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Design of fluorescent nanocapsules as ratiometric nanothermometers

Natalia G. Zhegalova, Sergey A. Dergunov, Steven T. Wang, Eugene Pinkhassik, and Mikhail Y. Berezin

Department of Radiology, Washington University in St. Louis School of Medicine, St. Louis, MO 63110, USA

Abstract

We have developed a novel design of optical nanothermometers that can measure the surrounding temperature in the range of 20–85°C. The nanothermometers comprise of two organic fluorophores encapsulated in a crosslinked polymethacrylate nanoshell. The role of the nanocapsule shell around the fluorophores is to form a well-defined and stable microenvironment to prevent other factors besides temperature from affecting the dyes' fluorescence. The two fluorophores feature different temperature-dependent emission profiles where a fluorophore with relatively insensitive fluorescence (rhodamine 640) serves as a reference while a sensitive fluorophore (indocyanine green) serves as a sensor. The sensitivity of the nanothermometers depends on the type of nanocapsules-forming lipid and is affected by the phase transition temperature. Both the fluorescence intensity and the fluorescence lifetime can be utilized to measure the temperature.

TOC image

Keywords

nanothermometers; nanocapsules; indocyanine green; fluorescence lifetime

1. INTRODUCTION

Nanothermometry of a biological subject is an emerging area that recently attracted attention for its ability to assess temperature on a very small preferably nanoscale^[1] with a recent excellent review^[2] where traditional methods based on contact thermometers, thermocouples, other sensors (i.e. electrodes, tissue impedance,^[3]) or MRI changes^[4] cannot be applied. A number of nanothermometry methods are based on the use of optical mechanisms that warrant a quick feedback of the system limited only by the speed of light and the response of electronics. Known approaches rely on optical mechanisms of reporting from mostly endogenous reporters and include Raman spectroscopy^[5], infrared microscopy^[6], and black body infrared thermometry.^[7] Despite the number of technical temperature measuring methods at the nanometer scale, increasing depth and resolution in biological tissues remains a formidable challenge.^[8] If developed, these techniques could open up a number of possibilities including the measurement of temperature within a single cell, understanding the mechanism of immune modulation, the modeling of temperature activated biological processes, and augmenting the family of thermal treatment of diseases such as thermal ablation of cancer.^[9]

Recently, exogenous temperature sensitive contrasts and nanoparticles whose signals (fluorescence intensity, lifetime, polarization) predictably reflect the thermal environment have been recognized as potential sensors for thermal measurements in cells and living tissue. The nanoparticles' design is especially attractive since it amplifies the signal by using multiple sensors and can be potentially combined with a therapeutic load. Such interests stimulated the conception of a number of fluorescent nanothermometers based on

nanodiamonds^[10], lanthanide nanoparticles,^[11] quantum dots,^[12] gold nanorods,^[13] and polymers with encapsulated fluorophores.^[14]

Most of the published approaches with fluorescence-based thermometry rely on the change in the fluorescence intensity and are therefore susceptible to concentration artifacts. To address this problem, fluorescence lifetime, fluorescence polarization, and fluorescence ratiometric methods have been proposed.^[14a, 15] Thus, we recently investigated a two-fluorophore system where two fluorophores emitting at two different wavelengths were covalently conjugated to each other.^[16] In that approach, a fluorescent visible dye, coumarin, with a strong temperature sensitivity ($6.6\% \cdot K^{-1}$) was conjugated to a dye rhodamine 640 (Rho640) with almost no thermal sensitivity ($<0.1\% \cdot K^{-1}$). The overall response of the construct, however, showed only a marginal thermal sensitivity of $0.5\% \cdot K^{-1}$, near the limit of a recently introduced “quality threshold” for thermometers.^[2] In the current work, we present a new design based on combining two fluorophores trapped in one hollow nanocapsule. We used hollow nanocapsules prepared by polymerization within the hydrophobic interior of the bilayers of liposomes^[17] formed by two different saturated lipids, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC). In these nanocapsules the inner leaflet of lipids remain entrapped in the nanocapsules.^[17b] We employed indocyanine green (ICG), a well-known infrared dye, often used as a standard in NIR spectroscopy^[18] and FDA approved for imaging that was previously demonstrated to show thermal sensitivity of $\sim 1\% \cdot K^{-1}$ when encapsulated in a polyacrylate nanoshell.^[19] A second fluorophore, Rho640, was used as a reference dye. By this design, the nanocapsule shell around the dyes will create a well-defined and stable microenvironment reflecting only temperature while preventing other factors from affecting the dyes' emission.

