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ORIGINAL PAPER

Behaviour of Fluorescence Emission of Cyanine Dyes, Cyanine Based Fluorescent Nanoparticles and CdSe/ZnS Quantum Dots in Water Solution Upon Specific Thermal Treatments

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Abstract Fluorescence techniques are widely used as detection methods in a wide range of biological imaging and analytical applications. The purpose of this work is to determine a measurement method which leads to a comparison between different classes of fluorophores in term of stability of the fluorescence signal upon thermal treatment cycles. This kind of investigation can determine whether the fluorophore performance is affected by heating/cooling cycles and to what extent. The fluorophores considered in this work were organic fluorophores belonging to the family of indocyanine dyes (IRIS3 by Cyanine Technologies S.p.A.) in their molecular form or encapsulated within silica nanoparticles, and CdSe/ZnS carboxyl quantum dots (Qdots 565 ITK by Invitrogen). The NIST Standard Reference Material® SRM 1932 fluorescein solution was used in the certified concentration as reference material in order to evaluate the repeatability of the used spectrofluorimeter. The proposed measurement protocol allows to characterize all kind of fluorophores upon thermal treatments. This allows direct comparison of their performance under temperature changes, giving useful guidelines for the selection of the most suitable fluorophore for the envisaged application. Moreover the method appears to be a promising tool for the characterisation of reference fluorescent materials. The experimental results demonstrate that each fluorophore class shows a specific behaviour. The experimental data analysis points out an important hysteresis effect for quantum dots that was not detected for cyanine molecules and was only slightly detected for cyanine doped silica nanoparticles.

Keywords Fluorescence · Cyanine · Quantum dots · Dye doped silica nanoparticles · Thermal behaviour

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Introduction

Fluorescence techniques are widely used as detection methods in a wide range of biological imaging and analytical applications. Different types of fluorescent markers have traditionally been used for imaging purposes [1], but organic fluorophores are the most exploited, because of the large number of commercially available molecules with interesting photophysical features. Moreover, their spectroscopic properties can be tuned by proper synthesis in accordance with actual application needs. At the same time there is a growing interest in developing new kinds of biomarkers, characterized by high photostability, high biocompatibility, easy handling and bioconjugation, low sensitivity to environmental effects (minimal or no changes in properties of both the biomarker and the biomolecule) and high photoemission intensity [2–7].

Quantum dots and fluorescent nanoparticles fulfill some of these requirements, like good photostability and low sensitivity to environmental factors [8, 9].

The discovery of new photoluminescent materials along with the advances in detectors development, made possible a wide spread of new methodologies for non invasive in vivo visualization of specific molecular target inside the cells. Most of the techniques are based on the detection of a fluorescent signal emitted from fluorescent probes properly excited. However, the results of some of the most widely used fluorescence-based methods, e.g. Real Time PCR (Polymerase Chain Reaction), are based on the measurement of fluorescence from molecules in critical and/or dynamic chemical-physical conditions (high temperature and rapid temperature variations). Therefore a detailed knowledge of the behaviour of various types of fluorophores in different chemical-physical condition is highly desirable.

The ability to carry out a quantitative and reproducible study, in order to compare results from different analyses or from different techniques, is based on the possibility to measure in an accurate, reproducible and standardized way the fluorescence spectrum parameters of a fluorophore.

Several comparisons between markers have been done during past years [10, 11] mainly regarding standard photophysical parameters (e.g. quantum yields, fluorescence lifetimes, molar extinction coefficient) and applicative aspects (e.g. bioconjugation, solubility, stability in cell media and in different environment, photostability).

At the best of our knowledge, no standardised measurement protocol for the direct comparison of different classes of fluorophores in term of fluorescence behaviour upon specific thermal treatment has been developed in the literature.

This work presents an attempt to fill this gap by describing a measurement method which allows comparing different classes of fluorophores in term of stability of the fluorescence signal upon thermal treatment cycles. The investigation carried out was aimed to determine whether and to what extent the fluorophore performances are affected by heating/cooling cycles. Various types of fluorophores have been considered and measured in this study: two molecular fluorophores, a water soluble trimethine indocyanine dye (IRIS3) and the NIST Standard Reference Material® SRM 1932 fluorescein solution and three different kind of nanoparticles, non porous cyanine dye loaded silica nanoparticles (NPNPs), mesoporous cyanine dye loaded silica nanoparticles (NPPs) and CdSe/ZnS carboxyl quantum dots (QD).

