TCSPC – Time Correlated Single Photon Counting

TEM – Transmission Electron Microscopy

TEOS – Tetraethyl Orthosilicate

UBNP – Ultrafast and Bio- Nanophotonics

W. D. – Working Distance

Chapter 1 - Motivation and objectives

Modern nanotechnology emerged after Feynman's talk in 1959 "There's Plenty of Room at the Bottom: An Invitation to Enter a New Field of Physics" (1). He described a novel investigation area, based on manipulating and controlling things at an atomic level, something unknown at the time and that would eventually gain massive importance on the world.

Nanotechnology has then made way into our everyday lives, with applications in the medical field, agriculture and food industries (2), environmental concerns (3), cosmetics (4), electronics (5), among many others (6). In relation with this project, it relies on applying nanotechnology to the medical field, where many ground-breaking accomplishments have taken place already. These include (but are not exclusive to) the development of targeted drug-delivery, diagnostics involving the development of bio-sensors, improved bio-imaging, genetic therapy, or a conjugation of many of these applications (7).

For this work, the main goal was to synthesize silica-shell gold nanorods and modify their surface with Rhodamine B isothiocyanate (RhBITC), in order to transform them into a temperature generating/fluorescence detection hybrid. While this hybrid may have many future uses, this project was focused on studying its effects on cancerous cells, and due to their heat generation, the cells would become more sensitive to other kinds of treatment and are destroyed more easily, adjusting the approaches and preventing doing harm to surrounding healthy cells.

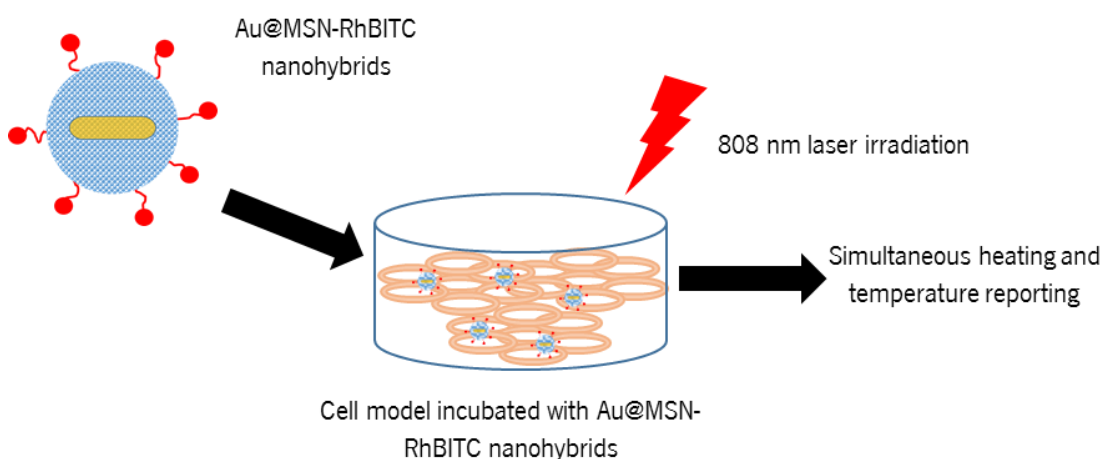


Figure 1 – Representation of cellular heating after Au@MSN-RhBITC nanohybrids internalization.

This Master's thesis is divided into five chapters, according to different aspects of each project's part. This thesis will start with a small introduction to nanotechnology, as well as the objectives and thesis organization (**Chapter 1 - Motivation and objectives**), followed by a brief theoretical overview (**Chapter 2 - Introduction**), on several topics. Firstly cancer, since it was the derivation of the cells used on the practical work, to contextualize the situations in which these hybrids are thought to be useful and give a summary of similar approaches in nanotechnology. Following that, a more detailed insight on both thermal therapies for cancer and on the hybrids' different constituents will be given. **Chapter 3 - Materials and Methods** will be used to describe all the materials and methods used in this work. Lastly, the results obtained for both characterization and *in vitro* assays will be presented (**Chapter 4 - Results and discussion**), with focus on their photothermal conversion, biocompatibility and fluorescence lifetime conversion, concluding with some future perspectives for the usage of these hybrids (**Chapter 5 - Conclusion and future perspectives**). At the end are all the references used to support this work, as well as some **Annexes** comprising of more detailed results.

Chapter 2 - Introduction

2.1. Cancer, research and therapeutic approaches

2.1.1. Cancer as a worldwide disease

Cancer is currently one of the leading causes of death in developed countries, with predictions from the Global Cancer Observatory (8,9) that its incidence will keep on rising (Figure 2), as life expectancy increases and new treatments arise for other diseases, as well as prevention measures becoming more disseminated (10). Cancer is no exception, although the task gets more intricate since there is no universal cure for every type of cancer.

Estimated number of incident cases from 2018 to 2040, all cancers, males and females, all ages

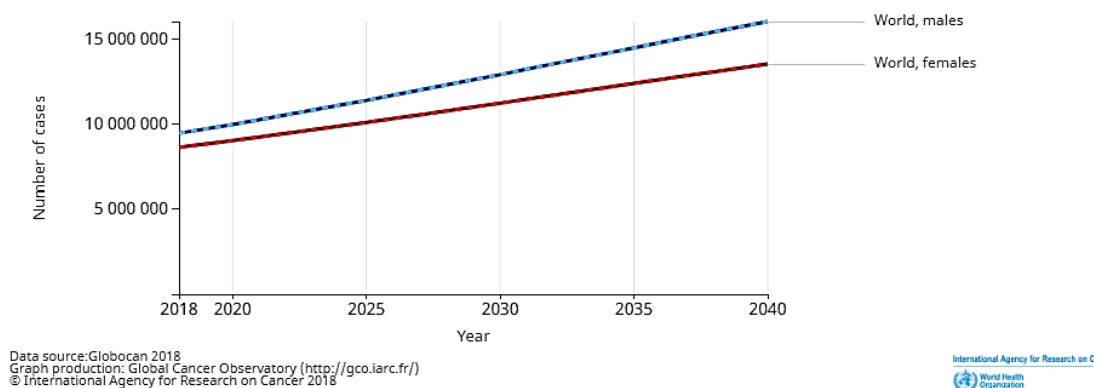


Figure 2 – Estimated number of incident cases from 2018 to 2040, all cancers, males and females, all ages (adapted from the information gathered by Ferlay et al. (8) for the Global Cancer Observatory with a model based on a publication by Bray et al. (9)).

Cancer can be caused by a number of factors, both preventable and non-preventable, and are usually driven by a combination of both kinds. The preventable types of factors are caused by external conditions, such as smoking, excessive drinking, poor diet and/or obesity, pollution, radiation or chemical exposure, infections by viruses (HPVs, HTLV-1, hepatitis B and C, Epstein-Barr, KSHV and MCPyV (11)) (12). In terms of non-preventable types, these are caused by environmental and/or genetic factors of the individual. In this case, it is recommended frequent

and early screenings, in order to detect cancer as soon as possible to increase the chances of successful treatment.

Cancer is seen as an epidemic, mainly due to the number of cases related to the preventable factors (13), and can also be affected by the development level of a country. The great majority of cancers have a higher incidence in developed countries (14), but this may not reflect the true comparison between development levels, due to a number of reasons: less developed countries offer less to none opportunities of cancer screening; preventable infections are less acknowledged and/or there are less resources to halt their dissemination/progression; their health records are not as accurate; other factors that can contribute to an incorrect comparison of the incidences around the world (10).

Not only is it of interest to study the causes and evolution' predictions for cancer, but it is crucial to understand the biological mechanisms within. Cancer emerges after the origination of carcinogenesis, which is a process where normal cells are transformed into cancerous cells, after an unexpected chromosomal change takes place, either caused by external or internal factors. This alteration is then passed on as the now-cancerous cell divides, creating a cellular aggregate with unfit functions that overwhelm the resources meant for healthy cells, destroying them and spreading through the organism in order to keep unmaintainable cells alive (15).

Taking into account the present and future predictions for the state of cancer incidence across the world, it is imperative to keep studying the risk factors, and find solutions to adjust the treatments and develop new therapies.

2.1.2. Cancer diagnosis and therapeutics

According to the Portuguese Cancer League (16), frequent cancer diagnosis methodologies encompass biological fluids' (blood, urine, etc.) analysis, biopsies or imaging, the latter which can be done by several techniques, like X-rays, computerized axial tomography, ultrasounds, magnetic resonance imaging, positron emission tomography, among many others. Most of the methodologies presented are considered to be invasive and must be done in a complementary basis, as many of them alone are inconclusive and not enough to support the existence of cancer. It has been of interest to upgrade the existing approaches of diagnosis, as well as developing new

ones, in order to minimize the invasiveness and optimize the sensitivity of the methods applied to each patient.

A similar scientific effort is being done to be applied to cancer therapeutics, who currently still present many harmful side effects. Therapies that are already in use in Portugal include surgical excision, radiotherapy, chemotherapy, immunotherapy, hormonal therapies or stem-cell transplant (17). These mostly aim for cancer cure, but many times are utilized to reduce symptoms and stop cancer propagation, which can be achieved by employing only one approach, or several in a combined manner.

Chemotherapeutic agents are usually designed to affect deoxyribonucleic acid (DNA) replication or protein synthesis, effectively stopping the tumor cells from dividing, which are usually done in a few high-dose applications, but can also be of slow nature with lower-doses (18). Chemotherapy presents serious disadvantages, mainly drug resistance by tumors cells, rendering the treatment ineffective after continued application, or even resistance from the beginning in some cells' types (18,19). Apart from cell resistance to chemotherapeutic drugs, Nygren (19) also refers poor vascularization of the tumor (translating into tumors lacking drug exposure) and increased drug metabolism (meaning low drug exposure overall) to be pitfall factors for chemotherapy.

Radiotherapy has similar aims as chemotherapy, being used to disrupt DNA of cells and preventing their proliferation as well. However, radiotherapy relies on the application of high-energy radiation (X-rays, gamma rays or electron/proton/neutron beams) instead of applying chemical-based options. Radiation can act directly on DNA, by damaging the strands, or indirectly, by damaging the strands only after creating free oxygen radicals by ionizing or exciting the water in the cells. Radiotherapy is a very important modality for cancer treatment, but the high toxicity and the distress on healthy cells' associated to it has urged improvements that can decrease such toxicity, mainly by studying methods that require less radiation to be applied in each round (20).

Other treatment options include removal of the tumor by surgery, which can only be done in localized tumors, immunotherapy, where the patient's own immune system is stimulated to eliminate cancer cells (which unfortunately only works in a subset of cancers and in a fraction of patients) (21), hormonal therapies, mainly used for prostate and breast cancer treatment. In these kinds of cancers, endocrine therapy works by suppressing hormones related to the progress of cancer, mostly estrogens and testosterone (22,23), but the side effects associated to this treatment (22,24), as well as acquired-resistance (22) and low application on other types of cancer still hinder

the widespread use of endocrine therapies. Lastly, another kind of therapy currently in use in Portugal is based on stem-cell transplant, where after destroying the patient's diseased and also healthy blood cells on the bone marrow, stem-cells are ministered to promote the development of healthy cells to replace the destroyed ones. This treatment is very physically and psychologically demanding on the patient, and as with any transplant, it may occur rejection or infections, among other consequences, that render the transplant unsuccessful (17,25). Besides specific side effects and difficulties for each treatment, they are all mostly short-term solutions, with the re-occurrence of cancer being an extremely likely scenario in the upcoming years after diagnosis.

2.1.2.1. Ongoing research to develop novel diagnostics and therapies

Since these conventional therapies still present severe pitfalls in their application, it has been a long-lasting challenge for the scientific community to overcome them. Many new solutions are rising every day, based on genetic therapy, microbiology, nanotechnology, among many others, but it is still lacking a solution that can be diverse to be a therapy in many cancer types, while simultaneously precise enough to deliver a specific treatment for each type.

Nanotechnology has been regarded as a possible resolution for that seeming illogicality, as nanotechnology-based solutions can be custom developed for specific purposes, promising more efficient targeting for certain types of cancer, not only as a diagnosis, but frequently as a physical and/or chemical-based therapeutic solution as well (controlled drug release, thermal ablation, photodynamic therapy, gene therapy, among others) (26). Nanomedicine-based cancer therapies benefit from the enhanced permeability and retention (EPR) effect of immune cells in tumor microenvironment, as they control the accumulation, retention and distribution within the tumor of the nanoparticles applied (27). Currently, a few nanotechnology-based solutions are already on the market, mainly based on liposomes, polymeric micelles, nanospheres, nanocapsules and nanotubes (28).

2.2. Hyperthermia-based therapies

2.2.1. Hyperthermia as a cancer therapy

Hyperthermia is characterized as being the condition in which the body temperature has been raised above 38.2°C, as the normal temperature has been defined as being in the 37°C - 38°C range (29,30). Differences between a healthy person's body temperature and a diseased individual had been reported (31) prior to Coley's experimentations (32), where hyperthermia was used as a cancer therapy for the first time, to promote the regression of sarcoma after inoculation with *Streptococcus erysipelatis*, provoking fever and thus releasing an anti-tumor response. While hyperthermia may have positive effects in many diseases, including cancer, as per instance provoking apoptosis in cancer cells (by proteins denaturation and/or formation of oxygen reactive species), acting alongside chemotherapy (increasing blood flow to enhance drug delivery and immune cell migration) or radiotherapy (increasing sensibility to X-rays) (33), among others, it can also have some harmful consequences. Hyperthermia may be the cause of neurological, cognitive and physical disorders, such as seizures, unconsciousness, memory, comprehension issues (34), arrhythmias, sweating, hypertension (35) and many others.

Hyperthermia may be of natural body response, in other words, fever (associated with inflammation, sepsis, drug-induced, endocrine factors or derived from brain injury (29)), or promoted by external elements. In the scope of this project, only external factors-mediated and localized hyperthermia is of interest, instead of the natural occurring type. This form of hyperthermia may then be directed to the tumor by radiation, only reaching the most superficial tumors, or reach the most deep located ones by inserting an electrode (36). When conjugated, the heat generated from the electrode forms a barrier between healthy and burnt tissue, which can prevent the propagation of the radio frequency (37). Besides these two most common methods, innovative solutions are being tested to induce hyperthermia, like ultrasounds, magnetic fields, radiofrequency and light-based processes, being the latter the most important for this project, as it is safer, less invasive, more profitable and with least side effects (38).

As a cancer approach, hyperthermia has many advantages, starting with the fact that abnormal cells cannot tolerate higher temperatures for as long as healthy cells, which makes them a good target for therapy (39). Next, hyperthermia can also be used to increase oxygenation, pH variation,

tissue elasticity and ion diffusion, which can help in pain relief (33). Finally, hyperthermia does show apoptotic properties, but is not recommended to be used for that sole purpose, as it may affect surrounding healthy cells, if the temperature reached becomes excessively high (33,40), and it is best to combine it with more conventional therapies. Hyperthermia becomes then a secondary solution, with an effort to increase the efficiency of the primary approach.

In terms of nanotechnology applied to hyperthermia treatments, it can make use of magnetothermal or photothermal nanoparticles (33), the latter being the focus of this project.

2.2.2. Photothermal nanotechnology-based therapies

Photothermal therapies (PTT) are based on the use of a photoabsorbing agent, also known as photothermal conversion agents (PTAs) (41) capable of generating heat from light (42). This agent must absorb light in the near infra-red region (NIR), as it is the optimal wavelength absorption region by the human tissues (43), further explained in 2.2.3. This procedure is used predominantly in cancer research, where several types of PTAs have already been under study. These must be biocompatible, nontoxic, allow easy surface modification and have good photothermal conversion efficiency as well as stability (41). Gold is one of the most common materials for PTAs, due to its surface plasmon resonance at specific photo-frequencies (taking shapes as nanorods, nanocages, spheres, nanoshells, nanoflowers or nanorings) (44–46), but other commonly-used materials include carbon (in its graphite-related structures like graphene or nanotubes with single or multi-walls), rare earth doped nanocrystals (33) or organic-derived (e.g. sodium humate) (41), among many others.

The PTAs are frequently used for cancer research, but they may have other types of applications. Hu *et al.* (47) studied PTT as a bactericidal approach, employing gold-covered nanocapsules to apply radiation and reduce the dosage of drug to eliminate *E. coli* BL21 in a combined and synergistic manner. This successful experiment may allow the decrease in use of antibiotics, decreasing the appearance of antibiotic-resistant organisms as well. Another study was performed by Beytollahi *et al.* (48), where PTT was used to destroy biofilm provoked by *Streptococcus mutans*. This bacterium is responsible for dental caries, and by using PTT mediated by indocyanine green under a diode laser, the appearance of colonies was significantly decreased, making it a suitable alternative to control caries. In a very different manner from the previous

examples, Maitland *et al.* (49) characterized the photothermal properties of shape-memory polymer micro-actuators in an effort to provide an alternative for treating stroke. This was eventually achieved by Small IV *et al.* (50), using a device based on Maitland *et al.*'s work (49) that would retrieve the blood clot by changing its shape after heating. The polymer would suffer 810 nm laser irradiation and with the increase in temperature, change its shape to a corkscrew, capturing the blood clot and restoring blood flow.

PTT presents many advantages to cancer treatment when compared to traditional approaches, being less invasive, and dealing a smaller amount of damage to the surrounding healthy cells, as it can have higher selectivity by doing specific targeting (51). PTT also does not require oxygen singlet formation (which is the case for photodynamic therapy), and by making use of a long and less energetic wavelength irradiation, it is safer for cell and tissue application (52).

Many challenges still surround these approaches, such as low photothermal conversion efficiency, reduced stability of the photoabsorbing agents, poor cellular uptake, tumor resistance of the photothermal treatment or even metastasis (41), as well as over-heating, which can be prevented by conjugating PTT in a co-therapy system (53).

2.2.3. Near-Infrared (NIR) laser and its interaction with biological tissue

Near-infrared laser radiation is used in photothermal therapy due to its tissue penetrating properties, but also for being absorbed less than other types of radiation (54). Human tissue has two biological windows for irradiation, due to several absorption bands provoked by the tissues' different constituents. The first biological window goes from 700 – 980 nm, starting in the absorption band of hemoglobin and ending at the absorption band of water. The second biological window encompasses 1000 - 1400 nm, in which the limits both refer to water absorption bands (33). The use of NIR radiation at 808 nm falls into the first biological window absorption, which makes it ideal to be absorbed by the skin, as well as to generate heat from the gold nanorods when irradiated (55,56).

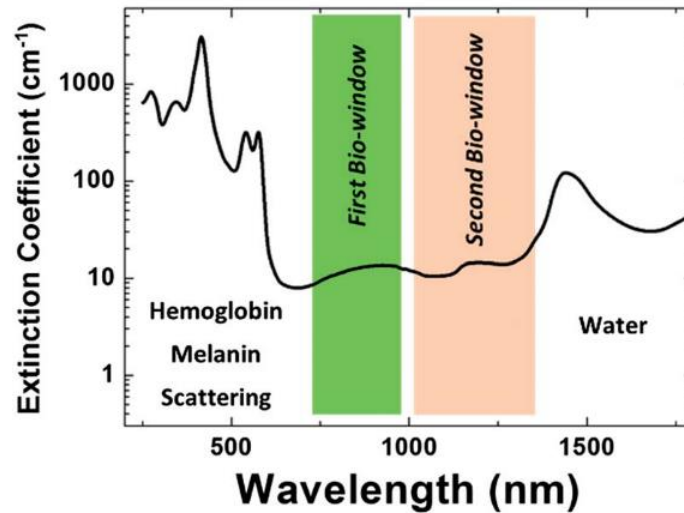
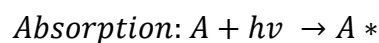


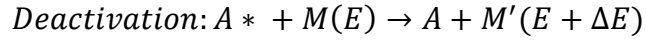
Figure 3 – Extinction coefficient of a representative tissue, along with indication of spectral extensions of the two biological windows (Adapted from Jaque et al. (33)).

Boulnois has stated (57) four types of interaction between light and tissue: photochemical, photoablative, electromechanical and thermal. Photochemical interaction refers to long exposures of light at low power densities, absorbed by molecules in the tissue, which can be naturally present or posteriorly added, resulting on the foundation of photodynamic therapy (57,58). Photoablative interaction consists on cutting tissue using ultra-violet light irradiation, where photons cause dissociation of biopolymers without necrosis and minimal thermal interaction (57). Electromechanical light/tissue interaction is caused when a high energy short term light pulse creates a local high electric field, which causes dielectric breakdown of the tissue, as well as forming microplasma¹, creating a shock wave when it expands (57,58). Lastly, Boulnois explains thermal light/tissue interaction, which is the most relevant in the scope of this project, and also the most frequent in surgical application of lasers. Attained by focusing a high energy density laser beam into a small area, the heat can remove tissue, injure or control bleeding. To comprehend how light is transformed into heat, Boulnois (57) summarizes it in the following reactions: Equation 1 that indicates the conversion of light into heat, denoting its absorption and Equation 2 that indicates the conversion of light into heat, denoting its deactivation.



Equation 1

¹ Boulnois defines “microplasma” as an ionized volume with a very large free electron density (57).



Equation 2

After light irradiation, a molecule that Boulnois designated as “A” absorbs a photon of energy, being promoted to a vibrational excited state, labeled as “A*”. After colliding with a collisional partner that belongs to the surrounding medium (designated “M”), the internal energy of a “A*” is transferred to “M”, increasing its kinetic energy (transforming “M” into “M’”), which is released under the form of heat, increasing the temperature. Boulnois also refers that the penetration depth depends on the wavelength applied and different type of tissue that will undergo irradiation, being essential to differentiate and select the outcome intended (57).

Though seemingly simple at the molecular scale, tissue irradiation with light is in fact a much more complex process, as multiple light scattering must always be considered as well (58). There are a few models that study the distribution of optical radiation through the tissues, such as the Kubelka-Munk, the Monte-Carlo and the diffusion model (59).

In terms of cellular optics, Gazdaru *et al.* (60) describe cells as having an anisotropic absorbing media, with a refractive index greater than that of air, which is responsible for partial radiation reflection and for radiation refraction into tissues. Inside cells, the organelles like mitochondria or collagen fibers are the main scatterers of laser radiations, although other organelles (Golgi apparatus, lysosomes and ribosomes, among others) may also be responsible for some scattering. Furthermore, Gazdaru *et al.* (60) refer different kinds of scattering made by cells components, being either elastic or inelastic. The former may be submitted to either Mie’s (when the dimension of the scattering particles is comparable to the wavelength of the radiations e.g. mitochondria) or Rayleigh’s (dependent on: incident wavelength; dimension of scattering centers; and on the differences between the refractive indexes/cytosol. It’s present in collagen fibers) theory, while the latter is much weaker in cells and tissues, being either of Raman or Brillouin scattering.

2.2.4. Fluorescence-based nanothermosensing

Fluorescence-based techniques were among the pioneers for intracellular temperature measurement, with organic commercial probes like Nitrobenzoxadiazole (NBD) and Laurdan

attaching to cell membrane and detecting temperature changes in a 2°C resolution for the former and 0.1°C – 1°C for the latter (61,62). Beyond the work with these compounds, further formulations were developed, mostly concerning the coupling of organic ligands onto metal based nanoparticle, due to the fact that organic compounds alone can be affected by environmental changes like pH, swelling, protein denaturation and/or cell membrane rupture (63).

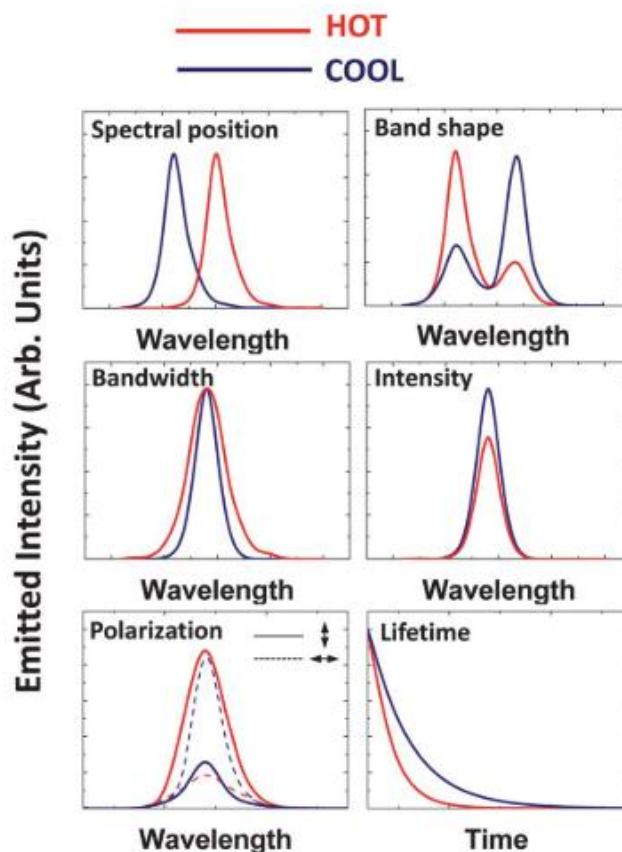


Figure 4 – Different cases of luminescence changes depending on temperature (Adapted from Jaque *et al.* (43)).

For temperature based studies involving luminescence, one can use several parameters, indicated by Jaque and Vetrone (43), such as intensity, band-shape, spectral, polarization, bandwidth and fluorescence lifetime, illustrated in Figure 4. Spectral luminescence thermometry depends on the energy that separates the electronic states responsible for emission, as they will be responsible for the position of the bands. This measurement is independent of concentration, therefore not affected by phenomena like photobleaching of the fluorophores, producing the best results in systems where even small temperatures would generate higher spectral shifts (43). These may be used in peak fraction approaches, as per used by Savchuk *et al.* (64), who showed a red-shift of the emission spectra as the temperature of wt-GFPs rose.

Regarding temperature-induced spectral band-shape changes, these occur when the electronic states responsible for emission are so close in energy that they are thermally coupled as well, which usually happens in samples with two emitting centers that quench or transfer energy between each other, or samples with one emitting center, where the energy levels get re-distributed (44). In the work of Kulinich *et al.* (65), the effect of temperature on the fluorescent properties of merocyanines was verified to reflect on their position and shape of the absorption and fluorescence bands, mainly by changing the polarity and viscosity of the medium in which they are inserted.

The width of fluorescence emission peak typically increases with temperature, as the density of phonons increases as well, leading to a broadening of the emission peak (43). In the work of Zhang *et al.* (66), the temperature sensing properties of a Ce³⁺-doped yttrium aluminum garnet by fluorescence properties was demonstrated by i) shifts in the emission band, ii) spectral bandwidth and intensity ratio.

As for intensity changes related to temperature, the number of counts per second changes, which can cause the emission spectrum to change in intensity as well, which is caused by thermal activation of luminescence quenching mechanisms and/or increases in non-radiative decay probabilities (43). Intensity may also change due to factors unrelated to temperature (e.g. photobleaching), in which may be used one of the alternative fluorescence-based methods mentioned above. In terms of fluorescence lifetime thermometry, this is based on the time that it takes to decay to 1/e of the initial value of intensity after a pulsed excitation. This decay depends on many factors, including temperature, like phonon-assisted energy transfer processes and multiphoton decays (43). Intensity measurements are considered simpler for a laboratory setting (67), while fluorescence lifetime is considered more robust (68). The intensity signal presents dependence on intensity of laser excitation (69), which prevents a quantitative sensing of analytes without wavelength-ratiometric probes (67). Whilst fluorescence lifetime has no laser power dependency, it is dependent however on other parameters, such as concentration, having longer fluorescence lifetimes as the concentration decreases until it plateaus (70).

Another fluorescence parameter that can show temperature dependency is the fluorescence polarization, often called fluorescence polarization anisotropy (FPA), which will happen when a fluorescent molecule, excited with a linearly polarized laser, re-emits the absorbed photon via the fluorescence emission process, not rotating before said emission. As a process dependent on the viscosity of the solvent, as well as the temperature molecular movement and rotation, this will lead

to an overall change in polarization fluorescence emission. Since an increase in Brownian motion induces molecular rotation, in dependence of increase in temperature, the polarization anisotropy will decrease, while it reaches its peak in absence of molecular motion (43). Pikulik *et al.* (71) demonstrated the dependence of fluorescence polarization to temperature in phthalimides, where the polarization decreased, as the temperature increased. Thompson *et al.* (72) reported the first use of a protein-dye hybrid as a FPA based thermoprobe, by conjugating small fluorescent dyes to larger proteins. This leads to a slower molecular rotation of the dye, increasing the dynamic range of FPA temperature sensitivity, which has an optimal ratio between fluorescence lifetime of the emitter and the size of the fluorescence emitting system.

2.2.4.1. Intracellular nanothermosensing

Cells present some intrinsic temperature variation derived from their metabolic activities (73,74), and in cells where some pathology (e.g. tumor cells) is present, the metabolic activity is higher and temperature will be higher as well (74). Beyond their own temperature, cells can be heated from external sources, such as hyperthermia treatments, magnetic, or photothermal hyperthermia. In this case, knowing the temperature reached inside the cells will be useful to set the exposure parameters to light, in *in vivo* assays. While the generation of hyperthermia within cells is of high importance, the measurement of the reached temperature cannot be neglected either, as knowing the temperature will prevent overheating and damages on the healthy cells, as well as make possible the study of diseases where cellular temperature can be seen as an indicating factor of severity (e.g. localizing and monitoring the progression of tumors by their heat generation) (61,75).

Temperature measurement is usually done by methods at a scale incompatible with an individual cell's size (e.g. microcalorimetry) or by methods incompatible with living cells, but so has been done successfully when testing a cluster of cells (76). It is then necessary to create new strategies when referring to a single cell extent, making sure the method is accurate enough and at the same time, able to maintain the cells alive whilst undergoing thermal reading. To have a thermometer sensible enough for intracellular temperature measurement, it must possess several features: high spatial resolution and repeatability, measurements must be done in real time and in a user-friendly manner, as well as the thermometer itself must be independent from concentration

and any other environmental changes (75). The measurements can be then be done in one of two manners: contact or non-contact, with the latter making use of luminescence properties of compounds.

When analyzing methods involving contact with the cells, one of the methods that has been most studied has been based on the variation of a typical thermocouple into a dimension compatible with cell size. Watanabe *et al.* (77) developed the first thermocouple-based approach, microfabricating a micropipette' tip capable of performing Seebeck's effect, the functioning base of thermocouples (75). The tip is capable of generating voltage according to the temperature of the sample, being converted into temperature by a digital thermometer or by comparing values on references tables. By Watanabe *et al.* (77) managing to place such thermocouple on a micropipette's tip, it allowed for direct contact with cells, as the tip only had a diameter of 1 μm . Similar experiments involving size-adjusted thermocouples have been conducted by Wang *et al.* (78) and Shrestha *et al.* (79) as well. Another contact method include the development of a pico-calorimeter, where temperature related changes alter the resonant frequency of the resonator, which Inomata *et al.* made of silicon (75,80).

Relating to the non-contact temperature measurement techniques, these are mostly based on luminescence properties, but not exclusively, like the work of Gao *et al.* (81), where they utilized a method based on photoacoustic microscopy to measure the temperature of a single cell for the first time. In a most common way, non-contact temperature measurements are usually done by resorting to fluorescent probes, which can be one of the following: small and purely organic probes, metal complexes with organic ligands, thermoresponsive synthetic polymers, quantum dots, purely inorganic, or organic macromolecules like DNA- or Green Fluorescent Proteins (GFPs)- based (61). The first report on resolving temperature maps and measuring from within biological cells using luminescence nanothermometry approaches were done in 2012 by Okabe *et al.* (73), while the pioneering work of Donner *et al.* (82), used GFPs as a temperature nanoprobe, based on fluorescence polarization anisotropy, which sensing mechanism was previously described (section 2.2.4), while using GNRs as a heating component. This temperature-assay method presented itself as biocompatible, with high spatial resolution and good temperature accuracy, presenting itself as less prone to artifacts, as per instance, ones caused by photobleaching.

2.3. Nanomaterials used for heating and nanothermometry

2.3.1. Gold in Medicine

Gold has been used for medicinal applications (also known as chrysotherapy or aurotherapy) for millenniums, with evidence proving its use back in ancient China and Egypt, and keeping its presence in medieval medicinal practices. In late 19th century, breakthrough discoveries regarding gold salts influence in tuberculosis diagnosis and treatment allowed for gold to be used in a more vast and efficient array of diseases, such as rheumatoid arthritis and lupus erythematosus, well into the 20th century (83–85). Nowadays, gold is known to have many harmful side effects, and its uses in bulk as therapy are limited, as its long term effects are largely unknown (86).

However, gold has gained new attention with a more secondary role in treatment, as nanoscience reinvents traditional methods of therapy, being the use of gold as a nanoparticle matrix one of the most interesting and studied, due to its attractive physical and chemical properties. In fact, gold nanoparticles may have a wide assortment of chemical, optical and electromagnetic characteristics, strongly depending on their shape and size (83), which may be controlled by rigorous regulation of experimental formation conditions (reducing agent, reaction time, temperature, capping agent², among others) (87). These differences in size or chemical/physical characteristics may influence their cellular uptake, as well as their toxicity and biological interaction (83).

Gold nanoparticles enter cells through a receptor- mediated clathrin-dependent endocytosis pathway, depending greatly on their size and shape, with rod-shaped nanoparticles having a slower uptake when compared to spherical nanoparticles (83,88). Studies have shown that gold toxicity also depends greatly on size, as smaller particles (1-2 nm) were observed to have higher cytotoxicity than larger particles (15 nm) in many diverse conditions, which is thought to be caused by differences in the uptake kinetics and/or cellular target specificities, as well as from interactions with the cell membrane (89). Currently, gold nanoparticles are being used in several medical approaches, such as diagnostics (bio-imaging and analytical methods) and many different kinds of

² Capping agent is an amphiphilic molecule with a polar head group and a nonpolar hydrocarbon tail, responsible for providing colloidal stability, preventing agglomeration and stopping uncontrolled growth (166).

therapeutics (photothermal, photodynamic, as drug-carrying and/or immunological agents, among others) that make use of their easily tunable properties (90).

Gold nanoparticles have been used in photodynamic therapy as carriers for photosensitizers (46), as drug-carriers for mainly anti-tumor drugs, albeit the use of antibiotics is also popular, and as immunological agents, with protein-encoding genes designed to serve as antigens to incite immune response (90). In terms of photothermal therapy, nanorods are the most common type of gold nanoparticles developed for this purpose (and their application and properties will be discussed in 2.3.2), but it can also be performed by nanoshells, nanostars and nanocages (44).

2.3.2. Gold nanorods in photothermal therapy

While there are many nanoagents capable of generating heat for photothermal therapies (see 2.2.2), gold nanorods are among the most popular for this purpose. This is due largely to them being plasmonic particles, which are capable of a photophysical phenomenon called localized surface plasmon resonance (LSPR), consisting on the interaction of the nanoparticles with light of resonant frequency (91). The free electrons in the conduction band of the metal oscillate in a collective and coherent manner, after absorbing light in resonance with the incident light (91,92). The scattering and absorption of light depends on the physical dimensions of the gold nanoparticle (smaller colloidal gold absorbs in shorter wavelengths, while larger colloidal gold absorbs in longer wavelengths), as well as on the medium of the colloidal gold (86), particle shape, structure and dielectric properties of the metal (91). The anisotropic shape of the nanorods causes their surface plasmon resonance to be red-shifted, when compared to spherical gold particles (91).

The nanorod shape allows the presence of two absorption bands (surface plasmon resonance bands), derived from a longitudinal band (strong long-wavelength) and a transverse band (weak short-wavelength) according to the different faces of the nanorods. These bands refer to the longitudinal or transverse electronic oscillation (93). The transverse band is insensitive to the aspect ratio of the nanorod and can be found at the visible region, while the longitudinal band is greatly dependent on the aspect ratio, shifting from the visible to the NIR region linearly as the aspect ratio increases (91,93,94).

Link and El-Sayed (95) provided an explanation on how does this plasmonic properties get converted into heat, based on two photophysical processes: electron-electron relaxation occurring on femto-seconds and electron-phonon relaxation occurring on the picoseconds. The absorbed light is converted into heat, forming a hot metallic lattice, which gets cooled off by phonon-phonon relaxation (91). This generated heat can be used for heating local environment, in this case, the intracellular medium, which can be particularly useful in destruction of unwanted cells, as per mentioned in 2.2.

2.3.3. Core/shell nanoparticles

The nanoparticles in a core/shell distribution may have either organic (O) or inorganic (I) components, in one of the following assemblies: O/O, O/I, I/O or I/I. In some special cases, the nanoparticle has more than one shell, or have a movable core with a hollow shell. The shells are mainly added to ensure stability of the nanoparticle, but can also have influence in biocompatibilization, drug-loading, dispersibility, controlled release or even in the economic aspect, by reducing the amount of precious materials that usually make up the core (96). Ultimately, the purpose of the nanoparticle is the factor that dictates its composition, as is not only of interest to apply already well-known particles to existing problems, but also develop novel structures to address more kinds of issues.

In terms of organic shells, they are often used to increase the biocompatibility and stabilization of the nanoparticles, being polyethylene glycol (PEG) one of the most common nanoshells used nowadays, as it can reduce opsonization³, nonspecific binding and allow specific groups at the NP surface to bind to cell receptors (97).

Concerning inorganic shells, the most common nanoshells used are based on silicon dioxide (generally known as silica). Other examples include the usage of metals or metal oxides (such as gold, nickel, cobalt, palladium, among others), as well as semiconductors or lanthanides for the shell. These allow for protection against oxidation, corrosion and allowing for a better

³ Opsonization - Process by which a foreign organism/particle becomes covered with opsonins (proteins), thereby making it more likely to undergo phagocytosis. If it indeed occurs, the foreign organism/particle is destroyed (167).

biocompatibilization and enhanced affinity due to the presence of amine and thiol groups (98), who have been proven to increase the binding to the nanoparticle's surface (99,100).

Silica is an inorganic compound, inexpensive, physically and chemically stable as well as harmless to the human body (101). The main advantages in using it to cover particles refer to its capacity in reducing bulk conductivity and increasing the suspension stability of the core particles, since silica is a very inert material. This inertness blocks the core surface without interfering with the redox reaction occurring, and can also modulate the position and intensity of the surface plasmon absorbance band since silica is optically transparent, which allows for a spectroscopic study of chemical reactions happening at core surface (96).

2.3.4. Mesoporous silica shells

In this project, an inorganic/inorganic assembly is used, as the inorganic silica shell covers an inorganic metallic compound (gold). The silica shell used is covered with mesopores that can be occupied with hydrophobic drugs, which is an important feature for controlled drug delivery (102,103), with proteins, DNA or RNA (ribonucleic acid) (104), among others, who can also be functionalized on its surface, as it is the case with rhodamine B in this project (found in 2.3.6). The need for transporting molecules of different characteristics can be accommodated (102), as these pores can also be tuned in terms of structure, volume, diameter and morphology (103). The mesoporous silica nanoparticles are also synthesized employing a simple process, another advantage for its use as a drug-delivery system, as they become cost effective (104).

The shell on the nanohybrids in current study present another characteristic that must be taken into account regarding the exposure time to the nanohybrids, which is its degradability. Paris *et al.* (103) have studied this aspect, observing a degradation after several days of dissolution in aqueous solution.

