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Design of Irreversible Optical Nanothermometers for Thermal Ablations

Tiffany P. Gustafson^a, Qian Cao^a, Steven Wang^a, and Mikhail Y. Berezin^a

Mikhail Y. Berezin: berezinm@mir.wustl.edu

^aDepartment of Radiology, Washington University in St. Louis School of Medicine, St. Louis, MO 63110, USA. Fax: +1 314 747 05191 Tel: 1 314 747 0701

Abstract

Nanothermometers composed from a gold nanorod core, temperature sensitive linker and fluorescent dye are reported. The nanothermometers have low fluorescence due to a self-quenching mechanism at temperature below 50°C and become highly fluorescence above 70°C.

Clinical thermometry of the human body is one of the most fundamental and successful techniques in healthcare. In spite of the technological advances, however, temperature measurements on a sub-millimetre scale remain a significant challenge.¹ The problem becomes especially apparent in thermal medical treatments such as tumour ablation when precise monitoring of temperature to minimize the damaging effects on healthy tissue and mapping of the treated tissue is essential for the efficacy and safety of these procedures.

To address this challenge, we present irreversibly thermally sensitive fluorescent nanoparticles. In our method, a non- or minimally fluorescent nanoparticle becomes irreversibly fluorescent when the temperature inside the target reaches a critical temperature. The irreversibility of fluorescence activation is important in highlighting treated regions and avoiding repetition of treatment over the same area. In the first example of this approach, we synthesized constructs composed of a gold nanorod core with a temperature sensitive linker and fluorescent dye. At normal body temperature, the nanoparticle's fluorescence was quenched rendering them invisible; upon heating the nanoparticle rapidly recovered fluorescence and became visible.

The ideal temperature for thermal treatment lies within the range of 60–80°C where tumour cells instantly die.^{2, 3} Hence, we aimed our efforts to design the nanothermometers to be sensitive to this temperature range. The overall design of the nanothermometers and the key steps are shown in Fig. 1. We utilized a gold nanorod core for its translational potential in laser^{2, 4} and radiofrequency⁵ ablations of tumours due to nanorods large absorption and scattering cross-sections.⁶

The synthesis of the nanothermometers involved several convergent steps. First, gold nanorods were prepared via an established procedure using a seed mediated synthesis in the presence of CTAB.⁷ The nanorods were synthesized with a maximum longitudinal absorption ranging from 700–850 nm (Fig. S1, ESI[†]), where λ_{max} was dependent on the aspect ratio of the rods as was determined by the amount of AgNO₃ used. As direct displacement of CTAB with peptide conjugates was found to be difficult, CTAB was first

Correspondence to: Mikhail Y. Berezin, berezinm@mir.wustl.edu.

[†]Electronic Supplementary Information (ESI) available: [Details of nanothermometers synthesis, TEM, optical measurements, optical spectra design of the imager, details of calculations]. See DOI: 10.1039/b000000x/

replaced by mPEG₂₀₀₀-thiol as described.⁷ This had little effect on the shape and optical properties of the nanorods (Fig. 2a,b and Fig. S1a,b, ESI[†]).

Second, the fluorescent dye 7-diethylaminocoumarin-3-carboxylic acid was conjugated to cysteine rich peptides.⁸ This dye absorbing at ca. 400 nm and emitting at 460 nm was employed as a temperature reporter. It was selected to avoid overlapping with the absorbance of the nanorods (transverse 532 nm and longitudinal surface plasmon resonances 650–800 nm) to be clearly seen in the nanoconstructs optical spectra. This selection allowed for method development and optimization of the ligand exchange procedure with the goal to have a high density of the dye on the surface of the gold. Coumarins, like many other organic fluorophores, are easily self-quenched at high concentrations as a result of intermolecular interactions and aggregation. Thus, a dense layer of coumarin on the gold nanorod surface was expected to be minimally fluorescent. In addition, plasmon effect from gold could further enhance dye quenching.