2. RESULTS AND DISCUSSION

2.1. Basic consideration

Organic fluorescent compounds, in general, are sensitive to temperature with a decreasing trend of quantum efficiency and fluorescence lifetime. Upon excitation, the double bonds forming the conjugate system decrease in bond order and become reminiscent of a single bond susceptible to rotation. For an ideal barrier-less rotation (such as an excited state of ICG), the rate of rotation is proportional to temperature according to the well-known Stokes-Einstein-Debye relationship^[20]; hence, it can be shown that the fluorescence lifetime is reversibly proportional to the temperature.^[21]

Correspondingly, given the molecules are chemically stable under excitation conditions, the fluorescence lifetime and fluorescence efficiency of the fluorophores with rotatable bonds in the excited state are inversely proportional to the temperature. The emission of ICG with a long heptamethine chain is, therefore, temperature-dependent.^[21–22] The fluorescence lifetime of encapsulated ICG in DMPC-templated matrix experienced a substantial decrease from 0.62 ns at 20°C to 0.49 ns at 70°C (Figure 3, the decays are shown in Figure S1, Supporting Information). In contrast, the emission of the encapsulated Rho640 with fixed N-C bonds was practically insensitive to temperature. This insensitivity of Rho640' fluorescence to temperature has been also pointed out by other researchers.^[23] The

fluorescence lifetime of Rho640 remained at $\sim 4.00 \pm 0.005$ ns within a broad temperature range (Figure 3, the decays are shown in Figure S2) rendering this compound an ideal reference.

2.2. Fluorescence intensity nanothermometers

One of the major objectives in developing nanothermometers is independence of thermal measurements from concentration. In living biological tissue, concentration of the contrast agent follows complex kinetics that takes into account the rate of extravasation from blood vessels to tissue, clearance pathways, etc.^[24] Fluorescence lifetime technique can potentially be an attractive way of measuring temperature since the fluorescence lifetime is independent of concentration artifacts.^[15b, 25] From Figure 3, the fluorescence lifetime sensitivity of Rho640/DMPC was close to zero. The sensitivity of ICG in the DMPC-templated capsules was higher, but somewhat low with $S = 0.58\% \cdot K^{-1}$.

To increase the sensitivity while retaining concentration independence, we utilized fluorescence intensity of ICG as the reporting parameter and a second fluorophore Rho640 as a reference, with both fluorophores integrated in the same nanocapsule. The two fluorophores forming the thermometers featured non-overlapping absorption/emission spectra (Figure S3) ensuring the absence of energy transfer that could complicate the temperature response, such as linearity. Fluorescence intensities of the prepared nanothermometers were recorded from 20 to 85°C, a clinically relevant temperature range of thermal ablations of biological tissues.^[26] The optical properties of the individual dyes in DMPC-templated nanocapsules are also tabulated in Table 1.

Bathochromic shifts, such as a shift in emission of 9 nm (325 cm^{-1}) in Rho640 and 28 nm for ICG (445 cm^{-1}), suggested the change in the environment from a PBS buffer to a more hydrophobic interior of the capsule. Our previous studies of measuring interior environment^[19, 27] using fluorescence lifetimes suggested that the micropolarity of the capsules was close to that of ethanol.

