

Evaluation of the Suitability of Quantum Dots as Fluorescence Standards

Alex Knight, Joe Gaunt, Tim Davidson,
Victor Chechik, and Stuart Windsor

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National Physical Laboratory
Queens Road, Teddington, Middlesex, TW11 0LW

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Approved on behalf of the Managing Director, NPL
by Martyn Sené, Head, Division for Quality of Life

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1. Context

This report describes work carried out under the project “Quantum dots: a new fluorescence calibration standard”. This project was led by NPL as part of the DTI’s “Measurements for Biotechnology Programme” in response to ITT GBBK/C/019/00009. Also participating in the project were the University of York, represented by the laboratory of Dr. Victor Chechik in the Chemistry Department, and Optiglass, a company involved in the manufacture of fluorescent standards. Nanoco, an SME which specialises in the bulk production of quantum dots, also kindly provided some samples which were used in the project.

The work described herein was part of an ongoing effort at NPL to improve the provision of fluorescence standards for the life sciences. To this end, we investigated the potential of semiconductor quantum dots to act as fluorescence standard materials. This report describes the synthesis and characterisation of quantum dots and the development and testing of prototype standards materials made from them.

2. Introduction

2.1. *Fluorescence Measurement in the Life Sciences*

Fluorescence measurement is the most widely used readout modality in the life sciences today, from research at academic institutions to high-throughput screening by pharmaceutical companies. It may encompass spectral measurements or intensity measurements; polarisation or fluorescence lifetime; or the quantum yield of fluorescence compounds. Samples may be in cuvettes or multi-well plates; on microscope slides; or in flow cytometers. The detection may use an imaging detector, a photomultiplier tube or a photodiode, and may be monitored at equilibrium or as it changes over time. The most common types of measurement are briefly reviewed in this section.

Many of the methods used have been developed in an academic research context; consequentially such issues as quality control or even quantitative measurement were not the main priority. Indeed, within one experiment, an absolute calibration is often not necessary, as internal standards are typically used. As a result of these factors, together with the rapid expansion in fluorescence technology, the standards to support this type of measurement have lagged dramatically behind their applications.

A key requirement for many industrial applications of fluorescence is the *comparability* of measurements batch-to-batch and lab-to-lab; in addition, there is a demand for stringent QC. This has led to a requirement for standards materials for fluorescence, and it is this area that this project set out to address.

2.1.1. **Key Fluorescence Applications**

Some of the key biological applications of fluorescence are outlined in the following paragraphs, together with the measurement issues that each presents.

High-Throughput screening is now a fundamental aspect of drug discovery, and pharmaceutical companies invest huge resources in this process. Fluorescence is the readout modality in 68% of all high throughput screens. However, there are few standards and reference materials to support QC of these fluorescent measurements. This undermines comparability across all the measurements in a large screen, and indeed between screens. This diminishes confidence in the results obtained and thereby decreases the value of the data.

DNA Microarrays have developed over the past decade into a widespread technology that is still developing and expanding. They are commonly used to measure levels of expression of many thousands of genes in parallel. Target DNA is printed as an array of spots on a glass substrate. Fluorescently labelled tissue cDNA samples are then hybridised to the array and imaged using specialised scanners. There are many measurement issues in microarrays and fluorescence is one source of variation. It is likely that the performance of this technology could be improved by optimising the optical performance of the scanners and using standard artefacts, for example a standard fluorescent slide.

Cellular imaging is a key technology in the life sciences and is increasingly of importance to industry. Indeed, it is developing into a high throughput technology in the form of **cell-based assays** used by pharmaceutical manufacturers in the screening of lead compounds. Typically, fluorescence is used to follow the distribution of

fluorescent molecules within the cell, and is not measured quantitatively. However, the value of this type of imaging could be enhanced if the performance of optical systems and detectors was well-characterised in terms of resolution, linearity, and other parameters.

Flow cytometry is widely used in both research and clinical applications, to characterise populations of cells through the use of fluorescent probes. However, the measurements of fluorescence intensity are typically uncalibrated, and this can create problems in the comparability of data acquired on different instruments or at different times. Fluorescence intensity standards would be of great value in this area, and the first prototypes have been developed at NIST in the form of fluorescent beads.