The resulting peptide-dye conjugates were then added to the PEG protected gold nanorods to replace the PEG shell. TEM images of the gold nanorods with dye-peptide conjugates indicated a lack of aggregation and retention of their original shape and size following ligand exchange steps (Fig. 2c). Successful exchange/attachment was apparent from the UV/Vis spectra of the purified nanoconstructs which featured an absorption band at 400 nm originating from coumarin, and two bands at 532 and 700 nm originating from the gold nanorods (Fig. 2d). The observed broadening and small hypsochromic shift of the gold nanorods' longitudinal absorption suggested a change in the nanorods' surrounding medium. The compound was weakly fluorescent due to incomplete quenching of the dye (Fig. 2e).

The nanothermometer linker, which holds the dye to the Au surface, was the key to thermal activation and served two purposes: *i.* to suppress fluorescence by placing dye molecules close to each other, and *ii.* to function as a temperature sensor. For the latter, cysteine rich peptides were employed. The ability of gold nanoparticles to react with thiols is well known.⁹ The formed Au-S bond is moderately strong for a metal-non-metal interaction, with a homolytic strength of 40 kcal/mol¹⁰ and temperature-dependent dissociation. A typical Au-S bond is stable under anhydrous conditions up to 200–300°C but undergoes dissociation below 100°C in aqueous media.¹¹ This principle of Au-S bond thermal sensitivity was the basis of the presented nanothermometers.

Following nanothermometer preparation, peptide-dye conjugates **1–3** were evaluated to determine the effect of *i.* the number of thiols and *ii.* the placement of thiols, relative to one another, within the linker. The temperature release profile was recorded for each sample in PBS buffer using two temperature ramps — slow, linear ramp 0.9°C/min of heating, total 60 min and fast, non-linear ramp from 20 to 85°C, total 8 min (Fig. S3a, ESI[†]). While the first is better suited for kinetic measurements of peptide-dye conjugate release, the latter is more relevant for thermal ablations in clinics. Corresponding data were obtained using a standard fluorescence spectrophotometer with a temperature controlled cuvette holder and by using a thermal ablation imager (for details see ESI[†]).

Fluorescence anisotropy data confirmed almost complete departure of the dye-peptide conjugate from the nanorods (Table 1). For example, in compound **1** the fluorescence anisotropy decreased from a relatively high value to a lower value after a heating-cooling cycle and became close to that of free dye-peptide conjugate. Such a decrease in fluorescence anisotropy was attributed to the faster mobility of a free dye-peptide conjugate compared to a slowly rotating nanorod. Despite the release, the nanoparticles did not aggregate. This stability was attributed to remaining PEG on the gold surface. PEG-SH

forms stronger bonds with gold than other thiols, including cysteins and cystamines¹² thus preventing aggregation after the release.

As a general trend, fluorescence intensities for all three compounds under slow temperature conditions were significantly enhanced at higher temperatures with only small changes in absorption spectra (Fig. 2d,e, and Fig. S4, ESI[†]). Subsequent cooling of the solution with nanothermometers did not lead to the original quenched fluorescent state, which suggests the entropy driven irreversible nature of Au—S bond cleavage. In fact, we observed further increase of the fluorescence during cooling. This enhancement was attributed to the general temperature dependent properties of fluorescent dyes with rotatable bonds.¹³

Thermal sensitivity of compounds **1–3** demonstrated the importance of linker design on their release profiles. The data indicated a massive release of compound **1** which contained a single thiol at low temperature from 40 to 55°C. (Fig. S4, ESI[†]). Compound **2** which contained two thiols next to each other was more stable in this range (Fig. S4, ESI[†]). Geometrical consideration suggests that an attachment of both cysteines on the gold surface introduces a conformational strain. To release the strain, compound **3** was synthesized by adding a flexible spacer, a single glycine residue, between the two cysteine residues. As expected, **3** demonstrated a thermal activation at higher temperature near 70°C (Fig. S4, ESI[†]), indicating stronger binding of the linker to the gold surface.