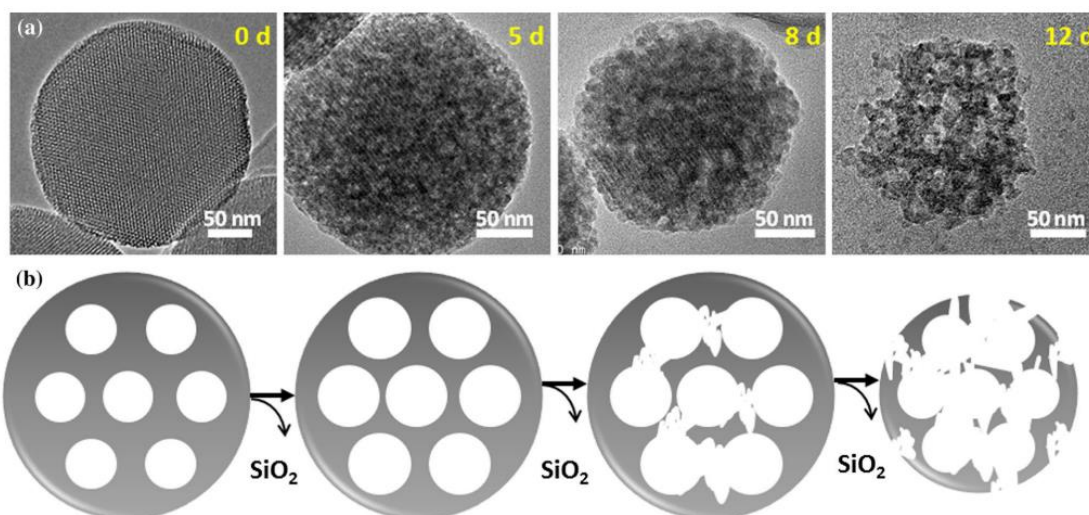


Figure 5 – **(a)** - TEM micrographs of MSNs after suspension in PBS for different periods of time (from left to right): 0, 5, 8, 12 days; **(b)** - schematic representation of the dissolution process of MSNs in aqueous medium (Adapted from Paris et al. (103)).

This dissolution of the shell is one attractive aspect for possible *in vivo* application as well, since after degradation silica turns into free silicon species (monosilicic acid byproducts) that can be excreted by urine (103).

2.3.5. Synthesis of mesoporous silica-covered gold nanorods

The nanohybrids' formation and silanization for this project was performed by Dr. Juan Luis Paris. The formation was performed by seed-mediated growth, where the gold nanorods are formed after association of three components: a growth solution, gold seeds and additives, like for instance AgNO_3 (105), which affects yield, crystal structure, morphology and optical properties (106).

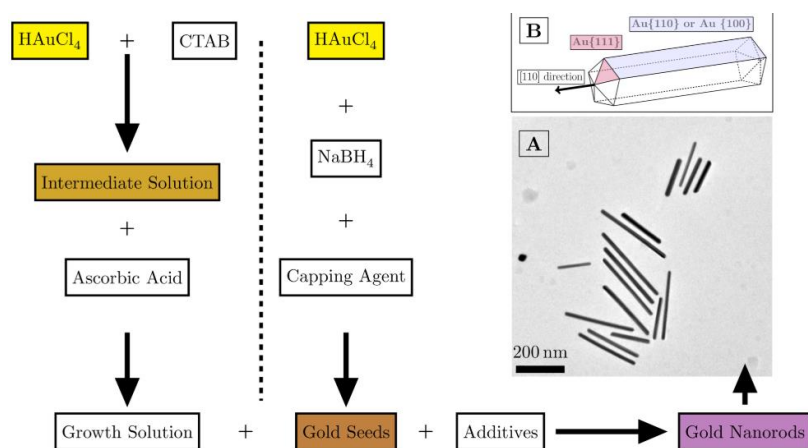


Figure 6 – Gold nanorods formation from gold seeds method (Adapted from Chhatre et al. (105)).

Referring to the preparation of the growth solution, the first steps involve joining chloroauric acid (HAuCl_4) to cetyltrimethylammonium bromide (CTAB), which will act like a surfactant to the gold ions (105), transforming them into solid gold. This happens due to the bonding of the cationic surfactant to the nanoparticle's surface, reducing the energy, while stabilizing the solution and preventing aggregation (107). Since CTAB is cyto- and environmentally toxic, new alternatives are being researched, such as, for instance, dodecylethyldimethylammonium bromide (C12EDMAB) (108). After formation of what Chhatre *et al.* (105) call the *Intermediate solution*, ascorbic acid is added since it will have an effect on the particles' shape and promote anisotropic growth, by reducing the gold (109), resulting in the *Growth Solution*. Combining this with the gold seeds and the additives will lead to the formation of the gold nanorods. This is a process that must be highly controlled in terms of physical and chemical conditions of preparation, as it is of utmost importance to guarantee an anisotropic shape on the hybrids core, essential for heat generation (as explained on 2.3.2). To coat the gold nanorods with the mesoporous silica shell, a protocol described by Villaverde *et al.* (110) with some minor modifications was used. The excess reagents from the prepared GNRs solution were removed by centrifugation, re-suspending in CTAB. These particles are then added to a solution of ethanol with CTAB and TEOS (Tetraethyl Orthosilicate) to form the silica shell itself. To allow posterior binding of molecules, the surface of the shell must be functionalized, which in the scope of this project is explained in 3.1.1.

2.3.6. Luminescence properties of Rhodamine B

Rhodamine B is a very soluble and toxic organic dye from the xanthene class, known for its red color and fluorescent properties (111). It is chosen as the fluorescent component of the nanohybrids in use for this project, and shall serve as the luminescent nanothermosensor. A binding assay used for similar organic dyes before (112) was followed, where the dye is added after the gold gained its nanorods shape and after it's coated with silica, in which the isothiocyanate group (becoming RhBITC) makes it easier to bind the dye to the shell, as it links to APTES' amine groups by thiourea linkages (112). The presence of the isothiocyanate group does not influence

⁴ Cofhydrolysis and polymerization of TEOS (tetraethoxysilane) with APTES (3-aminopropyltriethoxysilane) forms the silica shell (168).

the fluorescence emission spectra of standard RhB, and will always be referred to those spectra in this work.

The absorption spectrum of RhB shows a peak at 558 nm, while the fluorescence emission maximum is found at 585 nm. A 561 nm laser will be used for excitation and suited filter sets to avoid the detector's saturation, thus slightly blocking part of the emission spectrum (Annexes - 1 section, Supplementary figure 1). Rhodamine B was chosen as an intracellular thermometer due to the dependence of its fluorescence lifetime on temperature, as per verified by Chaze *et al.* (69), where the fluorescence lifetime of RhB decreases as the temperature increases, also confirmed by Mercadé-Prieto *et al.* (68). In the specific case of RhB, pH also has influence on fluorescence lifetime, due to RhB's spirocyclic structure. In acidic conditions, the fluorescence is stronger, as it opens the spirocyclic form, while it closes when the pH becomes more basic, reducing emission intensity (113).

Chapter 3 - Materials and Methods

3.1. Au@MSN-RhBITC hybrids fabrication and nanocharacterization methods

In the following section, the labelling procedure and several spectroscopic methods that were used to characterize the particles before applying them as heating sources and nanothermometers to cancer *in vitro* assays, will be described.

3.1.1. Au@MSN-RhBITC hybrids synthesis

As per mentioned in 2.3.5, the formation of the gold nanorods and subsequent silanization were performed by Dr. Juan Luis Paris by the process described, while the removal of the silica from the mesopores and the adding of the RhB to the surface were performed at INL.

After formation, the gold nanorods were covered with a mesoporous silica shell, using CTAB once more, but not as a stabilizer, being used as a template for silica instead. After adding the silica, the nanoparticles may be dried and stored (114), or continue the process, in order to extract the excess CTAB from the pores, hollowing and allowing them to be filled with other molecules. In this project, the Au@MSN nanorods were prepared in advance and were dried, so there was a need to re-disperse them in toluene and functionalizing with aminopropyl groups before progressing to the removal of CTAB. To achieve this, the prepared Au@MSNs were dispersed in dry toluene under a N₂ atmosphere and placed at 85 °C under magnetic stirring, adding APTES and leaving the sample to react overnight under an inert atmosphere. Afterwards, the Au@MSNs were washed with toluene and ethanol removing the surfactant by washing with an ethanolic solution of NH₄NO₃ (10 mg/mL NH₄NO₃ in 95 % ethanol). After several washes, RhBITC was added in a proportion of 1.38 mg to 20 mg of Au@MSN and some washes were needed to remove the molecules that didn't adhere to the amine groups on the silica shell, thus becoming the final product denominated Au@MSN-RhBITC. The nanohybrids were dried and kept in powder form, to avoid silica shell degradation, as per explained in 2.3.4 section.

3.1.2. Hybrids nanocharacterization

To better understand the physical and chemical properties of the Au@MSN-RhBITC nanohybrids, these must be characterized by several different techniques that will allow to know size, fluorescence and their correct assembly, among others. The methods used are comprised of Transmission Electron Microscopy (TEM), Dynamic Light Scattering (DLS), UV-Vis-NIR absorption spectroscopy and fluorescence spectroscopy⁵, which are described in more detail in the following sections.

3.1.2.1. Transmission Electron Microscopy

TEM is a microscopy technique in which an electron beam reaches a sample, scattering electrons, generating contrast and producing an image (115). This technique is very useful in detecting very small objects, as they cannot be perceived by light microscopy. A distinctiveness in this method is the fact that samples must be placed under vacuum conditions, in order to allow the movement of electrons without influence of the electromagnetic fields produced by exterior currents and charges (116). This is a rather important feature, since these microscopes rely on solenoids to generate a magnetic field, which affects the electron beam's focal length similarly to light and convex glass lens found in standard microscopes, focusing the electron beam (117).

Experimental settings used:

For this characterization, the equipment used was JEOL 2100 HT 200kV TEM (Cryo & Tomography). A drop of 200 µg/ml was pipetted onto a Formvar/Carbon Supported Copper Grids (size 400 mesh, thickness: 10 nm Formvar and 1 nm Carbon) grid, and for the imaging 200 kV electron beam was used. After focusing and some sample scanning, a characteristic area of the sample was selected for TEM image data acquisition.

⁵ Characterization experiments were performed by Dr. Juan Luis Paris and results analyzed by MSc. candidate.

3.1.2.2. Dynamic Light Scattering

The Dynamic Light Scattering is a method used to determine the hydrodynamic size of particles dispersed in a solution (118). DLS is based on the scattering of the incident light by the particles, as the angle of scattering is dependent on the particle size, as smaller particles scatter less light than larger particles (119). This angle can be measured by a detector, usually at 90° or one at 173° (depending on its position in relation to the sample), since it's the angles at which the dust contamination detection is lowest (120).

Experimental settings used:

A Horiba Dynamic Light Scattering System SZ-100Z instrument was used in the following parameters: 90° angle detection, five repetitions of measurements with 60 seconds integration time on 200 µg/ml Au@MSN and Au@MSN-RhBITC hybrids (in water) samples, of which 1 ml was placed in a disposable plastic cuvette. The results were compiled and represented using Excel.

3.1.2.3. UV-Visible-NIR absorption spectroscopy

Spectroscopy is a group of techniques that comprise the study of the interactions between electromagnetic radiation and a matter of interest, and different kinds of techniques can be divided according to what they study, which can be the energies involved, the type of transitions that can occur between energy levels of a molecule subjected to incident radiation and whether the molecules assessed absorb or emit the energy (121). The absorption of electromagnetic radiation causes the molecule to transition from a lower-energy state to a higher-energy state, happening only when the incident radiation has equal energy to the one separating the molecule's energy states (121). Kumar (121) also refers specifically that UV-Visible radiation promotes the molecule's valence electrons to a higher-energy state, while infrared radiation changes the molecule's vibrational energy. In Figure 7, an adapted Jablonski diagram (122) illustrates the energy levels of a phosphorescent molecule, where absorption is indicated by the arrow "A".

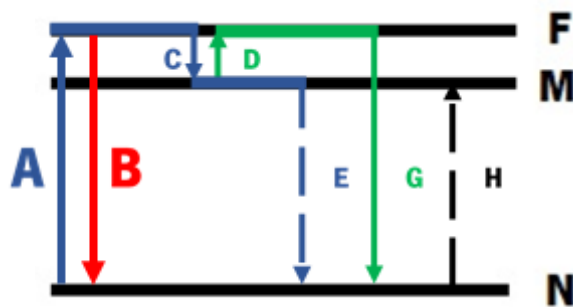


Figure 7 – Diagram illustrating energy levels of a phosphorescent molecule (*F* and *N* are stable energy levels, while *M* is a metastable energy level), with Anti-Stokes processes: **A** – Absorption, **B** – Fluorescence, **C** – Transition to metastable level, **D** – Thermal excitation, **E** and **G** – Phosphorescence and **H** – Absorbance of very small transition probability. Adapted from Jabłoński (122).

In the case of UV-Visible-NIR absorption spectroscopy, this approach is based on the absorbance of the wavelength (in the range of 400 to 900 nm) by the samples, which can vary due to differences in composition and size of the particles in solution, although it can also be used to measure concentration and aggregation level with a few changes to the standard model used (123).

Experimental settings used:

The absorption for both Au@MSN and Au@MSN-RhBITC was assessed using a UV-Vis-NIR spectrometer (LAMBDA 950, Perkin-Elmer), in a quartz cuvette (1 cm per side and clear in all sides) and the results were compiled and represented using Excel.

3.1.2.4. Fluorescence spectroscopy

Fluorescence spectroscopy is suited for the study of a different type of interaction between electromagnetic radiation and the molecules, when compared to the absorption spectroscopy. In this case, instead of studying the absorption of radiation by molecules of interest, it is studied the emission of radiation by said molecules, during their decay from the relaxed electronic excited to the electronic ground state manifold, indicated in Figure 7 by the arrow “B”. Regarding fluorescence, since there is always loss of energy by other processes other than radiative, the energy of the emitted light is lower than the absorbed light, meaning that the emission occurs at higher wavelengths than the absorption ones, since they are less energetic (121).

Experimental settings used:

For this experiment, a microplate reader (Synergy H1, Biotek) was used. The excitation was set at 560 nm, and experiments performed at room temperature ($\approx 25\text{ }^{\circ}\text{C}$), while the fluorescence emission wavelengths measured for both Au@MSN and Au@MSN-RhBITC were between 590 – 700 nm using while the results were compiled and represented using Excel.

3.2. Functional characterization of the nanohybrids

In order to perform functional characterization of the nanohybrids, firstly a photothermal conversion study will be executed, to understand how much do the nanohybrids in solution heat after irradiation with an 808 nm laser. Afterwards, the thermal sensitivity of the nanohybrids will also be assessed, to understand how temperature influences their fluorescence lifetime.

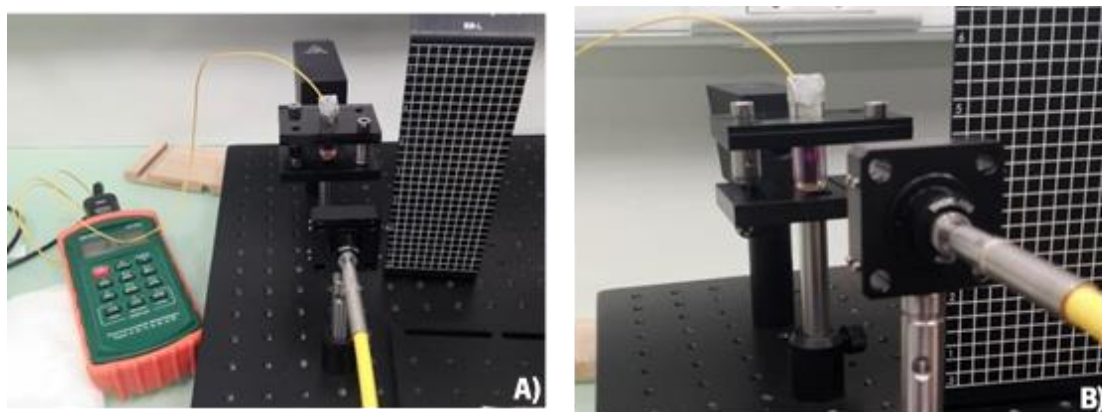
3.2.1. Photothermal conversion study

The photothermal conversion refers to the ability certain materials have to absorb photons and convert that energy into heat (124). To assess the temperature of the sample, a thermocouple was dipped on the cuvette, away from the laser's incident point, to avoid a direct heating and incorrect measurements. This temperature assay method is based on Seebeck's effect, which is focused on the measurement of heat by the contact between the thermocouple's tip and the sample, converting it into electricity (75). The tip of the thermocouple is made of two metallic wires that convert the temperature into voltage, allowing the comparison of the voltage values with a temperature reference table, or automatically convert them with a digital thermometer, which is the method used and is visible on Figure 8.

Experimental settings used:

In frame of this project, a photothermal conversion set-up was upgraded by adding an acrylic holder designed to fit a small cuvette (provided by Filipe Camarneiro) and hold it in the 808 nm laser excitation beam (dst11-DILAS-50W-808nm-400 μ -0.22NA-t19286-503 device (type 503) with M1F4S22-808.5-50C-SS5.2 laser assembled, OsTech Electro Optical Instruments), which

contained the nanohybrids at different concentrations (0; 50; 100; 200 and 500 $\mu\text{g/mL}$) dispersed in water. Both the sample with and without RhBITC were assessed, to understand if the RhBITC had any influence on the heating component. An 808 nm laser was used to irradiate the cuvette, in order to generate heat, and it was irradiated until the maximum temperature was achieved, at two different laser powers of 1.3 W and 1.8 W, as measured with a power meter (1917-R, Newport) at the output of the laser-coupled fiber, based on previous studies (46,125). Afterwards, the laser was turned off and the cooling down of the sample was also timed, as a mean to obtain a photothermal dependency curve on concentration, as well as the time it takes to reach the peak temperature and how long it takes to cool down the sample. The sample with the same concentration was re-heated, to verify the stability of the hybrid over multiple heatings. For the temperature recording, a thermocouple probe (type K) connected to a thermocouple data logger (421509, Extech Instruments) was used, and the results were compiled and represented using Excel.



*Figure 8 – Photothermal conversion set-up. **A** – Thermocouple placement and data logger with general overview of the irradiation set-up; **B** – Close-up of sample irradiation with 808 nm laser.*

3.2.2. Thermal sensitivity of the nanohybrids

The fluorescence lifetime will be calibrated in dependency of temperature (126), with the RhB's fluorescence lifetime being expected to decrease with the increase of temperature (68), allowing the attainment of a calibration curve. Using this calibration curve, it is possible to convert the cell's fluorescence lifetimes after exposure to the nanohybrids into a temperature measurement, thus

developing an intracellular thermometer. To achieve this goal, fluorescence lifetime characterization will be performed.

3.2.2.1. Time correlated single photon counting (TCSPC) based fluorescence lifetime microscopy

The thermal sensitivity of the nanohybrids is measured using the time correlated single photon counting (TCSPC) technique, and the fluorescence lifetime of RhBITC (present in the nanohybrids in study) that differs with changes in the sample's temperature. TCSPC is a type of fluorescence spectroscopy, based on time resolution. Since reconstructing a photon fluorescence decay from a single excitation cycle would not be achievable, TCSPC uses a periodic laser pulse as the source of excitation, occurring in several cycles in which photons may be detected and their fluorescence decay profiles reconstructed, taking into account the time resolution (127).

Wahl (127) refers TCSPC as a “start-stop” measurement (Figure 9), from which the information for the photon-arrivals histogram is obtained. After a laser pulse, the time between excitation and emission of a photon is measured, and then sorted into a histogram with a range of time bins. As time passes, the counts will drop in an exponential matter, as the likelihood of finding a molecule that has yet to emit fluorescence lessens, as the population of excited molecules decreases over time.

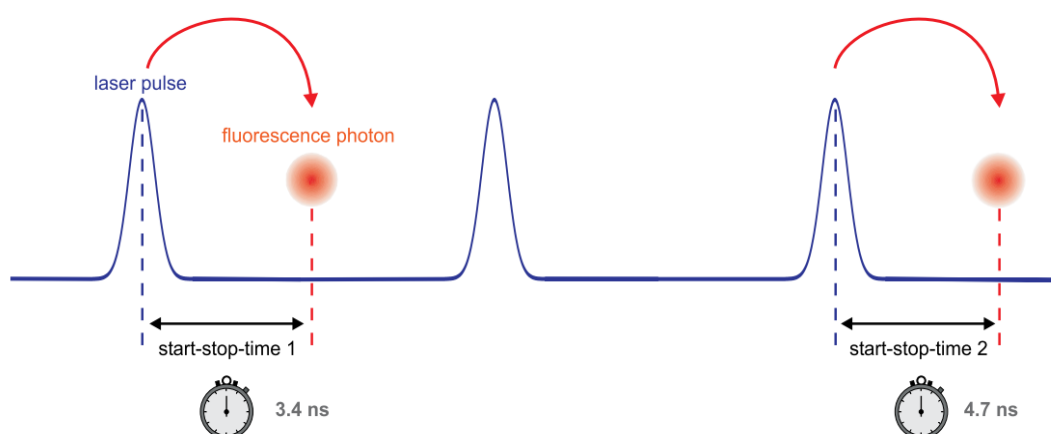
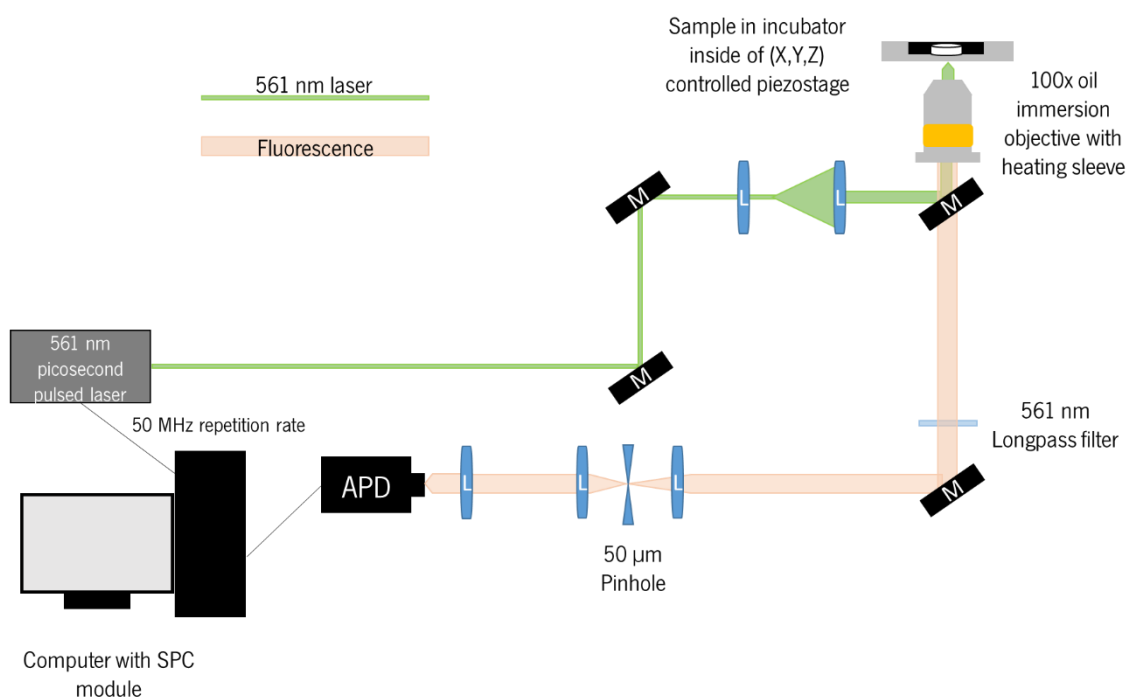


Figure 9 – Measurement of start-stop times in time-resolved fluorescence measurement with TCSPC. Adapted from Wahl (127).

Experimental settings used:

In this experiment, a custom-built FLIM set-up developed at UBNP was used. This set-up (Figure 10) is an inverted confocal microscope (RM21™ - MCL) comprised of a microstage (with a travel distance of 25 mm in X/Y and 50 mm in Z) and a nanostage (with a 200 μm travel distance in X/Y/Z), with a top-stage UnoStage Okolab incubator. This incubator allows to place a sample on the stages and control its temperature, CO₂ levels and humidity, which is useful for cells live imaging, as it maintains cells ideal environment conditions. The laser used is a 561 nm picosecond pulsed laser (BDS-561 SMY Picosecond Diode Laser, Becker & Hickl), which was chosen due to its spectral properties fitting the RhB's excitation spectrum. This laser enters the microscope through a periscope and a beam expander, which has two lenses with different focal lengths in order to match the objective back aperture. The laser is reflected on a dichroic mirror (Di02-R561, Semrock) to the 100x oil objective (N.A. 1.45, W.D. 0.13, PlanApo, Nikon) that focuses the laser on the sample placed inside of the incubator. After hitting the sample, the emitted fluorescence is captured by the objective and sent through the dichroic mirror, which is followed by a 561 nm long pass filter (561 Edge Basic Longpass Filter, AHF). This fluorescence is focused through a 50 μm pinhole, as in accordance to the microscope's confocal properties, and is then focused into a single photon counting Avalanche Photodiode (APD) detector, which is used to record time-dependent distribution of emitted photons after each laser pulse (128) in combination with the Single Photon Counting (SPC) electronics module (SPC130, Becker & Hickl), to which the Sync signal of the picosecond pulsed laser source is attached to allow TCSPC.



*Figure 10 – Diagram of the optics involved in the FLIM measurements (**M** represents mirrors, while **L** represents lenses). The 561 nm laser enters the microscope passing by two lenses with different focal lengths. The laser is reflected on a dichroic mirror, focusing it on the sample placed inside of the incubator. The emitted fluorescence from the sample is captured by the objective and sent through the dichroic mirror, which is followed by a 561 nm long pass filter. This fluorescence is focused through a 50 μm pinhole and later enters the APD detector after focusing through two lenses.*

To obtain said calibration curve (that related fluorescence lifetime information to temperature), Au@MSN-RhBITC hybrids dispersed in Dulbecco's Modified Eagle Medium (DMEM) with no phenol red at a concentration of 200 $\mu\text{g}/\text{mL}$ were placed in 70 μL wells on a Petri dish, and incubated on the top-stage incubator. Several set points were programmed at the incubator (25; 30; 35; 40; 45 and 50°C), stabilizing the temperatures for 10 minutes before measurement. FLIM images were recorded at those temperatures. Since the incubator is open on the bottom to fit the objective, a heating sleeve was placed around the objective to guarantee the least possible heat loss.

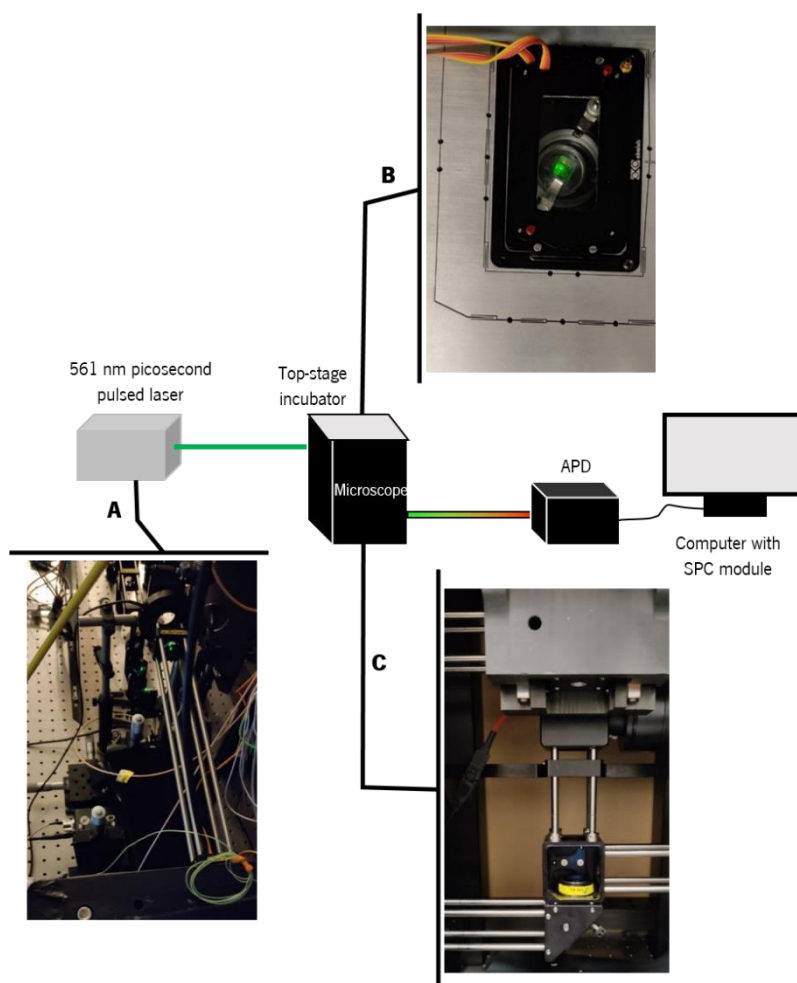


Figure 11 – Schematic representation of the set-up for FLIM imaging of the nanohybrids. In **A** is observed the 561 nm laser irradiation entering the set-up. In **B** the 561 nm laser is seen irradiating the sample, inside the top-stage incubator, from where the signal exits through the objective (not visible in the pictures), to the dichroic mirror and the 561 nm LP filter, as seen in **C**, to reach the detectors, also partially visible in **A**. The detector records the time-dependent distribution of emitted photons after each laser pulse, and TCSPC is performed after synchronization with the SPC module.

Data analysis

Each measurement was taken three times, and using the Nanophotonics Toolbox developed at UBNP for MATLAB, with main contributions of Ricardo Adão, they were converted into fluorescence lifetimes. The algorithm used was Enderlein, using the modelled IRF function and a fit offset of 175 time bins, selecting the peak in a Gaussian fit, in which the predominant fluorescence lifetime was selected, as well as the FWHM. The Enderlein algorithm used was

developed by Enderlein and Erdmann (129), as a way to perform appropriate deconvolution and fitting curves to decay registered in the fluorescence lifetime in TCSPC. Besides taking into account the Instrument Response Function (IRF), that contains information about the time profile of the excitation and characteristics of the system, this algorithm also takes into account the statistical character of the measured data (129).

The data is collected with a binning on the temporal axis of 1024 bins. The TCSPC card has a minimum time window of 50 ns, which can be reduced to increase time resolution by increasing the time to amplitude converter (TAC) gain of the card. In this experiment, the TAC gain used was set at 2, changing the time window to 25 ns over 1024 time bins. The laser repetition rate used was of 50 MHz, which means that for every 20 ns, a laser pulse occurs. In Figure 12, the raw data is shown with intensity (counts per 20 ms) per channel. For the fitting, the first 175 bins were excluded as indicated by the red line. The offset was chosen in order to allow for a better fit of the algorithm's curve, as there would be fluctuating peaks after the maximum (where the fit would automatically start), that would hinder the curve fit, as it would include parts dominated by IRF.

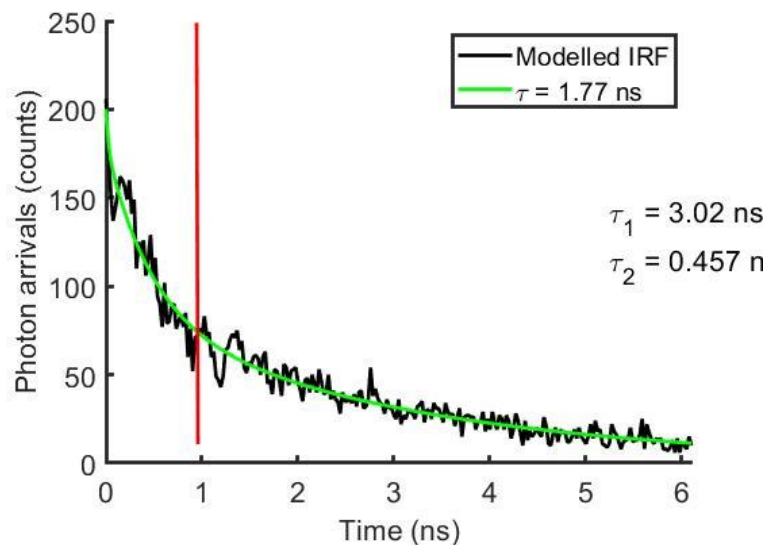


Figure 12 – Curve fit using Enderlein algorithm, with a red line indicating the start of offset at 175 time bins (around 1 ns).

Each repetition from the same condition was averaged and represented in a graph, where is possible to obtain a linear equation that fits the decay, which will then be used to convert the fluorescence lifetimes of the cells into temperature.

3.3. *In vitro* studies

In this study, two cancer cell lines were used for *in vitro* live cell testing, HeLa and A431 cells. HeLa cells belong to the first immortal cell line established, harvested from cancerous cervical tissue (130). These cells are known for their fast proliferation and for having an active version of telomerase during cell division. Shortening of telomeres is associated to cell aging and consequent death, which in the case of HeLa cells, does not happen since telomerase continues to act perpetually, leading to tumor growth (131). A431 cells belong to human epidermoid carcinoma, with a particular feature of having a great number of high affinity epidermal growth factor receptors (EGFR) in the cell membrane (132), which are responsible for tumor growth, invasion and metastasis (133). These factors are highly controlled in healthy cells, to maintain cell proliferation under homeostasis standards, while in cancer cells these factors are under continuous activation due to a mutation that causes an increase in EGFR ligands in the tumor microenvironment (133).

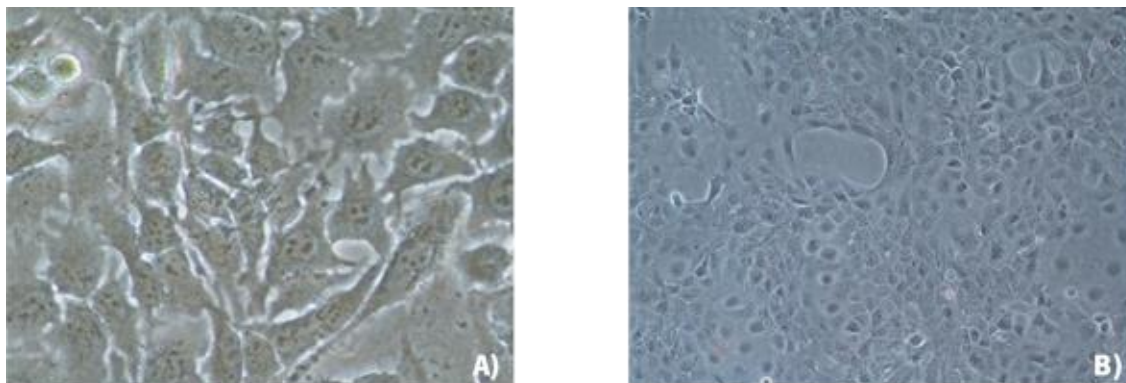
3.3.1. Cell culture

For the cell culture task, the cells used were of HeLa and A431 cell lines. Both these cell lines are considered adherent, and their maintenance protocol is equivalent. The cells were kept in a growth flask of ≈ 10 mL volume, at 37.5 °C, 5% CO₂. The media used are DMEM High Glucose for A431 cells, Minimum Essential Medium (MEM) for HeLa cells, both from Biowest® and supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin. The cell's medium is changed every time the confluence goes to a minimum of 80%. In the case of fluorescence-based experiments, the cells were placed in phenol-red-free DMEM, in order not to interfere with the gold nanorods' fluorescence and increase the background noise.

Experimental settings used:

In each passage, consumed medium is discarded, the cells are washed three times with 5 mL of Phosphate Buffer Solution 1X (PBS) (Corning®) each time to remove debris, and 1 + 1 mL of trypsin-EDTA is applied after washing (2 mL are applied to make sure the whole flask is covered, and 1 mL is immediately discarded to avoid cellular death by excess trypsin exposure). After a few

minutes of incubation with trypsin, which are variable according to the confluence shown, 9 mL of medium are added to inactivate the trypsin and the cells are re-suspended. The cells are re-seeded at the proportion cell suspension/medium needed. The cell lines were previously established and maintained by the UBNP group at INL, and were maintained in liquid nitrogen ($\approx -200\text{ }^{\circ}\text{C}$) until they were required for experiments.



*Figure 13 – **A** – HeLa cells observed at 40x magnification; **B** – A431 cells observed at 10x magnification. Images obtained with Nikon Digital Sight DS-FI1.*

3.3.2. Biocompatibility assay

The biocompatibility assay was performed by Trypan Blue exclusion test cell viability assay. In this test, the cells are regarded as viable by having a clear cytoplasm, or nonviable by having blue cytoplasm (134).

The procedure is started by seeding cells at a 10000 cells/well density, leaving them to incubate for 24 hours before incubation with the nanohybrid. This was done to ensure the cells would adhere to the 96-well microplate they were seeded on, and were on ideal growth conditions to internalize the nanohybrid. There were three plates seeded, one plate per incubation time (24; 48 and 72 hours). After the initial incubation, each well is washed three times with PBS before applying the nanohybrid, which was applied at three different concentrations: 50; 100 and 200 $\mu\text{g/mL}$, as well as maintaining a control well without Au@MSN-RhBITC exposure. Each concentration had three wells to serve as replicas, in every microplate. The nanohybrid was dispersed in medium without FBS, to avoid foam formation.

After each incubation time, the medium with nanohybrid was taken out, and the wells were washed with PBS, discarding it and replacing with trypsin, to detach the cells and collect them into eppendorfs, to be centrifuged for 5 minutes, 6000 rpm. The supernatant is discarded and replaced with 100 µl of PBS to resuspend the pellet. From this solution, 10 µl are combined with 10 µl of Trypan Blue dye solution, and from this mix, 10 µl are used for cell counting at a hemocytometer. Two calculations were performed with the cell counting data using Equation 3 (calculating the total number of cells per well) and Equation 4 (calculating the percentage of alive cells per well).

$$Total\ number\ of\ cells/well = \frac{Number\ of\ cells\ (total)}{4} \times 2 \times 10000 \times \frac{1}{10}$$

Equation 3

$$Alive\ cells\ (percentage/well) = \frac{Number\ of\ alive\ cells}{Total\ number\ of\ cells} \times 100$$

Equation 4

These calculations were performed for each well, and then for the three replicas of the same conditions the results were averaged. The results were compiled and represented using Excel.

3.3.3. Live cell confocal microscopy

Conventional microscopy presents several pitfalls regarding live cell imaging, where the whole field of sample is illuminated and visualized. The method represents a minor optical section and does not allow for 3D imaging. Confocal microscopy focuses a light excitation beam through the microscope objective to a focal volume that is diffraction-limited. Then, fluorescence emission is detected back through the same objective, and only the emission from that excitation volume reaches the detector, as a pinhole aperture is chosen to selectively and exclusively let light emitted from the focal volume to pass through, while blocking any other light outside of the focal plane. This allows to obtain very high quality images, with 3D optical sectioning. Besides only focusing on

an individual plane, confocal microscopy also allows to perform a “stack” of confocal image planes, scanning in different sample planes and reconstructing the scans in a 3D image, thereby representing a sample volume (135).

Experimental settings used:

In order to verify the internalization of the nanohybrids, confocal microscopy was performed⁶ after 12 and 24 hours of exposure in both HeLa and A431 cell lines (7500 cells per well of total volume of 70 μ L. Concentration of the nanohybrid was adjusted to 200 μ g/mL). Thirty minutes before confocal imaging, LysoHunt Blue DND-22 was added at a concentration of 50 nM to each well, in order to stain lysosomes. After that period of incubation, the medium containing the nanohybrids and the LysoHunt was removed and replaced with fresh medium, which for the success of this experiment, had to be DMEM without phenol red, so it wouldn't interfere with the sample's fluorescence.