All fluorophores were dissolved/suspended in distilled water and fluorescence spectra were acquired after each step of a specific thermal cycle. Dependence of the fluorescence intensity upon the thermal treatment was investigated, along with the correlation of the observed behaviour with the fluorophore concentration. Good stability of the experimental results has been obtained. The measurement protocol presented here is therefore applicable to characterise all kinds of fluorophores and appears to be a promising tool for the characterisation of reference fluorescent materials.

Materials and methods

A comparison of the performances of different classes of fluorophore with emission spectra in the same region, around 565 nm, has been performed. A typical organic fluorophore, two kind of dye-doped silica nanoparticles characterised by different architecture and quantum dots have been chosen:

- Cyanine IRIS3 dyes family fluorophore (by Cyanine Technologies S.p.A.)
  - in its molecular form;
  - encapsulated within ellipsoidal silica mesoporous nanoparticles (NPP);
  - encapsulated within spherical silica non-porous nanoparticles (NPNP);
- CdSe/ZnS carboxyl quantum dots (QD) Qdot® 565 ITK™ (by Invitrogen).

The quantum dots chosen are composed by a CdSe core encapsulated in a crystalline shell of ZnS and an amphiphilic polymer coating [12] and show low toxicity [13]. The quantum dots are suspended at the nominal concentration of 8 μmol/l in 50 mmol/l borate buffer at pH 9.

All fluorophores were characterized in water solution.

The repeatability of the spectrofluorimeter used to characterize the different fluorophores has been measured by using the NIST Standard Reference Material® SRM 1932 fluorescein solution at the certified concentration of $(60.67 \pm 0.40)\,\mu mol \cdot kg^{-1}$ [14, 15]. The SRM 1932 fluorescein solution at the certified concentration has been also characterized upon the specific thermal treatment described in this paper.

IRIS3 cyanine dye, porous and non porous cyanine dye loaded silica NPs were provided by Cyanine Technologies and used as received. CdSe/ZnS carboxyl quantum dots Qdot® 565 ITK™ were purchased by Invitrogen and used as received. The SRM 1932 fluorescein solution were purchased by NIST and used as received. The water used to dilute the solutions was bidistilled with a Millipore Apparatus.

Sample preparation

Stock solution of the quantum dots was already prepared by the dealer with a nominal concentration of 8 μmol/l, whilst for the other fluorophores stock solutions were prepared by dissolving/suspending fluorophores in deionized water. Stock solutions concentration was checked by weighting method.

The fluorophores have been tested in water solution at five nominal concentrations: 100 nmol/l, 50 nmol/l, 10 nmol/l, 5 nmol/l, 1 nmol/l. These samples were prepared by diluting with water a stock solution preventively ultrasonicated for at least 5 min in order to avoid fluorophores agglomeration. The dilutions were prepared just before each measurement and concentrations were checked by weighting method. Before each measurement the prepared solution was placed again in an ultrasonic bath for at least 5 min.

Non porous and mesoporous cyanine dye loaded silica nanoparticles concentration can be related: i) to the amount of encapsulated cyanine, thus rendering the fluorescence intensity value directly comparable with the cyanine in solution in term of fluorescence yield of each cyanine molecule; ii) to the number of suspended nanoparticles, thus allowing comparing the different fluorophores as "single fluorescent units/probes".

Whilst the first approach is interesting from a molecular point of view, the second one allows characterizing the fluorophores in a more practical condition of use.

In the present paper silica nanoparticles concentrations are related to the quantity of encapsulated cyanine molecules and not to the number of suspended nanoparticles.

The SRM 1932 fluorescein solution was used in the certified concentration as reference material and thus it was not diluted.

Fluorescence acquisition

Measurements were carried out on a Horiba Jobin Yvon Fluorolog 3 instrument, equipped with a 450 W Xenon Lamp, a Hamamatsu R928 photomultiplier detector and a temperature controller working in the 5–90 °C range with a ±0.1 °C accuracy. For proper excitation of IRIS3, NPNP, NPP, QD565 and SRM1932 Fluorescein solution the excitation source (Xenon lamp) was filtered with a monochromator peaked at 520 nm, with a FWHM of about 20 nm. Excitation and emission slits of the monochromator were set at 5 nm and 10 nm respectively.

The intensity spectra collected from the spectrofluorimeter are referred to the ratio between the fluorescence intensity measured through a photomultiplier placed at the end of the double monochromator emission branch, and the signal related to the instantaneous filtered excitation lamp intensity. The wavelengths spectral resolution was 1 nm while the intensity was measured in arbitrary units (counts).

