Ag$_2$S Nanoheaters with Multiparameter Sensing for Reliable Thermal Feedback during In Vivo Tumor Therapy

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The emergence of luminescence nanothermometry in bio and nanomedicine has enabled achievements outside the reach of conventional techniques. For instance, it has provided real-time monitoring of in vivo thermal therapies of tumors, a mandatory requirement for these techniques to work safely and efficiently. However, the reliability of intratumoral thermal readings is currently in question due to the presence of artefacts caused by the inhomogeneous optical properties of biological tissues. This work demonstrates how it is possible to avoid, under specific conditions, these artefacts and reach precise and reliable in vivo intratumoral thermal feedback during in vivo photothermal treatments. The method proposed is based on the use of luminescent nanoparticles capable of multiparametric thermal sensing. The results demonstrate how the convergence of the different thermal readouts becomes a solid indicator of their reliability. It is shown how this new approach makes possible precise (thermal uncertainties below 1 °C) intratumoral thermal feedback, while simple, efficient, and minimally invasive in vivo thermal treatments of surface tumors is carried out. Results included in this work provide an ingenious route toward the consolidation of luminescence nanothermometry as a convincing technique for high sensitivity preclinical thermal sensing, while also constituting a step toward improved photothermal therapies.

1. Introduction

Self-monitored photothermal agents (S-MPThs) are a sort of optically excited nanoparticles (NPs) capable of simultaneous heat generation and remote thermal sensing.[8] They constitute a unique platform for developing minimally invasive, cost-effective and fully controlled hyperthermia therapies.[2] Particularly, when dealing with the in vivo thermal treatment of tumors, S-MPThs make possible real-time control over the intratumoral temperature that is required to be found in the therapeutic range.[3] This, in turn, avoids the extremes of having either insufficient or excessive heating, which could lead to either inefficient therapy or collateral damage, respectively.[4] The importance of intratumoral temperature monitoring is such that the Food and Drug Administration (FDA) has launched safety warnings in already approved thermal therapy devices. When using S-MPThs both heating and luminescence-based thermal readings are triggered by a single laser beam, so that intratumoral heating and thermal sensing could be achieved straightforwardly. Several examples do exist in the literature demonstrating successful thermal in vivo treatment of tumors with S-MPThs, based on either lanthanide-doped NPs or infrared-emitting quantum dots (QDs).[5] These systems combine high radiation-to-heat conversion efficiencies with appreciable luminescence thermal sensitivities. During last years, luminescence nanothermometry has established itself as an unrivalled technique for in vivo remote thermal sensing, a research area where alternative methods (such as magnetic resonance, infrared thermal imaging or ultrasound thermal imaging) have failed to provide accurate, real-time and cost-effective thermal readouts.[6]

Recent works, however, have raised serious concerns about the reliability of sub-tissue thermal measurements based on luminescent nanothermometers.[7] It has been demonstrated how the non-homogeneous transmission of tissues can induce relevant spectral distortions and lead to erroneous thermal readouts that could be as large as 10 °C.[8] As such, previous results provided by S-MPThs in literature have been put into
doubt. One of the most common pitfalls when dealing with intratumoral thermal reading is the fact that S-MPThs are surrounded by tumor tissues and, therefore, the detected spectrum will always be modulated by their optical transmission. Achieving artefact-free intratumoral thermal reading is not a trivial problem at all. At first glance, reliable thermal reading inside the tumor would require exact knowledge of the optical properties of the tumoral tissue to separate the tissue-induced spectral distortions from those purely caused by temperature variations. This assumption is not realistic due to the lack of knowledge of the optical properties of tumoral tissues and, particularly, of their thermal dependence. Alternatively, reliable intratumoral thermal reading could be achieved by using multiparametric luminescent nanothermometers (MP-LNThs). MP-LNThs are capable of providing multiple thermal readouts based on the analysis of different spectroscopic parameters such as intensity ratio, peak position, bandwidth, lifetime and/or emitted intensity. Synchronised acquisition of multiple thermal reading unfolds a completely new way to check the accuracy of luminescence thermometry. The basic argument is the following: if the measurements are artefact-free, then all the different thermal readings should converge. If the different thermal readouts diverge, however, it constitutes a clear indication that the luminescent nanothermometer is not working properly, namely that experimental artefacts are crucially affecting the measurements. Note that the absence of convergence obeys to the fact that the artefacts typically affect the thermal readouts, based on the analysis of the different spectroscopic parameters, in different ways.

In this work, we introduce Ag2S NPs as S-MPThs capable of simultaneous multiparametric thermal sensing (system schematically represented in Figure 1). Though their radiation-to-heat conversion efficiency and the multiparametric thermal sensing potential have been demonstrated/reported separately, in this work we combine them both to efficiently achieve in vivo photothermal treatment of tumoral tissues with a precise real-time thermal control. The discussion of the results, therefore, is divided into two main parts: (i) achieving efficient tumor treatment and (ii) providing an analysis approach to get precise thermal readouts. The implications that the novel multiparametric readout procedure, here proposed, will have on the next steps of in vivo luminescence nanothermometry are also discussed.