For multi-color imaging and co-localization studies, a confocal microscope (LSM780, Zeiss) was used, with images obtained with a 63x oil objective (Plan-Apochromat 63x/ N.A. 1.40 Oil DIC M27, Nikon). The excitation and detection channels were optimized for LysoHunt Blue DND-22 and RhBITC, irradiating the sample with 405 nm and 561 nm lasers, to excite each dye respectively, as LysoHunt Blue DND-22 has a peak emission at 422 nm while for RhBITC is at 568 nm (emission wavelengths presented for equivalent dyes of LysoTracker Blue and Rhodamine B respectively, consulted at AAT Bioquest Spectrum Viewer (136)). With the laser of 405 nm, the power was set at 100%, with a gain of 600 and a detection window set in the range of 411 to 530 nm. With the laser of 561 nm, the power was set at 100%, with a gain of 700 and a detection window set in the range of 612 nm to 740 nm.

The results obtained were analyzed with ImageJ, with the data associated to spectral channels represented in different colors and merged for the discussion of internal localization.

⁶ Experiment was performed by Filipe Camarneiro and results analyzed by MSc candidate.

3.3.4. Live cell FLIM microscopy for intracellular thermometry

In order to obtain an intracellular temperature sensing, cells were seeded in a 35mm glass μ -dish (ref. 81158, ibidi), at a 7500 cells/mL density, with one pair of silicon wells (ref. 80209, ibidi) placed inside, with a volume of about 70 μ l. The nanohybrids were added after 24 hours of cell incubation, at a concentration of 200 μ g/mL, placed at an exposure time of 12 and 24 hours. This procedure was done for HeLa and A431 cells.

For the intracellular heating process, an 808 nm laser was added to the FLIM set-up (described in 3.2.2.1). The laser output port was mounted on top of the inverted microscope, at approximately the same distance towards the sample, as the one used in the cuvette based experiments on photothermal conversion (3.2.1), which was about 13 cm, to irradiate the sample under the same conditions.

Experimental settings used:

The samples were irradiated at 808 nm, with 1.3 W and 1.8 W laser power, for several time intervals (3; 6; 9 and 15 minutes). The FLIM microscopy settings used were the same as used for calibration curve recordings (3.2.2.1).

The results obtained were analyzed with MATLAB, using the Nanophotonics Toolbox developed at UBNP, while the images that refer to cell area selection and temperature underwent additional formatting using Inkscape.

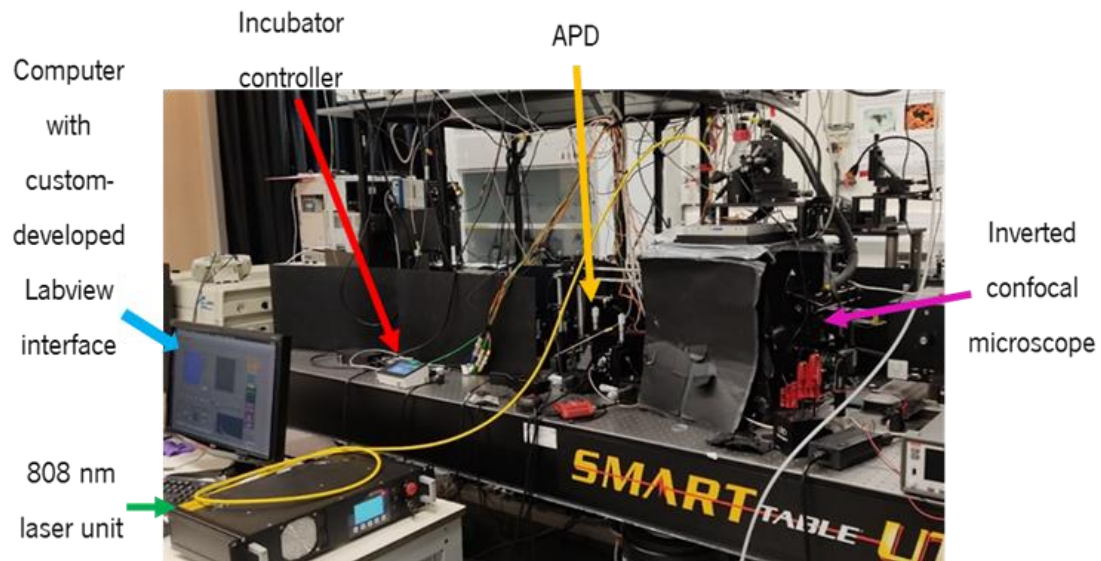


Figure 14 – Picture of the FLIM setup at INL's laser laboratory, with relevant components of the inverted FLIM set-up highlighted, including: the 808 nm laser added for photothermal experiments; the graphical user interface of a custom microscope control software and SPC module; the incubator controller; APD detectors alongside the inverted confocal microscope.

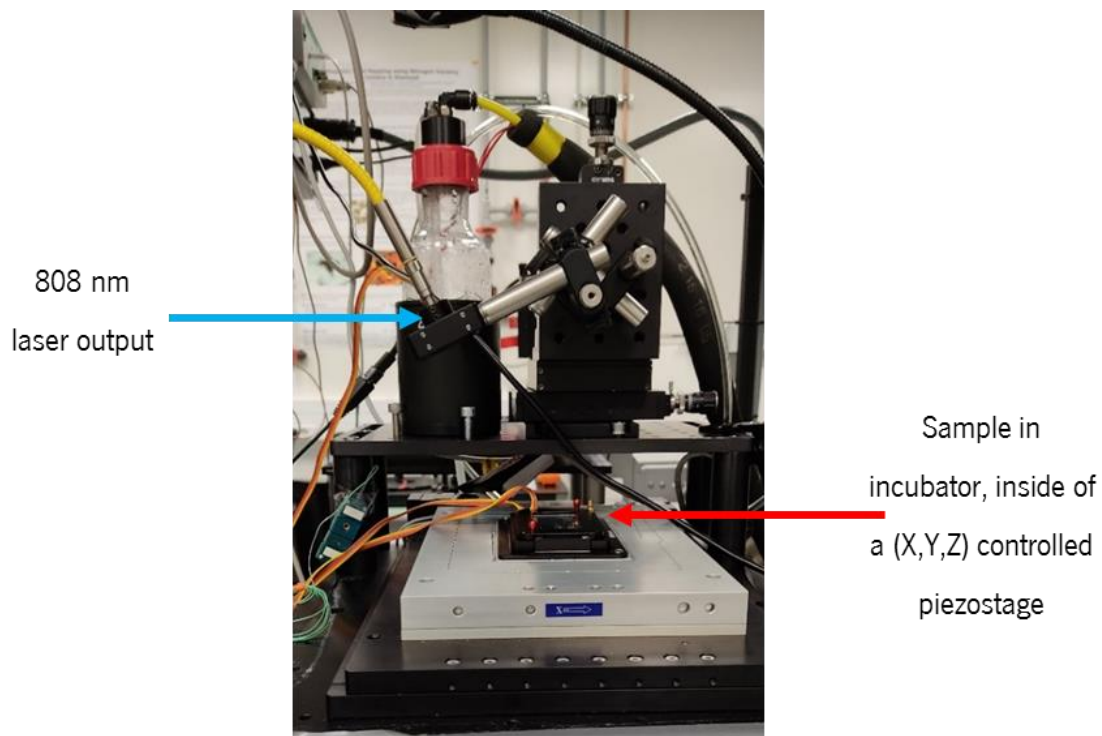


Figure 15 – Close-up of inverted FLIM set-up with 808 nm laser coupled on top for irradiation of the sample.

Chapter 4 - Results and discussion

4.1. Au@MSN and Au@MSN-RhBITC nanocharacterization

In this sub-section will be presented the results obtained after assessing the physicochemical properties of the hybrid nanoparticles, composed of a light absorbing gold nanorod core and a mesoporous silica shell. The influence of the presence of RhBITC on the nanohybrid was also taken into account for the characterization experiments, in which relevant parameters such as morphology, size, as well as optical properties, absorption and fluorescence properties of the hybrids were studied to test their suitability for cellular uptake and *in vitro* studies

4.1.1. Morphological characterization using TEM

For the TEM microscopy, it was of interest to observe the nanohybrids' size, as well as to understand their morphology, verifying the presence of an integer mesoporous silica shell with a nanorod core (Figure 16 and Figure 17).

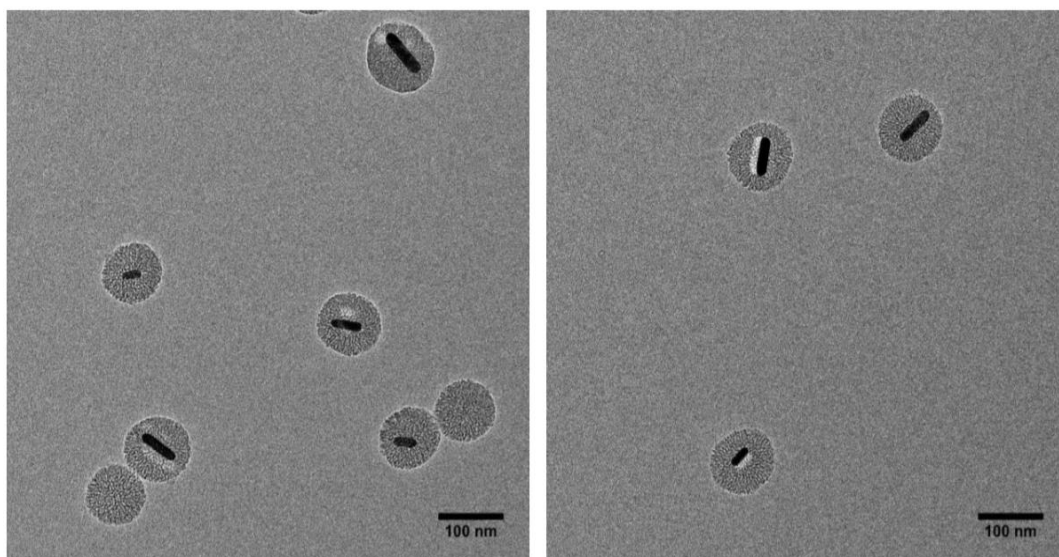


Figure 16 – TEM micrographs of Au@MSN particles. Sizes of the nanohybrids are noted to be around 100 nm, with pores visible around the nanoshell.

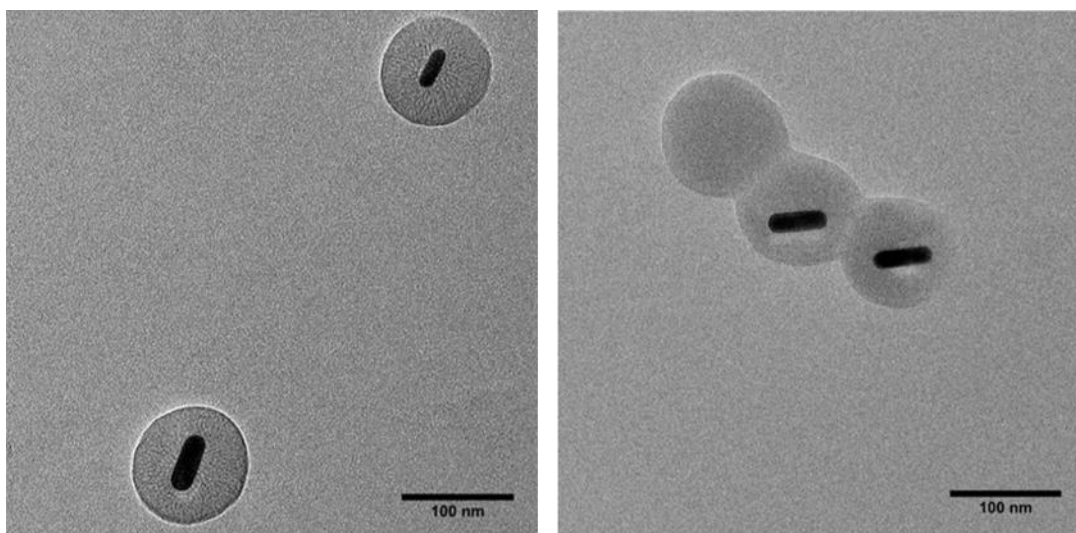


Figure 17 – TEM micrographs of Au@MSN-RhBITC particles. Sizes of the nanohybrids are noted to be around 100 nm, with pores less visible around the nanoshell, due to concealing done by the RhBITC functionalization.

In Figure 16 are represented the TEM micrographs obtained from the Au@MSN nanorods, where it is possible to verify the lack of aggregates, meaning the particles were successfully dispersed. The size of the particles is consistent with the sizes obtained through DLS analysis, with about 100 nm for each nanorod (including its shell), and the pores in said shell are notably evident, which can be valuable in future experiments that may make use of these pores to load drugs for targeted delivery (as long as their size is well-defined and very precise (137)). This confirms not only the goal in enveloping the nanorods with a shell, but also the success in forming its pores. In Figure 17 are represented the nanohybrids modified with RhBITC, showing the same size and shell properties as the unmodified hybrids, although also presenting more aggregation.

Something that is common to both samples is the presence of empty silica shells, which is explained by the shell formation process. After the formation of the gold nanorods, the silica is added and it envelops the particles in solution, as CTAB is added to provide a mold for the silica structure to be formed around the gold core. This structure is derived from TEOS, which is a silica based compound, and allows the formation of a shell with pores, whose size can be tuned by changing the concentration of both TEOS and surfactant (CTAB) (138), and by changing the process of formation, as per instance, the work of Jambhrunkar *et al.* (139), who developed a novel way based on vapor deposition to achieve angstrom-precise pore size. The nanorods will be then

coated with CTAB and posteriorly TEOS, and the excess of these reagents will form hollow shells. However, this is an infrequent occurrence, as presented in the results obtained.

Regarding the pores, both samples are considered to be mesoporous, although the pores are more easily seen in the sample without RhBITC, as the functionalization makes it harder to observe the mesoporous structure (140). Nonetheless, the structure is present in both formulations and may be used in future drug-loading applications.

The results obtained by TEM show the presence of the intended mesoporous properties of the nanohybrids, as well as verifying the integrity of the silica shell and the successful synthesis, as it presents very little aggregates, few empty silica shells and a consistent and suitable size for cellular application.

4.1.2. Hydrodynamic size characterization by DLS

The aim of this analysis was to confirm the nanohybrids' size, as DLS may also be used to assess their surface charge, useful to understand interaction of the cells with the nanohybrids (Figure 18).

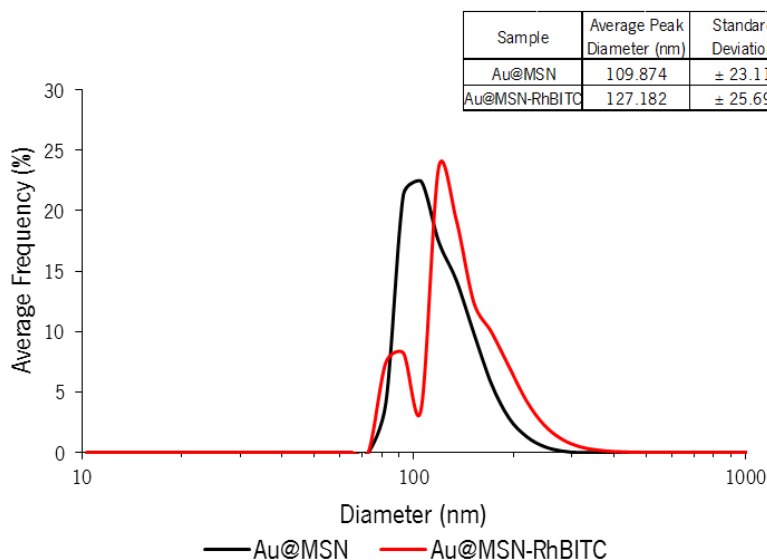


Figure 18 – Average size distribution of the Au@MSN and Au@MSN-RhBITC samples. Particle size is expected to be about 110 nm without RhBITC, and 127 nm with RhBITC.

In Figure 18, it is shown the distribution of sizes by frequency of the nanohybrids, both with and without RhBITC on their surface. To determine the average size of the nanorods, the peak sizes of

each repetition were averaged, and as shown in the table that accompanies the graph, the particle is expected to be about 110 nm without RhBITC, and 127 nm with RhBITC. More detailed graphs with each of the repetitions (five per sample), and graphs done with a linear scale can be found in the Annexes – 1 section (Supplementary figure 2, Supplementary figure 3 and Supplementary figure 4). The DLS measurement demonstrated that the nanorods with RhBITC are slightly larger than the ones without RhBITC, and this variance is not believed to have repercussions in terms of cellular internalization, as studies by Zhu *et al.* (141) suggest a maximum recommended nanoparticle diameter of 160 nm for HeLa cells specifically, while Rejman *et al.* (142) verified that non-phagocytic eukaryotic cells can internalize nanoparticles up to 500 nm when using energy-dependent processes. The sizes obtained by DLS measurement is consistent with the ones obtained in the TEM assay, thus appropriate for cellular application.

4.1.3. UV-Vis-NIR absorption spectroscopy analysis

To understand further the composition of the nanohybrids, their absorbance spectroscopy was studied (Figure 19), to confirm the presence of RhBITC attached, as it was not possible to do so in DLS or TEM measurements. The complete set of results can be found in Annexes - 3 section, in Supplementary table 1.

The results for both conditions show a peak in absorbance at about 780 nm, which is the region for the NIR plasmon absorption band, and one around 520 nm, which fall on the 510 – 540 nm range, characteristic for the surface plasmon of gold (140). The results from the sample containing RhBITC also show a third peak, in the 560-575 nm region, which is the same wavelength as the emission wavelength of rhodamine B.

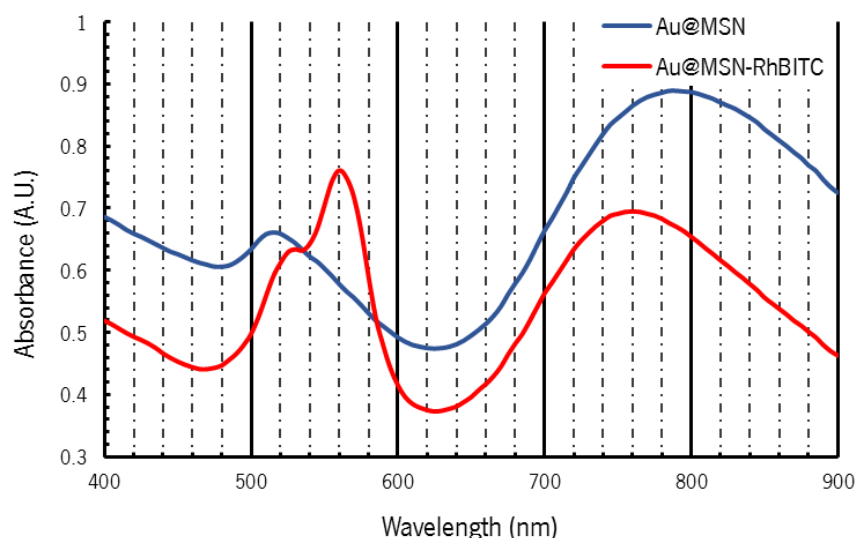


Figure 19 – Absorbance spectra taken on the bare mesoporous silica particles with gold nanorod core (Au@MSN) and on the RhBITC-loaded nanohybrids (Au@MSN-RhBITC). Both conditions show a peak in absorbance at about 780 nm (NIR plasmon absorption band), and one around 520 nm which is a characteristic region for the surface plasmon of gold. In the sample with RhBITC there is a third peak of absorbance in the 560-575 nm region, which is the same wavelength as the emission wavelength of RhB.

This allow the confirmation that RhBITC was successfully grafted to the nanoparticle's surface, as the nanohybrids present an absorbance peak absent from the nanoparticles without RhBITC.

4.1.4. Fluorescence spectroscopy

To complement the UV-Vis-NIR results, the presence of RhBITC on the nanohybrids was also assessed by fluorescence spectroscopy (Figure 20). The complete set of results can be found in Annexes - 4 section, in Supplementary table 2.

After excitation at 560 nm, which is within RhBITC's excitation wavelengths range, the Au@MSN-RhBITC hybrids show fluorescence, also within RhBITC's emission range (around 590 nm), while the Au@MSN particles do not show any fluorescence in the entirety of wavelength's range, which was expected, since they do not possess any intrinsic fluorescence by themselves.

This result proves the successful grafting of RhBITC to the Au@MSN particles' surface, as nanohybrids present fluorescence, a characteristic entirely nonexistent from the nanoparticles without RhBITC.

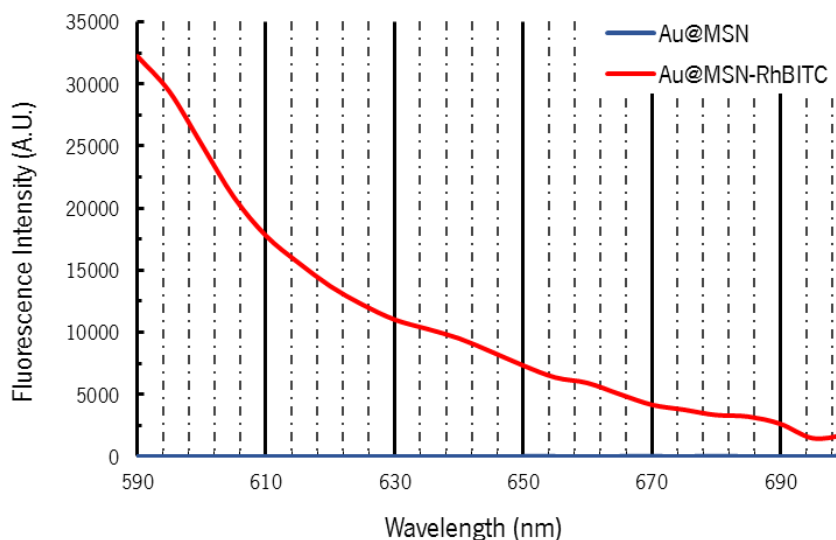


Figure 20 – Fluorescence emission spectra taken on the bare mesoporous silica particles with gold nanorod core (Au@MSN) and on the RhBITC-loaded nanohybrids (Au@MSN-RhBITC) upon excitation at 560 nm in aqueous solution at room temperature. Au@MSN-RhBITC hybrids show fluorescence within RhBITC's emission range (around 590 nm), while Au@MSN particles do not show any fluorescence.

In conclusion, the evaluated physicochemical properties of Au@MSN-RhBITC samples show an adequate size for cellular internalization, as well as optical properties that confirm the fluorescence and successful grafting of RhBITC to the nanohybrids' surface.

4.2. Functional characterization of the nanohybrids

After characterization, the next challenge is to confirm the nanohybrids' thermal heating aspect, owed to their light-absorbing gold core, and their temperature sensing characteristic, via RhBITC fluorescence lifetime dependence on temperature. In the following chapter, firstly will be discussed the results regarding the nanohybrids' ability to generate heat by irradiation with a NIR laser, and afterwards their ability to sense temperature by their fluorescence lifetime in a relevant biological temperature window (25 to 50 °C).

4.2.1. Photothermal conversion study

To understand the nanohybrids photothermal conversion, and its comparison between bare and dye-loaded particle, the sample was irradiated with a NIR laser until it reached a constant temperature, followed by blocking the laser path and allowing for the sample to cooldown. Several samples of different concentrations and irradiated at different laser powers were gathered. The time it took to reach peak temperature and cooldown points was also measured, and a representative graph of the results obtained can be found in Figure 21, while the results for every condition can be found in Annexes - 3 section (Supplementary figure 5 and Supplementary figure 6).

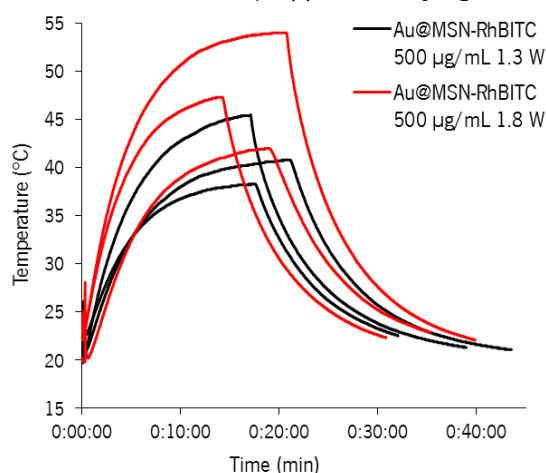


Figure 21 – Graph representing the data obtained on the photothermal conversion assay. The sample represented refers to Au@MSN-RhBITC nanohybrids at a concentration of 500 µg/mL, irradiated by two conditions of laser power (1.3 and 1.8 W). The sample reaches its highest temperatures at 1.8 W 808 nm laser power irradiation, taking around 20 minutes to reach their maximum temperature.

The results obtained in the photothermal conversion experiments depending on laser power, concentration and composition of the hybrid show a heat generation dependence' on both the concentration of nanohybrid and the laser power chosen to irradiate the sample. The control sample of H₂O (which was irradiated at 1.8 W laser power) heated poorly, so it was proven that the heat as generated by nanohybrids. The samples at higher concentration of nanohybrid reached higher temperatures over a similar period of time when compared to lower concentration of nanohybrid, which proves the amount of nanohybrid is directly correlated with temperature. In a similar way, a higher laser power irradiation was also proved to have an effect on reaching higher temperatures. In order to better understand how these factors impact the temperature reached by the nanohybrids, the minimum temperature was subtracted to the maximum temperature reached by the sample, so as to get the ΔT . Such is graphically represented in Figure 22 and Figure 23, with detailed results in Table 1. It is also possible to notice extreme fluctuation of temperatures in some of the first temperature recordings, which were left out when calculating the ΔT , as they were some human experimental errors concerning the adjustment of the thermocouple's position, as it incorrectly measured higher temperatures than the solution's actual temperature since it was directly in the laser path, with its position being adjusted in the seconds that followed.

Table 1 – Results for photothermal conversion ΔT in dependence of laser power, concentration of nanohybrid and nanohybrid composition.

			50 $\mu\text{g/mL}$	100 $\mu\text{g/mL}$	200 $\mu\text{g/mL}$	500 $\mu\text{g/mL}$
Au@MSN	1.3 W	ΔT ($^{\circ}\text{C}$)	1.20	2.50	9.23	9.97
		STDEV	0.432	1.25	2.17	1.00
	1.8 W	ΔT ($^{\circ}\text{C}$)	1.77	5.23	11.1	14.6
		STDEV	0.591	2.44	3.36	1.56
Au@MSN-RhB/TC	1.3 W	ΔT ($^{\circ}\text{C}$)	3.00	5.07	8.57	20.5
		STDEV	0.990	1.51	1.94	4.07
	1.8 W	ΔT ($^{\circ}\text{C}$)	5.17	8.80	12.0	26.2
		STDEV	0.974	1.14	1.40	4.21

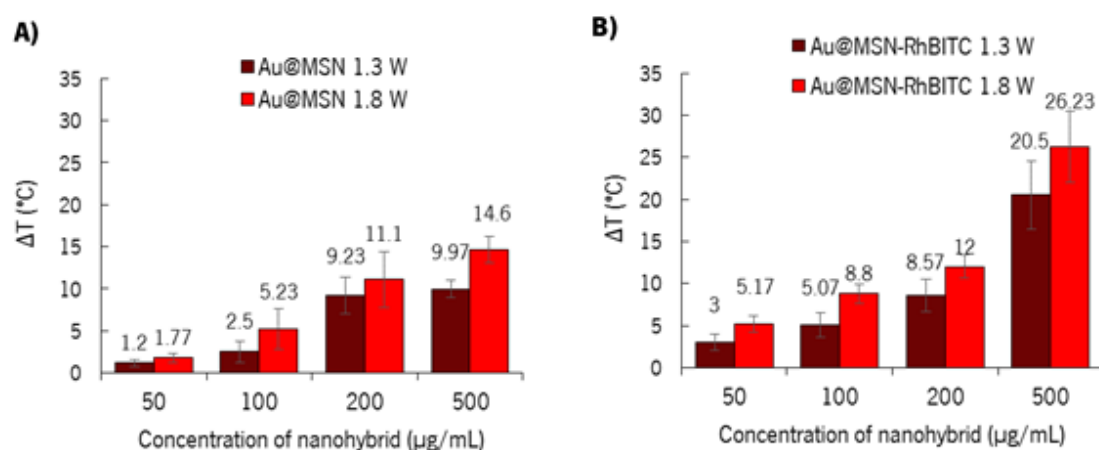


Figure 22 – Comparison of ΔT depending on different sample' concentrations and different laser power irradiation in **A** – Au@MSN nanohybrids and **B** – Au@MSN-RhBITC nanohybrids. In both samples, at higher laser power irradiation (1.8 W), higher ΔT is reached.

The effect of laser power is assessed in Figure 22, in which is possible to understand that samples at the same concentration and with the same nanohybrid composition present a higher increase in temperature in every condition when irradiated at a higher laser power. Therefore, higher laser power is directly related with a temperature rise, since there is an increase in photons, thus increasing the energy available to be absorbed by the sample.

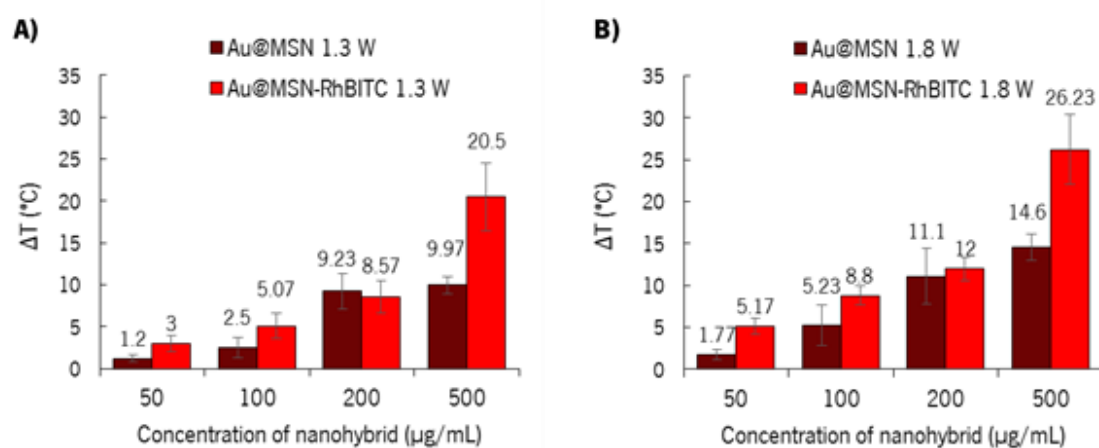


Figure 23 – Comparison of ΔT depending on different sample' concentrations and different nanohybrid composition in **A** – 1.3 W laser irradiation power and **B** – 1.8 W laser irradiation power. The samples containing RhBITC are shown to reach higher ΔT .

Regarding the influence of RhBITC on the temperature of the sample (Figure 23), both the bare (dark red) and RhBITC hybrids (red) are directly compared for 1.3 W of 808 nm illumination in Figure 23 - **A** and for 1.8 W in Figure 23 - **B**.

The nanohybrid sample containing RhBITC was shown to generate more heat, which is especially noticeable at its highest concentration (500 $\mu\text{g/mL}$), with a difference between samples of 10.53 $^{\circ}\text{C}$ and 11.6 $^{\circ}\text{C}$ for 1.3 W and 1.8 W conditions respectively. This difference can be explained by the fact that RhBITC is very soluble (111), which makes the nanohybrids easier to disperse in water (as was done for preparation of this solution) and slowing down particle aggregation. It was noticed that some deposition of the nanohybrids occurs at the bottom of the cuvette as the measurement progresses, which signifies that in more prolonged experiments there would be less nanohybrids in solution that would contribute to heat generation, as they are no longer exposed to the 808 nm laser, which was lined up to the middle part of the cuvette (Figure 8 - **B**). Since the photothermal conversion experiments were prolonged, this aspect may be deflected if the sample undergoes simultaneous stirring, although this movement may have other effects on sample temperature unrelated with laser irradiation. While this was not to be considered in the scope of this project, it may be studied in future experiments.

In conclusion, Au@MSN-RhBITC should be suitable to carry out photothermal therapy, since they were capable of increasing the temperature of the medium they are dispersed in when irradiated with a NIR laser. The extent of the heating obtained was proven to be directly dependent on laser power and concentration, and secondarily related to the nanohybrid composition. Although high ΔT 's were obtained, for the final aim of this project, the temperature rise should not exceed 10 $^{\circ}\text{C}$, as it will surpass the level of hyperthermia wanted and enter levels of temperature already known to cause irreversible injury (over 48 $^{\circ}\text{C}$, as the irradiated tissue is normally at 37.5 $^{\circ}\text{C}$) (33). Accordingly, at the concentration of 500 $\mu\text{g/mL}$ the generated heat is already exceeding what the tissue could handle before irreversible injury, while at 50 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$ the heat that was generated may not be enough to enter a hyperthermia desired stage. With these results, it was then determined to use a 200 $\mu\text{g/mL}$ concentration for the next experiments, as it was proven to generate the ideal amount heat for the purpose aimed in this project.

4.2.2. Thermal sensitivity of the nanohybrids

The following step of the experiment was to understand how the fluorescence lifetime of the nanohybrids depended on temperature, in the same concentration used for the *in vitro* assays, by using an incubator to set the sample's temperature, while the image was taken by FLIM. To assess the fluorescence lifetimes, an analysis was performed on MATLAB using the Nanophotonics Toolbox. The algorithm used was Enderlein, with modelled IRF and a fit offset of 175 time bins. The image was ran through the FLIM Image command, and the result obtained would divide the counts by time bins, in which a Gaussian curve was fitted. The software fits the model to all pixels of the recorded image and the most occurring fluorescence lifetime was the one determined for the whole sample and placed in the graph as the fluorescence lifetime, in order to get the calibration curve, with three fluorescence lifetime points for each condition. These were averaged and the standard deviation calculated to serve as the error for each condition tested.

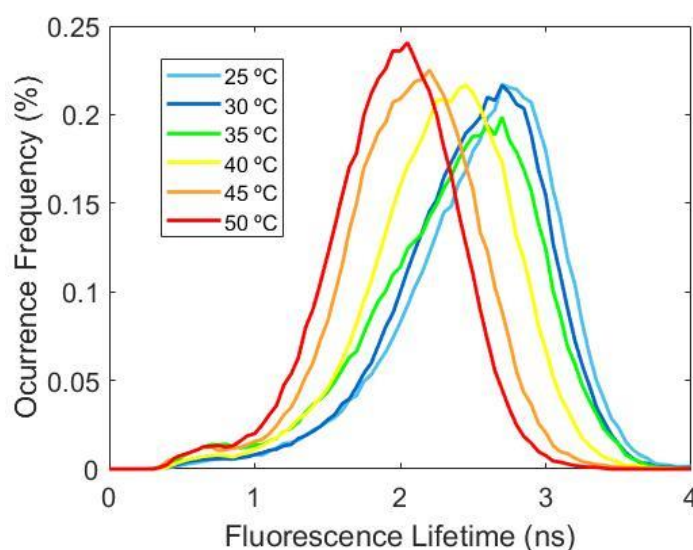


Figure 24 – Frequency of fluorescence lifetimes recorded for each temperature condition, in percentage. The lower temperatures show higher occurrences' percentage in higher fluorescence lifetimes values, while at higher temperatures, the occurrences befall in lower fluorescence lifetime values.

In Figure 24, the shift in occurrences of fluorescence lifetime depending on temperature is noticeable, as the fluorescence lifetime decreases with an increase in temperature. However, the temperature conditions' are not confined to a small distribution of fluorescence lifetimes, since there are fluorescence lifetimes that are occurring in more than one condition, although in different frequencies.

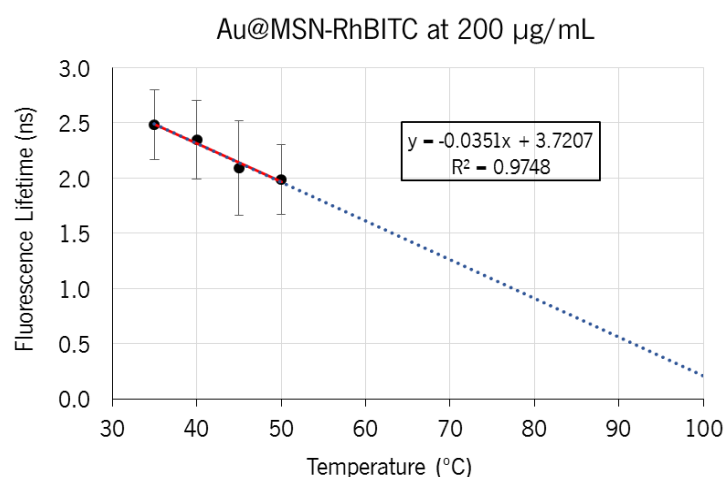


Figure 25 – Calibration curve of the Au@MSN-RhBITC nanohybrids. In red is represented the measured points and in blue the predicted fluorescence lifetimes when considering the calibration curve presented. The fluorescence lifetimes of the sample decrease as the temperature increases.

After selecting the most pertinent temperature points for *in vitro* and hyperthermia consideration (≥ 35 °C), the calibration curve was obtained, which shows a fluorescence lifetime decrease as the temperature of the sample increases (Figure 25). This decrease was expected, as per explained by Mercadé-Prieto *et al.* (68), who verified the same dependency for RhB. Each sample presents itself as heterogeneous due to absence of region selection and inclusion of nanohybrids' aggregates, as well as regions where the nanohybrids weren't present, causing the denoted error bars in Figure 25, as well as the big fluorescence lifetime distributions seen in Figure 24. However, since the imaging was always taken in the same area, the repetitions were homogeneous when in comparison, allowing for very little disparity in the sample's most occurring fluorescence lifetime. Along with the results obtained, a dotted curve was plotted, showing the results expected for higher temperatures up to 100 °C. The full display of FLIM images is found in Annexes - 6 section, Supplementary figure 7 to Supplementary figure 10, with Supplementary table 3 showing the fluorescence lifetime values used to obtain the calibration curve.

Besides assessing fluorescence lifetime, since the nanohybrids' sample was the same for every measurement, it suggests that the nanohybrids may be used for repeated experiments, as they continue exhibiting fluorescence throughout the increase in temperature.

Equation 5 represents the linear fit obtained of the calibration curve:

$$y = -0.0351x + 3.7207$$

Equation 5

After adjusting to the temperature converting function, where z is the temperature in °C and τ the fluorescence lifetime (in ns), the equation for the fluorescence lifetime conversion into temperature is denoted by Equation 6:

$$z = \frac{(\tau - 3.7207)}{-0.0351}$$

Equation 6

One aspect that could influence this calibration curve is the pH of the sample. We have found that alterations in pH induce differences in fluorescence lifetime of the nanohybrids (results that can be found in the Annexes – section 6, Supplementary figure 11), which may be interesting to analyze further, as intracellular environment also present different pHs in the various organelles (143). Expanding into this subject could show how the pH causes artifacts and changes in the fluorescence lifetime throughout the cell, and perhaps lead to different calibration curves for each organelle. This would require testing the nanohybrids fluorescence lifetime' dependence on the pH by using extremely precise pH measuring tools, as well as organelle-specific staining, to find the areas in which to apply each calibration curve.