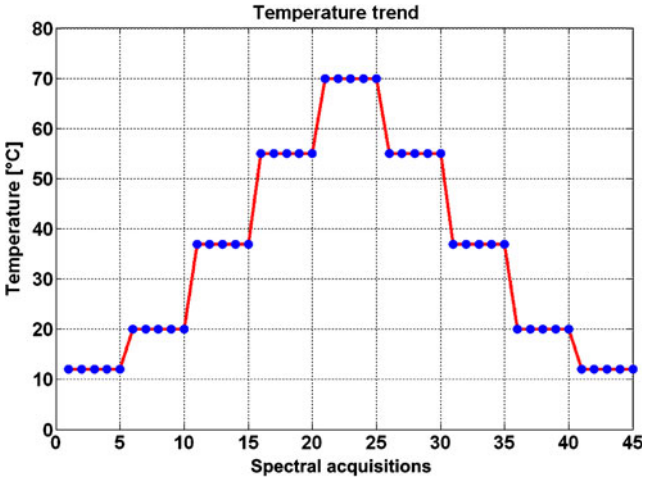

Fig. 1 In red, the temperature steps used in the thermal cycle over the time. The blue spots represent the spectral data acquisition (five spectral acquisitions per temperature step)

Definition of the thermal cycle

The range of work temperatures and concentrations was the same for each considered fluorophore and was chosen in a way to cover a wide range of practical applications.

For every step, temperature was held constant for at least 15 min collecting five spectra. Fluorophores were excited only during spectral measurements and spectral acquisition time was about 1 min. This time interval has been chosen in order to maximize the number of spectra in an acceptable experimental time.

Temperature cycle was from 12 °C to 70 °C and back to 12 °C in 9 steps (12 °C, 20 °C, 37 °C, 55 °C, 70 °C) as it can be seen in Fig. 1.

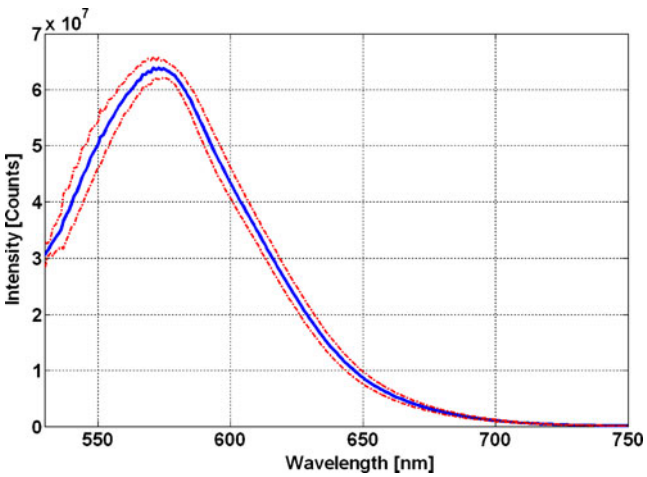

Fig. 2 Average emission spectrum of SRM1932 Fluorescein solution placed at 10.5 °C and excited at 520 nm (*blue curve*) and the confidence interval calculated through the standard deviation of the 35 collected spectra at the same conditions (*red curve*)

Table 1 Relative repeatability values of emission intensity at 565 nm (Concentration: 100 nmol/l; T=37°C)

Fluorophore	% Repeatability
QD	4.37
Cyanine IRIS3	0.26
NPNP	1.77
NPP	2.08

For each class of fluorophores the measurement protocol was repeated three times, in order to have a more precise measure.

In order to preserve the same measurement conditions and to avoid any possible fluorophore degradation, it is very important to perform all the measurements in a short period of time after the preparation of the sample.

Results and discussion

The measurement uncertainty has been evaluated. The stability of the Standard Reference Material over the time