Due to the small spectral overlap, negligible energy transfer between the two dyes was expected. Indeed, using fluorescence lifetime measurements of the donor in Rho640-ICG/DMPC with two encapsulated dyes together showed only a minor decrease of the fluorescence lifetime 0.09 ns of the potential donor (Rho640) (Table 1) which corresponds to <2% energy transferred (Eq. 1).

$$E_T = 1 - \frac{\tau_{da}}{\tau_d} = 1 - \frac{4.00}{4.08} = 0.02 \quad \text{Eq. 1}$$

2.3. Thermal sensitivity of individual dye/nanocapsules

First, we tested individual dyes encapsulated in nanocapsules. The fluorescence intensity profile of Rho640 vs. temperature in two types of nanocapsules (templated by DMPC or DLPC liposomes) demonstrated no change upon multiple cooling-heating cycles (Figure 4A), suggesting that Rho640 is a suitable candidate for a reference dye.

Indeed, the fluorescence emission of Rho640 in DLPC-templated nanocapsules demonstrates stable behavior and no changes in the emission were observed in the range from 20 to 85°C in a three heating-cooling cycle experiment. ICG's thermal dependence was more complex and dependent on the nature of the lipid. In the emission –temperature scan of ICG/DMPC, a sharp change in the emission within a relatively small temperature range from 20 to 28°C was clearly observed. Such change, apparently, corresponds to the phase transition temperature of DMPC with a chain 14:0 at +24°C.^[28] DLPC has a shorter chain (12:0) and its phase transition temperature occurs at –2°C,^[28] below the range of the measurements, and hence, no phase transition interference was observed (Figure 4B). As a result, converted emission intensity – temperature dependence of ICG/DLPC for this type of lipid-based nanocapsules was linear (see Figure S5). Noticeable decrease of the emission level from cycle to cycle is likely due to the low photostability of ICG during the experiment that was conducted for more than 1.5 hours of continuous irradiation.

2.4. Thermal sensitivity of two-dye DLPC nanocapsules

Encouraged by the successful results with individual dyes (Rho640 and ICG) in DLPC-templated nanocapsules and the linearity of both constructs response in DLPC matrix, we incorporated these dyes together with the molar ratio 1:1 in the DLPC-based shell (see the absorption spectra in Figure S5). As expected, the emission of the Rho640 remained stable upon heating and subsequent cooling cycles, while the emission signal corresponding to ICG decreased as temperature rose. The Rho640 emission was not dependent on temperature, we used its emission as a reference point. A representative cycle showing a ratio of Rho640 emission to the emission of ICG is given in Figure 5. The deviation from the linear behavior and a small hysteresis was due to higher photostability of Rho640 compare to ICG. The average increase of the emission in the physiologically and therapeutically important temperature range (20–85°C) was ca. $1.7\% \cdot K^{-1}$, almost three times higher than the sensitivity of encapsulated ICG measured with the fluorescence lifetime ($0.58\% \cdot K^{-1}$).

We envision the use of the proposed design in controlling thermal ablation procedures. Many of high sensitivity constructs are not suitable for this procedures since they include toxic compounds (i.e. quantum dots), or are limited in applicability to deep tissues because they do not reside in the red-NIR spectral range. In addition, the performance of many luminescent thermometers can also be affected by more than just temperature factors. These include the presence of ions, a change in pH, the level of oxygen, different viscosities, etc. The nanocapsules prevent these influences by creating a stable microenvironment that is affected by temperature only. Nanocapsules based on the presented materials have previously demonstrated low toxicity in vitro and in vivo^[19] and therefore are suitable for measuring temperature in biological tissues. Their sensitivity can be further enhanced by selecting a NIR dye with better thermal sensitivity, the work in this direction is currently underway.