ELISA's (enzyme-linked immunosorbent assays) and other microtitre-plate based assays based on fluorescence are widely used in diagnostics, research and pharmaceutical sectors. These assays have general requirements for standardisation. Errors can arise from the plates themselves, liquid dispensing, and optical alignment, as well as the errors arising from the fluorescence measurement itself. However, a fluorescent reference material could be used to evaluate the cumulative effect of many of these factors.

Quantum yield measurement is primarily of interest to manufacturers of fluorescent probes, but it has broader implications for end-users and assay developers. The difficulty of making primary quantum yield measurements means that virtually all measurements are made by comparison to a “standard” material of known quantum yield. However, there are many potential sources of error including the measurement technique itself and the fact that no certified reference materials for this measurement exist.

Fluorescence lifetime measurement or time-gated measurements are increasingly of interest, particularly in imaging applications. There are many potential sources of systematic error in these measurements, particularly where more than one species is present. Therefore there is a need for reference materials, with known lifetimes, to calibrate this type of measurement.

2.1.2. Emerging applications areas

There are a number of novel applications of fluorescence where expansion can be expected in the future. In most cases the measurement issues are not yet clear, but it would be wise to monitor developments in these areas. Most of these new technologies are related to the key applications areas mentioned above; for example, **protein arrays** are a logical extension of DNA array technologies, and face many of the same measurement issues. Likewise, **cell-based assays** – whether in a well or array-based format – exhibit the same issues as conventional cellular imaging, but multiplied immensely by the “high throughput” nature of the system, and the need for quantitative results. Other emerging technologies focus on measuring fluorescence at the “quantal” level. **Single molecule fluorescence** techniques directly measure the fluorescence from single fluorophores; **fluorescence correlation spectroscopy** performs a similar trick, but instead looks for fluctuations in fluorescence as molecules diffuse in and out of a focal volume. Here the measurement issues are not yet clear, but may crystallise as the applications of these technologies mature.

2.1.3. Types of Biological Fluorescence Measurement

Fluorescence as a detection modality is used in a very diverse manner within the life science industry. It is important that any strategy to address measurement issues related to fluorescence reflects this diversity, since no single standard will meet all the requirements. Without even considering the details of the molecules involved, there are a number of ways in which we can broadly subdivide biological fluorescence measurements:

Spectral vs. single-point: in some applications, spectral data is obtained using, for example, monochromators and/or a photodiode array. However, in the higher throughput applications, only a single intensity data point is acquired per sample. The wavelength of fluorescence is usually selected by using a band-pass filter, but sometimes other types of filters, or monochromators, may be used.

Wavelength: most commonly used dyes are excited by visible light (typically in the blue or green) although there is an increasing movement towards longer wavelength dyes, some of which are excited and emit in the near infra-red. There is also an increasing tendency towards wavelength multiplexing of assays, where each assay is read out in a different spectral “band”.

Time resolved vs. steady-state: conventional fluorescence measurements are generally made under constant illumination – i.e. at “steady state”. However, lifetime-based measurements are becoming increasingly popular in a variety of areas such as drug discovery and cellular imaging. Fluorescence lifetime is a sensitive probe of a fluorophore’s environment, and may be measured using pulsed (time-domain) or modulated (frequency-domain) light sources. Often, however, lifetime is not measured *per se*, but used to distinguish signal from background. For example, so-called “time-gated” imaging of cells helps to separate the fluorescence of probe molecules from the background of cellular autofluorescence. Time-gated measurements have also found applications in industry, where problems are often encountered with background fluorescence from laboratory plastics (for example). Kinetic fluorescence measurements are often made over longer timescales – seconds to hours – to characterise the activity of molecules such as enzymes.

Imaging vs. scanning: conventional fluorimeters typically measure one, or a few, samples at any one time. Micro plates are typically read using mechanical scanning of the plate past a detector head (or *vice versa*). Increasingly, however, imaging modalities are being used – typically with CCD detectors. Imaging offers higher throughputs by imaging all wells in a plate simultaneously. Imaging is also used in fluorescence microscopy and microarray scanners, although in both cases the images may be acquired confocally, which actually involves rapid scanning. Imaging methods bring their own measurement issues, such as homogeneity of illumination, detector uniformity, and the image processing algorithms used to extract data.