To calculate the nanohybrids' relative sensitivity, which is defined by Equation 7 (62), where Q represents the quenching of fluorescence and T corresponds to temperature. The calculated sensitivity between 35 and 50 °C was around 0.23% °C⁻¹.

$$S = \frac{\left(\frac{\partial Q}{\partial T}\right)}{Q}$$

Equation 7

Coppeta and Rogers (144) have assessed several fluorescent dyes' sensitivity, including that of RhB, in which the fluorescence intensity was assessed in the range of 20 to 60 °C, reporting a change of $-1.54 \pm 0.03\%$ per degree °C, while Estrada-Pérez (145) mention a higher sensitivity (of 2.0% °C⁻¹) when using a conjugate of RhB with Rhodamine 110.

A novel nanohybrid established for intracellular temperature has previously been developed at UBNP, by Silva *et al.* (146), making use of the temperature-sensitive fluorescence lifetime of GFPs,

and incorporating them into the actin filaments of HeLa cells, thus reporting their temperature increase after undergoing magnetic hyperthermia treatment via incubation with magnetic nanoparticles. This nanohybrid showed a maximum sensitivity at 29°C, with a value of 0.23% °C⁻¹. Arai *et al.* (147) have studied mitochondria temperatures by fluorescence thermometry using a dye called MitoThermo Yellow, reporting up to 2.8% °C⁻¹ in sensitivity. Savchuk *et al.* (64) have also established a mitochondrial thermometer with a sensitivity of 2.2% °C⁻¹ based on the peak shift parameters.

These results suggest that the sensitivity obtained was lower than what is usually employed as a intracellular fluorescent thermometer, but changes in pH that influence the degradation and affects affinity of the RhBITC to acidic organelles (lysosomes, endosomes, protein pumps, Golgi complex, among others (148,149)) may have influenced the success on using RhBITC as a temperature sensor in this project. A pH-controlled environment would be the key in the next testing steps of this experiment, to ensure the best outcome in using RhBITC as a temperature sensor.

4.3. *In vitro* studies

In order to evaluate the interaction between the Au@MSN-RhBITC nanohybrids and the *in vitro* cell models, as well as the intracellular thermometry measurements, several assays were performed. The biocompatibility test allowed to understand if the nanohybrids could be used while maintaining cell viability, while live confocal microscopy was used to understand their intracellular localization. Lastly, live cell FLIM microscopy allowed for cellular imaging dependent on its fluorescence lifetime, allowing for simultaneous intracellular thermometry.

4.3.1. Biocompatibility assay

After characterizing the nanoparticles and demonstrating their functionality for local heating and temperature sensing, it is of interest to assess their biocompatibility, to guarantee their success on future *in vitro* live cell applications.

It's noticeable in Figure 26 – **A** that all the conditions for HeLa cells experienced cell growth, as they all increased the number of cells in each well. However, the large variability in the measurement, possibly related to the seeding process, prevents the drawing of conclusions regarding the effect of the nanoparticles on cell proliferation. In any case, since the main goal of this experiment was to evaluate the effect of the nanoparticles on cellular viability (which is a relative measurement and will not be affected by this issue), it was performed an analysis on the results related to the percentage of cells dead and alive after treatment. Figure 26 - **B** shows the percentage of cellular viability after nanoparticle incubation with cells. All of the tested conditions show a cell viability percentage above 89%, which indicates that the nanoparticles are well tolerated by the cells even at 200 µg/mL and up to at least 72 h.

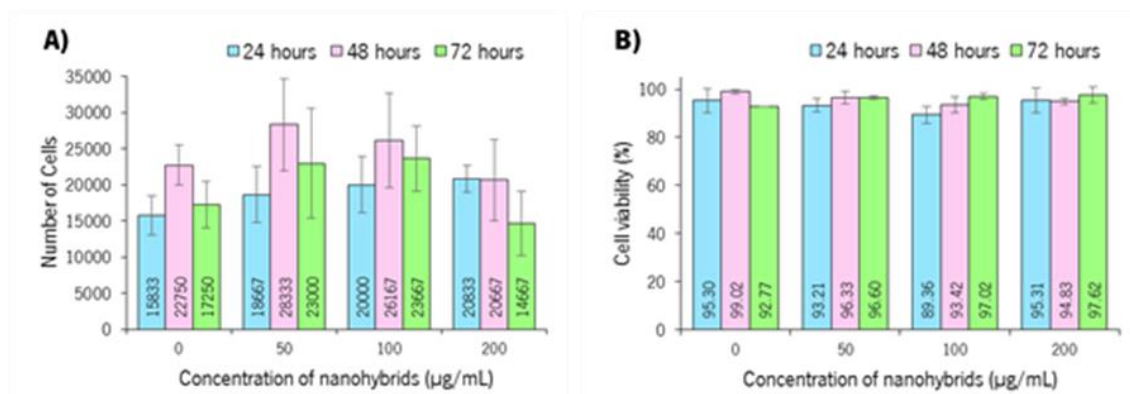


Figure 26 – **A** – Number of HeLa cells per condition; **B** – Cell viability (percentage of live cells). The cells retain cellular viability up to at least 72 hours, as they present percentages of living cells over 89 % for every condition of exposure time and concentration of Au@MSN-RhBITC nanohybrids tested.

Contrary to what was observed on HeLa cells, A431 cells showed a noteworthy difference in growth at different time periods, considering that all wells started with a density of about 10000 cells per well, a growth of 193%, 700% and 1500% correspond to 24, 48 and 72 hours respectively (Figure 27 – **A**). For HeLa cells, the growth was 88%, 145% and 97% for the same conditions respectively. This may be due to differences in each cell lines' properties, as HeLa cells have a doubling time of about 40 to 48 hours (150) , which means after that period they may have reached their maximum growth and confluency and may start to die, whilst A431 cells take 80 to 100 hours to double (151), meaning that at 72 hours, the cells are still in doubling process, showing a higher growth in the end. In terms of biocompatibility with the nanohybrids, the results obtained showed slightly better results to the ones attained from HeLa cells, with every condition showing a cellular viability of over 94% (Figure 27 - **B**).

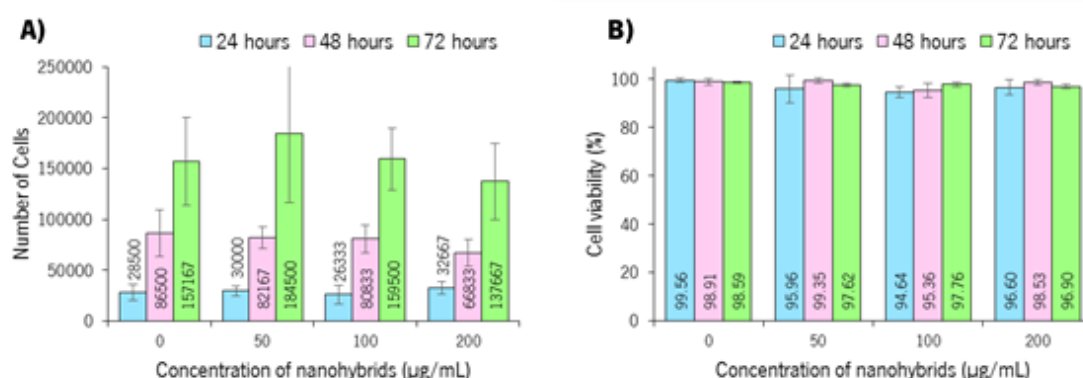


Figure 27 – **A** – Number of A431 cells per condition; **B** – Cell viability (percentage of alive cells). The cells retain cellular viability up to at least 72 hours, as they present percentages of living cells over 94 % for every condition of exposure time and concentration of Au@MSN-RhBITC nanohybrids tested.

While for both cell lines cytotoxicity seems to be very low, as per the ISO10993-5 standard that indicates that biocompatibility is achieved with a cell viability of over 70% (152), there are some issues associated with the method in which the results are obtained from. The cell viability test used, known as the Trypan Blue exclusion test of cell viability, relies on membrane integrity that prevents the dye from entering on living cells (134), and has been used for evaluating nanoparticles' cytotoxicity in numerous articles (153,154). However, many additional factors other than nanoparticle-caused damage may destroy the membrane or disrupt its integrity, as well as other kinds of toxicity may happen and go undetected, which makes this assay fairly unreliable. Unfortunately, there were many incompatibilities with other tests available, which made this the only solution obtainable. For instance, using the MTT test meant that there could be an enhancement of exocytosis of the formazan crystals by the presence of the mesoporous silica shell (155), leading to incorrect results, while MTS test absorbs in the same wavelength as gold (at 490-500 nm) (156,157), as well as Alamar Blue®, which has the same absorption wavelength as RhB (158,159) and resazurin whose absorption spectrum coincides with the emission spectra of RhB (160). In Annexes - 7 section is presented Supplementary table 4 and Supplementary table 5, that contain the full results for the biocompatibility assays.

In summary, these results prove that these nanohybrids are biocompatible and well-suited for *in vitro* assays at concentrations of up to 200 µg/mL.

4.3.2. Live cell confocal microscopy

To further understand nanohybrids' internalization, cells were incubated with nanohybrids at 200 µg/mL, as well with a lysosomes-specific dye, to visualize the location of the nanohybrids after internalization, which was done at 12 and 24 hours for each cell line (Figure 28 and Figure 29).

Figure 28 and Figure 29 show the results obtained from confocal imaging, in which both cell lines were incubated with LysoHunt Blue DND-22. The dye was excited at 405 nm laser irradiation, while laser irradiation at 561 nm was used to excite the RhBITC in the Au@MSN-RhBITC nanohybrids. The goal was to see places where the fluorescence would appear in both conditions, in order to confirm if the nanohybrids were internalized and if they were still present and maintained some integrity after 24 hours of exposure. The images shown already merge both wavelengths of irradiation, and in red are shown the results obtained by irradiation of the LysoHunt, while in blue

are the Au@MSN-RhBITC nanohybrids. The individual images, as well as camera images, may be found in the Annexes – 8 section (Supplementary figure 12 to Supplementary figure 15).

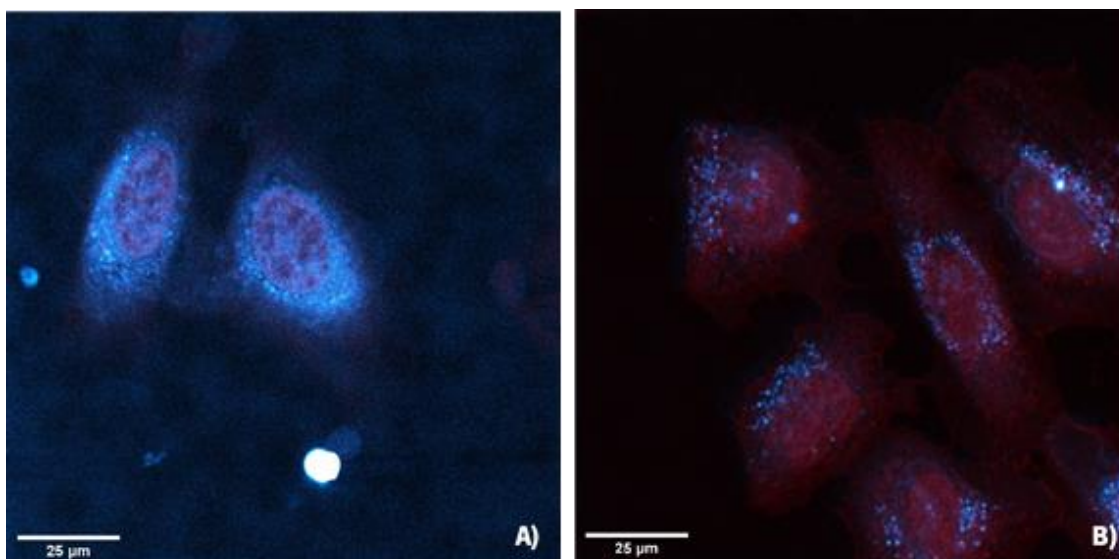


Figure 28 – HeLa cells with Au@MSN-RhBITC nanohybrids at 200 $\mu\text{g/mL}$, exposed in **A** – 12 hours and **B** – 24 hours. In red is represented LysoHunt Blue DND-22 dye, while in blue are represented Au@MSN-RhBITC nanohybrids.

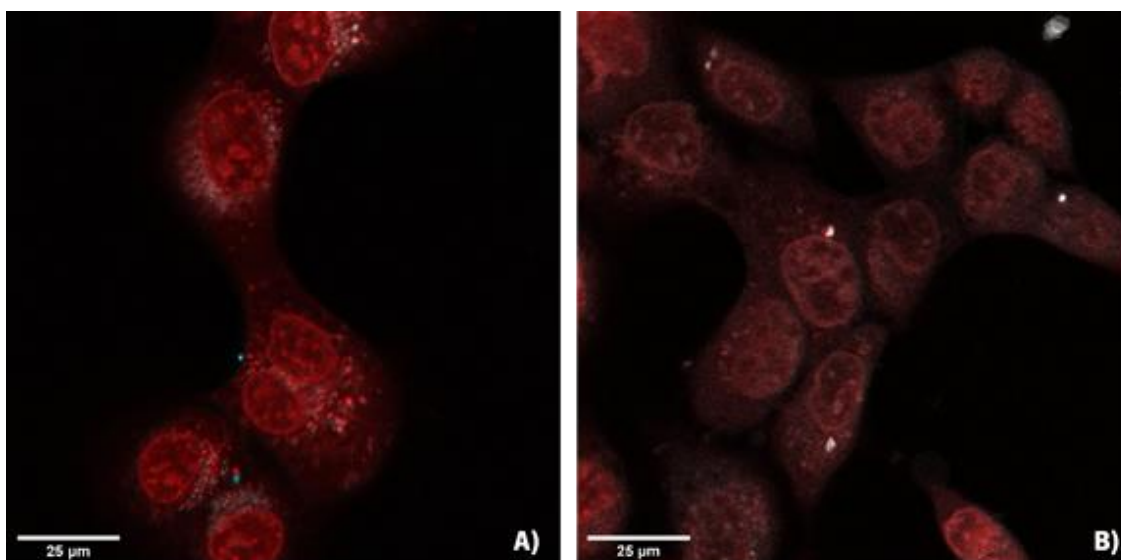


Figure 29 – A431 cells with Au@MSN-RhBITC nanohybrids at 200 $\mu\text{g/mL}$, exposed in **A** – 12 hours and **B** – 24 hours. In red is represented LysoHunt Blue DND-22 dye, while in blue are represented Au@MSN-RhBITC nanohybrids.

The use of the dye allowed to stain lysosomes, which are organelles responsible for receiving and degrading macromolecules that enter the cell, due to their acid hydrolases within (161). If internalization of the nanohybrids occurred, lysosomal activity would in the same area of the nanoparticles, meaning their fluorescence would overlap. The lysosomes can be seen in red in full extension of the cell area. In Figure 28 and Figure 29 it is possible to see the fluorescence of the nanohybrids inside of the cell, as they are within the area marked in red by the lysosomes, meaning that they don't remain exclusively in the extracellular environment. The location of the nanohybrids is also more substantial in proximity to the nucleus, but not inside of it, which suggests that while the nanohybrids penetrate the cellular membrane, they cannot penetrate the nuclear membrane.

Comparing between cell lines, HeLa cells show high fluorescence surrounding the nucleus in both time conditions, while the same is not observed for A431 cells, which seem to have more concentration of nanohybrids next to the nucleus at 12 hours, and very little at 24 hours (although still present). In the HeLa cells however, the fluorescence of the Au@MSN-RhBITC seems to be higher than the lysosomes' fluorescence, which may be likely due to a change in focal plane.

In conclusion, the cells from both cell lines were confirmed to uptake the nanorods, as there are many overlapping fluorescence areas, staying inside the cell for at least 24 hours, although they seem to have their fluorescent properties heightened at 12 hours, as the particles have been less exposed to silica shell-degrading factors, when in comparison with 24 hours exposure. Further assays will be beneficial to assess uptake dynamics, Au@MSN-RhBITC intracellular stability and expulsion. This could be studied by comparing cellular membranes' labelling, which can be labelled by free-diffusion RhBITC that has detached from the nanohybrid surface due to degradation, in comparison with localized intensity spots that correspond to intact nanohybrids inside of lysosomes. Since this degradation can take days to occur (Figure 5), it would be of interest to gather images at time points at a span of different days, to pin-point the exact time at which this degradation starts to occur intracellularly. Beyond degradation, cellular expulsion could also be tested after cellular uptake, by performing exosome labelling.

4.3.3. Live cell FLIM microscopy for intracellular thermometry

After determining the Au@MSN-RhBITC nanohybrids heating efficacy and knowing the concentrations at which they are biocompatible with the selected cell lines, it is of interest to explore how their heating capacity and intracellular temperature reporting capability evolves upon 808 nm laser irradiation. Previously it was obtained a calibration curve (Equation 6) that will allow to convert the fluorescence lifetime into temperature, by the process illustrated in Figure 30.

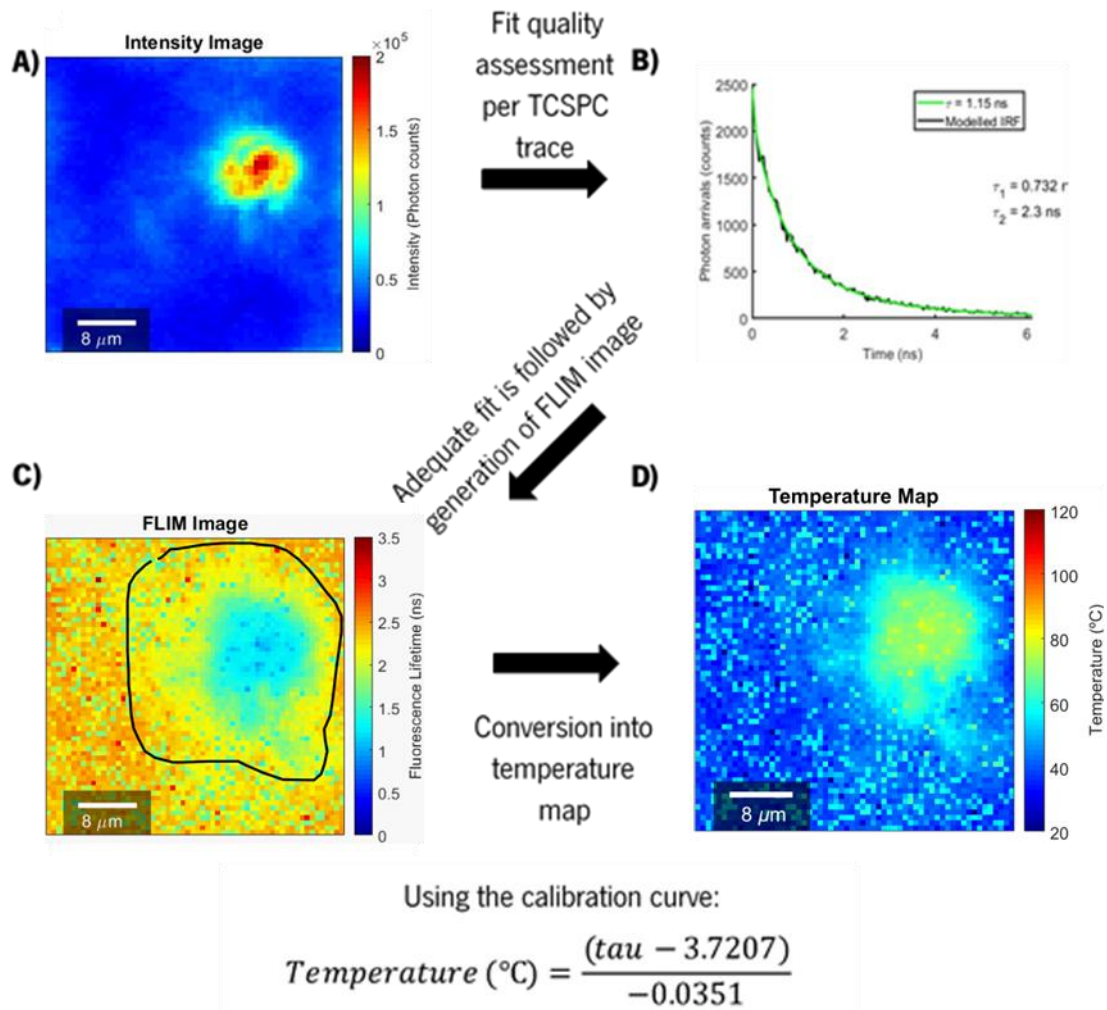


Figure 30 – General scheme on how the FLIM results were processed. In **A** is represented the intensity image, obtained directly from the APD detector. In **B** is shown the curve fitting, to understand if the algorithm used was adequate for the sample, obtaining **C**, which represents the distribution of fluorescence lifetimes. After obtaining the FLIM image, two analysis could be done: the first is visible in **C**, which is selecting the cell region (in black), and in **D** is the image obtained by converting the FLIM image into temperature by using the calibration curve. Example represented by HeLa cells without 808 nm laser irradiation at 12 hours exposure time to nanohybrids.

The process represented in Figure 30 was done for each condition, with at least three measurements for each one. However, due to such plentiful results, a representative sample of the cell model prior to heating, and another after heating 3 minutes at 1.3 W was chosen (while the remaining conditions may be found at the Annexes - 8 section, comprising of measurements for both cell lines at 12 and 24 hours of exposure to the Au@MSN-RhBITC, with irradiation with an 808 nm laser at either 1.3 W or 1.8 W, for 3, 6, 9 or 15 minutes).

4.3.3.1. Fluorescence lifetime measurements

Prior to irradiation with the 808 nm laser, the cells' fluorescence lifetime was measured only in dependence of the top-stage incubator's temperature, as a way to initially understand how the RhBITC could report the intracellular temperature before initiating the gold nanorod mediated heating.

In Figure 31 – **Condition 1** are shown HeLa cells that were incubated for 12 hours with Au@MSN-RhBITC prior to 808 nm laser irradiation. The FLIM image presents fluorescence lifetime values mostly in the range of 1 to 2.5 ns, while in the selected region (which is outlined by what is seen on the intensity image), the most-occurring fluorescence lifetime value is at 2.17 ns, which using the calibration curve mentioned in Equation 6 translates into 44 °C. Other peak values of fluorescence lifetime occur at 1.68 ns (translating into 58 °C) and 1.28 ns (translating into 70 °C).

In Figure 31 – **Condition 2** are shown HeLa cells that were incubated for 12 hours with Au@MSN-RhBITC, after undergoing 808 nm laser irradiation, at 1.3 W laser power for 3 minutes. The FLIM image presents fluorescence lifetime values mostly in the range of 1 to 2.2 ns, while in the selected region, the most-occurring fluorescence lifetime value is at 2.26 ns, which using aforementioned calibration curve translates into 42 °C, while the other peak value of fluorescence lifetime occurs at 1.88 ns (translating into 52 °C). After laser irradiation, the fluorescence lifetime has increased in the overall FLIM image, and consequently on the peak fluorescence lifetime values that occur within the selected areas. It is possible to see on both images a concentration of nanohybrids (presenting higher intensity values) around a structure, which correlating with confocal images previously shown, is believed to be the cell nucleus, as in these images there is a concentration of nanohybrids around it as well. This structure presents a shorter lifetime than the rest of the selected area, which can be considered as the cell's cytoplasm.

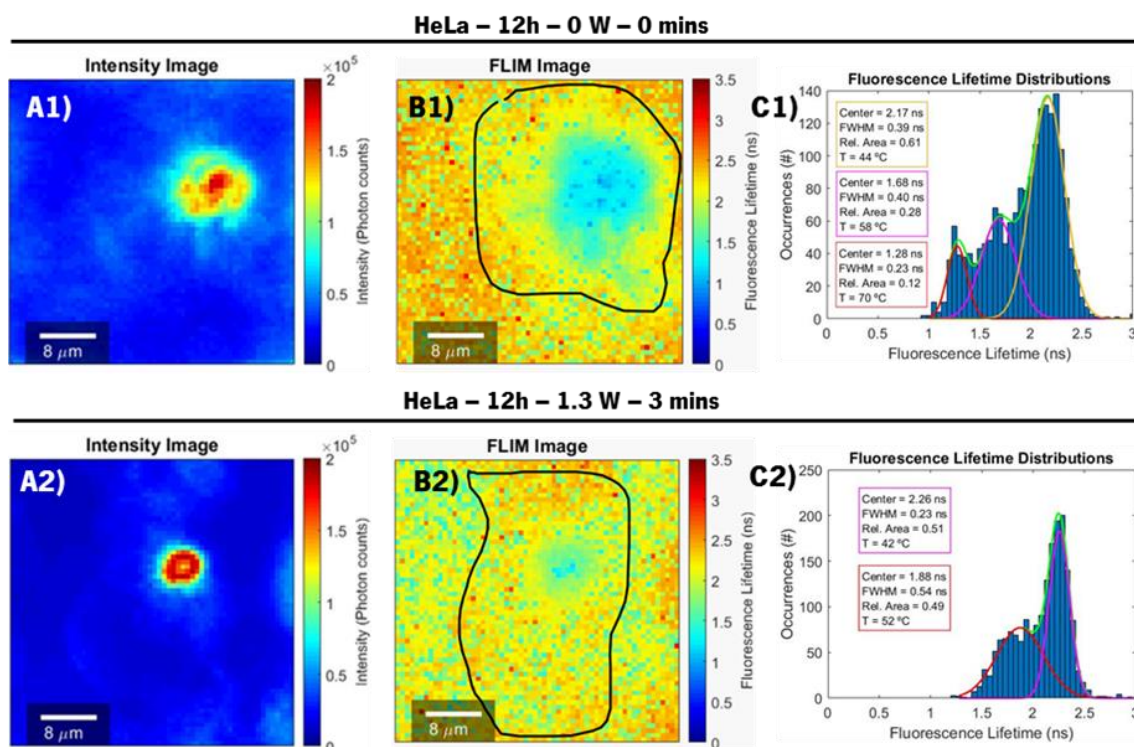


Figure 31 – Fluorescence intensity and FLIM characterization of Au@MSN-RhBITC nanohybrids: **A** – Intensity image, **B** - FLIM image that represents fluorescence lifetimes found in the measurement as well as cell region selection, **C** - Histogram of fluorescence lifetime values in highlighted region, which matches the extension of a cells - in accordance with the intensity distributions observed in **A**). The samples are both 12 h nanohybrid incubation with HeLa cells, and in **Condition 1** (top row) is represented the absence of 808 nm laser irradiation, while **Condition 2** (bottom row) undergoes 808 nm laser irradiation, 1.3 W for 3 minutes.

In Figure 32 – **Condition 1** are shown HeLa cells that were incubated for 24 hours with Au@MSN-RhBITC prior to 808 nm laser irradiation. The FLIM image presents a wide variety of fluorescence lifetime values, with higher fluorescence lifetime in the background (around 2.5 ns), while lower lifetime values are encountered in somewhat circular structures spread throughout the image. In the selected region (0 – 2 ns), the occurrences are insufficient to present a robust Gaussian fit, meaning that the structures selected do not present a prevalent fluorescence lifetime. In Figure 32 – **Condition 2** are shown HeLa cells that were incubated for 24 hours with Au@MSN-RhBITC, after 808 nm laser irradiation, at 1.3 W laser power for 3 minutes. The FLIM image presents fluorescence lifetime values mostly in the range of 0.5 to 1.5 ns, dominated by the selected region, with a small part of fluorescence lifetime values between 2 to 3 ns in the background. In the selected region, the most-occurring fluorescence lifetime value is at 0.90 ns,

which using the calibration curve translates into 80 °C, while the other peak value of fluorescence lifetime occurs at 1.19 ns (reporting 72 °C).

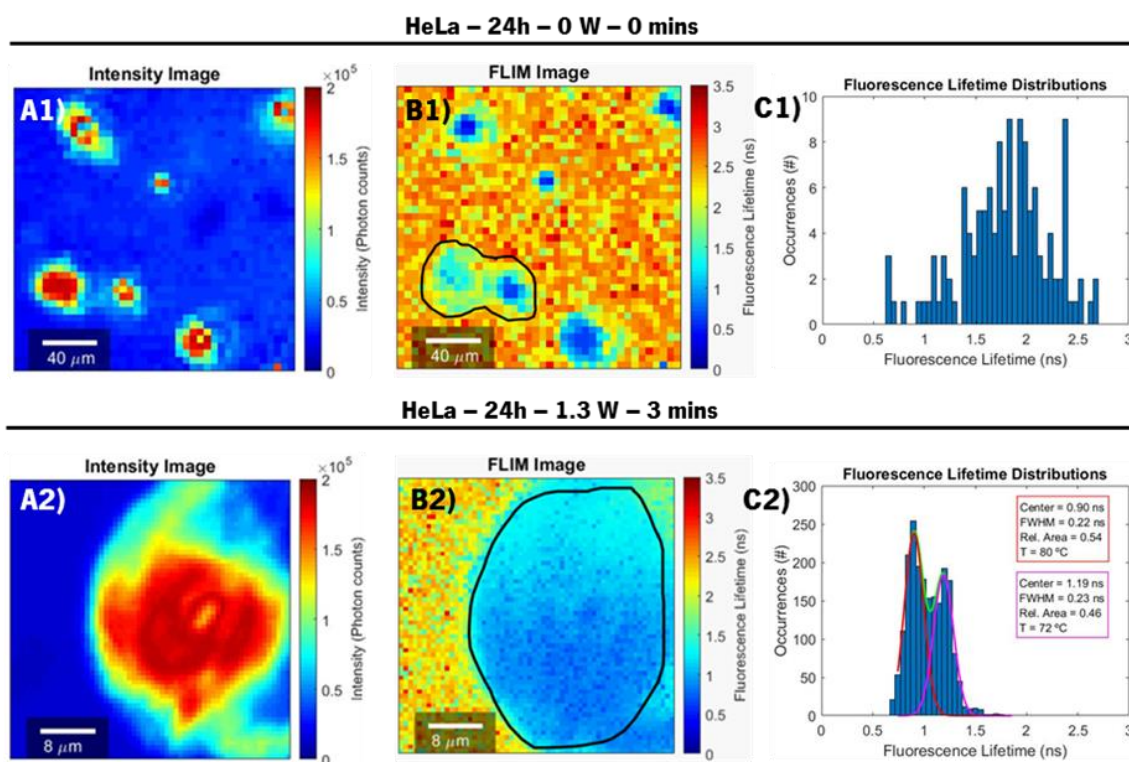


Figure 32 – Fluorescence intensity and FLIM characterization of Au@MSN-RhBITC nanohybrids: **A** – Intensity image, **B** - FLIM image that represents fluorescence lifetimes found in the measurement as well as cell region selection, **C** - Histogram of fluorescence lifetime values in highlighted region, which matches the extension of a cells - in accordance with the intensity distributions observed in **A**). The samples are both 24 h nanohybrid incubation with HeLa cells, and in **Condition 1** (top row) is represented the absence of 808 nm laser irradiation, while **Condition 2** (bottom row) undergoes 808 nm laser irradiation, 1.3 W for 3 minutes.

For A431 cells measurement that were incubated for 12 hours with Au@MSN-RhBITC prior to 808 nm laser irradiation (Figure 33 – **Condition 1**), the most occurring lifetimes in the background range between 2 to 2.5 ns, which is within the same range as reported for HeLa cells in the same zone. Inside of the selected region, three fluorescence lifetimes are most prevalent, being the most occurring 0.69 ns (translating into 86 °C), followed by 1.05 ns (translating into 76 °C) and finally by 1.66 ns (translating into 59 °C). In Figure 33 – **Condition 2** are shown A431 cells that were incubated for 12 hours with Au@MSN-RhBITC after 808 nm laser irradiation, at 1.3 W laser power for 3 minutes. The FLIM image presents a higher fluorescence lifetime in the background (around

2 ns), while lower lifetime values are encountered within the selected region (0.5 – 1.5 ns). From the Gaussian fit in the fluorescence lifetimes in that region, the most occurring fluorescence lifetime is at 1.06 ns (corresponding to 76 °C), while two more peaks are observed (1.23 ns reporting 71 °C and 1.47 ns reporting 64 °C).

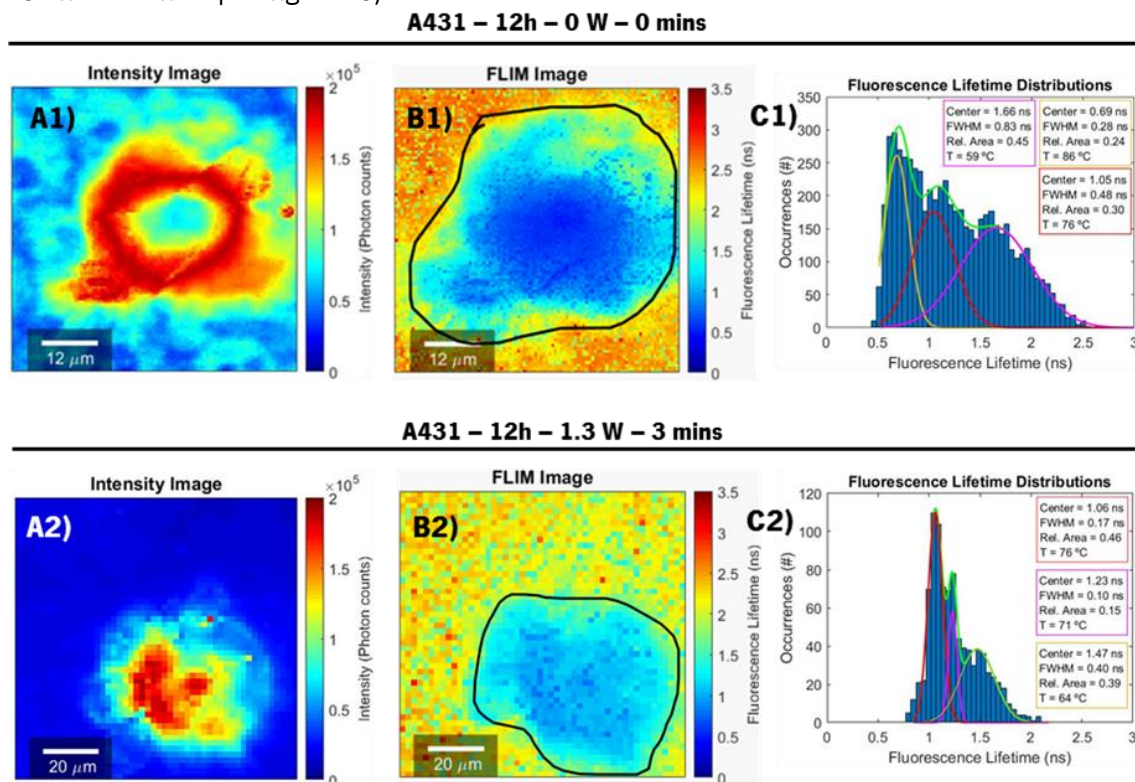


Figure 33 – Fluorescence intensity and FLIM characterization of Au@MSN-RhBITC nanohybrids: **A** – Intensity image, **B** - FLIM image that represents fluorescence lifetimes found in the measurement as well as cell region selection, **C** - Histogram of fluorescence lifetime values in highlighted region, which matches the extension of a cells - in accordance with the intensity distributions observed in **A**). The samples are both 12 h nanohybrid incubation with A431 cells, and in **Condition 1** (top row) is represented the absence of 808 nm laser irradiation, while **Condition 2** (bottom row) undergoes 808 nm laser irradiation, 1.3 W for 3 minutes.

For A431 cells measurement that were incubated for 24 hours with Au@MSN-RhBITC prior to 808 nm laser irradiation (Figure 34 – **Condition 1**), the fluorescence lifetimes are more homogenous than on previously shown samples, with the full image having fluorescence lifetime values predominantly between 2 to 2.5 ns. The selected region presents a uniform fluorescence lifetime distribution as well, with a peak at 2.27 ns that reports 41 °C. In Figure 34 – **Condition 2** are shown A431 cells that were incubated for 24 hours with Au@MSN-RhBITC after 808 nm laser irradiation, at 1.3 W laser power for 3 minutes. The background presents itself as having

homogenous fluorescence lifetime, within the range of 2 to 2.5 ns. For the selected region, two different cellular locations may be distinguished. The first, which is believed to be the nucleus due to higher concentration of nanohybrids around it (also visible in confocal images presented as Figure 29), reports a shorter fluorescence lifetime, at 0.89 ns (translating into 81 °C). The other region, the cytoplasm, reports a higher fluorescence lifetime, at 2.29 ns (translating into 41 °C). Lastly, from the Gaussian fit one more peak is shown, which corresponds to background that was included during cell selection, which reports 1.88 ns (translating into 52 °C).

In summary, the fluorescence lifetime reported in the different cell conditions for the background seem to be fairly consistent, which indicates that the FLIM analysis has been correctly collected. Some cellular heterogeneity has also been shown, indicating that the nanohybrids' intracellular distribution is not uniform, concentrating mostly in the surroundings of the nucleus, as per shown in Figure 28 and Figure 29 as well.

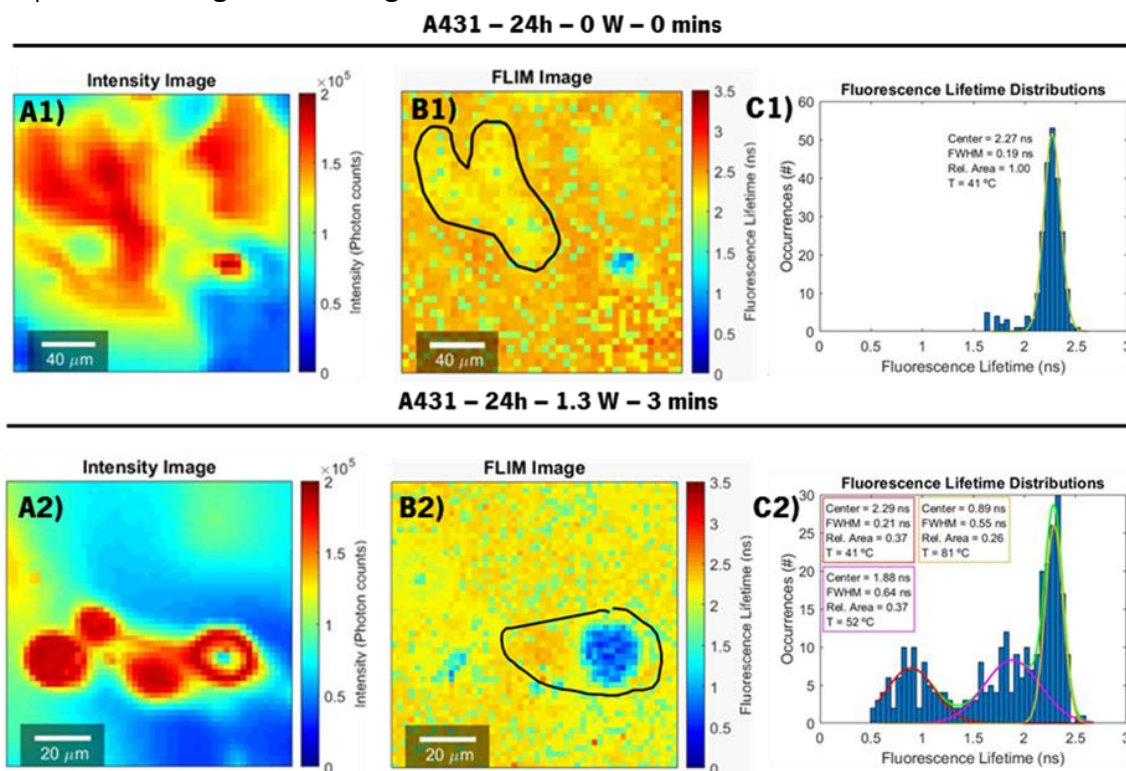


Figure 34 – Fluorescence intensity and FLIM characterization of Au@MSN-RhBITC nanohybrids: **A** – Intensity image, **B** - FLIM image that represents fluorescence lifetimes found in the measurement as well as cell region selection, **C** - Histogram of fluorescence lifetime values in highlighted region, which matches the extension of a cells - in accordance with the intensity distributions observed in **A**). The samples are both 24 h nanohybrid incubation with A431 cells, and in **Condition 1** (top row) is represented the absence of 808 nm laser irradiation, while **Condition 2** (bottom row) undergoes 808 nm laser irradiation, 1.3 W for 3 minutes.