3. CONCLUSIONS

In the presented approach, the nanothermometer was constructed from two fluorophores integrated in a nanocapsule. ICG with a temperature-sensitive fluorescence served as a

sensor while rhodamine 640, with almost no sensitivity, served as a reference point. The ratio of the two emissions correlated with the temperature of the environment, providing the basis for thermometry. Such a ratiometric approach eliminates many concentration artifacts caused by the difference in the probes' distribution in tissue such as rates of clearance and photobleaching that are especially critical in heterogeneous biological media. The choice of lipid in building the nanocapsule was important to eliminate phase transition induced changes in the emission. The thermometer was found to be reversible after heating-cooling cycles as the emission mostly returned to the original level. The sensitivity of the construct was $\sim 1.7\% \cdot K^{-1}$, due to the ~ 200 nm size of the construct, we expect it to report temperature on a diffraction-limited sub-millimeter scale. The optical nature of the construct also implies a fast response. Future work will focus on synthesis and testing of near-infrared fluorophores with higher sensitivities to temperature and the search of a more appropriate deep tissue dye as a reference. Such reference dye should ideally be working in the 700 nm emission range, which is more suitable for biological applications.

4. EXPERIMENTAL SECTION

Details of materials, dynamic light scattering (DLS) and thermal measurements are described in Supporting Information.

Synthesis of nanocapsules

The procedure was similar to the previously published.^[19] Lipids 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) or 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC) (160 mg) were dissolved in 0.4 mL of chloroform in a test tube, then t-BMA (32 μ L, 0.2 mmol), BMA (32 μ L, 0.2 mmol), EGDMA (32 μ L, 0.17 mmol) and an initiator 2,2-dimethoxy-2-phenyl-acetophenone (3 mg, 0.01 mmol) were added. Chloroform was evaporated using a stream of purified argon to form a lipid/monomer mixture. The mixture was further dried under vacuum to remove traces of solvent. The solution of ICG (200 μ M) and/or Rho640 (200 μ M) in 8 mL of Tris buffer (pH 8.8) was added to the test tube with the lipid/monomer mixture and incubated at 35°C for 30 min. During this period, the mixture was briefly vortexed every 5 min. ICG was not stable in high intensity UV light. To minimize the degradation of the dye during the polymerization process, ascorbic acid was added to the reaction mixture (5 mg/mL). The suspension was extruded 20 times at 35°C through a track-etched polyester Nucleopore membrane (Sterlytech) with 200 nm pore size using a Lipex stainless steel extruder (Northern Lipids).

The sample was irradiated for 1.5 hours with UV light ($\lambda=254$ nm) in a photochemical reactor (10 lamps, 32W each; the distance between the lamps and the sample was 10 cm) using a quartz tube with a path length of light of approximately 3 mm. A short path length is important for efficient polymerization in the presence of dye. After polymerization, non-entrapped dyes were removed by using size exclusion chromatography. The sample was passed through a Sephadex G-50 column (10 mL) twice to ensure complete removal of the free dyes. The faster-eluting nanocapsule fraction was collected.

TEM and SEM studies

Electron microscopy images (Figure 6) were obtained with an FEI Inspect F50 STEM scanning electron microscope (Hillsboro, OR) at a working voltage of 30 kV.

For TEM analysis, a drop with the sample was carefully placed on a 300-mesh carbon grid and excess sample was wiped away with filter paper. Then, a drop of 2% uranyl acetate was added to the grid to negatively stain the sample. After 2 min, the excess liquid was wiped off. For SEM analysis, a drop of sample was placed on SEM pin stub specimen mount covered with double coated carbon conductive tabs and dried under vacuum. The studied samples were coated with a 5 nm gold- layer using EMS 590 X sputter (Hatfield, PA).