2. Results and Discussion

2.1. Ag2S NPs Photothermal Agents for In Vivo Tumor Treatment

As already mentioned, Ag2S NPs have been previously characterized as photothermal transducers. Their potential use for in vivo tumor ablation, however, is here evaluated. The Ag2S NPs used along this work were provided by SINANO Ltd (China). Details about their physical properties are provided in the Section S1 of Supporting Information. Section S2, on the other hand, includes the characterization of Ag2S NPs as robust photothermal transducers with a radiation-to-heat conversion efficiency above 90% caused by a fluorescence Quantum Yield below 1% (Section S3). The absorption cross section of Ag2S NPs has been measured to be 3.46 × 10⁻²² cm² at 800 nm. Comparison of these numbers to those corresponding to other PPT agents (see Table 1) numbers suggest the great potential of Ag2S NPs for in vivo photothermal treatment of tumors. To verify it, the experimental procedure schematically shown in Figure 2 was adopted. For in vivo experiments, we employed C57Bl/6 mice. This particular strain was selected due to its strong (black) pigmentation. In these conditions, such a highly absorbing tissue provides a strong autofluorescence background that significantly complicates the experimental procedure. In other words, we here evaluate the potential use of Ag2S NPs for in vivo photothermal treatment of tumors under the most adverse circumstances.

Throughout this work, we investigated four different situations: i) tumor inoculated with Ag2S NPs and not exposed to any photothermal treatment (“Ag2S+Tumor”), ii) tumor inoculated with Ag2S NPs and subjected to a photothermal treatment (“Ag2S+Tumor+Laser”), iii) tumor subjected to a photothermal treatment (“Tumor+Laser”), and iv) tumor not exposed to any kind of treatment (“Tumor”). In all the cases, tumor inoculation was performed by subcutaneous injection of 150 µL of a sterile PBS solution containing 75 µL of murine melanoma cells (cell concentration of 10⁶ mL⁻¹). In both “Ag2S+Tumor” and “Ag2S+Tumor+Laser” cases, 75 µL of Ag2S NPs PBS solution
Figure 2. Schematic representation of the in vivo photothermal treatment of tumors developed in this work. The Ag₂S NPs are used as dual agents capable of laser-induced heating and multiparameter and accurate intratumoral thermal reading during treatment. Tᵣ, Tₛ, and Tᵢ are the intratumoral thermal readouts obtained from the analysis of the intensity ratio (ratiometric analysis), the analysis of the peak position and the analysis of the emitted intensity, respectively.

Table 1. Main properties of the most representative nano-sized systems already used for in vivo photothermal therapy of tumors.

| System            | Thermal feedback | Sᵣ [% °C⁻¹] | Multi-sensing | ηh [%] | σabs@ 800 nm (cm²) | Ref.  |
|-------------------|------------------|-------------|---------------|--------|--------------------|------|
| NdVO₄              | NO               | 2.67        | NO            | 72.1   | 2.5 × 10⁻²⁰        | [5e] |
| SWCNTs            | NO               | 0.005       | NO            | 3.6    | 1 × 10⁻¹²          | [28] |
| Au@Gd₂O₃          | NO               | 1.24        | NO            | 55.7   | 4.3 × 10⁻¹¹        | [29] |
| Au nanorods        | NO               | 1.16        | NO            | 50     | 2.3 × 10⁻¹⁵        | [30] |
| Polypyrrole        | NO               | 0.19        | NO            | 44.7   | 8.5 × 10⁻¹²        | [31] |
| CdTe              | NO               | 0.08        | YES           | 14     | 5.83 × 10⁻²⁴       | [32] |
| MWCNTs            | NO               | 0.12        | NO            | 16.6   | 9.8 × 10⁻²²        | [33] |
| Carbon nanodots    | YES              | 1.79        | NO            | 30.6   | 2.09 × 10⁻²¹       | [34] |
| NaYF₄: Yb,Er@PDA/ICG | YES            | 1.11        | NO            | 15.9   | 9.11 × 10⁻²¹       | [35] |
| LaF₃: Nd          | YES              | 0.2         | YES           | 12.5   | 5.44 × 10⁻²⁰       | [36] |
| Ag₂S-PEG           | YES              | 4           | YES           | 93.2   | 3.46 × 10⁻²²       | This work |
(1.5 mg mL⁻¹) were added to the cells. This, in turn, is justified by the fact that simultaneous injection of cancer cells and thermal agents avoids the requirement of intratumoral injection of photothermal transducers and warranties their homogeneity within the tumor. Nevertheless, for the sake of completion, tumor targeting after intravenous administration of Ag₂S NPs was also investigated in an extra experiment (Section S4 of Supporting Information). Visualization of tumor vasculature just after injection was verified. Permanent accumulation of Ag₂S NPs within the tumor, however, was not observed. We state that this is due to the lack of a proper biofunctionalization. After all, the Ag₂S NPs here utilized were simply functionalized with PEG. Though it provided them with long circulation times it did not confer tumor targeting capabilities. Nevertheless, one needs to emphasize that tumor targeting with Ag₂S NPs has been demonstrated in the past. Thus, for the sake of the discussion, this will not be considered to be inside of the scope of the present work.