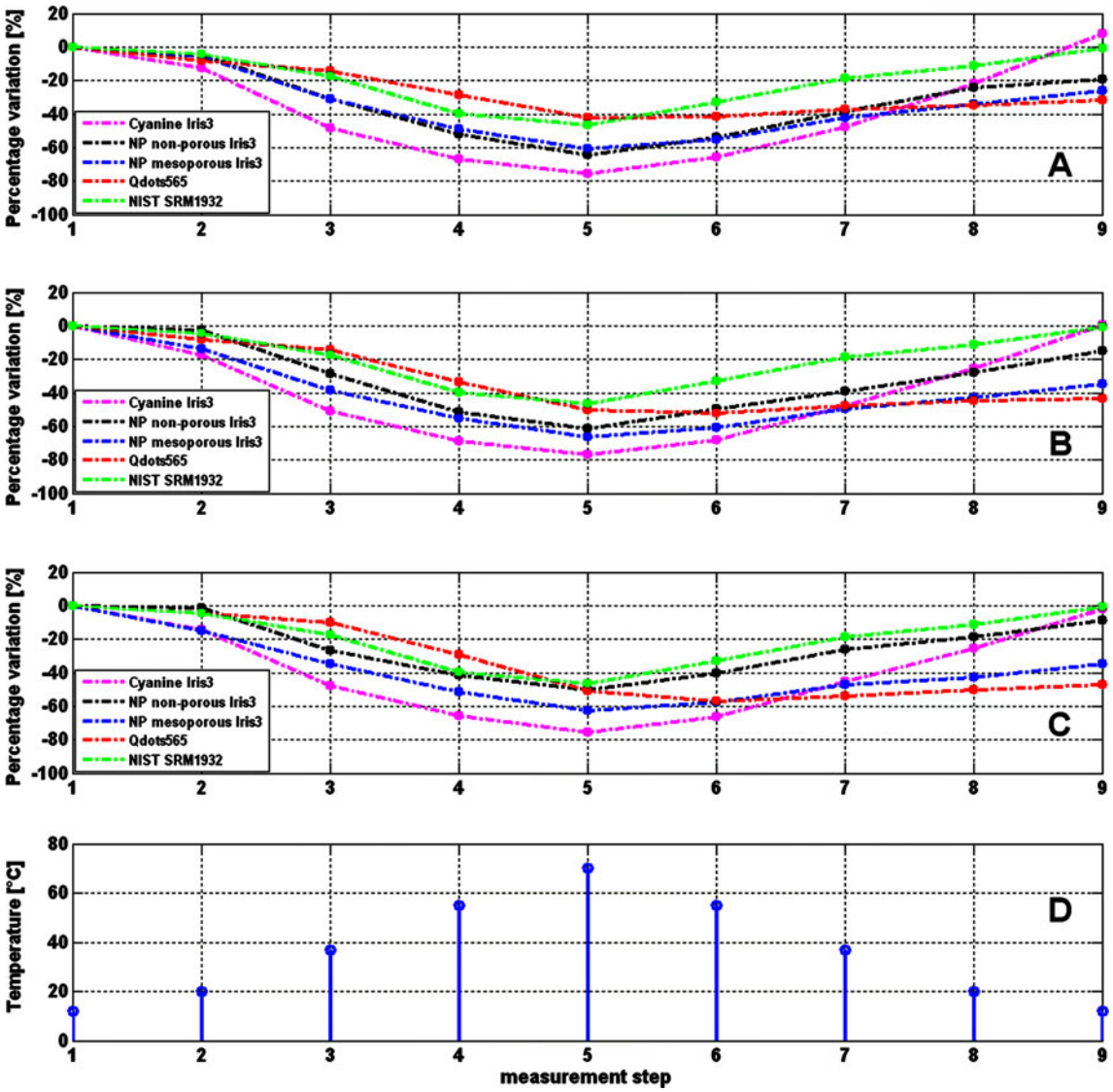

Fig. 3 The emission intensities variations relative to the initial emission intensity of each fluorophore at 565 nm are plotted as a function of the temperature steps of the thermal cycle at three different concentrations except for SRM1932 fluorescein that is used as reference at the certified concentration of (60.67±0.40) µmol·kg^{-1}. **a** Percentage variations of emission intensities for each fluorophore at the concentration of about 10 nM. **b** Percentage variations of emission intensities for each fluorophore at the concentration of about 5 nM. **c** Percentage variations of emission intensities for each fluorophore at the concentration of about 1 nM. **d** Temperature values in function of the measurement steps

allows to quantify precisely the term of uncertainty lied to the measurements repeatability. The spectrofluorimeter repeatability has been evaluated by calculating the standard deviation of 35 spectra of the SRM 1932 fluorescein solution collected kept at 10.5 °C, using the excitation and emission set-up described above. The repeatability value relative to the average intensity measured at the wavelength of 565 nm was about 4.22% (Fig. 2).

All the different fluorophore classes show a variation on the fluorescence intensity with the temperature.

The repeatability of the five spectra collected at every temperature step for each material has been evaluated by calculating the standard deviation of the emission intensity at 565 nm. The obtained values do not exceed the instrumental repeatability, demonstrating that eventual photobleaching and/or quantum yield variations during the measurement timeframe are negligible. The relative repeatability values obtained for all fluorophores at 37 °C and 100 nmol/l concentration are reported in Table 1.