Optical measurements

UV/Vis spectra of samples were recorded on Beckman Coulter DU 640 UV-visible spectrophotometer and Olis DM245 spectrophotometer equipped with CLARITY integrating cavities for removal of scattering induced by the nanocapsules of ~200 nm size. The spectra with conventional cavity are given in Figures S3 and S5 in the Supporting Information. Steady state fluorescence spectra were recorded on a Nanolog spectrofluorometer (Horiba Jobin Yvon, Inc.) and processed using the FluorEssence software. Fluorescence intensities (S, counts per second) were normalized to the intensity of light (R, microamp) (S/R) to minimize the fluctuations over the time of recording. The fluorescence lifetime of the dyes was determined using the time-correlated single photon counting (TCSPC) technique with excitation sources NanoLed 560 nm/em. 605 nm, and NanoLed 740 nm/em. 815 nm similar to the method described previously.^[27] Samples with nanocapsules were diluted with water to four different concentrations so that their absorbances were between 0.1 and 0.3 au. The concentrations of the samples were calculated using Beer-Lambert's law and the known molar absorptivities of ICG.^[27]

Data analysis

Since signal from Rho640 did not significantly change with temperature, a ratiometric analysis using rhodamine's fluorescence as a reference and ICG fluorescence as a sensor according to the Eq. 2 was conducted:

$$Q = \frac{F_{Rho640}}{F_{ICG}} \quad \text{Eq. 2}$$

where F_{Rho640} is the emission of the construct for Rho640 at em/ex: 520/600 and ICG channel ex/em: 750/810 nm.

The temperature sensitivity over the studied temperature range was defined according to the Eq. 3^[2]:

$$S = \frac{\Delta Q}{Q_T \Delta T} \times 100\%, \quad \% \cdot K^{-1} \quad (\text{percent change per degree}) \quad \text{Eq. 3}$$

where Q_T is fluorescence ratio (or fluorescence lifetime) at low temperature, $Q -$ corresponds to the quenching of fluorescence and equal to the change in the fluorescence intensity ratio (or fluorescence lifetime), $T -$ temperature span.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Structures of rhodamine 640 (Rho640) and ICG used to construct nanothermometers.

Figure 2.
Structures of lipids DLPC (12:0) and DMPC (14:0)

Figure 3.

Fluorescence lifetime thermal sensitivity of ICG/DMPC and Rho640/DMPC in 1x PBS at ex/em. 560/605 nm for Rho640 and 740/815 nm for ICG. Sensitivities $S_{Rho640} = 0\% \cdot K^{-1}$, $S_{ICG} = 0.58\% \cdot K^{-1}$

Figure 4.

A: Thermal sensitivity of Rho640/DLPC. B: Thermal sensitivity of ICG/DMPC-NC and ICG/DLPC-NC. Fluorescence intensities (S, counts per second) were normalized to the intensity of light (R, microamp) (S/R) C: temperature ramp. Three heating-cooling cycles are shown.

Figure 5.

Temperature-dependent emission of Rho640-ICG/DLPC emission: ratiometric dependence of the nanoconstructs' emission intensity as a function of temperature (Heating/cooling cycle from 20 to 85 °C, one cycle out of three is shown). $S=1.7\% \cdot K^{-1}$

Figure 6.

Electron micrograph and DLS of nanocapsules. (a) and (c) TEM images and DLS of polymer nanocapsules made from DLPC and DMPC, respectively. DLS data showed objects with an intensity weighted average size of about 190 ± 20 nm (DLPC) and 180 ± 20 nm (DMPC), with PDI about 0.15–0.19 for both types of nanocapsules. (b) and (d) SEM image of polymer nanocapsules made from DLPC and DMPC, respectively. The average size of nanocapsules isolated after the polymerization of monomers and measured by SEM and TEM was identical to the average size of vesicles observed by DLS.

Table 1
Fluorescence lifetime values of ICG and Rho640 free and in DMPC nanocapsules in PBS at T=25 °C.

Sample	Abs	Em	τ_1 , ns	τ_2 , ns	f_1 , %	f_2 , %	τ_{fit} , ns	χ^2
ICG	779	802	<0.2	–	–	–	–	–
ICG/DMPC	807	807	0.62	–	97	–	–	1.27
Rho640	576	585	4.08	–	100	–	4.0	0.94
Rho640/DMPC	585	594	4.00	–	100	–	4.0	1.09