The presence of Ag₂S NPs within the tumor was checked by the acquisition of infrared (λ_ex = 800 nm, λ_em = 1200 nm) fluorescence images (see Figure 3a). In both “Ag₂S+Tumor+Laser” and “Tumor+Laser” cases, the photothermal treatment was applied 5 days after inoculation and consisted of 4-min-long irradiation with an 808 nm laser beam (1 W/cm²). The time courses of tumor size in these four target cases are shown in Figure 3b. The tumor evolution obtained for “Tumor+Laser”, “Tumor”, and “Ag₂S+Tumor” are virtually identical. This fact evidenced that the Ag₂S NPs by themselves have no decremental effect on the behaviour of tumoral tissues and, for the irradiation density used in this work (1 W/cm²), the heating induced by the tissue’s light absorption is not large enough to cause any therapeutic effect. This is in accordance with our experimental observations that reveal moderate tumor heating (< 6 °C) in absence of Ag₂S NPs (see next section that includes the measurements performed with a thermal camera). Figure 3b reveals complete tumor removal for the “Ag₂S+Tumor+Laser” case. The tumor eradication is supported by the optical pictures included in Figure 3c. Note that 25 days after inoculation there is no evidence of tumor presence. We state at this point that the radiation-to-heat conversion ability of Ag₂S NPs has produced “fast and severe” intratumoral heating that has induced irreversible damage on tumoral cells leading to what is typically referred to as “tumor ablation”. This, in turn, results in a total treatment that does not require any complementary technique (such as chemotherapy). Optical pictures corresponding to the “Tumor” (Figure 3d), “Ag₂S+Tumor” (Figure 3e), and “Tumor+Laser” (Figure 3f) cases evidence the continuous tumor development that forces the sacrifice at day 15 after inoculation. Fully treated mice survived more than 25 days after tumor inoculation without showing any evidence of tumor reappearance. It is important to note that the fluorescence images included in Figure 3a also reveal that 17 days after inoculation, the intratumoral injected Ag₂S NPs were completely cleared. This is at variance with the long-term retention of Ag₂S NPs within
the tumor when no treatment was applied (Section S5 of Supporting Information). This reveals that tumor ablation favours the leakage of Ag$_2$S NPs from the tumor to the bloodstream very likely caused by cell lysis and re-vascularization of healthy tissues. The clearance pathway of Ag$_2$S NPs has not been investigated in this work. Nevertheless, according to previous works, it is very likely produced through the reticuloendothelial system.$^{[18]}$

Finally, complete tumor removal was additionally checked by histological studies. Sections of non-treated (Figure 4a–f), and treated tumors were studied (Figure 4g–l), both with staining method for hematoxylin-eosin, which reveals microscopic morphology; and also with Ki67 immunohistochemistry, which is an indicator of active cell proliferation (Figure 4c,f). In non-treated-tumor hematoxylin-eosin stained sections (Figure 4a,b,d,e) we observed encapsulated tumor cells where we can see melanin pigmentation (Figure 4d). The Ki67 immunohistochemistry (Figure 4c,f) shows sections presenting a clear necrotic core zone of the tumor and a radial layer of proliferating cells. It is also evident the unpecific staining with this antibody for the melanin deposits, and more importantly, it is clearly noticeable the proliferative cell layer at the outer zone of the tumor and the central zone with necrosis and cell fibrosis (Figure 4c,f). We also analyzed a normal and healthy skin section observing the epidermis and proliferative hair follicles (Figure 4e).

The histological study of the treated-tumor sections (Figure 4g–l) were also studied using hematoxylin-eosin staining method and Ki67 immunohistochemistry. In this case, in the hematoxylin-eosin stained sections a scar area is clearly visible, both in the epidermis and at inferior layers (Figure 4g–i). There was a high number of immune cells infiltration and red blood cells (Figure 4g–i,k). It was also visible a thickening of the skin associated with a loss of architectural structure of the epidermis (Figure 4g–i). In addition, it was observed a high index of cell proliferation with fibroblastic morphology, visible in the scar and perivascular areas, non-associated to tumoral growth as it was the case observed in the non-treated tumor sections

![Figure 4](https://www.afm-journal.de/fig/4.png)

**Figure 4.** a–f) Images of non-treated tumor sections. a,b,d) Hematoxylin-eosin staining and c,f) Ki67 immunohistochemistry show encapsulated tumor sections. a,b,d) Necrotic areas are present in the core of the tumor, as well as a proliferative surrounding cell layer. Proliferative areas are evident in the non-treated tumor sections using Ki67 antibody (c,f). e) Skin section showing proliferative hair follicle. g–l) Images of treated-tumor. In the hematoxylin-eosin images of treated tumor sections no evidence of tumor areas were found. g–l) Stained sections show a clear scar and skin thickening. g–l) Appreciable structural loss is observed both in epidermis and deep layers of the skin. j,k) Magnification microphotograph show immune cell infiltration of the tissue and blood cells. l) Ki67 antibody immunohistochemistry of sections of the tumor-treated show few proliferation cells surrounding blood vessels normally present in injured and regenerative tissue. j) Non-tumor proliferative cells were evidenced by Ki67 antibody in the tumor-treated sections. Any association with tumor growth is visible in tumor-treated sections. Magnification 10× and 40×. Scale bar: 100 mm.
This proliferation is probably associated with the scar formation process due to fibroblastic proliferation and high collagen secretion typical of a fibrotic process. No melanin deposits were observed associated with tumoral aggregates in the treated-tumor sections. Not even any tumor aggregates were present in the sections studied (Figure 4i).

Proliferation index was obtained in both cases (treated and non-treated tumors) using ImageJ software studying representative sections of the tissue. In the non-treated case Ki67 gives a value of 9.179% for the images studied of the tumor versus a value of 0.33% for the normal skin sections studied. In the case of the tumor-treated mice, the proliferation index for Ki67 obtained was 0.488% in skin sections and 1.065% for the scar (tumor) sections studied. These results can be taken as a good overview of the effectiveness of the treatment.