The fluorescence intensity at 565 nm for all considered fluorophores decreases along with the increase in temperature. In Fig. 3 the intensities variation relative to the initial emission intensity of each fluorophore are plotted as a function of the temperature steps of the thermal cycle. This is a suitable method for evaluating eventual hysteresis effect on the emission intensity of fluorophores. The hysteresis on this parameter is very important when a big thermal cycle is inherent in the measurement process. The

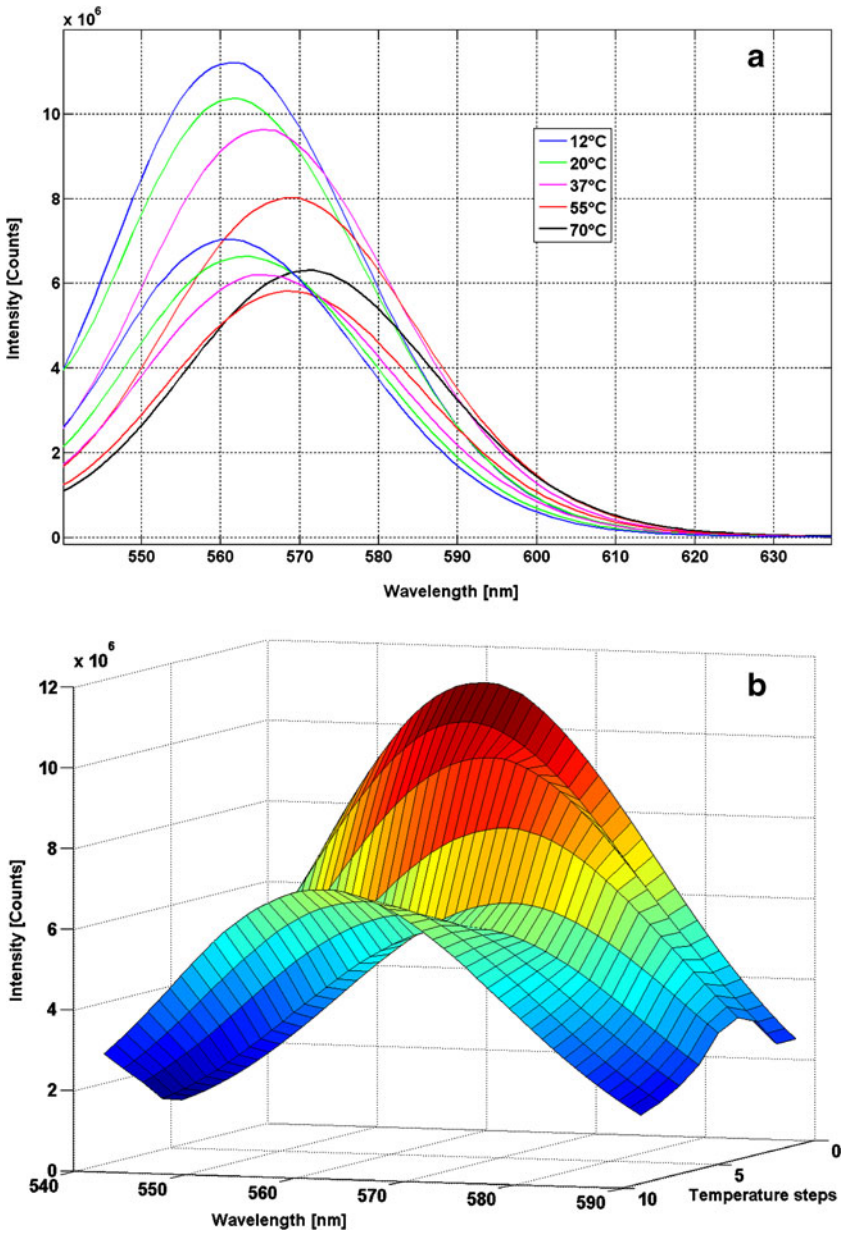

Fig. 4 **a** Average spectral emissions of Qdots565ITK at 5nM nominal (checked by weighting method at 3.7 nM) for the different temperatures of the thermal cycle. 12 °C (blue spectra), 20 °C (*green spectra*), 37 °C (*magenta spectra*), 55 °C (*red spectra*) and 70 °C (*black spectrum*). **b** Average spectral emission of the same Qdots565ITK solution over the different temperature steps of the thermal cycle in 3D

results show that cyanine IRIS3 and SRM 1932 fluorescein solution do not show hysteresis effect; in fact emission intensity at 565 nm at the end of the thermal cycle has a value similar to the initial one. Both non-porous and mesoporous cyanine dye doped silica nanoparticles (NPNP and NPP) at the end of the thermal cycle show a slight hysteresis effect on the emission intensity, while for quantum dots there is a strong hysteresis effect and the emission intensity is clearly lower than the initial one.