However, regarding cellular selection some issues were encountered, mainly due to the fact that fluorescence lifetime has increased after 808 nm laser irradiation, which was unexpected, since it has been established that RhB's fluorescence lifetime decreases as the temperature increases (68), and it was established in 4.2.1 that 808 nm laser irradiation increased the temperature of the solution.

To guarantee success on this experiment there is a need to gather results before and after laser irradiation on the same cell, which has yet to be achieved since with changes in temperature and possible over-irradiation, the cells detach from the bottom of the well and were verified to change location in between measurements.

4.3.3.2. Temperature conversion from fluorescence lifetime imaging

Another type of analysis performed beyond the fluorescence lifetime obtained and its distribution from the FLIM imaging, was the conversion of said fluorescence lifetime into temperature, by using Equation 6 in order to do so. In this analysis, the full FLIM image has been converted into temperature values, with a discussion focused on the overall representation.

In Figure 35, the temperature maps of different conditions regarding the HeLa cell line (exposure time to Au@MSN-RhBITC nanohybrids and presence or absence of 808 nm laser irradiation) are shown. In all of the figures it is possible to see a predominant background in the range of 40 to 60 °C, which indicates a higher temperature than the one set at the incubator where the cells were kept throughout the measurement (37.5 °C). As this is seen at both the images with and without 808 nm laser irradiation, indicating that the calibration curve is reporting higher temperatures than it was expected. Nevertheless, in the regions that concentrate further nanohybrids (in the surroundings of what is believed to be the cell's nucleus as per seen in confocal imaging), the temperature was measured as higher than the one verified on the background (around 60 °C), and in the structure that is regarded as the nucleus, the temperature reaches its peak, in the ranges of 60 to 80 °C.

Comparing between exposure times to nanohybrids, the temperature seems to reach higher values in the 24 h exposure time, which could be expected due to the cells further internalizing the nanohybrids over time. Regarding laser irradiation, the images that reference HeLa cells prior to

laser irradiation show a broad range of temperatures other than the background, with regions reaching up to 80 °C in 12 h nanohybrid exposure and > 90 °C in 24 h nanohybrid exposure. After 808 nm laser irradiation, the temperature seems to be lower throughout the sample, as the temperatures do not exceed 70 °C in 12 h nanohybrid exposure, and are < 90 °C in 24 h nanohybrid exposure.

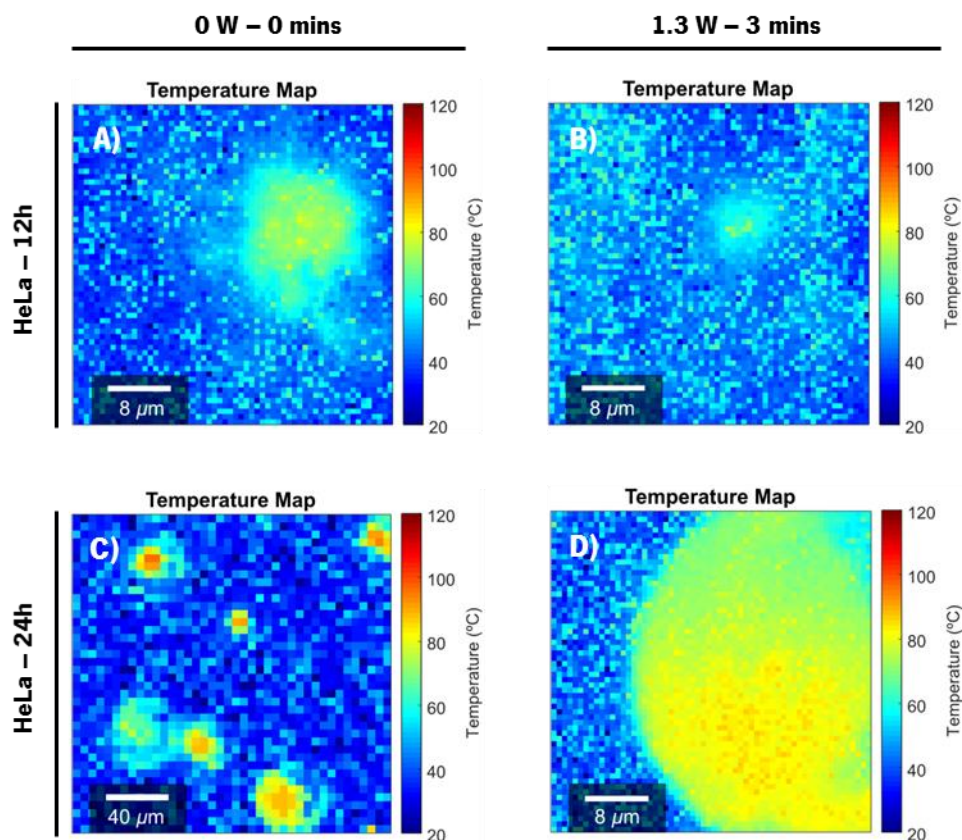


Figure 35 – Temperature maps resulting from converting FLIM images using the calibration curve determined in Equation 6 for Au@MSN-RhBITC nanohybrids incubated for **A** - 12 h with HeLa cells, without 808 nm laser irradiation; **B** - 12 h with HeLa cells, with 808 nm laser irradiation for 3 minutes at 1.3 W laser power; **C** - 24 h with HeLa cells, without 808 nm laser irradiation, **D** - 24 h with HeLa cells, with 808 nm laser irradiation for 3 minutes at 1.3 W laser power.

In Figure 36, the temperature maps of different conditions regarding the A431 cell line (exposure time to Au@MSN-RhBITC nanohybrids and presence or absence of 808 nm laser irradiation) are shown. In all of the figures it is possible to see a predominant background in the range of 40 to 60 °C as well, staying in accordance to what was observed for HeLa cells at this location. In the regions next to what is believed to be the cell's nucleus the temperature was measured as higher than the one verified on the background (around 70 °C), and in the structure that is regarded as the nucleus, the temperature reaches its peak, in the ranges of 80 to 90 °C.

After 808 nm laser irradiation, the temperature seems to be lower, from what is observed in the 12 h measurements, as the image regarding cells prior to laser irradiation depicts temperatures up to 90 °C, while the measurement taken after laser irradiation does not exceed 80 °C. The same cannot be correlated for 24 hours images, as the temperature before laser irradiation is fairly homogenous, and the area where the measurement took place differs greatly from the area gathered after 808 nm laser irradiation. This difference in measurement location is better observed in the intensity images presented in Figure 33 and Figure 34.

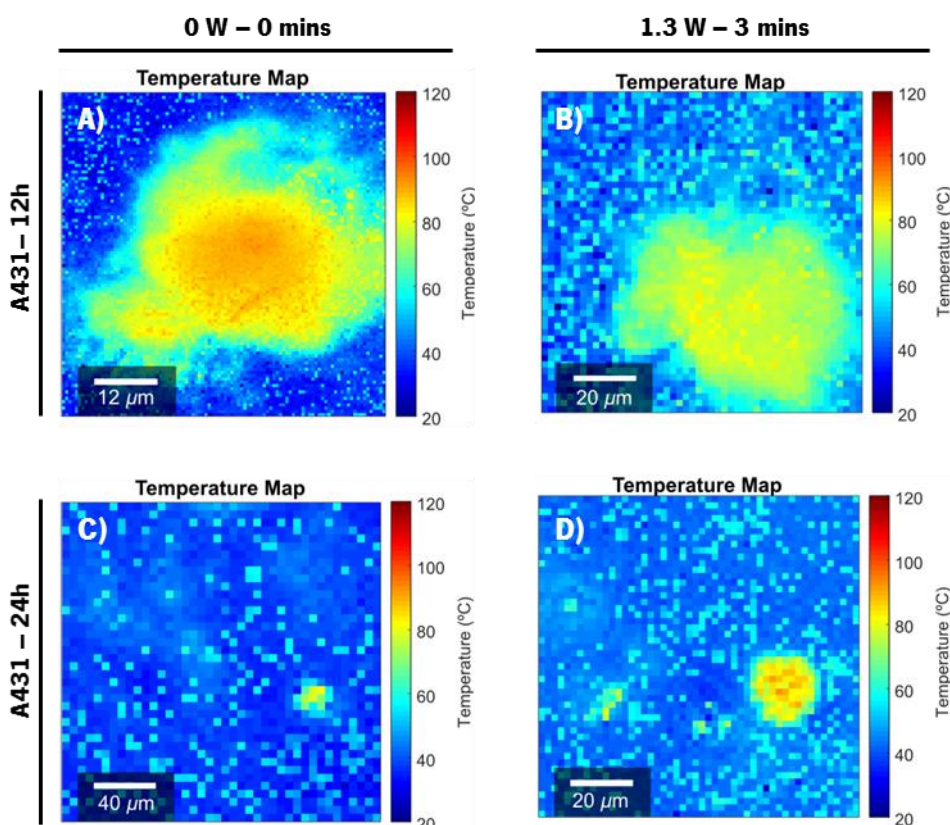


Figure 36 – Temperature maps resulting from converting FLIM images using the calibration curve determined in Equation 6 for Au@MSN-RhBITC nanohybrids incubated for **A** - 12 h with A431 cells, without 808 nm laser irradiation; **B** - 12 h with A431 cells, with 808 nm laser irradiation for 3 minutes at 1.3 W laser power; **C** - 24 h with A431 cells, without 808 nm laser irradiation, **D** - 24 h with A431 cells, with 808 nm laser irradiation for 3 minutes at 1.3 W laser power.

For both HeLa and A431 cells, their starting temperature was measured to be around 35 °C, and ideally shouldn't reach over 48 °C after laser irradiation, as to not enter irreversible tissue damage temperatures. However, even in the samples prior to 808 nm laser irradiation and therefore deprived of further heat generation, the temperature had far exceed such values, which

is an indicator of issues most likely regarding the method of conversion of fluorescence lifetime into temperature.

The main goal of this experiment was to understand if the nanohybrids could generate heat intracellularly, as well as report the temperature they could reach by their fluorescence lifetime, which was achieved. While the results presented seem promising, there are still many pitfalls that should be taken into account to get more optimized and accurate results. One aspect that could have contributed for such varying and unexpected temperatures could be the fact that the same sample was overly irradiated, as this process occurred always in the same place of the same well, in consecutive measurements. Not only had this meant that the cells were exposed to non-sterile surroundings for a long time, this irradiation caused evaporation of the medium on some conditions, which meant the temperature reached was much different than predicted, drying the cells and making them prone to cellular death from reasons that would not be considered for *in vivo* conditions. The images in annex (Supplementary figure 16 to Supplementary figure 41) have most likely suffered over irradiation and the results obtained may be compromised, as many figures depict cells with a round shape (which in A431 and HeLa cell lines is a dead cell's characteristic), as well as showing changes in cell location in consecutive measurements, due to detaching of the cell from the dishes' surface, which is another indication of poor cellular viability.

To overcome this pitfall, ideally there would be one prepared silicon well with nanohybrids-incubated cells for each condition of exposure and laser irradiation, to avoid accumulation of damaging factors throughout the experiment. Beyond this change in preparation, the sample may be over irradiated from the experiment's own settings, as 1.3 W or even 3 minutes may be too intense for cellular survival. While at photothermal conversion experiment these seem suited parameters, the differences between higher sample volume and presence of cells may be crucial factors that were not accounted for previously, meaning that a wider range of conditions must be tested to ensure a robust profile of laser power/irradiation time dependency.

While the calibration curve decays in a manner that was expected from previous works, some problems have arisen from its use as well, which means that its optimization is a crucial step for this experiment's future success. An aspect that could have influenced the calibration curve success in temperature conversion were the changes in the environmental setting that have taken place during different exposure times.

One of the aspects that was considered was the pH of the solution used for calibration. This factor has an influence on the fluorescence lifetime, as a change in pH influences the degradation of RhBITC molecules, which was analogously observed by Chang *et al.* (162) with RhB molecules, hindering a correct assessment of fluorescence lifetime. Besides degrading RhBITC molecules, pH also has an effect on gold nanorods' shape during synthesis, as per verified by Cheng *et al.* (163), that mention basic solutions change the rod shape, meaning that the ability of heating (characteristic of the rod shape), is severely compromised. A pH-dependent experiment regarding Au@MSN-RhBITC's fluorescence lifetime dependence on temperature was done by adding drops of acetic acid until pH had changed (measured by Universal Indicator Paper - pH 1-11), in nanohybrids at a starting pH of 8. In Supplementary figure 11, a graph describing the results from this experiment is shown. While the decrease in fluorescence lifetime with the rise of temperature is confirmed in most conditions, at pH 6 the slope seems to have an opposite tendency, increasing as temperature increases. It was also verified that the fluorescence lifetime presented different values as the pH changed within the same temperature mark. This indicates that these nanohybrids fluorescence lifetime's dependency on pH should be tested much further, in order to build a complete profile on this dependency, as cell environment pH changes over time and this should be accounted for when measuring intracellular temperature. Another point that could be of interest would be the use of multiple calibration curves, as to understand the temperature of each intracellular organelle, as they differ in pH and could report different temperatures throughout the measurement.

Another aspect that can be considered is the ionic strength, as per mentioned to have influence in the fluorescence parameter by Savchuk *et al.* (64) as well as photobleaching, inhomogeneous concentration and excitation power instability variations in the surrounding medium. As the measurement takes place, the variations in the cellular medium will accumulate over time, which can also be determinant of the calibration curve's success. To overcome such pitfalls, these parameters must be equivalent throughout all of the measurements, including those of cellular and of calibration nature.

Concerning the intrinsic cellular temperature, Lervik *et al.* (164) have studied the thermal properties of proteins, and verified that proteins can sustain large thermal gradients throughout their structure, due to their low heat conductivity when compared to water. This may explain heat spots in the cell vicinity, as a higher presence of proteins could sustain the heat longer, than when in comparison with the medium. Here there is also some presence of proteins included in the cell

medium (due to FBS), and it would be of interest to perform an experiment in which the cells would be cultured in FBS-free medium or even PBS, as to get a higher denotation of such heat spots caused by cell's intrinsic proteins. Besides proteins, the cell as a whole also presents thermal conductivity, which allowed Park *et al.* (148) to use as a differentiating parameter between live and dead cells, as the former presented a lower thermal conductivity than the latter. In terms of intracellular gradients, the information available is still very limited, and a recent study by Howard *et al.* (165) simulated what could be the intracellular fluids motion and transportation caused by differences in organelles' intrinsic temperature, by convection currents instead of random diffusive motion. In the occurrence that these simulations get verified *in vitro*, it may be an important factor to take into account in the scope of this experiment, as it will allow to understand better how the temperature influences intracellular transport, which is useful in terms of nanoparticle cellular distribution for drug delivery.

In conclusion, while the nanohybrids in study have reported temperature based on fluorescence lifetime measurements, the result shown differ from what was expected in terms of cellular survival, indicating problems that could be due to wrongful temperature conversion, for which further factors that may influence the calibration curve used must be studied.

Chapter 5 - Conclusion and future perspectives

The nanohybrids in study were successfully synthesized and characterized, and their size was shown to be appropriate for cellular application. They were verified to be biocompatible on HeLa and A431 cell lines, with viabilities over 89 % in every condition tested, which may also pave way for possible future *in vivo* studies.

The nanohybrids were also proved to have heating properties after irradiation with an 808 nm laser, as their photothermal conversion results show heat generation dependent on laser power and nanohybrid concentration. The highest temperature increase was obtained was at the highest concentration and laser power irradiation (500 µg/mL of Au@MSN-RhBITC at 1.8 W 808 nm laser irradiation), reaching 26.23 °C.

Following the nanohybrids' characterization and demonstration of their biocompatibility, a calibration curve of their fluorescence lifetime in dependency of temperature was obtained at relevant *in vitro* temperatures, in both typical and hyperthermia circumstances (from 35 to 50 °C).

Finally, after proving their internalization by fluorescence imaging, the cells were irradiated with an 808 nm laser at different laser powers and exposure times, obtaining intensity and FLIM images, as well as an intracellular temperature map, reported by resorting to the calibration curve previously obtained. The results obtained show a successful uptake of the nanohybrids by both cell lines, although the derived temperature maps report temperature values incompatible with cellular survival.

This incorrect temperature reporting may have been caused by the usage of a calibration curve that has not been perfected for this application, as well as sample over-irradiation, which should be a priority for advancement when repeating this experiment. For this purpose, a more extensive research on the nanohybrids' temperature dependence on factors other than the ones tested should be done (such as ionic strength or pH), as it is likely a key source of error in this conversion, making use of optical characterization to perfect the calibration curve as well. Additionally, the experiment should ideally be repeated in independent wells for each condition, as over-irradiation has caused cell drying and possible death that could be avoided. While fresh medium could be added throughout the experiment to replace what is evaporated, it may hinder the reliability on the

concentration used, which has already been proven to be an important factor for the heating obtained.

Nevertheless, the results obtained in both biocompatibility and photothermal conversion experiments are encouraging, as they prove that further *in vitro* tests may take place using the heating properties shown by this work, and while challenges were encountered with the temperature sensing unit results, in this work is also possible to already understand some of the pitfalls and use the proposed strategies to circumvent these issues in future experiments.

Furthermore, the mesoporous hybrid nanostructures proposed in this work could be used in the future to additionally deliver a chemotherapeutic agent, by drug-loading the pores of the mesoporous silica shell, combining hyperthermia with chemotherapy in one nanohybrid, which will not only increase the sensitivity of tumor cells to treatment with the heating component, it could simultaneously target-delivery the most adequate drug, increasing the chances of survival by reducing the side effects from generalized treatment application.

References

1. Feynman RP. There's Plenty of Room at the Bottom: An Invitation to Enter a New Field of Physics. 1959 p. 22–36.
2. Mishra S, Keswani C, Abhilash PC, Fraceto LF, Singh HB. Integrated approach of Agri-Nanotechnology: Challenges and future trends. *Front Plant Sci.* 2017;8(471):1–12.
3. Guerra FD, Attia MF, Whitehead DC, Alexis F. Nanotechnology for environmental remediation: Materials and applications. *Molecules.* 2018;23(7):1–23.
4. Aziz ZAA, Mohd-Nasir H, Ahmad A, Setapar SHM, Peng WL, Chuo SC, et al. Role of Nanotechnology for Design and Development of Cosmeceutical: Application in Makeup and Skin Care. *Front Chem.* 2019;7(739):1–15.
5. Zhang A, Lieber CM. Nano-Bioelectronics. *Chem Rev.* 2016;116(1):215–57.
6. M R, Tn D, T V, K S. Applications of Nanotechnology. *J Nanomedicine Biother Discov.* 2015;5(1):1–5.
7. Caruso F, Hyeon T, Rotello VM. Nanomedicine. *Chem Soc Rev.* 2012;41(7):2537–8.
8. Ferlay J, Ervik M, Lam F, Colombet M, Mery L, Piñeros M, et al. Global Cancer Observatory: Cancer Tomorrow. Lyon, France: International Agency for Research on Cancer [Internet]. Global Cancer Observatory. 2018. Available from: <https://gco.iarc.fr/tomorrow>
9. Bray F, Møller B. Predicting the future burden of cancer. *Nat Rev Cancer.* 2006;6(1):63–74.
10. Kanavos P. The rising burden of cancer in the developing world. *Ann Oncol.* 2006;17(Supplement 8):15–23.
11. Krump NA, You J. Molecular mechanisms of viral oncogenesis in humans. *Nat Rev Microbiol.* 2018;16(11):684–98.
12. Organization WH. Cancer prevention [Internet]. 2020. Available from: <https://www.who.int/cancer/prevention/en/>
13. Arem H, Lofftfield E. Cancer Epidemiology: A Survey of Modifiable Risk Factors for Prevention and Survivorship. *Am J Lifestyle Med.* 2018;12(3):200–10.
14. Ma X, Yu H. Global Burden of Cancer. *Yale J Biol Med.* 2006;79(3–4):85–94.
15. Peters JM, Gonzalez FJ. The Evolution of Carcinogenesis. *Toxicol Sci.* 2018;165(2):272–6.
16. Cancro LPC o. Diagnóstico [Internet]. 2015 [cited 2020 May 13]. Available from: <https://www.ligacontracancro.pt/diagnostico/>
17. Cancro LPC o. Métodos de Tratamento [Internet]. 2015 [cited 2020 May 13]. Available from: <https://www.ligacontracancro.pt/metodos-de-tratamento/>
18. Mihlon F, Ray CE, Messersmith W. Chemotherapy agents: A primer for the interventional radiologist. *Semin Intervent Radiol.* 2010;27(4):384–90.

19. Nygren P. What is cancer chemotherapy? *Acta Oncol (Madr)*. 2001;40(2–3):166–74.
20. Baskar R, Lee KA, Yeo R, Yeoh K-W. Cancer and radiation therapy: Current advances and future directions. *Int J Med Sci*. 2012;9(3):193–9.
21. Yang Y. Cancer immunotherapy: harnessing the immune system to battle cancer. *J Clin Invest*. 2015;125(9):3335–7.
22. Reinbolt RE, Mangini N, Hill JL, Levine LB, Dempsey JL, Singaravelu J, et al. Endocrine Therapy in Breast Cancer: The Neoadjuvant, Adjuvant, and Metastatic Approach. *Semin Oncol Nurs*. 2015;31(2):146–55.
23. McLeod DG. Hormonal Therapy: Historical Perspective to Future Directions. *Urology*. 2003;61(2A):3–7.
24. Hellerstedt BA, Pienta KJ. The Current State of Hormonal Therapy for Prostate Cancer. *CA Cancer J Clin*. 2002;52(3):154–79.
25. Mahla RS. Stem Cells Applications in Regenerative Medicine and Disease Therapeutics. *Int J Cell Biol*. 2016;7:1–23.
26. Gmeiner WH, Ghosh S. Nanotechnology for cancer treatment. *Nanotechnol Rev*. 2014;3(2):111–22.
27. Shi Y, van der Meel R, Chen X, Lammers T. The EPR effect and beyond: Strategies to improve tumor targeting and cancer nanomedicine treatment efficacy. *Theranostics*. 2020;10(17):7921–4.
28. Sutradhar KB, Amin ML. Nanotechnology in Cancer Drug Delivery and Selective Targeting. *ISRN Nanotechnol*. 2014;2014:1–12.
29. Walter EJ, Hanna-Jumma S, Carraretto M, Forni L. The pathophysiological basis and consequences of fever. *Crit Care [Internet]*. 2016;20(1):1–10. Available from: <http://dx.doi.org/10.1186/s13054-016-1375-5>
30. O'Grady NP, Barie PS, Bartlett JG, Bleck T, Carroll K, Kalil AC, et al. Guidelines for evaluation of new fever in critically ill adult patients: 2008 Update from the American College of Critical Care Medicine and the Infectious Diseases Society of America. *Crit Care Med*. 2008;36(4):1330–49.
31. Squire W. Observations on the Temperature of the Body in Health and Disease. *British Med J*. 1871;1(524):32.
32. Coley WB. Contribution to the knowledge of sarcoma. *Ann Surg*. 1891;14(3):199–220.
33. Jaque D, Maestro LM, del Rosal B, Haro-Gonzalez P, Benayas A, Plaza JL, et al. Nanoparticles for photothermal therapies. *Nanoscale*. 2014;6(16):9494–530.
34. Walter EJ, Carraretto M. The neurological and cognitive consequences of hyperthermia. *Crit Care [Internet]*. 2016;20(199):1–8. Available from: <http://dx.doi.org/10.1186/s13054-016-1376-4>
35. Wakim KG. Bodily Reactions to High Temperature. *Anesthesiology*. 1964;25(4):532–48.
36. Wust P, Hildebrandt B, Sreenivasa G, Rau B, Gellermann J, Riess H, et al. Hyperthermia in combined treatment of cancer. *Lancet Oncol*. 2002;3:487–97.

37. Mulier PMJ, Hoey MF. Method and Apparatus for RF Ablation and Hyperthermia. 1998. p. 38.
38. Murugan C, Sharma V, Murugan RK, Malaimengu G, Sundaramurthy A. Two-dimensional cancer theranostic nanomaterials: Synthesis, surface functionalization and applications in photothermal therapy. *J Control Release* [Internet]. 2019;299(February):1–20. Available from: <https://doi.org/10.1016/j.jconrel.2019.02.015>
39. Glazer ES, Curley SA. The Ongoing History of Thermal Therapy for Cancer. *Surg Oncol Clin N Am* [Internet]. 2011;20(2):229–35. Available from: <http://dx.doi.org/10.1016/j.soc.2010.11.001>
40. Chatterjee DK, Diagaradjane P, Krishnan S. Nanoparticle-mediated hyperthermia in cancer therapy. *Ther Deliv*. 2011;2(8):1001–14.
41. Hu J-J, Cheng Y-J, Zhang X-Z. Recent advances in nanomaterials for enhanced photothermal therapy of tumors. *Nanoscale*. 2018;10(48):22657–72.
42. Cheng L, Wang C, Feng L, Yang K, Liu Z. Functional Nanomaterials for Phototherapies of Cancer. *Chem Rev*. 2014;114:10869–939.
43. Jaque D, Vetrone F. Luminescence nanothermometry. *Nanoscale*. 2012;4(15):4301–26.
44. Riley RS, Day ES. Gold nanoparticle-mediated photothermal therapy: applications and opportunities for multimodal cancer treatment. *Wiley Interdiscip Rev Nanomedicine Nanobiotechnology*. 2017;9(4):1–25.
45. Kennedy LC, Bickford LR, Lewinski NA, Coughlin AJ, Hu Y, Day ES, et al. A New Era for Cancer Treatment: Gold-Nanoparticle-Mediated Thermal Therapies. *Small*. 2011;7(2):169–83.
46. Kim HS, Lee DY. Near-Infrared-Responsive Cancer Photothermal and Photodynamic Therapy Using Gold Nanoparticles. *Polymers (Basel)*. 2018;10(961):1–14.
47. Hu B, Zhang L-P, Chen X-W, Wang J-H. Gold nanorod-covered kanamycin-loaded hollow SiO₂ (HKAurod) nanocapsules for drug delivery and photothermal therapy on bacteria. *Nanoscale*. 2013;5(1):246–52.
48. Beytollahi L, Pourhajibagher M, Chiniforush N, Ghorbanzadeh R, Raoofian R, Pourakbari B, et al. The efficacy of photodynamic and photothermal therapy on biofilm formation of *Streptococcus mutans*: An in vitro study. *Photodiagnosis Photodyn Ther* [Internet]. 2017;17:56–60. Available from: <http://dx.doi.org/10.1016/j.pdpdt.2016.10.006>
49. Maitland DJ, Metzger MF, Schumann D, Lee A, Wilson TS. Photothermal properties of shape memory polymer micro-actuators for treating stroke. *Lasers Surg Med*. 2002;30(1):1–11.
50. Small IV W, Wilson TS, Benett WJ, Loge JM, Maitland DJ. Laser-activated shape memory polymer intravascular thrombectomy device. *Opt Express*. 2005;13(20):8204.
51. Jung HS, Verwilt P, Sharma A, Shin J, Sessler JL, Kim JS. Organic molecule-based photothermal agents: An expanding photothermal therapy universe. *Chem Soc Rev*. 2018;47(7):2280–97.
52. Liu H, Ge J, Ma E, Yang L. Advanced biomaterials for biosensor and theranostics. In: *Biomaterials in Translational Medicine* [Internet]. Elsevier Inc.; 2019. p. 213–55. Available

from: <http://dx.doi.org/10.1016/B978-0-12-813477-1.00010-4>

53. Gai S, Yang G, Yang P, He F, Lin J, Jin D, et al. Recent advances in functional nanomaterials for light – triggered cancer therapy. *Nano Today* [Internet]. 2018;19:146–87. Available from: <http://dx.doi.org/10.1016/j.nantod.2018.02.010>
54. Hu K, Liu T, Chung K, Huang K, Hsieh C, Sun C-K, et al. Efficient Near-IR Hyperthermia and Intense Nonlinear Optical Imaging Contrast on the Gold Nanorod-in-Shell Nanostructures. *J Am Chem Soc*. 2009;131(40):14186–7.
55. Davaji B, Richie JE, Lee CH. Microscale direct measurement of localized photothermal heating in tissue-mimetic hydrogels. *Sci Rep* [Internet]. 2019;9(6546):1–12. Available from: <http://dx.doi.org/10.1038/s41598-019-42999-w>
56. Abadeer NS, Murphy CJ. Recent Progress in Cancer Thermal Therapy Using Gold Nanoparticles. *J Phys Chem C*. 2016;120:4691–716.
57. Boulnois J-L. Photophysical processes in recent medical laser developments: A review. *Lasers Med Sci*. 1986;1(1):47–66.
58. Patterson MS, Wilson BC, Wyman DR. The propagation of optical radiation in tissue I. Models of radiation transport and their application. *Lasers Med Sci*. 1991;6:155–68.
59. Stolik S, Delgado JA, Pérez A, Anasagasti L. Measurement of the penetration depths of red and near infrared light in human “ex vivo” tissues. *J Photochem Photobiol B Biol*. 2000;57:90–5.
60. Gazdaru D, Chilom C, Calin MA, Geanta C, Popescu A. Laser Radiation Propagation and Heat Transfer Into Cells and Tissues. *Rom J Biophys*. 2000;18(1):73–85.
61. Carlos LD, Palacio F. Thermometry at the Nanoscale: Techniques and Selected Applications. 2016. 534 p.
62. Brites CDS, Lima PP, Silva NJO, Millán A, Amaral VS, Palacio F, et al. Thermometry at the nanoscale. *Nanoscale*. 2012;4(16):4799–829.
63. Paviolo C, Clayton AHA, McArthur SL, Stoddart PR. Temperature measurement in the microscopic regime: a comparison between fluorescence lifetime- and intensity-based methods. *J Microsc*. 2013;250(3):179–88.
64. Savchuk OA, Silvestre OF, Adão RMR, Nieder JB. GFP fluorescence peak fraction analysis based nanothermometer for the assessment of exothermal mitochondria activity in live cells. *Sci Rep*. 2019;9(7535):1–11.
65. Kulinich A V., Ishchenko AA, Bondarev SL, Sukhodola AA. Effect of Temperature on the Spectral Fluorescent Properties of Positively Solvatochromic Merocyanines. *Theor Exp Chem*. 2018;54(5):331–8.
66. Zhang W, Wang G, Cai Z, Baxter GW, Collins SF. Spectral analysis for broadband fluorescence: temperature sensing with the YAG:Ce phosphor as an example. *Opt Mater Express*. 2016;6(11):3482.
67. Szmajcinski H, Lakowicz JR. Fluorescence lifetime-based sensing and imaging. *Sensors Actuators B*. 1995;29:16–24.

68. Mercadé-Prieto R, Rodríguez-Rivera L, Chen XD. Fluorescence lifetime of Rhodamine B in aqueous solutions of polysaccharides and proteins as a function of viscosity and temperature. *Photochem Photobiol Sci.* 2017;16(11):1727–34.
69. Chaze W, Caballina O, Castanet G, Lemoine F. The saturation of the fluorescence and its consequences for laser-induced fluorescence thermometry in liquid flows. *Exp Fluids* [Internet]. 2016;57(58). Available from: <https://doi.org/10.1007/s00348-016-2142-8>
70. Kristoffersen AS, Erga SR, Hamre B, Frette Ø. Testing Fluorescence Lifetime Standards using Two-Photon Excitation and Time-Domain Instrumentation: Rhodamine B, Coumarin 6 and Lucifer Yellow. *J Fluoresc.* 2014;24:1015–24.
71. Pikulik LG, Rudik KI, Kostko MY. Temperature dependence of the fluorescence polarization of complex molecules. *J Appl Spectrosc.* 1968;9(1):731–3.
72. Thompson SA, Martínez IA, Haro-González P, Adam AP, Jaque D, Nieder JB, et al. Plug and Play Anisotropy-Based Nanothermometers. *ACS Photonics.* 2018;5(7):2676–81.
73. Okabe K, Inada N, Gota C, Harada Y, Funatsu T, Uchiyama S. Intracellular temperature mapping with a fluorescent polymeric thermometer and fluorescence lifetime imaging microscopy. *Nat Commun.* 2012;3(705):1–9.
74. Gota C, Okabe K, Funatsu T, Harada Y, Uchiyama S. Hydrophilic Fluorescent Nanogel Thermometer for Intracellular Thermometry. *J Am Chem Soc.* 2009;131(8):2766–7.
75. Bai T, Gu N. Micro / Nanoscale Thermometry for Cellular Thermal Sensing. *Small.* 2016;12(34):4590–610.
76. Ying F, Zhaofu L, Shongsheng Q, Congyi Z, Hong X. Study on the thermosensitivity of a tumor cell by microcalorimetry. *Thermochim Acta.* 1997;303(2):203–7.
77. Watanabe MS, Kakuta N, Mabuchi K, Yamada Y. Micro-thermocouple probe for measurement of cellular thermal responses. In: 27th Annual International Conference of the IEEE Engineering in Medicine and Biology Society IEEE Engineering in Medicine and Biology Society Conference. 2005. p. 4858–61.
78. Wang C, Xu R, Tian W, Jiang X, Cui Z, Wang M, et al. Determining intracellular temperature at single-cell level by a novel thermocouple method. *Cell Res.* 2011;21(10):1517–9.
79. Shrestha R, Choi T-Y, Chang W, Kim D. A high-precision micropipette sensor for cellular-level real-time thermal characterization. *Sensors.* 2011;11(9):8826–35.
80. Inomata N, Toda M, Sato M, Ishijima A, Ono T. Pico calorimeter for detection of heat produced in an individual brown fat cell. *Appl Phys Lett.* 2012;100(15):1–4.
81. Gao L, Wang L, Li C, Liu Y, Ke H, Zhang C, et al. Single-cell photoacoustic thermometry. *J Biomed Opt.* 2013;18(2):1–5.
82. Donner JS, Thompson SA, Kreuzer MP, Baffou G, Quidant R. Mapping intracellular temperature using green fluorescent protein. *Nano Lett.* 2012;12(4):2107–11.
83. Thakor A, Jokerst J, Zaveleta C, Massoud T, Gambhir S. Gold Nanoparticles: A Revival in Precious Metal Administration to Patients. *Nano Lett.* 2011;11(10):4029–36.
84. Antonovych TT. Gold Nephropathy. *Ann Clin Lab Sci.* 1981;11(5):386–91.

85. Kean WF, Kean IRL. Clinical pharmacology of gold. *Inflammopharmacology*. 2008;16(3):112–25.
86. Vines JB, Yoon J-H, Ryu N-E, Lim D-J, Park H. Gold Nanoparticles for Photothermal Cancer Therapy. *Front Chem*. 2019;7(167):1–16.
87. Baptista P, Pereira E, Eaton P, Doria G, Miranda A, Gomes I, et al. Gold nanoparticles for the development of clinical diagnosis methods. *Anal Bioanal Chem*. 2008;391(3):943–50.
88. Chithrani BD, Chan WCW. Elucidating the Mechanism of Cellular Uptake and Removal of Protein-Coated Gold Nanoparticles of Different Sizes and Shapes. *Nano Lett*. 2007;7(6):1542–50.
89. Pan Y, Neuss S, Leifert A, Fischler M, Wen F, Simon U, et al. Size-Dependent Cytotoxicity of Gold Nanoparticles. *Small*. 2007;3(11):1941–9.
90. Dykman LA, Khlebtsov NG. Gold Nanoparticles in Biology and Medicine: Recent Advances and Prospects. *Acta Naturae*. 2011;3(2 (9)):34–55.
91. Huang X, El-Sayed MA. Plasmonic photo-thermal therapy (PPTT). *Alexandria J Med* [Internet]. 2011;47(1):1–9. Available from: <http://dx.doi.org/10.1016/j.ajme.2011.01.001>
92. Zeng S, Baillargeat D, Ho H-P, Yong K-T. Nanomaterials enhanced surface plasmon resonance for biological and chemical sensing applications. *Chem Soc Rev*. 2014;43:3426–52.
93. Alsultan AG, Mijan N-A, Taufiq-Yap YH. Nanomaterials: An Overview of Nanorods Synthesis and Optimization [Online First]. In: *Nanomaterials: An Overview of Nanorods Synthesis and Optimization* [Online First]. 2019. p. 1–24.
94. Huang X, El-Sayed IH, Qian W, El-Sayed MA. Cancer Cell Imaging and Photothermal Therapy in the Near-Infrared Region by Using Gold Nanorods. *J Am Chem Soc*. 2006;128(6):2115–20.
95. Link S, El-Sayed MA. Optical properties and ultrafast dynamics of metallic nanocrystals. *Annu Rev Phys Chem*. 2003;54:331–66.
96. Chaudhuri RG, Paria S. Core/Shell Nanoparticles: Classes, Properties, Synthesis Mechanisms, Characterization, and Applications. *Chem Rev*. 2012;112:2373–433.
97. Mahmoudi M, Lynch I, Ejtehadi MR, Monopoli MP, Bombelli FB, Laurent S. Protein - Nanoparticle Interactions : Opportunities and Challenges. *Chem Rev*. 2011;111:5610–37.
98. Wang L, Luo J, Fan Q, Suzuki M, Suzuki IS, Engelhard MH, et al. Monodispersed Core - Shell Fe₃O₄@Au Nanoparticles. *J Phys Chem B*. 2005;109(46):21593–601.
99. Mercier L, Pinnavaia TJ. Heavy Metal Ion Adsorbents Formed by the Grafting of a Thiol Functionality to Mesoporous Silica Molecular Sieves : Factors Affecting Hg (II) Uptake. *Environ Sci Technol*. 1998;32(18):2749–54.
100. Zhong Z, Patskovskyy S, Bouvrette P, Luong JHT, Gedanken A. The Surface Chemistry of Au Colloids and Their Interactions with Functional Amino Acids. *J Phys Chem B*. 2004;108(13):4046–52.