2.2. Reliable Intratumoral Thermal Reading by Multiparametric Thermal Sensing

Although the radiation-to-heat conversion efficiency of Ag$_2$S NPs allows for successful in vivo tumor treatment, a reliable intratumoral thermal readout is still required to provide real-time control to it. Such reliable intratumoral thermal reading becomes essential to understand the physiological origin of tumor destruction. This, in turn, can be achieved by the thermally sensitive properties of Ag$_2$S NPs.[19] Figure 5a includes the emission spectra obtained from a colloidal solution of Ag$_2$S NPs in PBS under 808 nm laser excitation, as obtained at different temperatures. An intense thermal quenching accompanied by a red-shift of this emission band due to the temperature increment in the bandgap energy is evidenced.[20] This simultaneous thermal quenching and spectral shift offer the possibility of single-shot multiparametric luminescence thermal reading. This constitutes and advantage over other photothermal agents that are not capable of providing any thermal feedback (see Table 1). Figure 5b–d shows the thermal dependence of the emitted intensity ($I(T)$), integrated in the whole wavelength range), peak position ($\lambda_p(T)$) and the ratio between the emitted intensities at 1225 and 1175 nm ($R(T)$), respectively.

As observed, the peak position and the intensity ratio show close-to-linear increments with temperature increase, while the emitted intensity decreases following a non-linear trend. The graphs in Figure 5b–d also include the corresponding relative thermal sensitivities obtained from the analysis of the different spectroscopic parameters, i.e.:

\[
S^I(T) = \frac{1}{I(T)} \frac{dI(T)}{dT}
\]

(1)

\[
S^{\lambda_p}(T) = \frac{1}{\lambda_p(T)} \frac{d\lambda_p(T)}{dT}
\]

(2)

\[
S^R(T) = \frac{1}{R(T)} \frac{dR(T)}{dT}
\]

(3)

The relative thermal sensitivity corresponding to the intensity, $S^I(T)$, is strongly temperature-dependent, varying from 5%
at room temperature to 4.1% at 50 °C. The wavelength-based relative thermal sensitivity, \( S^r(\lambda) \), on the other hand, is virtually temperature-independent. It can be verified that the emission band of our Ag\(_2\)S nanoparticles shifts at a rate of 1.65 nm/°C, which corresponds to an energy shift of \(-1.3\) meV per °C. This is very similar to the thermal shift rate previously reported for bulk Ag\(_2\)S (\(-1.2\) meV per °C). \(^{[21]}\) Consequently, it evidences that the average thermal spectral shift of our Ag\(_2\)S NPs is mainly given by the temperature dependence of the Ag\(_2\)S bandgap and that the possible effects caused by quantum confinement can be considered as of second order. This also agrees with the fact that 80% of our Ag\(_2\)S nanoparticles show radii above the Bohr radius of Ag\(_2\)S (Section S6 of Supporting Information). Finally, and similar to the integrated intensity, the ratiometric thermal sensitivity \( S^r_R \), is also found to be temperature-dependent, decreasing from 1.4% at room temperature to 1.0% at 50 °C. Data included in Figure 5, therefore, not only clearly certify the ability of Ag\(_2\)S NPs for multiparametric thermal sensing but also reveal non-negligible temperature dependencies in their thermal sensitivities.

To show how these thermally sensitive parameters could provide a reliable readout of the temperature, the emission spectra generated by Ag\(_2\)S NPs within the tumor during irradiation were recorded. This experiment, of course, involved the “Ag\(_2\)S+Tumor+Laser” mouse described in Figure 3. Figure 6a shows these emission spectra as obtained at different instants (\( t = 0 \) s corresponds to the beginning of the treatment and \( t = 240 \) s to its end). The signal-to-noise ratio (SNR) of the acquired spectrum varied in the 10–15 dB range. This, in turn, ensured an accurate study of the data (Supporting Information Section S7). The analysis of the intratumoral emission could then be used to get the time evolution of the internal temperature. Moreover, as pointed out in the introduction, recent works revealed that, before extracting thermal information from the emission spectra, it is mandatory to evaluate at which extent they are distorted by tissue extinction.\(^{[22]}\) Figure 6b shows the intratumoral emission spectrum of Ag\(_2\)S NPs as obtained at \( t = 0 \) (thus, expected to correspond to a temperature of 32 °C) together with the emission spectrum of an aqueous solution of Ag\(_2\)S NPs at 32 °C as obtained under the same experimental conditions. Both spectra were normalized by the area under the curve. Despite their similarity in the 1140–1300 nm wavelength range, some differences were observed for wavelengths shorter than 1140 nm. These differences also exist when the emission spectra are compared at higher temperatures (Supporting Information Section S8). Based on previous works,\(^{[22]}\) we attribute these differences to the tissue-induced spectral distortions caused by the particular attenuation (scattering and absorption).
absorption) of light in the tumor in this spectral region. Note that the corroboration of this attribution would require detailed knowledge of the optical properties of the tumoral tissue. Such a complex insight should constitute the scope of future works beyond the scope of this paper. Still, Figure 6b suggests that, depending on the thermometric parameter selected, the acquisition of intratumoral thermal readouts would be contaminated by tissue-induced spectral distortions. This will be more easily noticed with \( I(T) \) and \( R(T) \) than with \( \lambda_p(T) \) due to the fact that the first two parameters present numerous ways of being calculated (i.e., one has many options of defining the limits of integration of \( I \) and the combinations of wavelengths to compute \( R \)).