2.2. Fluorescence standards for the Life Sciences

The foregoing section indicates the vast scope of fluorescent measurements today. It is clear that a wide variety of standards materials are likely to be required, to cater for different spectral regions or different sample formats, for example. Existing demand for standards has been met by a range of fluorescence reference materials and artefacts. In the following section, we briefly review these standards and discuss their strengths and limitations. It is worth noting that other standards materials are available

which do not meet the needs of the life sciences. These are mostly materials such as tiles which have been developed for industries such as paper and fabrics, and the requirements here are very different, and hence beyond the scope of this report.

2.2.1. Existing Fluorescence Standards

Currently available fluorescence standards of which we are aware are summarised in Table 2.1. Other NMIs – for example BAM and PTB – are known to be working on standards materials also.

The “gold standard” intensity and spectral standard has always been considered to be the NIST standard reference material SRM 936, which is solid quinine sulphate dihydrate, which when made up correctly forms a traceable fluorescence standard, both for intensity and for spectral characteristics [1, 2]. Two more recent standards are based on fluorescein. NIST’s SRM 1932 is a solution for establishing a scale in units of molecules of equivalent soluble fluorophore (MESF) [3-5]. This allows for the effect of solvent conditions to be compensated for. Molecular Probes now produces a similar NIST-traceable standard.

These materials are all solution based and require careful handling, preparation and storage to obtain accurate results. Like other organic dyes, they are vulnerable to photobleaching. They are also of limited usefulness for wavelength calibrations, since the spectra are relatively broad.

MATECH’s standards are solid-state and stable, consisting of inorganic ions in a glass matrix. They are available in 96-well and 384-well microplate formats. They are also NIST-traceable. Molecular probes also makes microplate standards, but these are not traceable.

Sets of dyes are also available as spectral standards, again from Molecular Probes as liquids, or from Optiglass embedded in PMMA. These standards are useful for evaluating the spectral characteristics of instruments, but typically have relatively broad peaks and are not usable for intensity calibration; nor are they traceable. The PMMA embedded standards, however, do have the advantage that they are stable and do not require any preparation before use.

One of the most interesting standards currently available is produced by Photon Technology International, and consists of a single crystal of dysprosium-activated yttrium aluminium garnet. The excitation and emission spectra contain a number of narrow peaks, and this material is therefore ideal as a wavelength calibrant. It also exhibits high stability; but it is not clear whether the material is traceable or not.

Although the situation has improved somewhat since this project began, it is clear that available coverage is patchy. There are few existing wavelength standards, and the intensity standards that are available mostly require careful handling and are not ideal for routine use. There is clearly scope for improvement.

	Producer	Name	Application	Traceability?	Reference
NMI	NIST	SRM 936a: Quinine Sulfate Dihydrate	Generic solution intensity standard	✓	[1, 2]
		SRM 1932: Fluorescein	Solution intensity standard (MESF)	✓	[3-6]
Commercial	Photon technology International	FA-2036 Fluorescence Standard for UV-Vis-IR	Cuvette-type wavelength standard	?	[7, 8]
	MATECH	Fluorescent Reference Standards (range)	96 and 384 well plate standards	✓	[9, 10]
	Molecular Probes	Reference Dye Sampler Kit	Set of solution standards	✗	[11]
		Fluorescein NIST-Traceable Standard	Fluorescein solution standard	✓	[12]
		RediPlate™ Microplate Intensity Standards	96 well plate standards	✗	[13]
	Optiglass	6BF Fluorescent Reference Materials Set	Cuvette-type solid spectral standards	✗	[14]

Table 2.1 Existing fluorescence standards appropriate for the life sciences. Reflectance-type standards are not included.