101. Iskandar F, Lenggoro IW, Kim TO, Nakao N, Shimada M, Okuyama K. Fabrication and Characterization of SiO₂ Particles Generated by Spray Method for Standards Aerosol. *J Chem Eng Japan*. 2001;34(10):1285–92.
102. Lu J, Liong M, Zink JI, Tamanoi F. Mesoporous silica nanoparticles as a delivery system for hydrophobic anticancer drugs. *Small*. 2007;3(8):1341–6.
103. Paris JL, Colilla M, Izquierdo-Barba I, Manzano M, Vallet-Regí M. Tuning mesoporous silica dissolution in physiological environments: a review. *J Mater Sci*. 2017;52(15):8761–71.
104. Narayan R, Nayak UY, Raichur AM, Garg S. Mesoporous Silica Nanoparticles: A Comprehensive Review on Synthesis and Recent Advances. *Pharmaceutics*. 2018;10(118):1–49.
105. Chhatre A, Thaokar R, Mehra A. Formation of Gold Nanorods by Seeded Growth: Mechanisms and Modeling. *Cryst Growth Des*. 2018;18(6):3269–82.
106. Pérez-Juste J, Pastoriza-Santos I, Liz-Marzán LM, Mulvaney P. Gold nanorods: Synthesis, characterization and applications. *Coord Chem Rev*. 2005;249:1870–901.
107. Nikoobakht B, El-sayed MA. Preparation and Growth Mechanism of Gold Nanorods (NRs) Using Seed-Mediated Growth Method. *Chem Mater*. 2003;15:1957–62.
108. Allen JM, Xu J, Blahovec M, Canonico-May SA, Santaloci TJ, Braselton ME, et al. Synthesis of less toxic gold nanorods by using dodecylethyldimethylammonium bromide as an alternative growth-directing surfactant. *J Colloid Interface Sci* [Internet]. 2017;505:1172–6. Available from: <http://dx.doi.org/10.1016/j.jcis.2017.06.101>
109. Li P, Wu Y, Li D, Su X, Luo C, Wang Y, et al. Seed-Mediated Synthesis of Tunable- Aspect-Ratio Gold Nanorods for Near-Infrared Photoacoustic Imaging. *Nanoscale Res Lett*. 2018;13(313):1–8.
110. Villaverde G, Gómez-Graña S, Guisasola E, García I, Hanske C, Liz-Marzán LM, et al. Targeted Chemo-Photothermal Therapy: A Nanomedicine Approximation to Selective Melanoma Treatment. *Part Part Syst Charact*. 2018;35(1800148):1–10.
111. Jain R, Mathur M, Sikarwar S, Mittal A. Removal of the hazardous dye rhodamine B through photocatalytic and adsorption treatments. *J Environ Manage*. 2007;85(4):956–64.
112. Rittikulsittichai S, Singhana B, Bryan WW, Sarangi S, Jamison AC, Brazdeikis A, et al. Preparation, characterization, and utilization of multi-functional magnetic-fluorescent composites for bio-imaging and magnetic hyperthermia therapy. *RSC Adv*. 2013;3(21):7838–49.
113. Shen S-L, Chen X-P, Zhang X-F, Miao J-Y, Zhao B-X. A rhodamine B-based lysosomal pH probe. *J Mater Chem B*. 2015;3:919–25.
114. Sanz-Ortiz MN, Sentosun K, Bals S, Liz-Marzán LM. Templated Growth of Surface Enhanced Raman Scattering-Active Branched Gold Nanoparticles within Radial Mesoporous Silica Shells. *ACS Nano*. 2015;9(10):10489–97.
115. Winey M, Meehl JB, O'Toole ET, Giddings Jr TH. Conventional transmission electron microscopy. *Mol Biol Cell*. 2014;25(3):319–23.
116. Rose HH. Optics of high-performance electron microscopes. *Sci Technol Adv Mater*.

2008;9(014107):1–30.

117. Ruska E. The Development Of The Electron Microscope And Of Electron Microscopy. 1986 p. 355–79.
118. Bhattacharjee S. DLS and zeta potential – What they are and what they are not ? J Control Release [Internet]. 2016;235:337–51. Available from: <http://dx.doi.org/10.1016/j.jconrel.2016.06.017>
119. Costello MJ, Johnsen S, Gilliland KO, Freel CD, Fowler WC. Predicted Light Scattering from Particles Observed in Human Age-Related Nuclear Cataracts Using Mie Scattering Theory. Invest Ophthalmol Vis Sci. 2007;48(1):303–12.
120. Fischer K, Schmidt M. Pitfalls and novel applications of particle sizing by dynamic light scattering. Biomaterials [Internet]. 2016;98:79–91. Available from: <http://dx.doi.org/10.1016/j.biomaterials.2016.05.003>
121. Kumar P. Spectroscopy. In: Kumar A, Joseph J, editors. Fundamentals and Techniques of Biophysics and Molecular Biology. 2nd ed. New Delhi: Pathfinder Publication; 2018. p. 33–4.
122. Jabłoński A. Efficiency of anti-stokes fluorescence in dyes. Nature. 1933;131(3319):839–40.
123. Amendola V, Meneghetti M. Size Evaluation of Gold Nanoparticles by UV - vis Spectroscopy. J Phys Chem C. 2009;113(11):4277–85.
124. Jiang Q, Zeng W, Zhang C, Meng Z, Wu J, Zhu Q, et al. Broadband absorption and enhanced photothermal conversion property of octopod-like Ag @ Ag₂S core @ shell structures with gradually varying shell thickness. Sci Rep [Internet]. 2017;17782(December):1–11. Available from: <http://dx.doi.org/10.1038/s41598-017-18220-1>
125. Chuang YC, Lin CJ, Lo SF, Wang JL, Tzou SC, Yuan SS, et al. Dual functional AuNRs at MnMEIOs nanoclusters for magnetic resonance imaging and photothermal therapy. Biomaterials [Internet]. 2014;35(16):4678–87. Available from: <http://dx.doi.org/10.1016/j.biomaterials.2014.02.026>
126. Suhling K, Hirvonen LM, Levitt JA, Chung P, Tregidgo C, Le Marois A, et al. Fluorescence lifetime imaging (FLIM): Basic concepts and some recent developments. Med Photonics [Internet]. 2015;27:3–40. Available from: <http://dx.doi.org/10.1016/j.medpho.2014.12.001>
127. Wahl M. Time-Related Single Photon Counting The Principle of Time-Related. 2014.
128. Berezin MY, Achilefu S. Fluorescence Lifetime Measurements and Biological Imaging. Chem Rev. 2010;110(5):2641–84.
129. Enderlein J, Erdmann R. Fast fitting of multi-exponential decay curves. Opt Commun. 1997;134(1–6):25–30.
130. Landecker H. Immortality , in vitro : a history of the HeLa cell line. In: Biotechnology and culture: bodies, anxieties, ethics. 2015. p. 53–72.
131. Hongbao M. Hela Cells and Immortality. Cancer Biol. 2017;7(3):71–8.

132. Barnes DW. Epidermal Growth Factor Inhibits Growth of A431 Human Epidermoid Carcinoma in Serum-free Cell Culture. *J Cell Biol.* 1982;93(1):1–4.
133. Sasaki T, Hiroki K, Yamashita Y. The Role of Epidermal Growth Factor Receptor in Cancer Metastasis and Microenvironment. *Biomed Res Int.* 2013;2013(546318):1–8.
134. Strober W. Trypan Blue Exclusion Test of Cell Viability. *Curr Protoc Immunol.* 2015;111:1–3.
135. Nwaneshiudu A, Kuschal C, Sakamoto FH, Anderson RR, Schwarzenberger K, Young RC. Introduction to confocal microscopy. *J Invest Dermatol.* 2012;132(12):1–5.
136. AAT Bioquest. Spectrum Viewer [Internet]. 2019 [cited 2021 May 3]. Available from: <https://www.aatbio.com/spectrum/>
137. Wu S-H, Mou C-Y, Lin H-P. Synthesis of mesoporous silica nanoparticles. *Chem Soc Rev.* 2013;42(9):3862–75.
138. Liberman A, Mendez N, Trogler WC, Kummel AC. Synthesis and surface functionalization of silica nanoparticles for nanomedicine. *Surf Sci Rep.* 2014;69(2–3):132–58.
139. Jambhrunkar S, Yu M, Yang J, Zhang J, Shrotri A, Endo-Munoz L, et al. Stepwise Pore Size Reduction of Ordered Nanoporous Silica Materials at Angstrom Precision. *J Am Chem Soc.* 2013;135(23):8444–7.
140. Li Y, Song F, Cheng L, Qian J, Chen Q. Functionalized Large-Pore Mesoporous Silica Microparticles for Gefitinib and Doxorubicin Codelivery. *Materials (Basel).* 2019;12(766):1–19.
141. Zhu J, Liao L, Zhu L, Zhang P, Guo K, Kong J, et al. Size-dependent cellular uptake efficiency, mechanism, and cytotoxicity of silica nanoparticles toward HeLa cells. *Talanta* [Internet]. 2013;107:408–15. Available from: <http://dx.doi.org/10.1016/j.talanta.2013.01.037>
142. Rejman J, Oberle V, Zuhorn IS, Hoekstra D. Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. *Biochem J.* 2004;377(Pt 1):159–69.
143. Theillet F-X, Binolfi A, Frembgen-Kesner T, Hingorani K, Sarkar M, Kyne C, et al. Physicochemical Properties of Cells and Their Effects on Intrinsically Disordered Proteins (IDPs). *Chem Rev.* 2014;114(13):6661–714.
144. Coppeta J, Rogers C. Dual emission laser induced fluorescence for direct planar scalar behavior measurements. *Exp Fluids.* 1998;25(1):1–15.
145. Estrada-Pérez CE, Hassan YA, Tan S, Dominguez-Ontiveros EE. Experimental characterization of temperature sensitive dyes for laser induced fluorescence thermometry. *Rev Sci Instrum.* 2011;82(7):2–5.
146. Silva PL, Savchuk OA, Gallo J, García-Hevia L, Bañobre-López M, Nieder JB. Mapping intracellular thermal response of cancer cells to magnetic hyperthermia treatment. *Nanoscale.* 2020;12(42):21647–56.
147. Arai S, Suzuki M, Park SJ, Yoo JS, Wang L, Kang NY, et al. Mitochondria-targeted fluorescent thermometer monitors intracellular temperature gradient. *Chem Commun.*

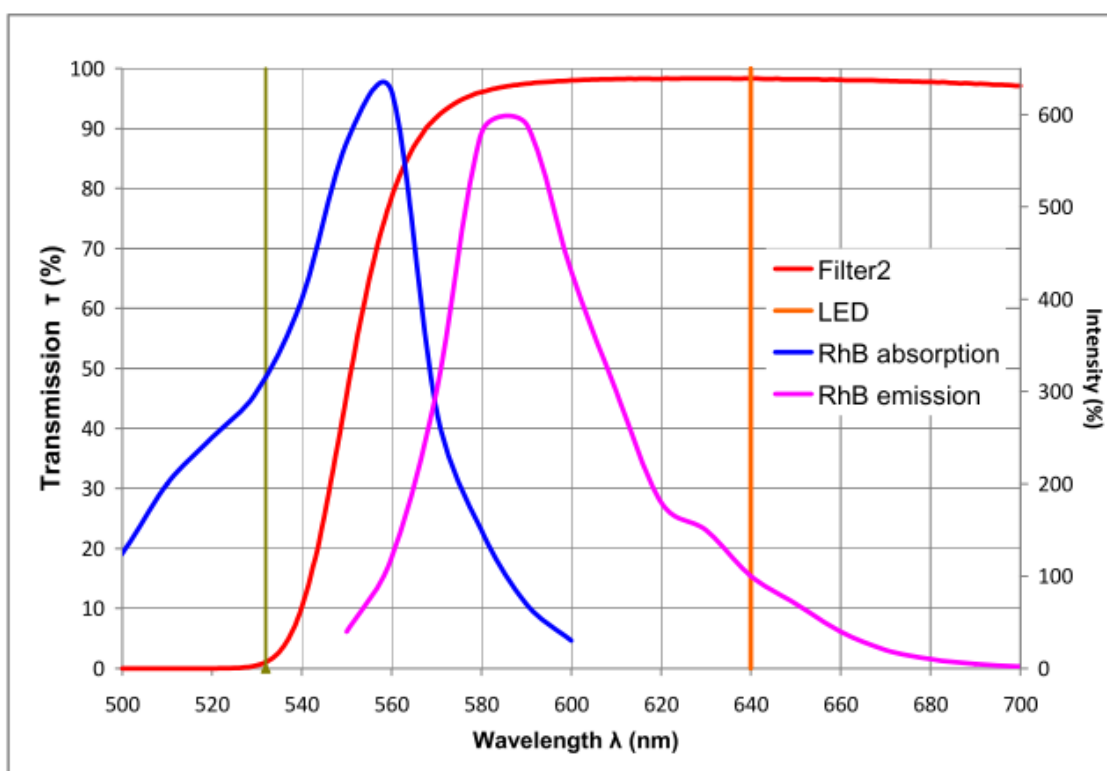
2015;51(38):8044–7.

148. Anderson RGW, Falck JR, Goldstein JL, Brown MS. Visualization of acidic organelles in intact cells by electron microscopy. *Proc Natl Acad Sci U S A*. 1984;81(15):4838–42.
149. Grabe M, Oster G. Regulation of Organelle Acidity. *J Gen Physiol*. 2001;117(4):329–44.
150. DSMZ. HELA - ACC 57 [Internet]. 2020 [cited 2020 Aug 16]. Available from: <https://www.dsmz.de/collection/catalogue/details/culture/ACC-57>
151. DSMZ. A-431 - ACC 91 [Internet]. 2020 [cited 2020 Aug 16]. Available from: <https://www.dsmz.de/collection/catalogue/details/culture/ACC-91>
152. Sunthornpan N, Watanabe S, Moolsradoo N. Corrosion resistance and cytotoxicity studies of DLC, TiN and TiCN films coated on 316L stainless steel. *J Phys Conf Ser*. 2018;1144(1):0–4.
153. Jesus VPS, Raniero L, Lemes GM, Bhattacharjee TT, Caetano Júnior PC, Castilho ML. Nanoparticles of methylene blue enhance photodynamic therapy. *Photodiagnosis Photodyn Ther* [Internet]. 2018;23(May):212–7. Available from: <https://doi.org/10.1016/j.pdpdt.2018.06.011>
154. Choi SJ, Oh JM, Choy JH. Toxicological effects of inorganic nanoparticles on human lung cancer A549 cells. *J Inorg Biochem* [Internet]. 2009;103(3):463–71. Available from: <http://dx.doi.org/10.1016/j.jinorgbio.2008.12.017>
155. Fisichella M, Dabboue H, Bhattacharyya S, Saboungi M-L, Salvétat J-P, Hevor T, et al. Mesoporous silica nanoparticles enhance MTT formazan exocytosis in HeLa cells and astrocytes. *Toxicol Vitro* [Internet]. 2009;23(4):697–703. Available from: <http://dx.doi.org/10.1016/j.tiv.2009.02.007>
156. Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ, et al. Cell Viability Assays. In: *Assay Guidance Manual*. 2016. p. 1–25.
157. Vigderman L, Khanal BP, Zubarev ER. Functional Gold Nanorods: Synthesis, Self-Assembly, and Sensing Applications. *Adv Mater*. 2012;24(36):4811–41.
158. Houde S, Iliescu MS, Fraser R, Lemay S, Ciocan GD, Deschênes C. Experimental and numerical analysis of the cavitating part load vortex dynamics of low-head hydraulic turbines. In: *ASME-JSME-KSME 2011 Joint Fluids Engineering Conference, AJK 2011*. 2011. p. 171–82.
159. Răz B, Iten M, Grether-Bühler Y, Kaminsky R, Brun R. The Alamar Blue ® assay to determine drug sensitivity of African trypanosomes (*T. b. rhodesiense* and *T. b. gambiense*) in vitro. *Acta Trop*. 1997;68:139–47.
160. Bioquest A. Resazurin and Rhodamine B spectrum [Internet]. 2020 [cited 2020 Jul 20]. Available from: <https://www.aatbio.com/spectrum/>
161. Luzio JP, Pryor PR, Bright NA. Lysosomes: Fusion and function. *Nat Rev Mol Cell Biol*. 2007;8(8):622–32.
162. Chang YH, Yao YF, Luo H, Cui L, Zhi LJ. Magnetic Fe₃O₄-GO nanocomposites as highly efficient Fenton-like catalyst for the degradation of dyes. *Int J Nanomanuf*. 2014;10(1–2):132–41.

163. Cheng J, Ge L, Xiong B, He Y. Investigation of pH Effect on Gold Nanorod Synthesis. *J Chinese Chem Soc.* 2011;58(6):822–7.
164. Lervik A, Bresme F, Kjelstrup S, Bedeaux D, Rubi JM. Heat transfer in protein – water interfaces. *Phys Chem Chem Phys.* 2010;12(7):1610–7.
165. Howard R, Scheiner A, Cunningham J, Gatenby R. Cytoplasmic convection currents and intracellular temperature gradients. *PLoS Comput Biol.* 2019;15(11):1–17.
166. Gulati S, Sachdeva M, Bhasin KK. Capping Agents in Nanoparticle Synthesis: Surfactant and Solvent System. *AIP Conf Proc* 1953. 2018;1953(1):1–4.
167. Owens DE, Peppas NA. Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. *Int J Pharm.* 2006;307:93–102.
168. Gao X, He J, Deng L, Cao H. Synthesis and characterization of functionalized rhodamine B-doped silica nanoparticles. *Opt Mater (Amst)* [Internet]. 2009;31(11):1715–9. Available from: <http://dx.doi.org/10.1016/j.optmat.2009.05.004>

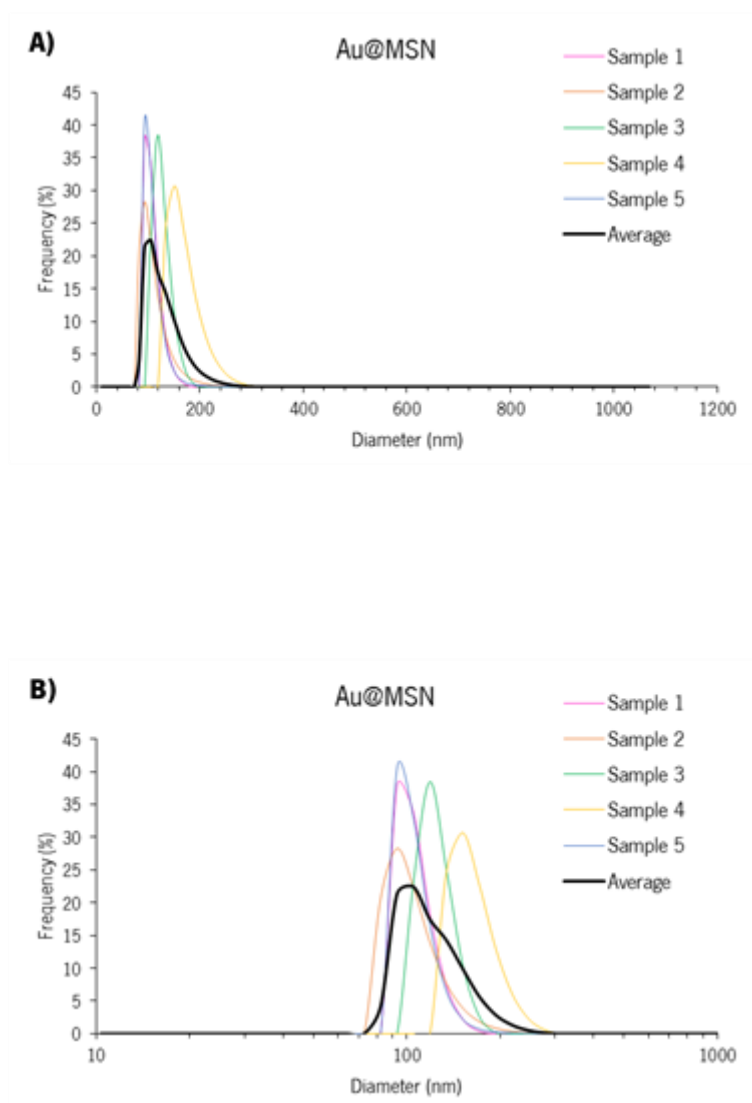
Annexes

1. Luminescence properties of Rhodamine B

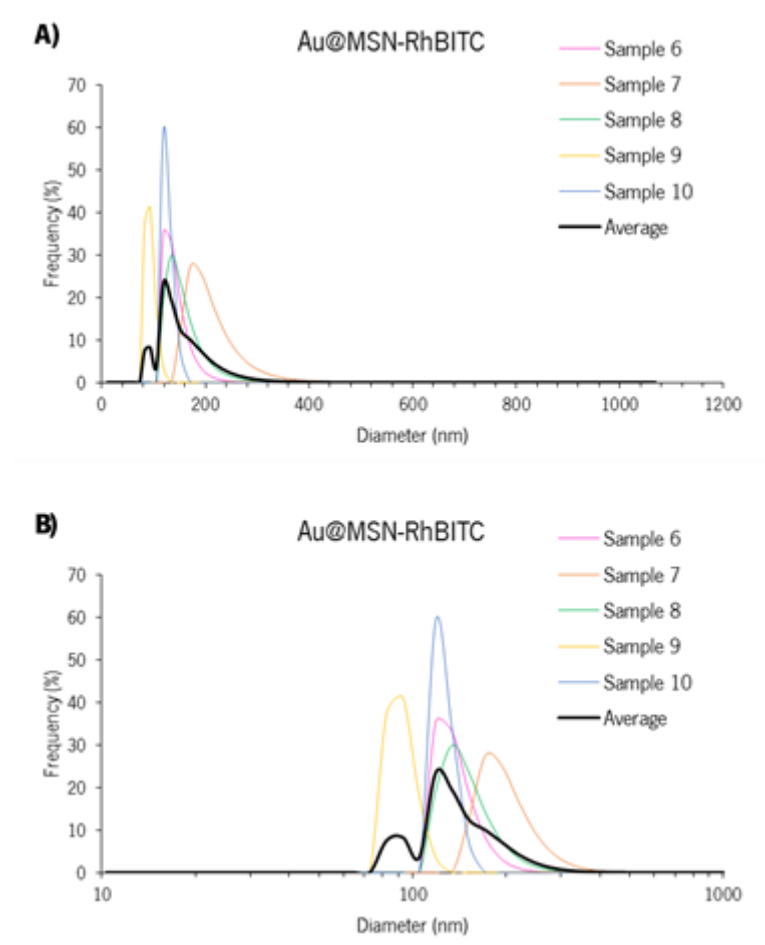


Supplementary figure 1 – Absorption spectrum of RhB (Adapted from Houde et al. (158)).

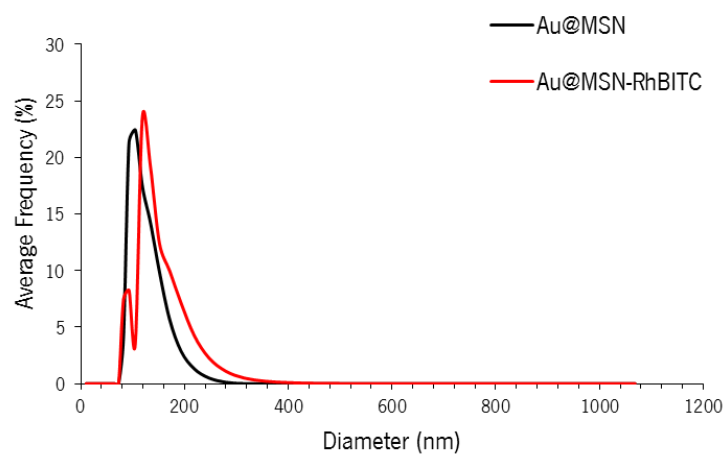
2. DLS results



Supplementary figure 2 – Detailed distribution of sizes for each sample of Au@MSN in **A** – Linear and **B** – Logarithmic scale.



Supplementary figure 3 – Detailed distribution of sizes for each sample of Au@MSN-RhBITC in **A** – Linear and **B** – Logarithmic scale.



Supplementary figure 4 – Average frequency of sizes of Au@MSN and Au@MSN-RhBITC particles in linear scale.

3. UV-Vis-NIR absorption spectroscopy analysis

Supplementary table 1 – Measured absorbance in the 400-900 nm range, for Au@MSN and Au@MSN-RhBITC (subdivided into 5 tables).

Wavelength	Absorbance (A.U.)	
	Au@MSN	Au@MSN-RhBITC
400	0.686	0.519
405	0.68	0.513
410	0.673	0.506
415	0.665	0.499
420	0.659	0.493
425	0.654	0.488
430	0.648	0.482
435	0.642	0.475
440	0.636	0.466
445	0.63	0.459
450	0.626	0.453
455	0.621	0.448
460	0.616	0.444
465	0.612	0.441
470	0.609	0.441
475	0.606	0.443
480	0.606	0.447
485	0.608	0.456
490	0.614	0.466
495	0.623	0.48

Wavelength	Absorbance (A.U.)	
	Au@MSN	Au@MSN-RhBITC
600	0.492	0.415
605	0.485	0.396
610	0.48	0.385
615	0.477	0.379
620	0.475	0.375
625	0.474	0.373
630	0.475	0.374
635	0.477	0.377
640	0.481	0.381
645	0.487	0.388
650	0.495	0.396
655	0.504	0.407
660	0.514	0.417
665	0.526	0.429
670	0.541	0.444
675	0.561	0.464
680	0.579	0.482
685	0.596	0.498
690	0.617	0.519
695	0.641	0.541

Wavelength	Absorbance (A.U.)	
	Au@MSN	Au@MSN-RhBITC
500	0.634	0.498
505	0.648	0.525
510	0.658	0.558
515	0.661	0.59
520	0.659	0.612
525	0.652	0.629
530	0.644	0.634
535	0.633	0.632
540	0.622	0.643
545	0.614	0.667
550	0.603	0.705
555	0.591	0.743
560	0.578	0.761
565	0.566	0.749
570	0.556	0.718
575	0.544	0.663
580	0.531	0.59
585	0.519	0.523
590	0.51	0.479
595	0.501	0.442

Wavelength	Absorbance (A.U.)	
	Au@MSN	Au@MSN-RhBITC
700	0.664	0.563
705	0.683	0.58
710	0.704	0.598
715	0.725	0.616
720	0.749	0.634
725	0.767	0.648
730	0.785	0.66
735	0.803	0.671
740	0.82	0.68
745	0.835	0.688
750	0.845	0.691
755	0.855	0.694
760	0.865	0.695
765	0.873	0.694
770	0.879	0.691
775	0.883	0.688
780	0.886	0.683
785	0.889	0.676
790	0.889	0.67
795	0.888	0.663

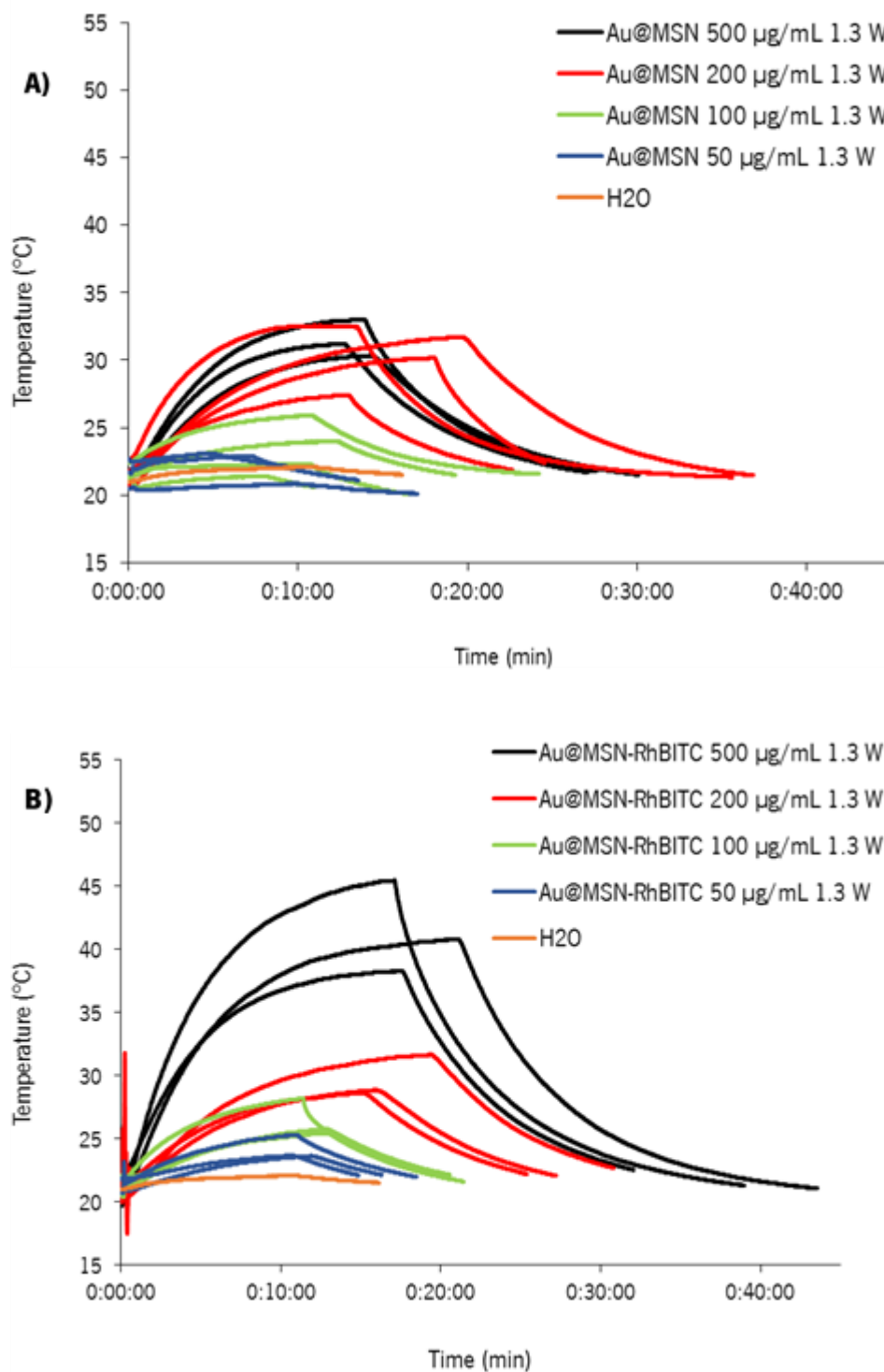
Wavelength	Absorbance (A.U.)	
	Au@MSN	Au@MSN-RhBITC
800	0.887	0.654
805	0.884	0.645
810	0.88	0.635
815	0.876	0.626
820	0.87	0.616
825	0.865	0.607
830	0.86	0.597
835	0.853	0.588
840	0.846	0.578
845	0.838	0.568
850	0.827	0.556
855	0.818	0.547
860	0.809	0.538
865	0.8	0.529
870	0.79	0.519
875	0.781	0.511
880	0.769	0.501
885	0.76	0.492
890	0.746	0.48
895	0.734	0.47
900	0.725	0.463

4. Fluorescence spectroscopy

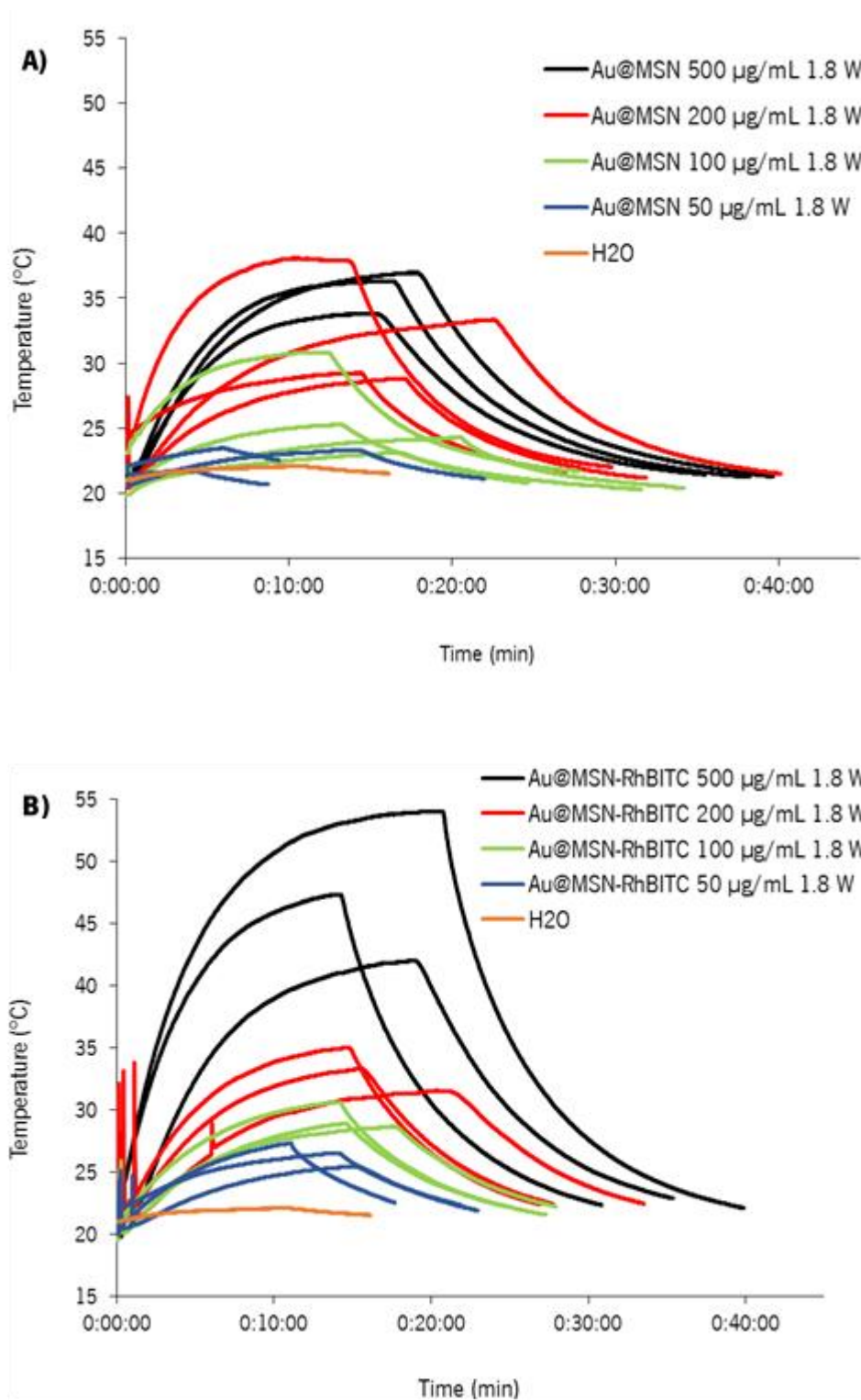
Supplementary table 2 – Measured fluorescence intensity for Au@MSN and Au@MSN-RhBITC.

Wavelength	Fluorescence Intensity (A.U.)	
	Au@MSN	Au@MSN-RhBITC
590	5	32228
595	4	29372
600	4	25115
605	3	20887
610	6	17776
615	7	15618
620	9	13711
625	3	12223
630	6	11021
635	7	10279
640	12	9511
645	4	8442
650	14	7325
655	10	6356
660	6	5917
665	10	5013
670	18	4159
675	0	3763
680	36	3333
685	0	3209
690	9	2620
695	0	1466
700	18	1704

5. Photothermal conversion study



Supplementary figure 5 – Photothermal conversion over time in dependence of nanohybrids concentration at 1.3 W laser power irradiation in **A** – Au@MSN nanohybrids and **B** – Au@MSN-RhBITC nanohybrids.

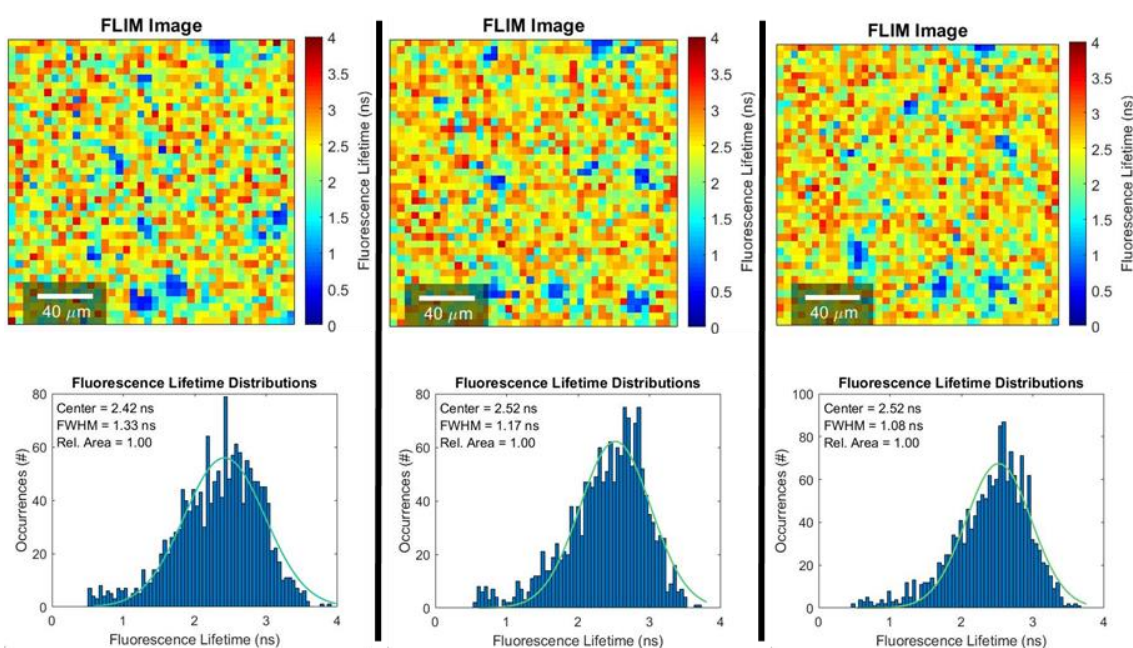


Supplementary figure 6 – Photothermal conversion over time in dependence of nanohybrids concentration at 1.8 W laser power irradiation in **A** – Au@MSN nanohybrids and **B** – Au@MSN-RhBITC nanohybrids.

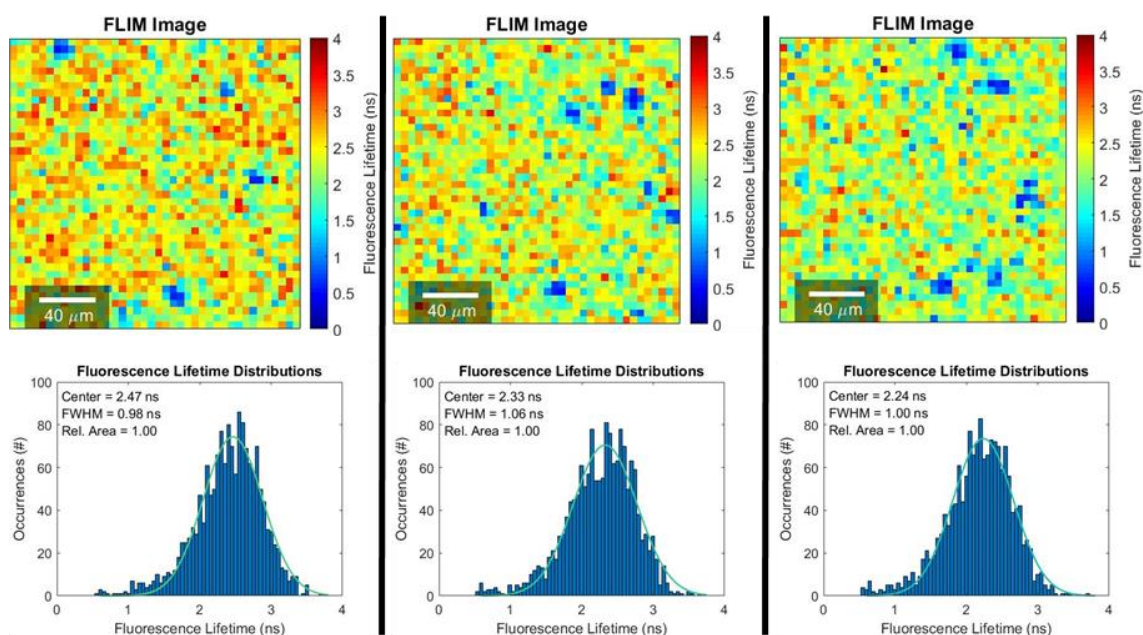
6. Thermal sensitivity of the nanohybrids

Supplementary table 3 – Fluorescence lifetime of the nanohybrids in dependence of temperature, used for obtaining the calibration curve.