To illustrate how the tissues can affect the thermal readout, the intratumoral temperature has been calculated by applying no restriction whatsoever in the selection of the thermometric parameters. Thus, by being the most general possible, \( I \) was defined as the integrated intensity from 1100 to 1300 nm and \( R \) was defined as the ratio between the intensities at 1110 ± 5 nm and 1285 ± 5 nm (denoted as \( I_2 \) and \( I_1 \) in Figure 6b). These particular wavelengths (1110 nm and 1280 nm) were chosen because (as it has been traditionally thought) when dealing with ratiometric thermal measurements, the larger the difference between evaluated wavelengths, the better the readout would be.\(^{[23]}\) When following such a protocol, the tissue temperature increments calculated from the analysis of the variation of emission intensity, wavelength shift, and ratio were given by:

\[
\Delta T^{\text{gen}}_{(I)}(t) = \int_{t^\text{std}(I=0)}^{t} \frac{1}{S_{I^\text{std}}} \left| \frac{dI}{dt} \right| dI
\]

\[
\Delta T^{\text{gen}}_{(R)}(t) = \int_{t^\text{std}(R=0)}^{t} \frac{1}{S_{R^\text{std}}} \left| \frac{dR}{dt} \right| dR
\]

\[
\Delta T^{\text{gen}}_{(I,R)}(t) = \int_{t^\text{std}(I=0,R=0)}^{t} \frac{1}{S_{I^\text{std},R^\text{std}}} \left| \frac{dR}{dt} \right| dR
\]

where \( I^\text{std}(t = 0) \) and \( R^\text{std}(t = 0) \) were the intensity, ratio and peak position at the beginning of the heating cycle, respectively (which correspond to the ones at 32 °C, i.e., the initial intratumoral temperature). The integrals in (4)–(6) were all calculated after expressing the relative thermal sensitivity as a direct function of the corresponding thermometric parameter (instead of temperature). The time evolution of the multiparametric thermal readouts calculated by following this procedure are included in Figure 6c. Additionally, the surface temperatures, recorded with a thermographic camera, of a “control tumor” (tumor without Ag2S NPs) as well as of the tumor with Ag2S NPs are also included in Figure 6c. A comparison between these curves reveals the dominant role of the heating induced by Ag2S NPs over the one resulting from tissue absorption of light. More importantly, however, is the fact that Figure 6c disclose discrepancies (\( \Delta T \)) up to 7 °C between the thermal readouts obtained from the analysis of the different spectral parameters. That large thermal divergence reduces the reliability of the thermal measurements, as such large uncertainty is not acceptable when dealing with biological systems, where few degrees can have a great impact on the physiological behaviour of the live system under study. Though previous \textit{ex vivo} experiments already suggested the possibility of finding those large discrepancies, we must note that this is the first time that the disagreement between simultaneous thermal readouts obtained from different thermometric parameters is verified at the in vivo level.

To improve the reliability and robustness of the thermal measurements, the presence of tissue-induced spectral distortions should be avoided. This task, however, is not straightforward. After all, one is not able to know a priori the manner by which to compute \( I \) and \( R \) in such a way that the disagreement of thermal readouts is minimized. Thus, when the measurement has this ambiguous nature, one needs to thoroughly inspect all the possible combinations of thermometric parameters and look for the ones that minimize the discrepancy. However, as we already mentioned, one of the parameters used in the present study has only one way of being calculated: the peak position, \( \lambda_p \). Such a feature could be very useful in the sense that if its corresponding thermal readout is indeed correct, one can consider it as the standard by which all the other readouts must be compared. In the present case, this correctness seems to be suggested by the fact that there were no valleys in the spectra of Figure 6a. This means that, whatever the contribution of the tissue was, \( \lambda_p \) was minimally affected by it. Thus, in this work, we reasonably consider the thermal reading from \( \lambda_p \) as the standard one. Calibrations with an \textit{ex vivo} tumor seem to support this conclusion (Section S9 of Supporting Information). At the same time, we recognize that if the properties of the surrounding tissue were different (for instance, a higher absorption coefficient around 1200 nm), maybe this approach would not be applicable. In spite of this, even if the universality of the method is not guaranteed, when comparing with other complex approaches that take into account all of the tissue-induced effects, it can still be considered as more practical under some circumstances.

Once the standard thermal readout has been defined, one only needs to find the ways of computing \( I \) and \( R \) that minimize the deviation from it. The rationale comes from the fact that if the thermal reading from different thermometric parameters agree, this can only mean one of two things: (I) either the tissue-induced effects were completely avoided in the selection of the parameters or (II) the tissues are affecting the parameters in the exact same manner. A detailed discussion is expanded in Section S10 of Supporting Information. To follow this rationale, one needs first to select the thermal reading from \( \lambda_p \) (already included in Figure 6c) and consider it as a multidimensional vector, \( \vec{T} \). The dimension \( N \) of this vector would be equal to the number of time points in the dynamic measurement (in the present case, 285). Since the other two thermometric parameters, \( I \) and \( R \), presented many ways of being calculated, the result would be, in contrast, many multidimensional vectors, \( \vec{T}_I \), ..., \( \vec{T}_a \) and \( \vec{T}_R \), ..., \( \vec{T}_b \). Each of these vectors would correspond to the thermal dynamics according to the particular integration limits and the particular combination of wavelengths to compute \( I \) and \( R \), respectively.