The concentration plays only a slight influence on the hysteresis effect; quantum dots show the biggest dependence of hysteresis effect with concentration. Figures 3a-b-c show that percentage variations of the emission intensity for quantum dots increases along with the lowering of the concentration.

In the first part of the thermal cycle quantum dots have the lowest relative change on the fluorescence intensity at 565 nm, showing a minor influence of the increase in temperature on the emission intensity. In the second part of the thermal cycle, instead, quantum dots emission intensity does not go back to the initial value, showing permanent fluorescence intensity degradation.

Moreover, along with the increase in the temperature, the quantum dots spectra undergo a red-shift and the overall spectral amplitude decreases (Fig. 4a and b).

During the thermal treatment the spectra of the SRM 1932 fluorescein solution appear to be red-shifted and the related peak amplitudes in the spectra slightly decrease at higher temperature (Fig. 5a and b). When the temperature decreases

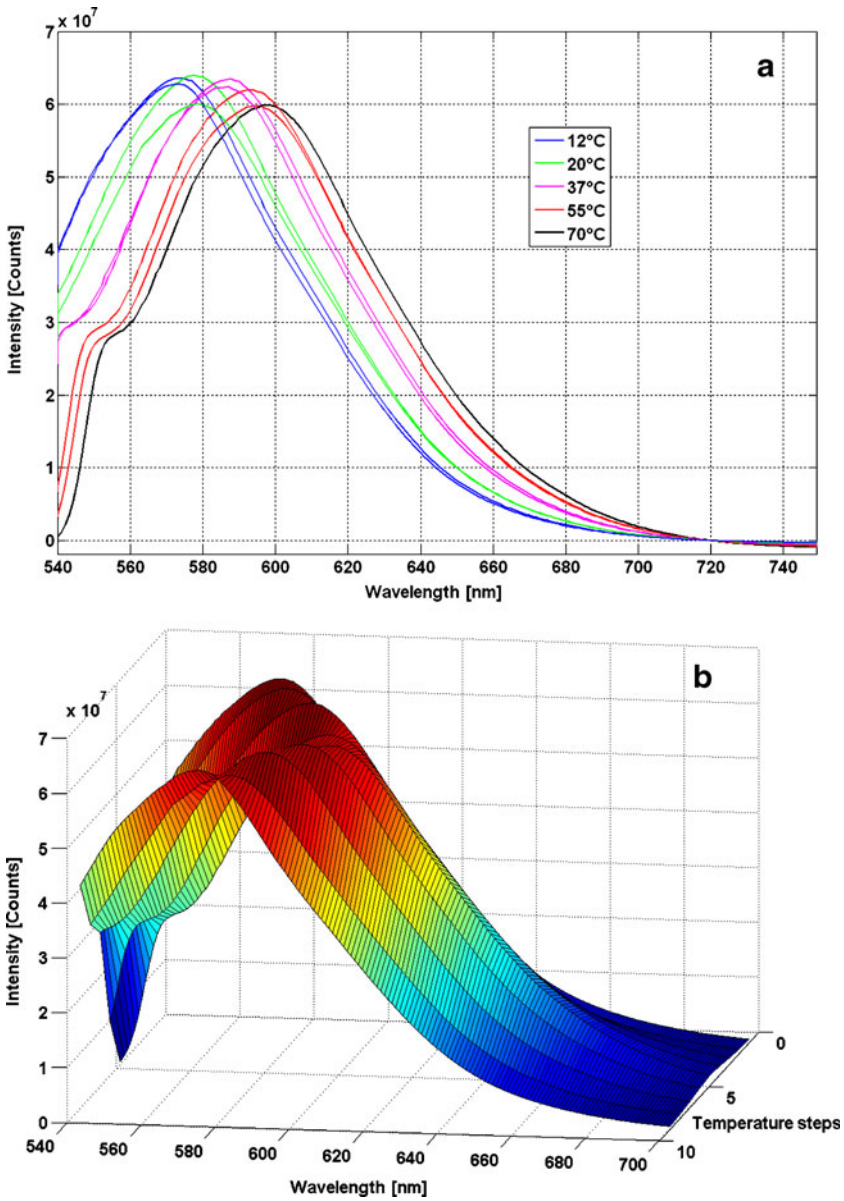

Fig. 5 **a** Average spectral emissions of NIST SRM1932 Fluorescein solution at (60.67± 0.40) µmol·kg^{-1} for the different temperatures of the thermal cycle. 12 °C (*blue spectra*), 20 °C (*green spectra*), 37 °C (*magenta spectra*), 55 °C (*red spectra*) and 70 °C (*black spectrum*). **b** Average spectral emission of the same NIST SRM1932 Fluorescein solution over the different temperature steps of the thermal cycle in 3D