2.2.2. Desirable Characteristics of materials

The goal of this project was to investigate new standards materials, which were in line with the requirements of the pharmaceutical and life sciences industries. There are a number of criteria that must be met to fulfil these requirements:

- The standard should take the form of a robust artefact, requiring no preparation and thus ideal for routine use.
- The standard should be stable in its primary characteristic (e.g. intensity or wavelength) over a reasonable period of time, for example 3-6 months.
- The standard should be resistant to changes in environmental conditions (such as temperature).
- The standard should be resistant to photobleaching. For an intensity standard, this is particularly the case. For a wavelength standard, it need not retain quantitatively the same intensity, as long as the signal-to-noise ratio remains sufficiently high and there is no shift in the emission spectrum.

- The material should be available in a number of formats – so that it can be used in a form appropriate to the measurement being controlled.
- For a wavelength standard, the material should have narrow spectral features, which permit accurate wavelength calibration.
- Ideally, the material should be traceable in its primary characteristic.

It will be apparent that many of the standards listed above fail to meet these criteria, due to a lack of traceability, flexibility of format, stability, or ease of use. A strong candidate for a new standard material, which meets the above criteria, is semiconductor nanocrystals or quantum dots, which are described in the following section.

2.3. Quantum Dots

The properties of bulk materials are naturally different from those of the isolated atoms of which they are composed. Furthermore, small amounts of such materials – for example, aggregates of a few hundred or thousand atoms – often exhibit properties that are different from either of these. This is typified by some semiconductor nanocrystals, which can behave as fluorescent quantum dots¹. Their optical properties are radically different from either the bulk or isolated atoms, and are strongly related to their size, as illustrated in Figure 2.1.

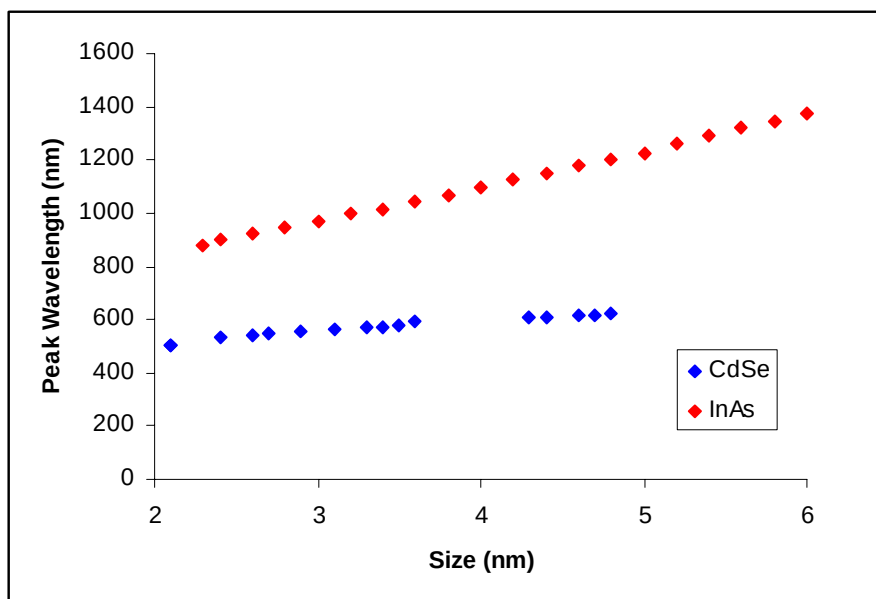


Figure 2.1 Variation of peak fluorescence wavelength with nanocrystal size. Data are taken from [15]. Note that the relationship differs for different semiconductor materials, allowing different wavelength regions to be used.

Emission of light from bulk semiconductors, such as in light emitting diodes (LEDs) is achieved by generating electron-hole pairs or *excitons*. When the electron and hole recombine, a photon of light is emitted. Excitons can be generated electrically (as in an LED) or optically. The energy (or wavelength) of the emitted photon is determined by the bulk properties of the material. The exciton has a characteristic dimension

¹ Quantum dots may also be produced by epitaxial growth on solid substrates; these quantum dots have rather different properties and are not discussed further in this report.

known as the Bohr radius; for example, in cadmium selenide the radius is 11.2 nm. If a nanocrystal is smaller than this radius, the “quantum confinement” of the exciton means that the wavelength of the emitted photon is strongly dependent on the size of the nanocrystal. Thus it is possible to tune the wavelength of light emitted by changing the size of the nanocrystal. As described below, these fluorescent nanocrystals have many other unique properties.