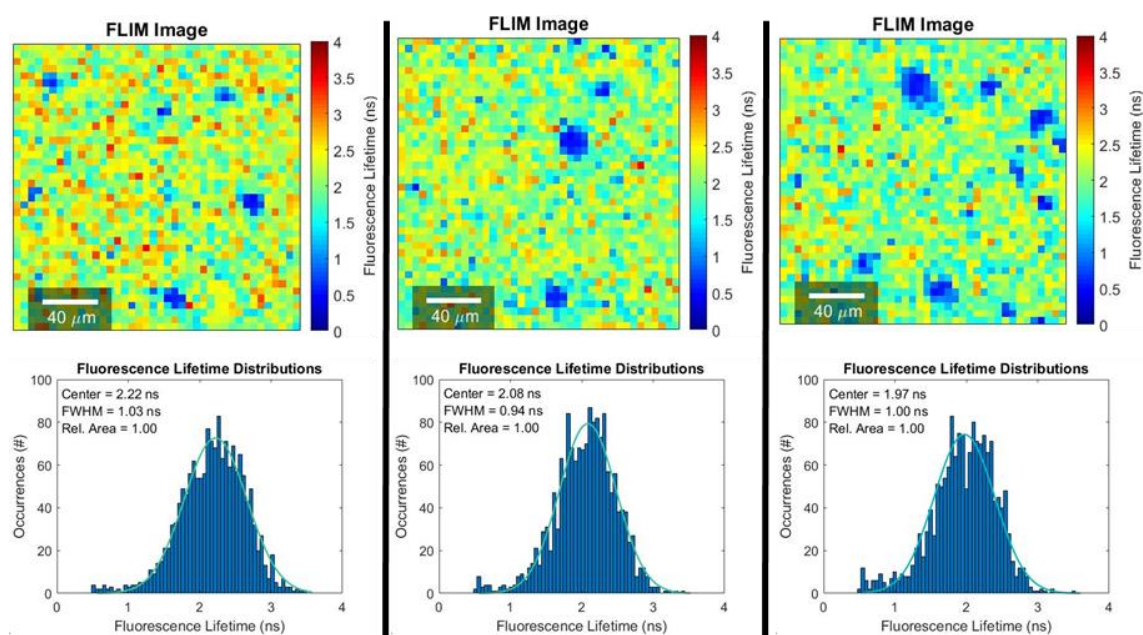
Temperature (°C)	Fluorescence Lifetime (ns)	FWHM	Fluorescence Lifetime (ns) - Averaged	Standard Deviation
35	2.42	1.33	2.4867	0.4291
35	2.52	1.17		
35	2.52	1.08		
40	2.47	0.98	2.3467	0.3162
40	2.33	1.06		
40	2.24	1.00		
45	2.22	1.03	2.0900	0.3323
45	2.08	0.94		
45	1.97	1.00		
50	2.09	0.91	1.9867	0.2936
50	1.98	0.93		
50	1.89	0.90		



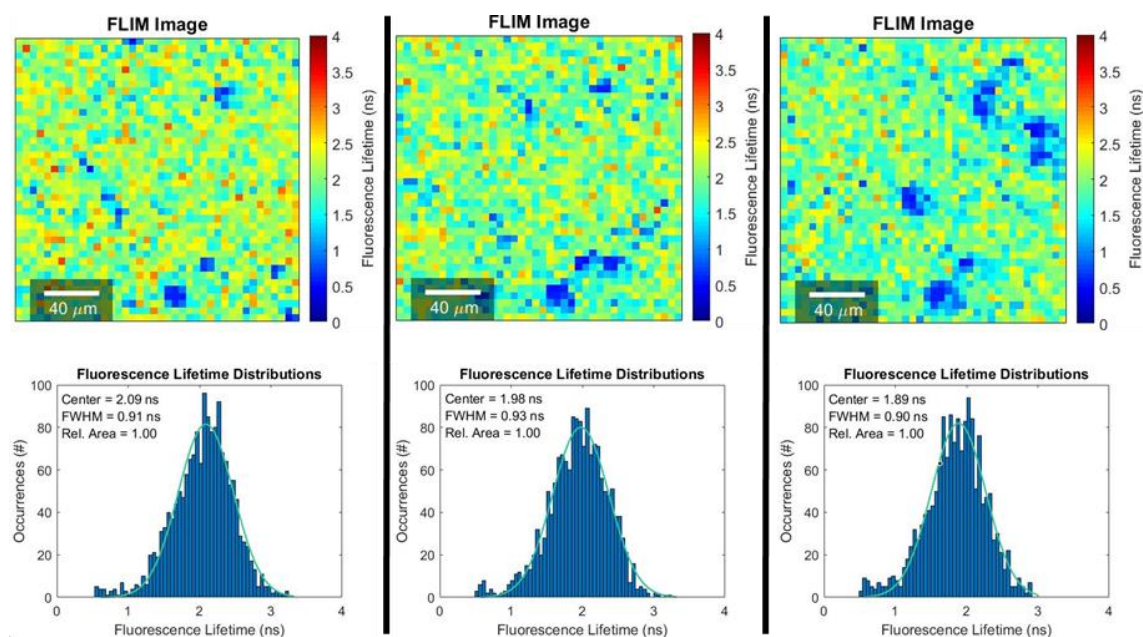
Supplementary figure 7 – FLIM and histogram image acquired in nanohybrids samples used to obtain the calibration curve. Sample at 35 °C.



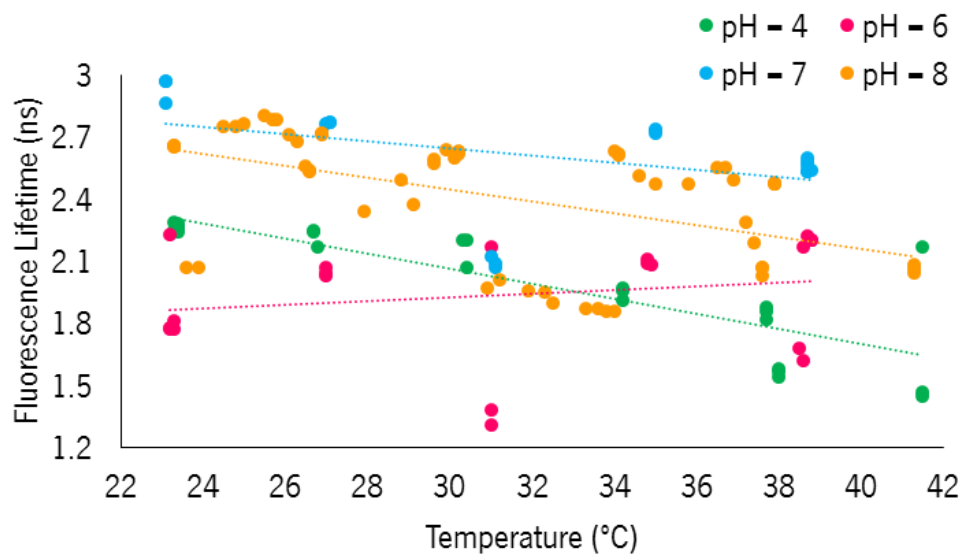
Supplementary figure 8 – FLIM and histogram image acquired in nanohybrids samples used to obtain the calibration curve. Sample at 40 °C.



Supplementary figure 9 – FLIM and histogram image acquired in nanohybrids samples used to obtain the calibration curve. Sample at 45 °C.



Supplementary figure 10 – FLIM and histogram image acquired in nanohybrids samples used to obtain the calibration curve. Sample at 50 °C.



Supplementary figure 11 – Au@MSN-RhBITC nanohybrids fluorescence lifetime' dependence on pH.

7. Biocompatibility

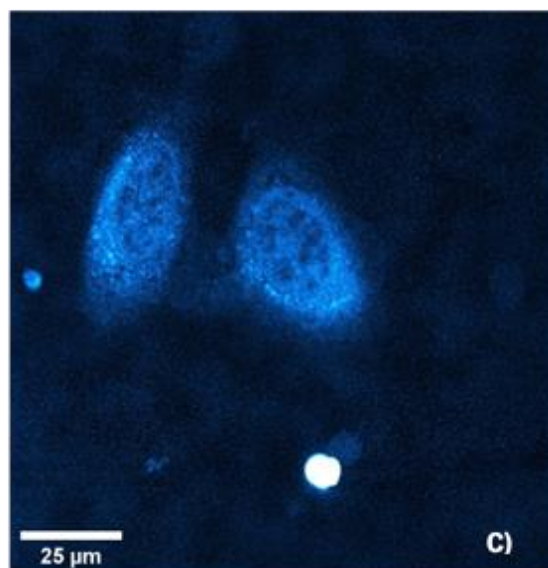
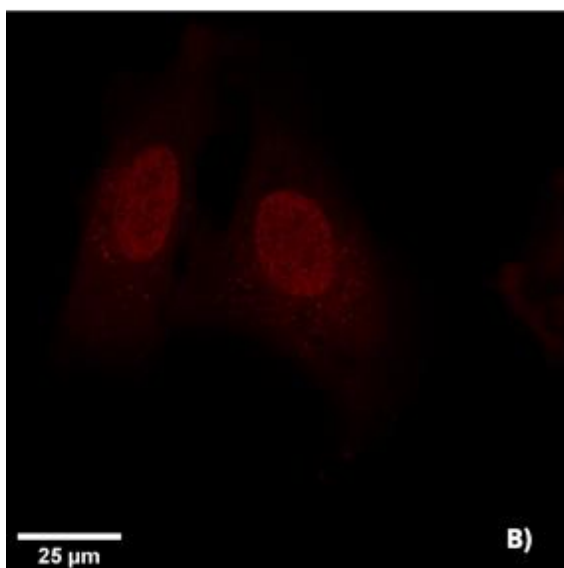
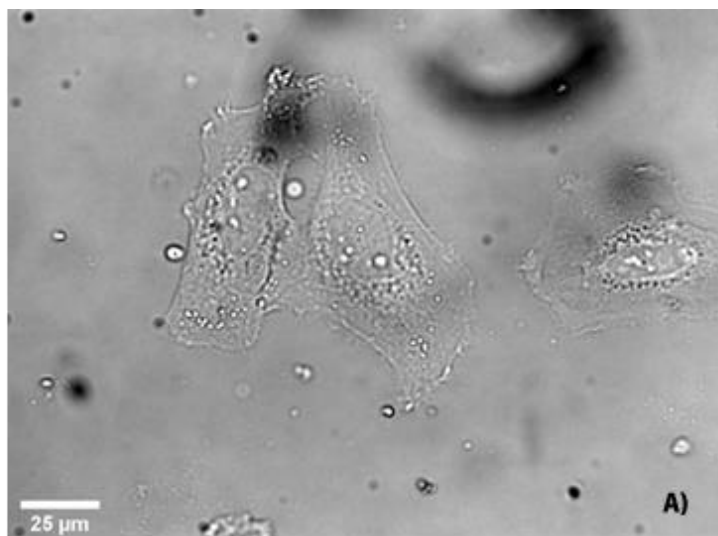
Supplementary table 4 – Results regarding the number of cells and the percentage of cell viability of HeLa cells.

HeLa cells			0 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL
Nr. of Cells	24 hours	Average	15833	18667	20000	20833
		STDEV	2718.25107	3880.14891	3894.44048	1840.8935
	48 hours	Average	22750	28333	26167	20667
		STDEV	2750	6355.22532	6599.66329	5632.24842
	72 hours	Average	17250	23000	23667	14667
		STDEV	3250	7648.52927	4496.91252	4496.91252
Cell viability (%)	24 hours	Average	95.30	93.21	89.36	95.31
		STDEV	4.94694	2.81317	3.52095	5.17621
	48 hours	Average	99.02	96.33	93.42	94.83
		STDEV	0.98039	2.57759	3.31525	1.23165
	72 hours	Average	92.77	96.60	97.02	97.62
		STDEV	0.08711	0.81682	1.30772	3.36718

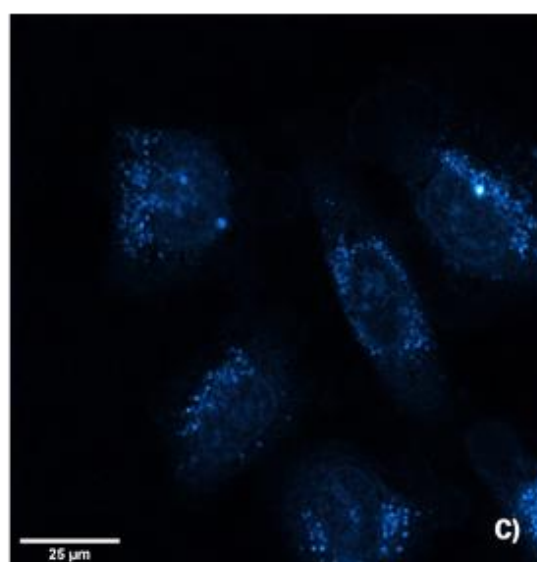
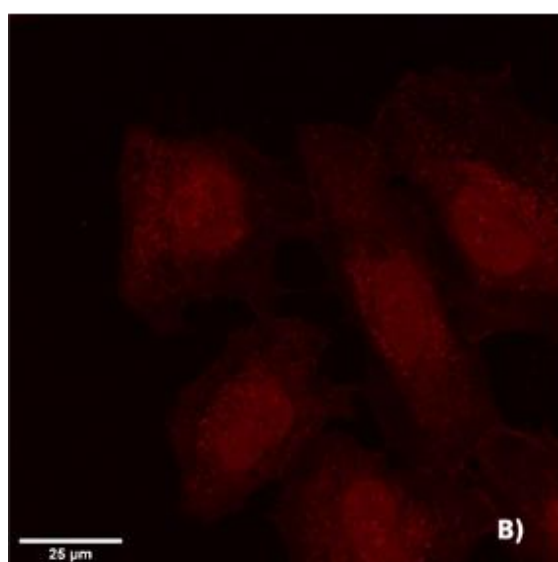
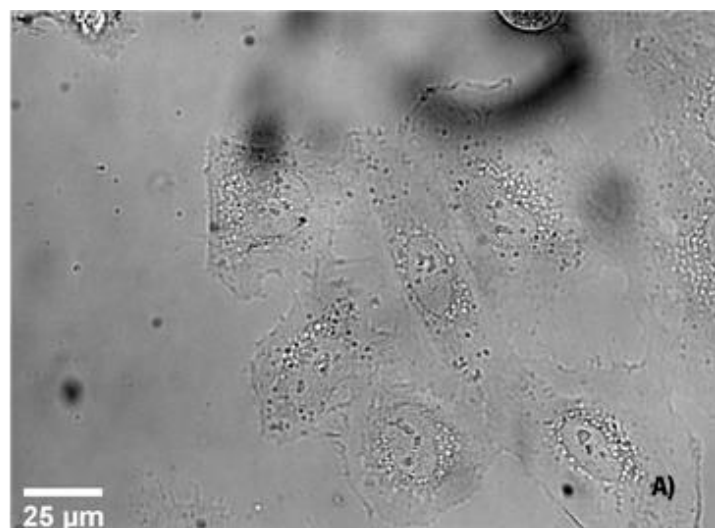
Supplementary table 5 – Results regarding the number of cells and the percentage of cell viability of A431 cells.

A431 cells			0 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL
Nr. of Cells	24 hours	Average	28500	30000	26333	32667
		STDEV	7937.25393	4822	9292	6007
	48 hours	Average	86500	82167	80833	66833
		STDEV	23302	10324	13475	13137
	72 hours	Average	157167	184500	159500	137667
		STDEV	43038.7422	67506	30327	37464
Cell viability (%)	24 hours	Average	99.56	95.96	94.64	96.60
		STDEV	0.7698	5.8221	2.1218	2.9985
	48 hours	Average	98.91	99.35	95.36	98.53
		STDEV	1.29281	1.1175	2.9711	1.1605
	72 hours	Average	98.59	97.62	97.76	96.90
		STDEV	0.41489	0.7504	0.7857	0.8823

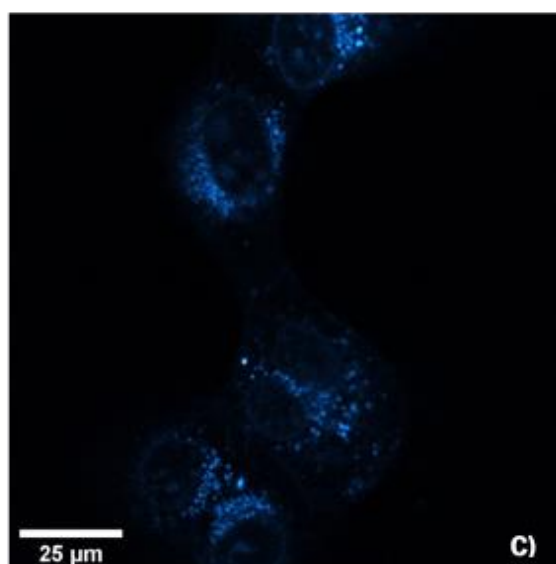
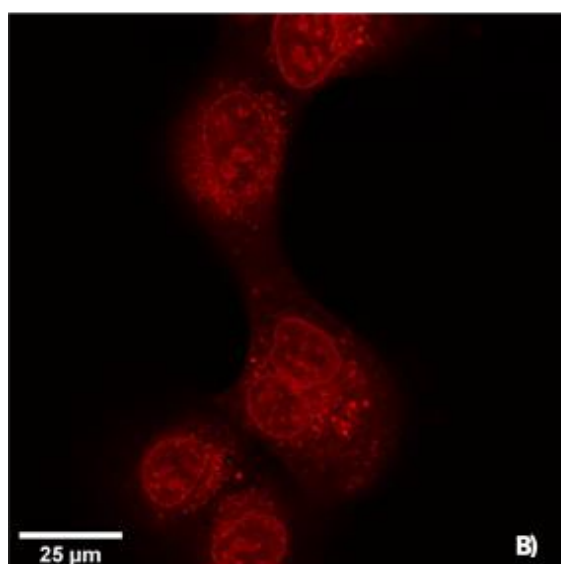
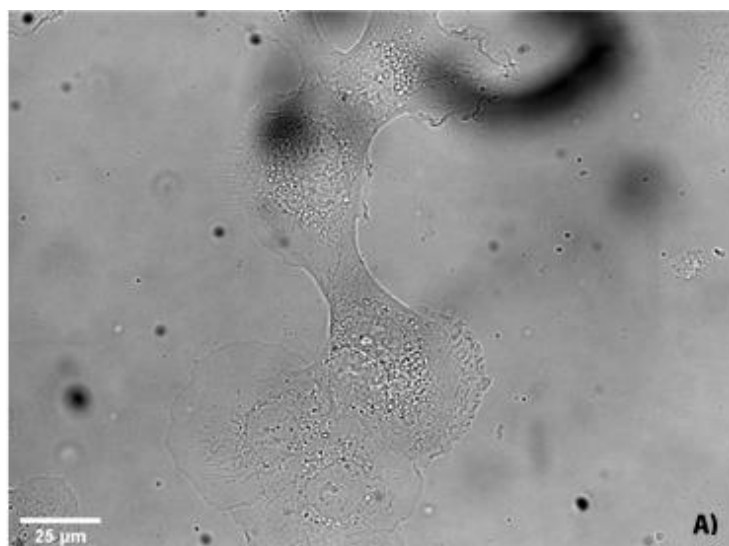
8. Confocal imaging



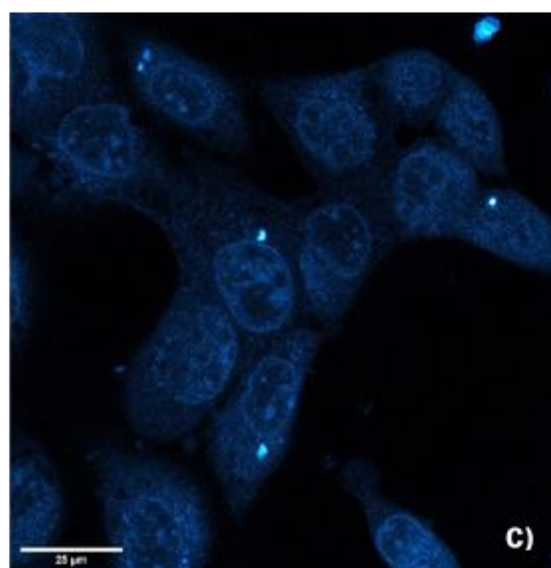
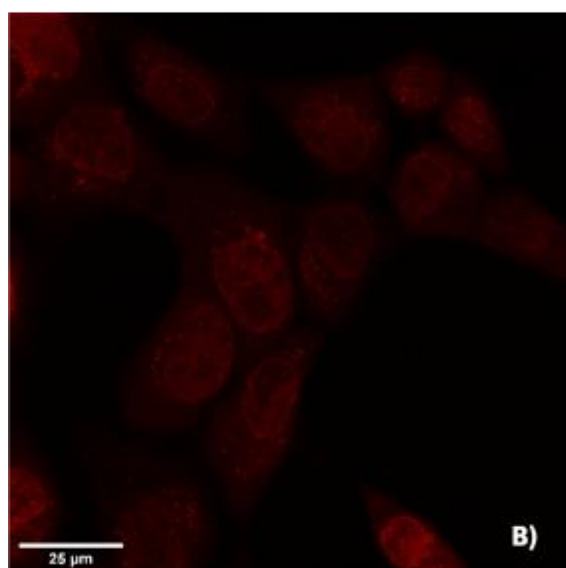
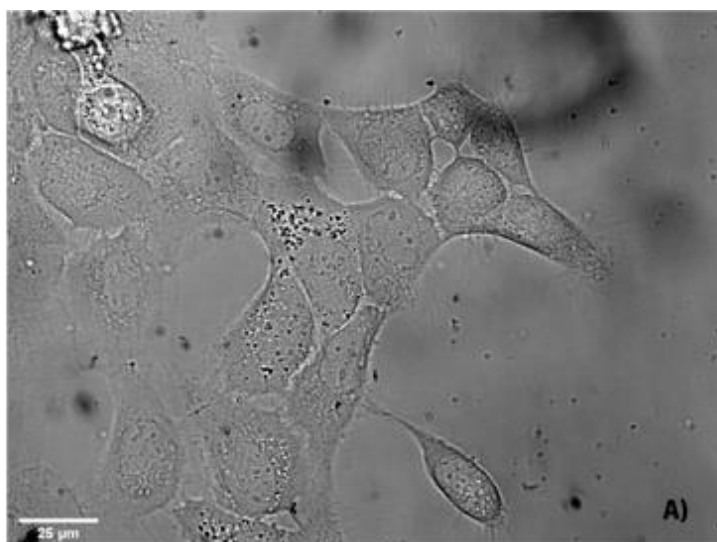
*Supplementary figure 12 – HeLa cells with Au@MSN-RhBITC nanohybrids at 200 μg/mL concentration – 63x Objective. **A** – Camera view at 12 hours exposure to Au@MSN-RhBITC nanohybrids; **B** – 405 nm laser irradiation at 12 hours exposure to Au@MSN-RhBITC nanohybrids; **C** – 561 nm laser irradiation at 12 hours exposure to Au@MSN-RhBITC nanohybrids.*



*Supplementary figure 13 – HeLa cells with Au@MSN-RhBITC nanohybrids at 200 $\mu\text{g/mL}$ concentration – 63x Objective. **A** – Camera view at 24 hours exposure to Au@MSN-RhBITC nanohybrids; **B** – 405 nm laser irradiation at 24 hours exposure to Au@MSN-RhBITC nanohybrids; **C** – 561 nm laser irradiation at 24 hours exposure to Au@MSN-RhBITC nanohybrids.*



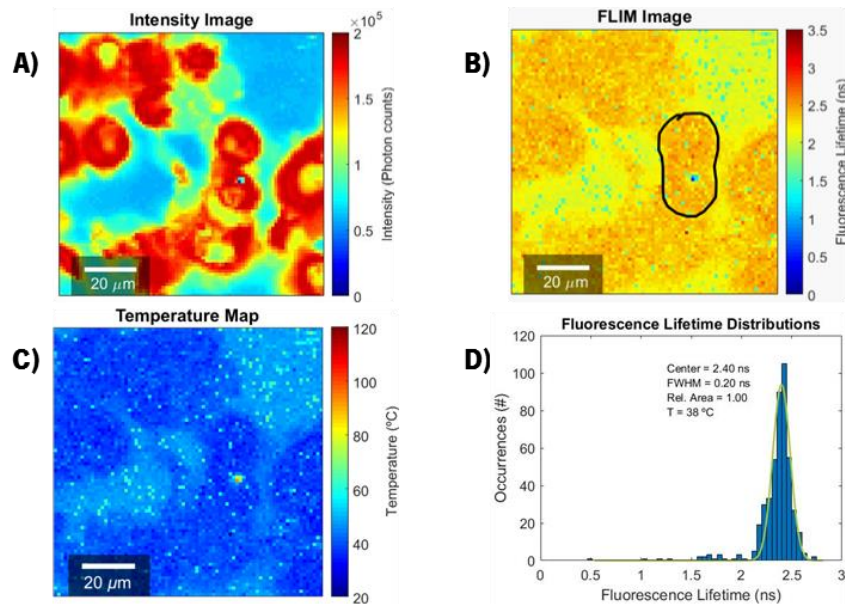
Supplementary figure 14 – A431 cells with Au@MSN-RhBITC nanohybrids at 200 $\mu\text{g/mL}$ concentration – 63x Objective. **A** – Camera view at 12 hours exposure to Au@MSN-RhBITC nanohybrids; **B** – 405 nm laser irradiation at 12 hours exposure to Au@MSN-RhBITC nanohybrids; **C** – 561 nm laser irradiation at 12 hours exposure to Au@MSN-RhBITC nanohybrids.



*Supplementary figure 15 – A431 cells with Au@MSN-RhBITC nanohybrids at 200 µg/mL concentration – 63x Objective.; **A** – Camera view at 24 hours exposure to Au@MSN-RhBITC nanohybrids; **B** – 405 nm laser irradiation at 24 hours exposure to Au@MSN-RhBITC nanohybrids; **C** – 561 nm laser irradiation at 24 hours exposure to Au@MSN-RhBITC nanohybrids.*

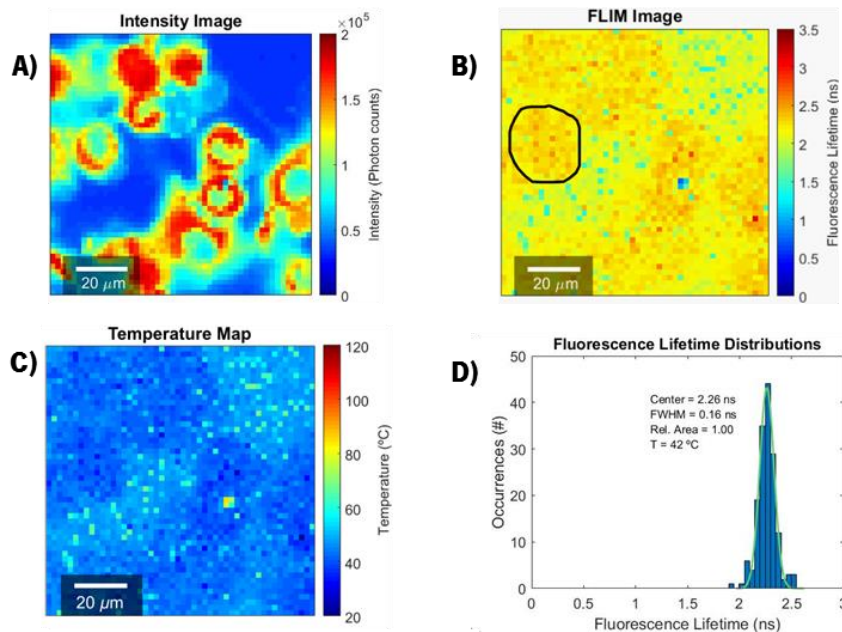
9. Live cell FLIM microscopy for intracellular thermometry

HeLa – 12h – 1.3 W – 6 mins



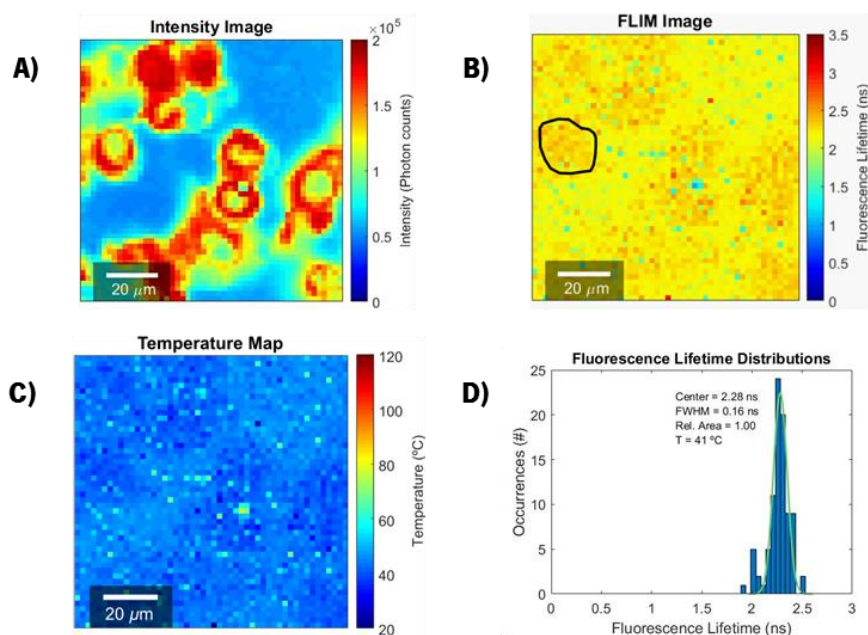
Supplementary figure 16 – Temperature map study based on FLIM characterization of Au@MSN-RhBITC nanohybrids incubated 12 h with HeLa cells, 808 nm laser irradiation, 1.3 W, 6 minutes. **A** – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected.

HeLa – 12h – 1.3 W – 9 mins



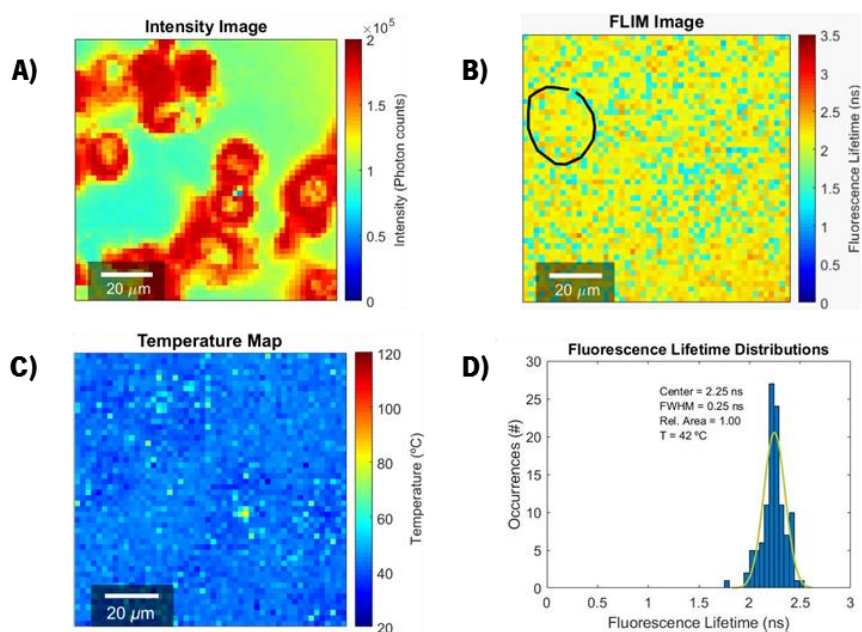
Supplementary figure 17 – Temperature map study based on FLIM characterization of Au@MSN-RhBITC nanohybrids incubated 12 h with HeLa cells, 808 nm laser irradiation, 1.3 W, 9 minutes. **A** – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected.

HeLa – 12h – 1.3 W – 15 mins



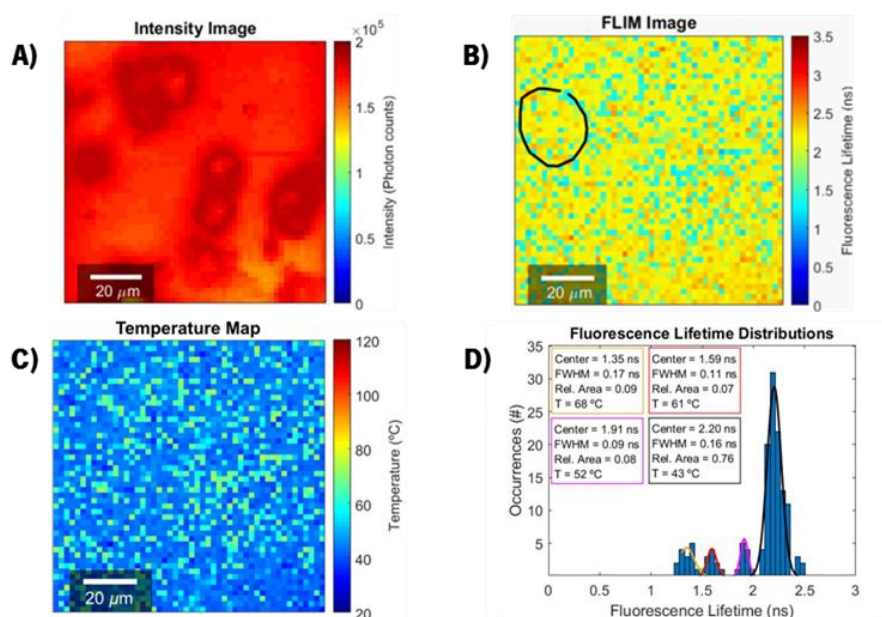
Supplementary figure 18 – Temperature map study based on FLIM characterization of Au@MSN-RhBITC nanohybrids incubated 12 h with HeLa cells, 808 nm laser irradiation, 1.3 W, 15 minutes. **A** – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected.

HeLa – 12h – 1.8 W – 3 mins



Supplementary figure 19 – Temperature map study based on FLIM characterization of Au@MSN-RhBITC nanohybrids incubated 12 h with HeLa cells, 808 nm laser irradiation, 1.8 W, 3 minutes. **A** – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected.

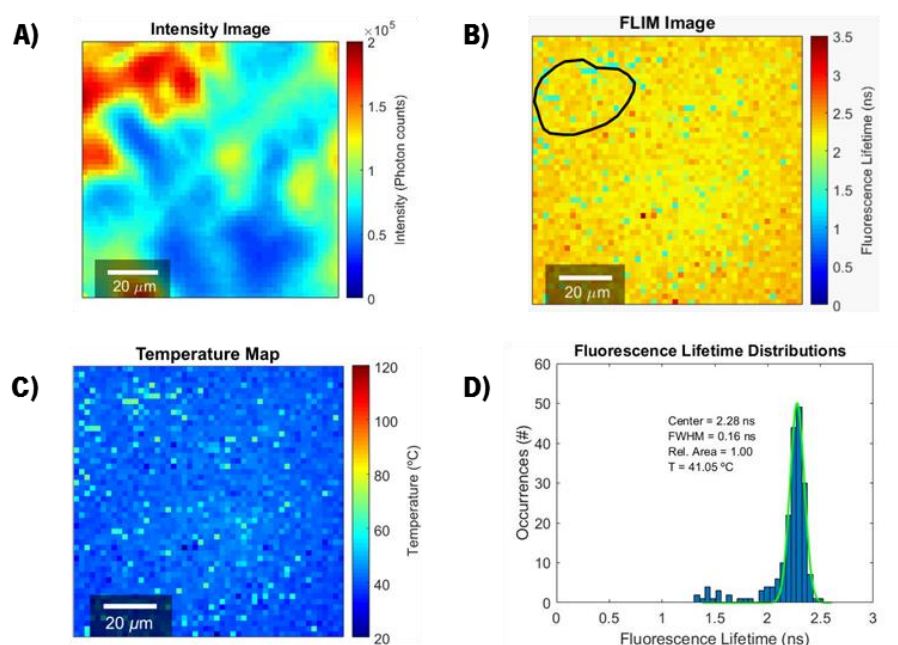
HeLa – 12h – 1.8 W – 6 mins



Supplementary figure 20 – Temperature map study based on FLIM characterization of Au@MSN-RhBITC nanohybrids incubated 12 h with HeLa cells, 808 nm laser irradiation, 1.8 W, 6 minutes.

A – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected.

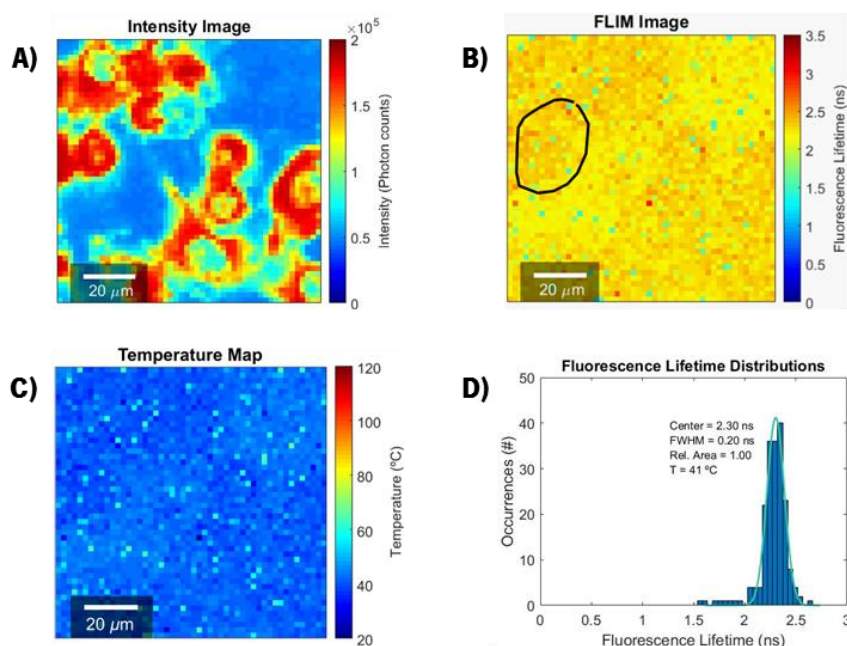
HeLa – 12h – 1.8 W – 9 mins



Supplementary figure 21 – Temperature map study based on FLIM characterization of Au@MSN-RhBITC nanohybrids incubated 12 h with HeLa cells, 808 nm laser irradiation, 1.8 W, 9 minutes.