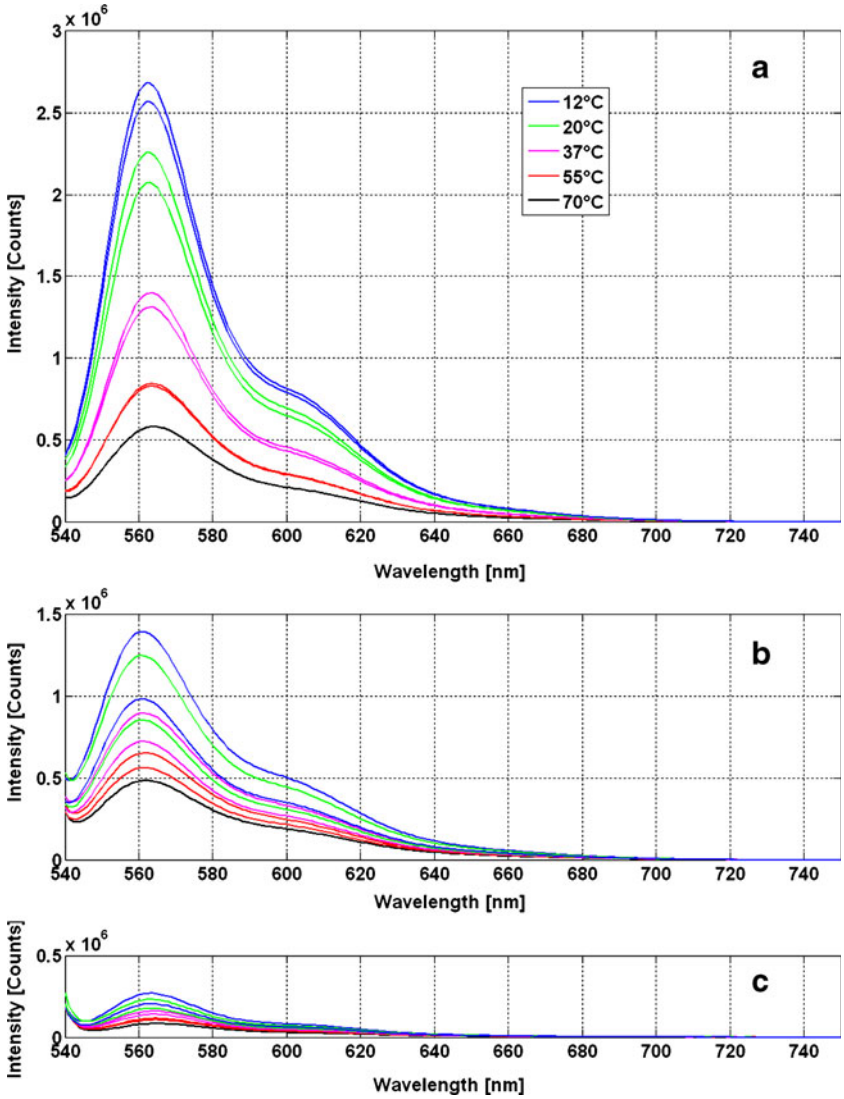

Fig. 6 **a** Average spectral emissions of Cyanine IRIS3 at 52.2 nM for the different temperatures of the thermal cycle. 12 °C (*blue spectra*), 20 °C (*green spectra*), 37 °C (*magenta spectra*), 55 °C (*red spectra*) and 70 °C (*black spectrum*). **b**) Average spectral emissions of Cyanine doped mesoporous silica nanoparticles (NPP) at 51 nM for the different temperatures of the thermal cycle. 12 °C (*blue spectra*), 20 °C (*green spectra*), 37 °C (*magenta spectra*), 55 °C (*red spectra*) and 70 °C (*black spectrum*). **c** Average spectral emissions of Cyanine doped non-porous silica nanoparticles (NPNP) at 53 nM for the different temperatures of the thermal cycle. 12 °C (blue spectra), 20 °C (*green spectra*), 37 °C (*magenta spectra*), 55 °C (*red spectra*) and 70 °C (*black spectrum*)