The key development in the application of this phenomenon was the development of methods for the synthesis of monodisperse nanocrystals in significant quantities [16-18]. This has unleashed investigation of an enormous diversity of applications. During the lifetime of this project, the first commercial products have reached the market.

2.3.1. Synthesis

To be of practical use (particularly as a fluorescence standard) a preparation of nanocrystals must be very uniform in size, or *monodisperse*. (A preparation is considered to be monodisperse if the root mean square (RMS) variation in diameter is less than 5%.) The production of monodisperse nanocrystals relies on a brief nucleation of the nanocrystals from a supersaturated solution, followed by a slower growth phase. If all the nanocrystals are nucleated simultaneously, they will all have similar growth rates and the resulting nanocrystal population will have a narrow size distribution. The size of the nanocrystals depends on the duration of the growth phase [18].

In practice this is achieved by a rapid injection of the raw materials to achieve a concentration above the nucleation threshold. Once nucleation occurs, and growth begins, the concentration drops below the nucleation threshold. If there is little growth during the nucleation phase, a “focussing” of the size distribution can occur, resulting in improved monodispersity. Over longer time scales, a process known as Ostwald ripening can degrade the size distribution; larger nanocrystals tend to grow at the expense of the smaller ones. Therefore tight control of the reaction conditions and rapid termination of the reaction are vital to achieving monodisperse nanocrystals of the desired size.

For semiconductor nanocrystals such as CdSe, the reaction typically involves the injection of precursors into a vigorously stirred flask containing a hot coordinating solvent. The precursors used are typically organometallic compounds; in this case cadmium stearate as the cadmium source and tri-*n*-octylphosphine selenide as the selenium source. The role of the coordinating solvent is to stabilise the surface of the nanocrystal as it grows, and to prevent aggregation; typically compounds such as tri-*n*-octylphosphine oxide and hexadecylamine are used. These compounds play an additional role in that they may form the “capping” groups of the nanocrystals and therefore determine their solubility and other properties. Typically, the capping groups give the nanocrystals solubility in organic, but not aqueous, solvents.

To modify the properties of the nanocrystals, the capping groups may be substituted. For example, to make the nanocrystals water-soluble, mercaptocarboxylic acids such as mercaptopropionic acid may be used as replacement capping groups. These compounds are bifunctional, with a polar group to make the nanocrystals water soluble, and the mercapto group which binds strongly to the nanocrystal surface. Chan and Nie [19] used mercaptoacetic acid to make nanocrystals water soluble, and to attach protein molecules to the nanocrystals using cross-linking agents.

Another widely used method for modifying the properties of the nanocrystals is the addition of an inorganic “shell” to the nanocrystal “core” [18]. For example, a shell of a second semiconductor with a wider band gap has been used to dramatically enhance the quantum yield and stability of the nanocrystals by eliminating non-radiative relaxation pathways; for example, CdSe nanocrystals with a ZnS shell may reach quantum yields of 50% [17, 20]. Silanes have also been added as a third layer in order to achieve water solubility and convenient derivatization [21].

A number of other modifications to the synthesis have been developed which allow modification of the nanocrystal shape. CdSe nanocrystals typically have an aspect ratio of 1.1 to 1.3 [16]. However, by fine-tuning the kinetics of nanocrystal growth, it is possible to make the nanocrystals grow preferentially along a particular axis of the crystal lattice. For example, nanocrystal rods, or “nanorods”, may be synthesised [22] with variable aspect ratios as high as 10 and are almost monodisperse (5% RMS in diameter and 10% in length [23]). These rods have some interesting properties; their fluorescence is linearly polarised [24] and the emission wavelength may be tuned by varying both width and length [23]. Extension of this principle has also enabled the production of arrows, teardrops and tetrapod shapes [25].

For fluorescence studies, nanocrystals are often studied in solution. However, they may also be prepared in the form of powders, or embedded in polymer or inorganic matrices [26, 27]. Another intriguing phenomenon is the self-assembly of nanocrystals into close packed assemblies, which may be glassy or crystalline in nature [18]. A number of papers have also demonstrated approaches to the controlled assembly of nanocrystals into different types of structure, including quantum-dot “molecules” [28-30]. CdTe quantum dots have also been shown to self-assemble into fluorescent nanowires [31].