With this mathematical notation, one can say that the optimal wavelength regions for multiparametric thermal sensing are found when \( \delta T = ||\vec{T}_a - \vec{T}_b|| / \sqrt{N} \) and \( \delta T = ||\vec{T}_a - \vec{T}_b|| / \sqrt{N} \) are minimized. Due to the numerous possibilities of calculating
I and R, this task was only made possible by computational efforts. The program obtained, from the spectra of Figure 5, the calibration for each possible thermometric parameter. Then, by observing the dynamics of this specific parameter, the program converted it to temperature units through the just mentioned calibration. In mathematical terms:

$$T_{\text{Int}} = \int_{t_0}^{t_j} \frac{1}{S_r} \frac{dI}{I}$$

$$T_{\text{R}} = \int_{r_0}^{r_j} \frac{1}{S_r} \frac{dR}{R}$$

Where $T_{\text{Int}}$ and $T_{R}$ are the j-th coordinates of the $T_{\text{Int}}$ and $T_{R}$ multidimensional vectors, respectively. While $t_0$ and $t_j$ refer to the initial and j-th instants in the dynamic measurement. Finally, the corresponding $\delta T$ was calculated and stored in a dataset. A simplified scheme of the algorithm is included in Figure 6d.

After going through all the possible outcomes of I and R, one obtains the results shown in Figure 7. As one can see in Figure 7a, the integrated intensity becomes more reliable when at least one of integration limits distances itself from the extremes (1100 nm and 1300 nm). This can be explained by the fact that the level of signal at the extremes is reduced. Furthermore, as we previously discussed, the tissue-induced attenuation seemed to preferably affect the wavelengths from 1100 nm to 1140 nm. This, in turn, resulted in a brighter region at the bottom left of the figure. By numerically searching for the best combination of inferior and superior wavelengths for the integration, one finds 1175 nm and 1260 nm to be optimal choices. They provide a deviation of only 1.2 °C from the standard readout. At this point, it is worth to emphasize that the diagonal going from bottom left to top right was replaced by the deviation values obtained when considering the absolute intensity at a certain wavelength as the thermometric parameter. This, in

**Figure 7.** Evaluation of thermal deviations of a) the luminescence intensity when integrated in the range going from the first to the second integration limit and b) the luminescence intensity ratio as calculated at different combinations of wavelengths. The deviations were obtained after taking the thermal reading from $\lambda_{\text{max}}$ as the standard. c) Time evolution of laser-induced tumor temperature increment during photothermal treatment as calculated from the analysis of the peak position ($T_\lambda$), emitted intensity ($T_I$), and intensity ratio ($T_R$). The wavelength range used for I and the combination used for R were the ones that minimized $\delta T$ according to preceding data. The analysis leading to figures (a) and (b) was performed by a code written by authors in MATLAB R2020a. The Maximum Deviation indicated in c) correspond to the maximum difference between the averaged values of the different temperatures in the steady state (times longer than 200 s).
turn, would provide a fair comparison between integrated and absolute intensity.

Figure 7b, on the other hand, shows that, in a general sense, the deviation of the reading provided by \( R \) is maximized near the identity line \( (\lambda_1 = \lambda_2) \). This is, of course, expected, since, independently of the temperature, \( R \) is always 1 when \( \lambda_1 = \lambda_2 \). Thus, to minimize \( \Delta T_R \), one has to distance the selection of wavelengths from this diagonal. Through numerical algorithms, one can identify the combinations \( \lambda_1, \lambda_2 \) that lead to minimum values of \( \Delta T_R \). By restricting the search to the first 2 minimal values of \( \Delta T_R \), we obtained \( I_{172} \pm 5 \text{ nm} / I_{1255} \pm 5 \text{ nm} \) and \( I_{170} \pm 5 \text{ nm} / I_{1256} \pm 5 \text{ nm} \), as the best ways of computing \( R \). Both of them presented a deviation of 0.9 °C from \( T_{\text{max}} \). This value is very similar to the one provided by the optimal integrated intensity and much better than the ones given by the coordinates around the identity line (i.e., \( \Delta T_R = 30 \text{ °C} \)).

To visually demonstrate how the new choice of \( I \) and \( R \) indeed provide a better agreement between the thermometric parameters, Figure 7c provides a comparison between their thermal readouts and the one calculated with \( \lambda_2 \). Once again, the surface temperatures, recorded with a thermographic camera, of a “control tumor” (tumor without \( \text{Ag}_2\text{S NPs} \)) as well as of the tumor with \( \text{Ag}_2\text{S NPs} \) were included. As one can see, the three thermal readouts not only present similar increasing curves but also converge to the same value. If one calculates the standard deviation of the mean, a value of 0.5 °C is found. Such an uncertainty, in turn, can be admissible for in vivo studies at the macroscopic scale.