For clinical applications the compounds must respond to a temperature increase over a relatively short time scale. The target temperatures in thermal ablations are usually reached between 2–3 min up to 10–15 min from the beginning of the treatment and are kept at that temperature for 5–15 min depending on the ablation method, type, and size of the tissue.¹⁴ To evaluate the performance of the nanothermometers under fast temperature rising conditions (shown in Fig. S3b, ESI[†]), a solution of nanothermometers carrying peptide–dye conjugate **2** in a glass vial was placed into a thermal ablation imager.

The results demonstrated that, under this rapid heating conditions, the nanoparticles become activated between 70 and 80°C – an ideal range for thermal ablation (Fig. 3 and Video S1, ESI[†]). The fluorescent enhancement after a complete heating-cooling cycle was more than 10 times at 37°C compare to the initial fluorescence and entirely irreversible. From this data the critical temperature sensitivity was determined to be 77.6°C (Fig. S7a and for details see ESI[†]). The control experiment with the dye–peptide conjugate did not reveal such reporting properties. Fluorescence of the free dye-peptide conjugate **2** followed the initial trend similar to the nanoparticle conjugate. The fluorescence intensity was largely suppressed above 55°C (Fig. 3 and Video S2, ESI[†]) and restored back to the original value after the cooling. Such reversible behaviour of the free-dye peptide was consistent with the general behaviour of fluorophores under reversible thermal conditions.

In a clinical setting, nanothermometers must respond quickly to the critical temperature, preferably within seconds to avoid overheating of the treatment area. Gold nanorods carrying peptide–dye conjugate **2** showed a response rate of 3.38% of fluorescence increase/deg^{−1}sec^{−1} at the critical temperature and above (Fig. S10, ESI[†]). This value provides a quantitative way of comparing future nanothermometers with improved design.

In summary, we present a new concept of clinically relevant nanothermometers. Gold-nanoparticle based constructs with temperature sensitive linkers, and a fluorophore showed low fluorescence at the body temperature ~37°C. The fluorescence was triggered at elevated temperatures. Irreversibility of the signal and the overall increase in fluorescence >10 times after a complete heating-cooling cycle suggested its potential applicability as nanothermometers. The nanothermometers reporting function could be adjusted by changing

the type of a temperature sensitive linker. This material could be potentially utilized with thermal ablation therapies to report temperature and better control the heating process. Future work will be focused on demonstrating this feasibility in vivo under laser ablation and radiofrequency heating and inclusion of NIR dyes for deeper light penetration. In addition, the kinetics and computational studies will be conducted to reveal the mechanism of the release and optimize the structure of the nanothermometer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

The basis for the nanothermometer response to heating is the emergence of fluorescence. Key steps of nanothermometers synthesis and the structures of dye-peptide conjugates.

Fig. 2. TEM of AuNR (A), CTAB coated AuNR (B) after ligand exchange with mPEG₂₀₀₀-thiol and (C) dye-peptide conjugate **2** coated nanothermometers. The average size of the AuNR at all stages of preparation was 50x15 nm. Optical properties of nanothermometers **2**, absorption (D) and emission (E) before and after heating-cooling cycle.

Fig. 3. Change of the temperature inside the vial under fast heating-cooling conditions for nanothermometers carrying peptide-dye conjugate **2** (red diamonds) and free peptide-dye conjugate **2** (green empty circles). Normalized to the initial intensity=10 au.

Table 1Fluorescence anisotropy (r) of gold-dye-peptide conjugate **1** and a corresponding gold free dye-peptide **1**

Sample	$r^{[a]}$	St. Dev.
Au-dye-peptide ^[b]	0.347	0.009
Au-dye-peptide ^[c]	0.153	0.014
Free dye-peptide	0.143	0.015

^[a] PBS buffer, T=20°C, ex/em. 410/440 nm,^[b] before heating,^[c] after a heating-cooling cycle.