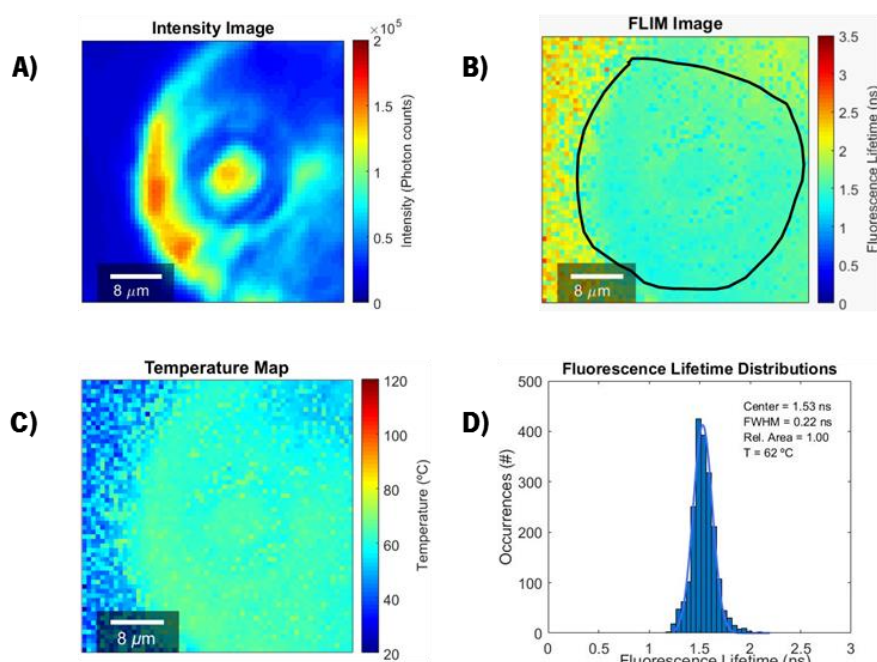
A – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected.

HeLa – 12h – 1.8 W – 15 mins



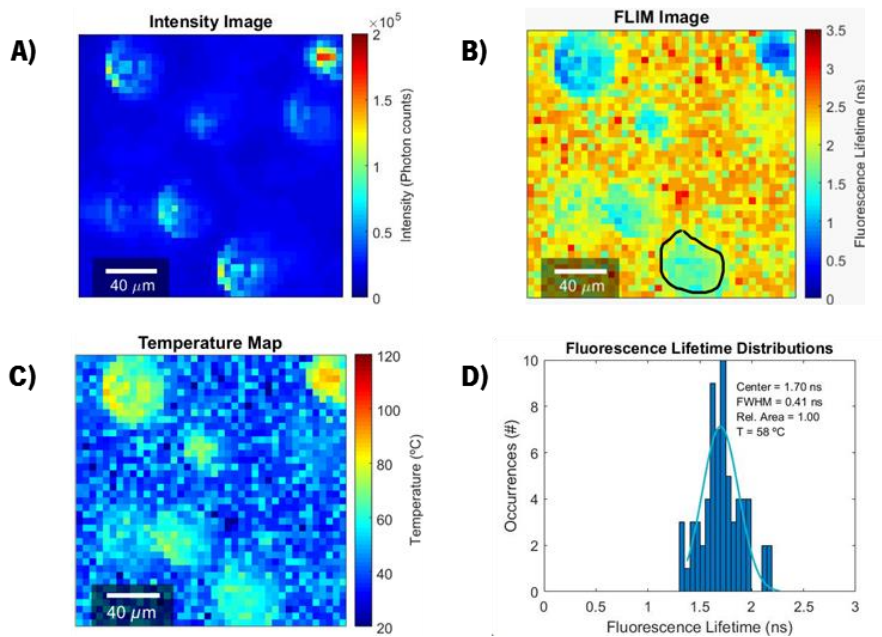
Supplementary figure 22 – Temperature map study based on FLIM characterization of Au@MSN-RhBITC nanohybrids incubated 12 h with HeLa cells, 808 nm laser irradiation, 1.8 W, 15 minutes. **A** – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected.

HeLa – 24h – 1.3 W – 6 mins



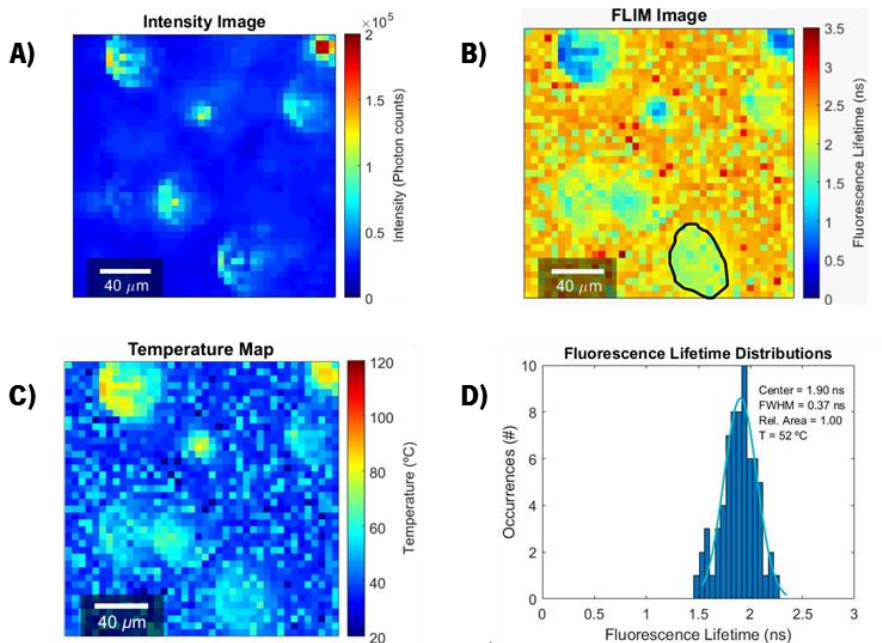
Supplementary figure 23 – Temperature map study based on FLIM characterization of Au@MSN-RhBITC nanohybrids incubated 24 h with HeLa cells, 808 nm laser irradiation, 1.3 W, 6 minutes. **A** – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected.

HeLa – 24h – 1.3 W – 9 mins



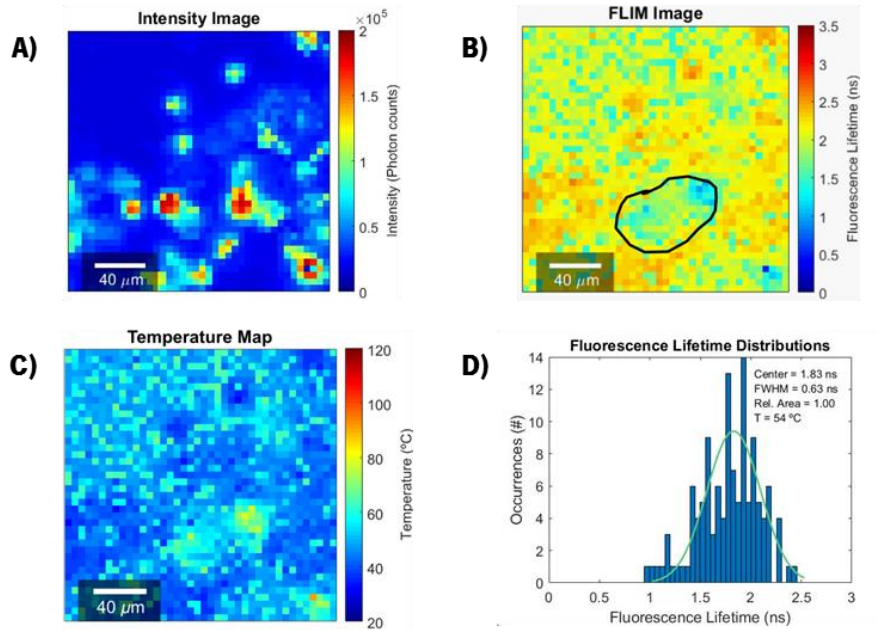
Supplementary figure 24 – Temperature map study based on FLIM characterization of Au@MSN-RhBITC nanohybrids incubated 24 h with HeLa cells, 808 nm laser irradiation, 1.3 W, 9 minutes. **A** – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected.

HeLa – 24h – 1.3 W – 15 mins



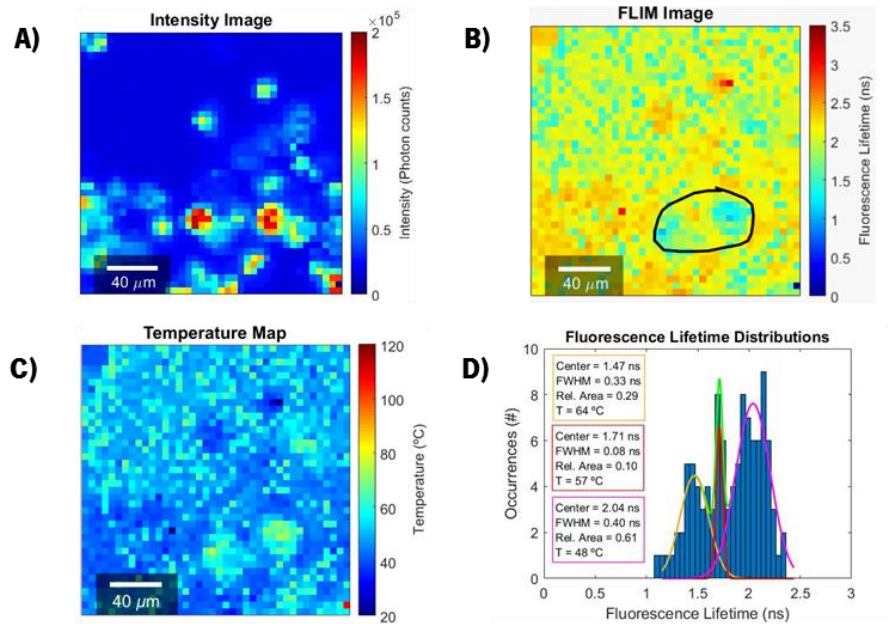
Supplementary figure 25 – Temperature map study based on FLIM characterization of Au@MSN-RhBITC nanohybrids incubated 24 h with HeLa cells, 808 nm laser irradiation, 1.3 W, 15 minutes. **A** – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected.

HeLa – 24h – 1.8 W – 3 mins



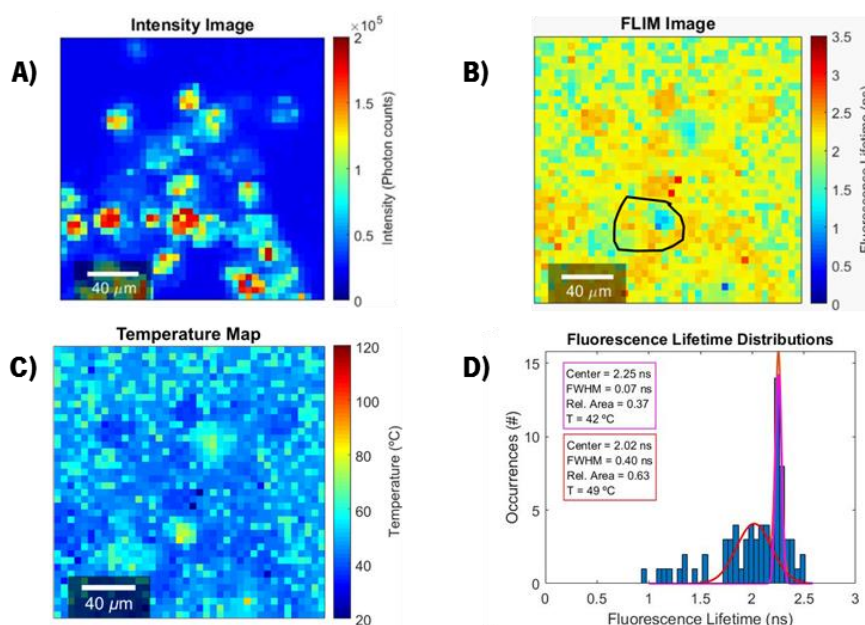
Supplementary figure 26 – Temperature map study based on FLIM characterization of Au@MSN-RhBITC nanohybrids incubated 24 h with HeLa cells, 808 nm laser irradiation, 1.8 W, 3 minutes. **A** – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected.

HeLa – 24h – 1.8 W – 6 mins



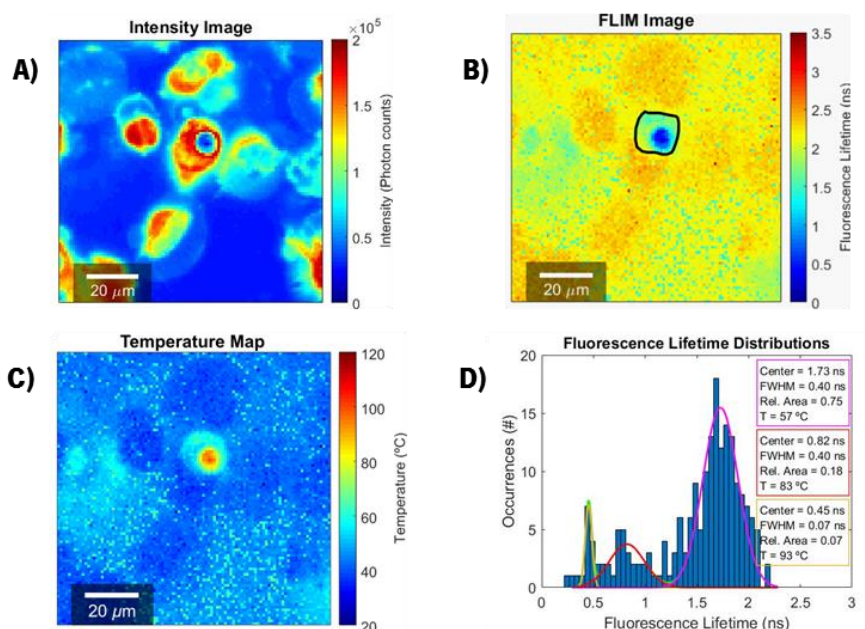
Supplementary figure 27 – Temperature map study based on FLIM characterization of Au@MSN-RhBITC nanohybrids incubated 24 h with HeLa cells, 808 nm laser irradiation, 1.8 W, 6 minutes. **A** – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected.

HeLa – 24h – 1.8 W – 9 mins



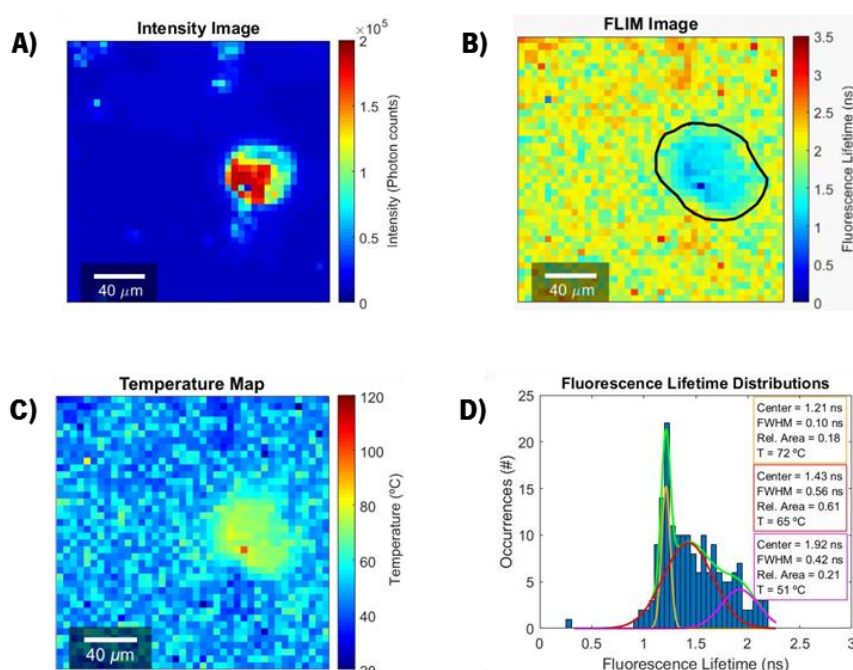
Supplementary figure 28 – Temperature map study based on FLIM characterization of Au@MSN-RhBITC nanohybrids incubated 24 h with HeLa cells, 808 nm laser irradiation, 1.8 W, 9 minutes. **A** – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected.

HeLa – 24h – 1.8 W – 15 mins



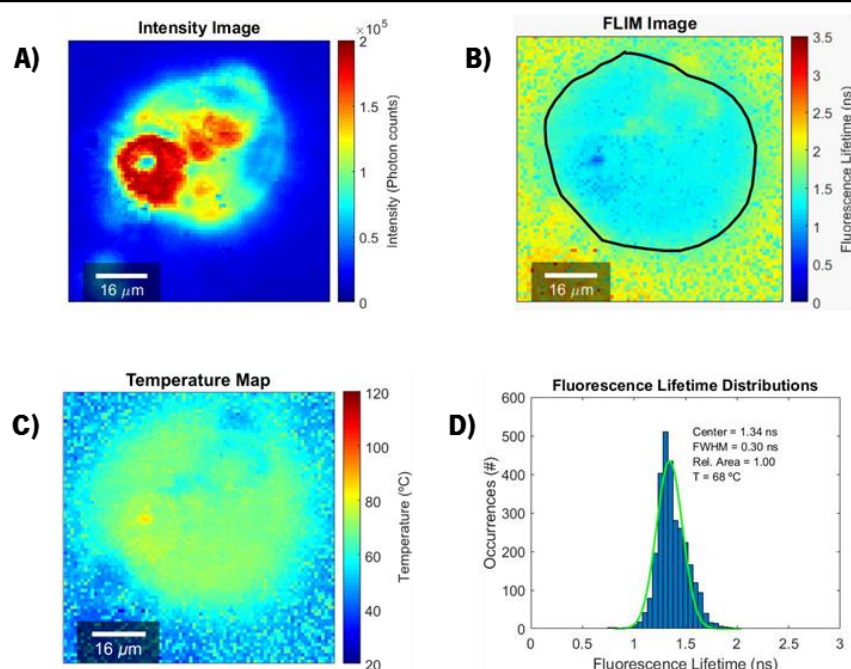
Supplementary figure 29 – Temperature map study based on FLIM characterization of Au@MSN-RhBITC nanohybrids incubated 24 h with HeLa cells, 808 nm laser irradiation, 1.8 W, 15 minutes. **A** – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected.

A431 – 12h – 1.3 W – 6 mins



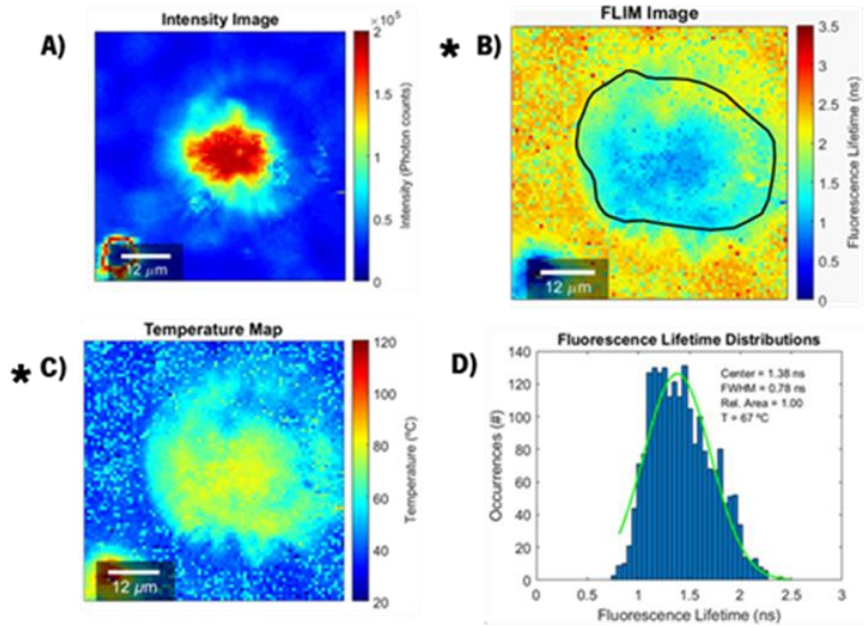
Supplementary figure 30 – Temperature map study based on FLIM characterization of Au@MSN-RhBITC nanohybrids incubated 12 h with A431 cells, 808 nm laser irradiation, 1.3 W, 6 minutes. **A** – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected.

A431 – 12h – 1.3 W – 9 mins



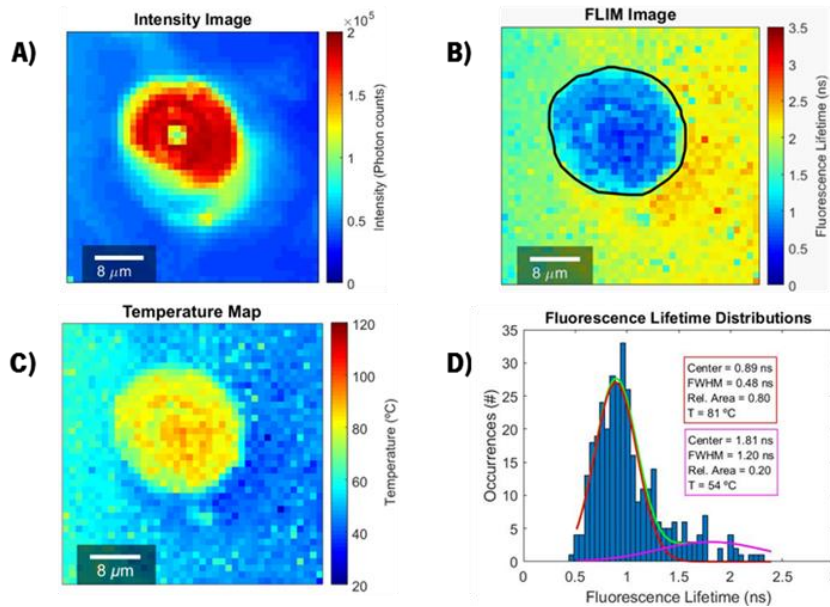
Supplementary figure 31 – Temperature map study based on FLIM characterization of Au@MSN-RhBITC nanohybrids incubated 12 h with A431 cells, 808 nm laser irradiation, 1.3 W, 9 minutes. **A** – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected.

A431 – 12h – 1.3 W – 15 mins



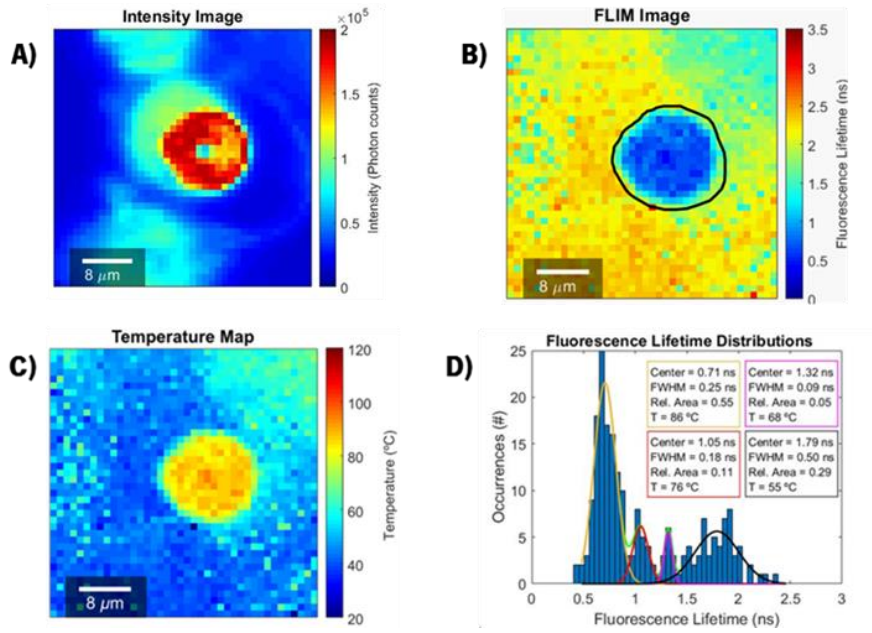
Supplementary figure 32 – Temperature map study based on FLIM characterization of Au@MSN-RhBITC nanohybrids incubated 12 h with A431 cells, 808 nm laser irradiation, 1.3 W, 15 minutes. **A** – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve (* indicates the existence of an outlier pixel reporting a temperature below 0 degrees, with a fluorescence lifetime above 5 ns), **D** – Histogram of fluorescence lifetime values in cell region selected.

A431 – 12h – 1.8 W – 3 mins



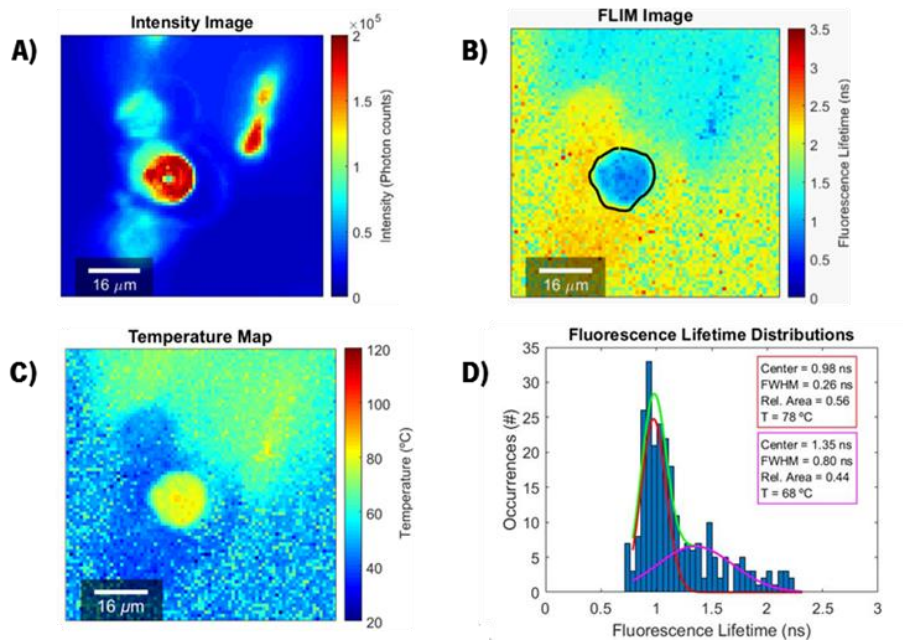
Supplementary figure 33 – Temperature map study based on FLIM characterization of Au@MSN-RhBITC nanohybrids incubated 12 h with A431 cells, 808 nm laser irradiation, 1.8 W, 3 minutes. **A** – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected.

A431 – 12h – 1.8 W – 6 mins



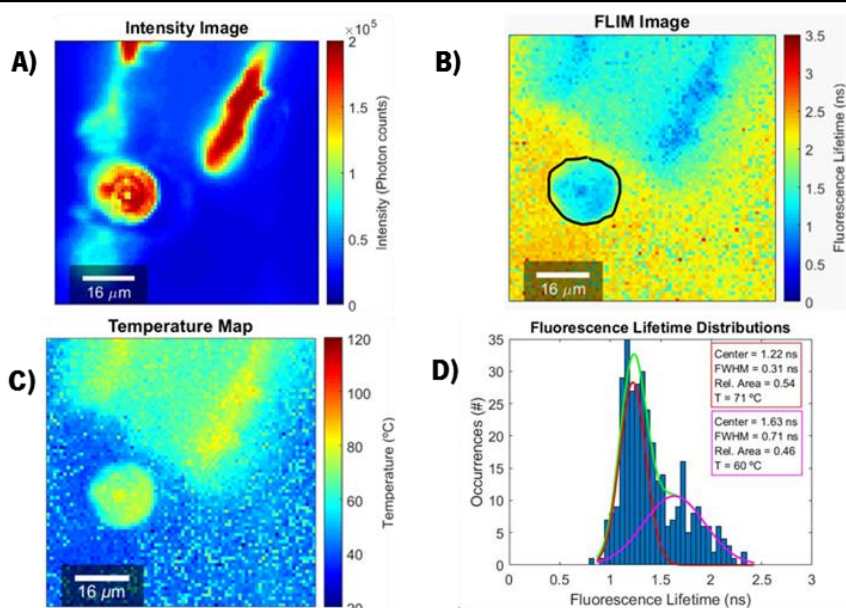
Supplementary figure 34 – Temperature map study based on FLIM characterization of Au@MSN-RhBITC nanohybrids incubated 12 h with A431 cells, 808 nm laser irradiation, 1.8 W, 6 minutes. **A** – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected.

A431 – 12h – 1.8 W – 9 mins



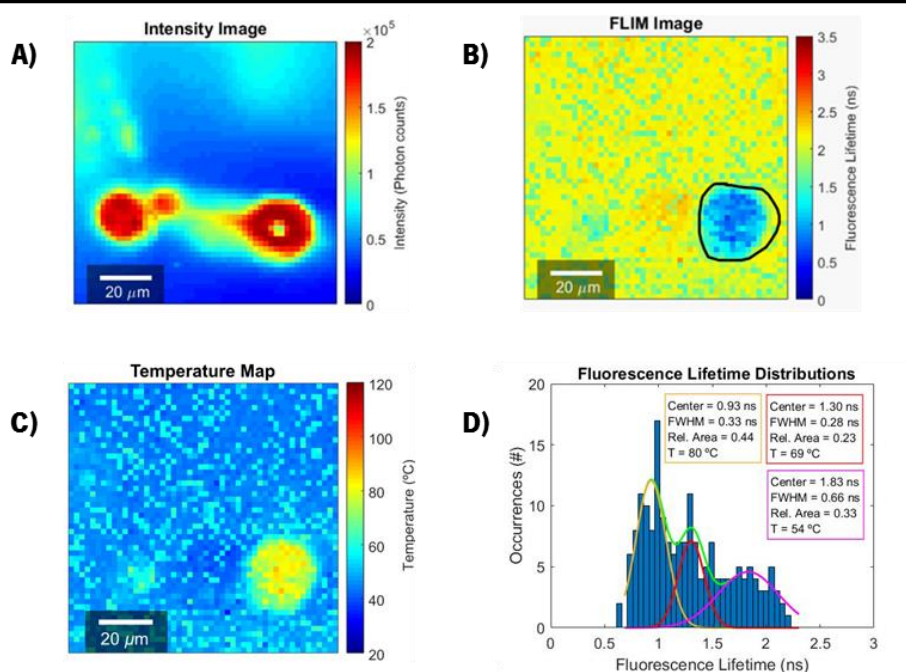
Supplementary figure 35 – Temperature map study on FLIM characterization of Au@MSN-RhBITC nanohybrids incubated 12 h with A431 cells, 808 nm laser irradiation, 1.8 W, 9 minutes. **A** – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected.

A431 – 12h – 1.8 W – 15 mins



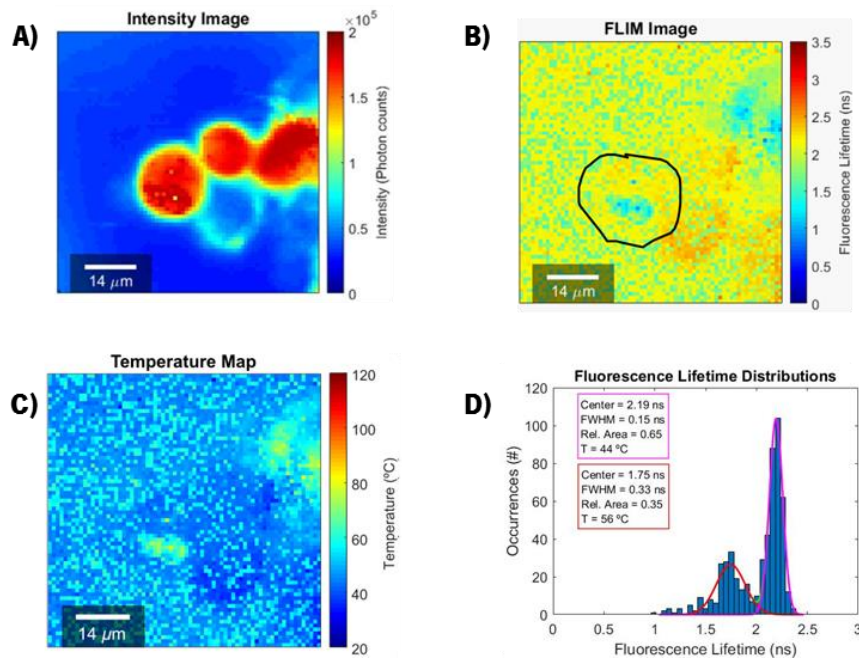
Supplementary figure 36 – Temperature map study based on FLIM characterization of Au@MSN-RhBITC nanohybrids incubated 12 h with A431 cells, 808 nm laser irradiation, 1.8 W, 15 minutes. **A** – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected.

A431 – 24h – 1.3 W – 6 mins



Supplementary figure 37 – Temperature map study based on FLIM characterization of Au@MSN-RhBITC nanohybrids incubated 24 h with A431 cells, 808 nm laser irradiation, 1.3 W, 6 minutes. **A** – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected.

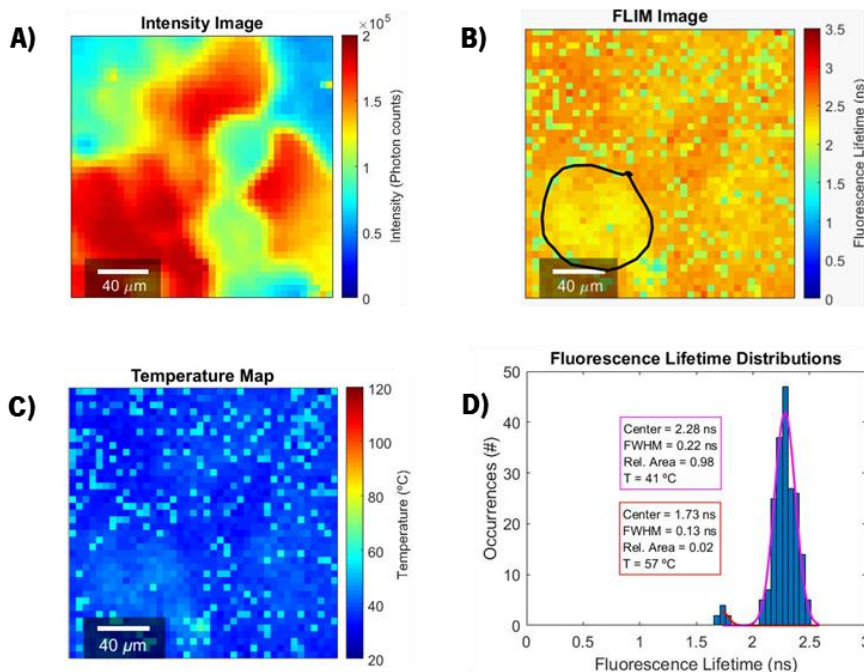
A431 – 24h – 1.3 W – 9 mins



Supplementary figure 38 – Temperature map study based on FLIM characterization of Au@MSN-RhBITC nanohybrids incubated 24 h with A431 cells, 808 nm laser irradiation, 1.3 W, 9 minutes.

A – Intensity image, **B** – FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected.

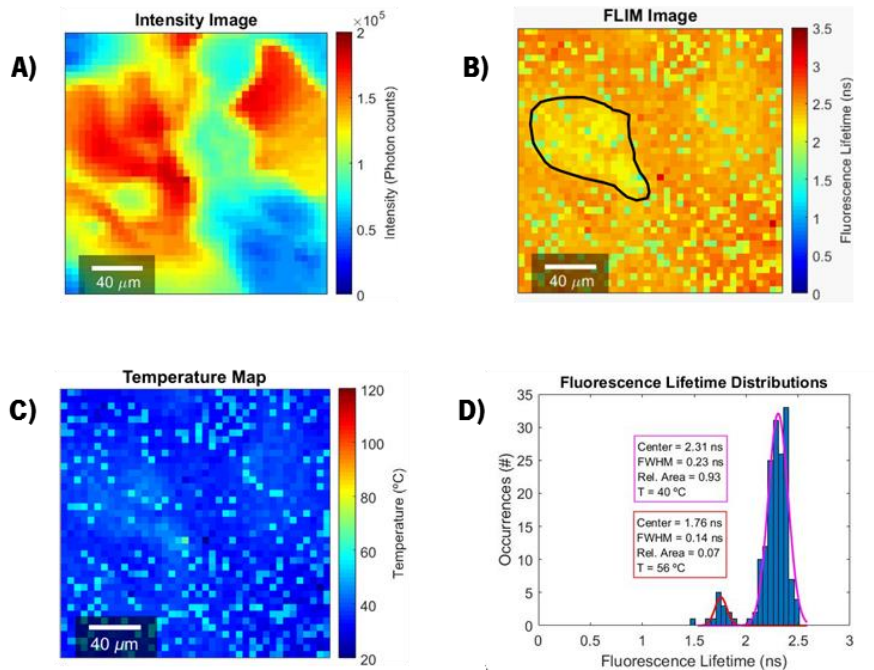
A431 – 24h – 1.3 W – 15 mins



Supplementary figure 39 – Temperature map study based on FLIM characterization of Au@MSN-RhBITC nanohybrids incubated 24 h with A431 cells, 808 nm laser irradiation, 1.3 W, 15 minutes.

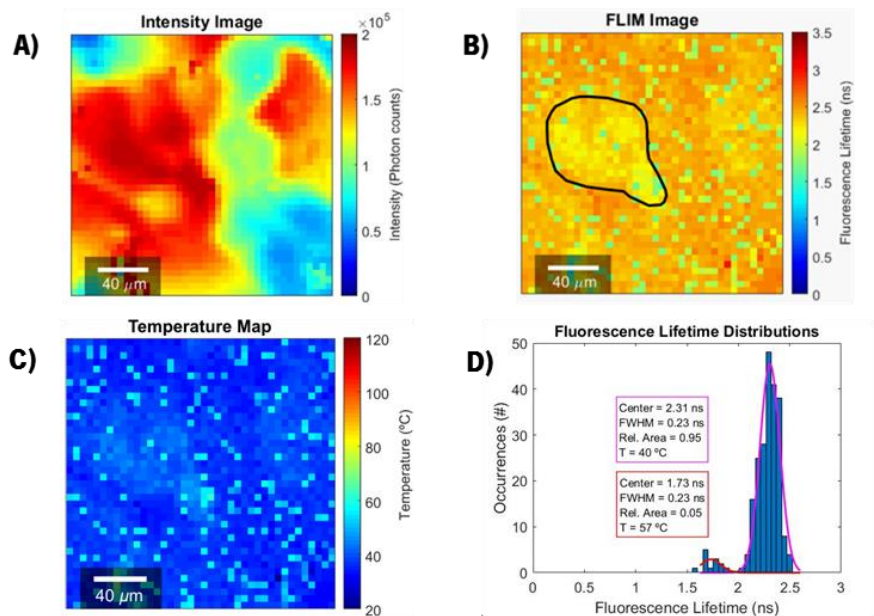
A – Intensity image, **B** – FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected.

A431 – 24h – 1.8 W – 3 mins



Supplementary figure 40 – Temperature map study based on FLIM characterization of Au@MSN-RhBITC nanohybrids incubated 24 h with A431 cells, 808 nm laser irradiation, 1.8 W, 3 minutes. **A** – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected.

A431 – 24h – 1.8 W – 9 mins



Supplementary figure 41 – Temperature map study based on FLIM characterization of Au@MSN-RhBITC nanohybrids incubated 24 h with A431 cells, 808 nm laser irradiation, 1.8 W, 9 minutes. **A** – Intensity image, **B** - FLIM image with cell region selection, **C** – Temperature map study derived from calibration curve, **D** – Histogram of fluorescence lifetime values in cell region selected.