As discussed in Supporting Information Section S10, this high level of concordance between the different thermal readout ways discussed above could be a consequence of (i) either the thermometric parameters were selected so that the thermal dependence of the optical properties of the tissue were completely avoided or (ii) the issue-induced effects have the same contribution on the estimations of temperature by different thermometric parameters. While the latter would imply an improvement in accuracy only, the former would entail an enhancement in accuracy as well. The question concerning which of these two conditions is factual can only be answered by a thorough consideration of the thermal dependence of scattering and absorption of light in several biological tissues. Such a study, however, is out of the scope of this work. Nevertheless, whichever case is true, the reliability of the thermal readout is improved when compared with the “traditional” procedure. For this reason, we present here this investigative approach as a safety step for the analysis of future in vivo luminescence thermometry studies. At this point, it is worth emphasizing that, due to stronger attenuation-induced effects, tumors that are more internalized than this point, it is worth emphasizing that, due to stronger attenuation-induced effects, tumors that are more internalized than melanoma will most likely request more elaborated protocols for the analysis of the data. In fact, Supporting Information S10 suggests that even a change in the skin colour might completely change the dependence observed in Figure 7.

Lastly, by the precise thermal readout provided by the \( \text{Ag}_2\text{S NPs} \), one can also infer the occurrence of changes in the properties of the tumoral tissue (as previous works have demonstrated with the analysis of heating or cooling curves).\(^{[23]}\) Transient thermometry has indeed used this principle to detect in advance tumor development or even to diagnosis ischemic tissues.\(^{[24]}\) In the simplest of the approaches, the temperature should follow an exponential trend with a characteristic time (\( \tau \)) that depends on the thermal and physical properties of the tissue under study (see Section S11 of Supporting Information). The analysis of the intratumoral heating curves included in Figure 6d reveals that, in our experimental conditions (tumor being subjected to heating over 30 °C), the heating curve does not follow an exponential trend (see Section S12 of Supporting Information). We state at this point that this discrepancy is a clear indicator that the physical properties (including thermal and optical properties) are changing during the treatment, thus leading to a time-dependent thermal characteristic time \( \tau \). This is in accord with the results included in the last section that demonstrates the complete tumor eradication due to the photothermal treatment.

3. Conclusion

In summary, the feasibility of having multiple readouts simultaneously using different thermometric parameters was explored and demonstrated with \( \text{Ag}_2\text{S NPs} \). The convergence between the different estimations of temperature (with uncertainties below 1 °C) in an intratumoral heating transient was verified, and hence proposed as a convincing indicator of the improved reliability of these luminescent thermometers. Results included in this work provide the community with a much-needed route to correct the effects of optical artefacts produced by biological tissues, within the framework of minimally invasive photothermal therapies. These luminescence thermometry properties were combined with over 90% radiation-to-heat conversion efficiency to provide controlled photothermal therapy of a tumor in small animal models. Though future studies might be necessary to completely account for the tissue-induced effects, this work certainly provides a timely and novel kind of contribution to the consideration of luminescence nanothermometry as a convincing technique for high-sensitivity preclinical thermal sensing.

4. Experimental Section

\( \text{Ag}_2\text{S NPs} \): \( \text{Ag}_2\text{S-PEG NPs dispersed in PBS} \) were offered by SINANO Int. (China).

**Physical Characterization:** X-ray diffraction pattern was recorded by a Philips X’pert diffractometer with Cu-K\( \alpha \) radiation at 45 kV and 40 mA. The data were collected from \( 2\theta = 20°-90° \) with a step size of 0.02° and a normalized count time of 1 s/step. Transmission electron microscopy (TEM) image was obtained by using a transparent electron microscope (TEM, TECNAI G2, the resolution is 0.2 nm) with an acceleration voltage of 200 kV

**Spectroscopic Characterizations:** The emission spectra were achieved by using an InGaAs CCD camera (Andor iDus DU490A) then the acquired luminescence signals were analyzed by an Andor Shamrock 193i spectrometer, while the aqueous suspension of \( \text{Ag}_2\text{S NPs in the quartz cuvette} \) was excited by the 808 nm LiMO laser diode (Lumics). Additionally, the spectrometer was equipped by the heating plate (heidolph, MX078-20-HD26) to study the temperature effect to the \( \text{Ag}_2\text{S NPs} \) emission. The time-evolution emission spectra of intratumoral \( \text{Ag}_2\text{S NPs} \) were acquired by the Andor Shamrock 193i spectrometer with Kinetic mode. The luminescence decay curves were recorded by exciting the aqueous suspension of \( \text{Ag}_2\text{S NPs} \) by using an Optical Parametric Oscillator (Quanta Ray, OPO) laser equipped with the heating plate...
Quantum Yield Measurement of Ag$_2$S NPs Dispersed in PBS:
The absolute emission quantum yields were measured at room temperature using the commercial C13534 system from Hamamatsu with an integrating sphere as a sample chamber and two multi-channel analysers for signal detection in the visible and in the NIR ranges. An external laser diode (808 nm, FC-808-3W, CNI Lasers) was used as the excitation source. The laser power was adjusted up to 0.4 W controlling the laser diode current. The illumination area is 0.0025 cm$^2$, according to the manufacturer. An empty cuvette was used as a reference and three measurements were made for each sample (the average is reported). The method is accurate within 10%. More details are included in Section S13 of Supporting Information.

Estimation of Ag$_2$S NPs Heating Capability under Different Power Densities: 300 µL aqueous solution of Ag$_2$S NPs (1.5 mg mL$^{-1}$) was put into an open quartz cuvette then excited with 808 nm laser (Lumics) under different power densities while the temperature variation was monitored by a NIR thermal camera (FLR 40Bx).