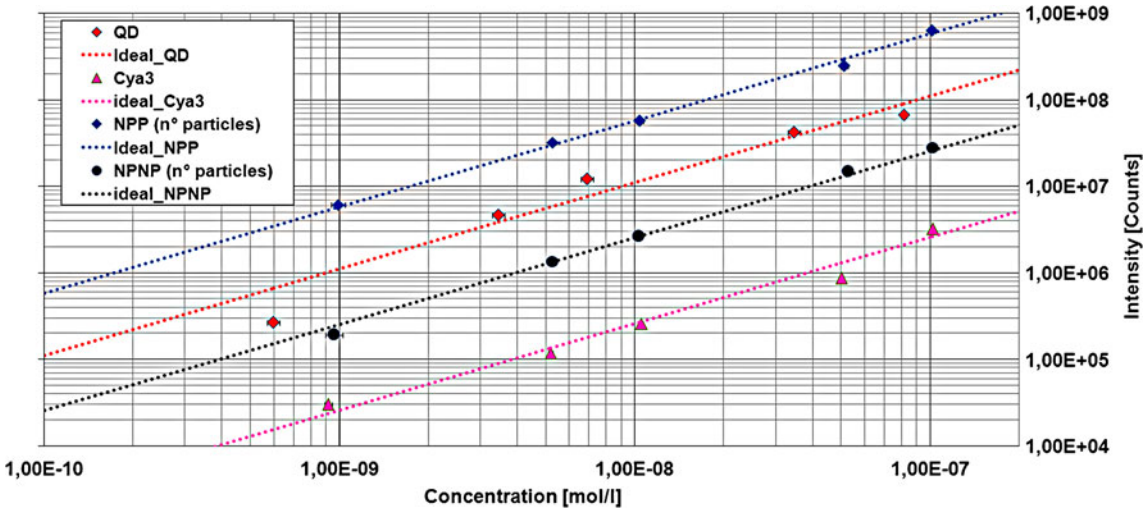

Fig. 7 Average emission intensities measured at 565 nm at 37 °C as a function of the concentration for each fluorophore, with concentrations referred to the number of nanoparticles also for NPPs and NPNPs

to the initial values the spectra undergo a blue-shift and spectra amplitudes recover their initial values.

Cyanine IRIS3 and related nanoparticles during the thermal cycle do not show spectral shifts but only a change in the overall spectral amplitude, as shown in Fig. 6.

All the fluorophore classes, except the SRM 1932 fluorescein solution, were compared also in terms of emission intensity related to the five concentrations chosen. In Fig. 7 emission intensities at 565 nm measured at the temperature of 37 °C are plotted as a function of the different fluorophores concentrations. For the silica nanoparticles, emission intensity is corrected for the filling factor, that expresses the average number of encapsulated cyanine molecules per nanoparticle. This way is possible to compare directly the silica nanoparticles with the quantum dot in term of number of particles.

The filling factor can be obtained computing the ratio between the number of cyanine molecules and silica nanoparticles. The number of silica nanoparticles is obtained computing the total mass of silica nanoparticles divided by the average weight of a single silica nanoparticle. This weight can be calculated multiplying the average nanoparticle volume with the specific weight of the nanoparticle related silica.

The computed filling factors were about 100 for nonporous silica nanoparticles and about 300 for mesoporous silica nanoparticles.

From the Fig. 7 is evident that Cyanine doped NPP exhibits a brightness nearly an order of magnitude greater than quantum dots, while cyanine doped NPNP exhibits a brightness that is lower by a factor three with respect to quantum dots and an order of magnitude higher than cyanine.

Conclusions

A reproducible method to compare different fluorophore classes evaluating the fluorescence emission performance as a function of temperature and concentration has been presented. Two molecular fluorophores, two different types of cyanine loaded nanoparticles and CdSe/ZnS carboxyl QDs have been investigated

The obtained results show that QDs exhibit a consistent hysteresis effect on the emission intensity after the thermal cycle, whereas in the first part of the cycle they show less dependence on the temperature with respect to the other fluorophores considered. On the basis of the results, we can therefore conclude that for applications where the temperature changes widely, like in PCR, cyanine based probes could guarantee a minor hysteresis effect and thus a minor degradation of the photoemission due to the thermal stress, leading to more reliable quantitative measurements.

Moreover, cyanine loaded nanoparticles show higher intensity emission than Cyanine molecules in their molecular form. Cyanine doped mesoporous silica nanoparticles exhibited the brightest emission between the fluorophores that have been characterized. The used measurement protocol is very general and reproducible and appears to be a promising tool for the characterisation of reference fluorescent materials.

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