Photothermal Conversion Efficiency Calculation of Ag$_2$S NPs through Experimental Data: The photothermal conversion efficiency ($\eta_{\text{h}}$) is a value to estimate the heating conversion ability of nanoparticles, namely the proportion of absorbed laser energy that is transformed into heat. The method of calculation followed here is reported by Roper et. al.[26] and the details are listed below:

The aqueous solution of Ag$_2$S NPs (1.5 mg mL$^{-1}$) was put into an open quartz cuvette then excited with 808 nm laser (Lumics, at a power of 505 mW) while monitoring the temperature change by a NIR thermal camera (FLR 40Bx). When the temperature of suspension reached a stable value (here it took 15 min), straight after turning off the laser to wait for the temperature relaxation until equal to the surrounding environment temperature.

The formula for calculating $\eta_{\text{h}}$ can be defined as

$$
\eta_{\text{h}} = \frac{hAT_{\text{max}} - T_{\text{eq}}}{P(1 - 10^{-\text{OD}})}
$$

(9)

where $h$ is the heating transfer coefficient, $A$ is the surface area of the quartz cuvette where the solution is placed. $T_{\text{max}}$ and $T_{\text{eq}}$ are the stable temperatures reached by the solution after continuous laser excitation for 15 min and the temperature of the surrounding environment, respectively. $Q_0$ represents the heat dissipated from the laser absorbed by the solvent container, while $P$ is the laser power and $OD$ is the optical density of the solution sample.

According to the results reported by Roper et. al., the value of $hA$ could be obtained through temperature relaxation time $\tau$ (determined from the cooling process shown in Figure 3b), which could be defined as

$$
hA = \frac{mC_p(\Delta T)}{\tau}
$$

(10)

where $m = 0.3$ g and $C_p = 4.184$ J g$^{-1}$·°C$^{-1}$ represent the mass and heating capacity of the solvent (PBS in our work) respectively. Then substitute the value of $m$ and $C_p$ into the formula (10) as well as the obtained $\tau = 65.17$ s, then the value of $hA$ is equal to 19.33 mW·°C$^{-1}$.

The temperature increment could be obtained from the heating-cooling curve (Figure 3b), which is 19.9 °C. Additionally, the laser power $P$ is 505 mW and the optical density (OD) is 0.50 at this concentration under 808 nm laser. And $Q_0$ was estimated as 63 mW through measuring the laser power lost after passing through the opening quartz cuvette filled with the same volume of the same PBS. Substituting all these values into the formula (9), then obtained the photothermal conversion efficiency of the studied solution of Ag$_2$S NPs is 93 ± 7%.

Cell Culture: The murine melanoma B16 is a spontaneously arising melanoma of C57BL/6 mice, from which the B16 cell line was established. We have used this commercially available cell line (ATCC® CRL-6322™, Manassas, VA, USA) and cultured with DMEM medium (Thermo Fisher Scientific), supplemented with 10% of fetal bovine serum and antibiotics (100 U mL$^{-1}$ penicillin, 100 µg mL$^{-1}$ streptomycin, all from Thermo Fisher Scientific), in a humidified incubator with 5% CO$_2$. After reaching 75–80% confluence, cells were trypsinized and centrifuged at 1000 g for 5 minutes. Cells were resuspended in 200 µL of sterile PBS and counted using a Neubauer chamber.

Animal Studies: All the experiments were conducted following the European Union directives 63/2010UE and Spanish regulation RD 53/2013. Both the internal Animal Ethics Committee of the Universidad Autónoma de Madrid and the external Committee of Ethics of Research of the Comunidad Autonoma de Madrid with reference number Proex 081/18 approved the use of these animals. We used 6 to 12-week-old female C57BL/6 mice to induce melanoma, a well-established and widely used tumor model.[27] Briefly, mice were anesthetized with isoflurane in 100% oxygen, placed in the prone position on the operating table and hair was removed with an electric shaver and hair removal cream to leave the back of the animal exposed for injection. With a 1-ml syringe with attached 27%G needle, we inserted the needle superficially, so that it was visible through the skin. Subcutaneous injection of 150 µL containing a dose of 1 × 10$^6$ B16 cells (which is 1.5 to 2 times the minimal tumorigenic dose in normal C57BL/6 mice) and 75 µL of Ag$_2$S-PEC NPs dispersed in PBS (1.5 mg mL$^{-1}$). The application resulted in a clear “bleb”. A palpable tumor appeared as soon as 4–5 days and was observed until it grew to an approximate size of 1 × 1 × 1 cm$^3$ in around 17 days. At this point, the tumors became necrotic in the top and began to ulcerate or bleed; and we ethically sacrificed the mice.

Histology Analysis and Immunohistochemistry: Tumors were fixed in 4% buffered formalin and embedded in paraffin wax. Dewaxed tissue sections were stained with Hematoxylin, and Ki67 immunohistochemistry using standard procedures. Ki67 antibody (1:200) staining have always been processed together with negative control in which no primary antibody were added to assess the specificity of the staining obtained. Proliferative index analysis was obtained by quantification using ImageJ software (tool for measure and color deconvolution) in representative 10X microscopy section images.

Statistical Analysis: For the tumour therapy experiments the sample size was $n = 3$ for each of the cases analysed in this work (“Tumor”, “Ag$_2$S-Tumor”, “No treatment”, and “Tumor+laser”). For statistical analysis on the emission spectra we used MATLAB R2020a under the license provided to Universidad Autónoma de Madrid.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

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