2.3.2. Properties

Due to their fundamentally different mechanism of fluorescence, nanocrystals have properties very different from those of organic fluorophores. In many respects, these properties make the nanocrystals suitable for a wide variety of applications, including lasers, LEDs and biological labelling (see section 2.3.3 below). The key features of nanocrystals, particularly with regard to biological applications, are summarised below.

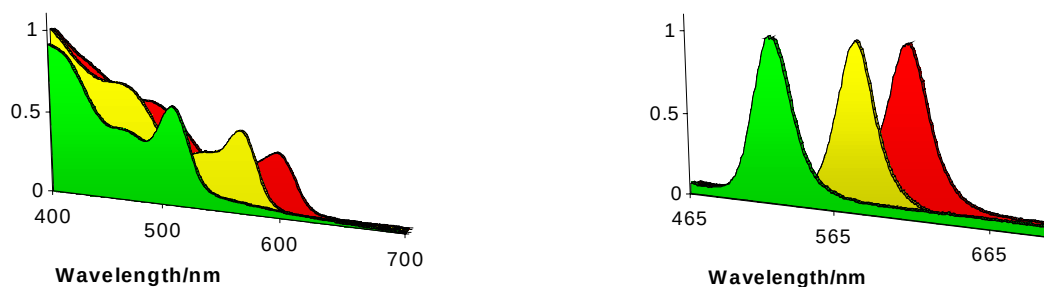


Figure 2.2 Normalised absorbance (left) and fluorescence (right) of CdSe/ZnSe nanoparticles.

Stability. Nanocrystals are typically very stable. Being composed of simple inorganic compounds they are relatively chemically inert, and those with outer shells are particularly resistant to photochemical damage. Because of their minute size, a

colloidal suspension of nanocrystals is generally stable and will not precipitate over time. Indeed, they cannot normally be sedimented by centrifugation.

Environmental sensitivity: the capping ligands of the nanocrystals should render them relatively insensitive to changes in environmental conditions. However, further studies are needed to evaluate this property for particular types of nanocrystals.

Emission spectrum: the emission spectrum of nanocrystals is very **narrow** compared to organic fluorophores; it may be as little as 12 nm FWHM for a single nanocrystal [19]. The emission wavelength of a nanocrystal depends on its size and therefore it is **tuneable**. Furthermore, a nanocrystal preparation can be prepared to be highly **monodisperse** (5% RMS variation in size) [32]. This means that the spectral width of a suspension of nanocrystals can also be very narrow, typically 32 nm or less [21]. Compared to organic dyes, nanocrystals lack a long "red tail" which can cause cross-talk in multicolour fluorescence experiments. These features of the emission spectrum make nanocrystals ideal for multiplexed fluorescence measurements.

Excitation Spectrum: the excitation spectrum of nanocrystals is very broad. This has the advantage that nanocrystals can be excited at any wavelength shorter than the emission peak. It further means that a mixture of nanocrystals with different emission peaks may be excited efficiently by light of a single wavelength, which facilitates simultaneous detection, imaging or quantitation. This is of particular benefit where a laser or spectral line source is being used for excitation. Nanocrystals also emit very **bright** fluorescence. This is because they have a high excitation cross section and can also have high quantum yields, comparable with those of many fluorescent dyes. Furthermore, it is possible to achieve a large difference between excitation and emission wavelengths, which means that it is much easier to separate the excitation and emission light and reduce background due to scattering. [21]

Fluorescence lifetime: nanocrystals have a moderately long fluorescence lifetime in the range of tens of nanoseconds, which is significantly longer than for organic dyes (1-5 ns) or cellular autofluorescence (2-3 ns). This permits their fluorescence to be distinguished from other sources of fluorescence in a fluorescence lifetime experiment. For example, Dahan *et al.* [33] used time gated imaging to distinguish nanocrystal fluorescence from a background of cellular autofluorescence.

Surface Chemistry: nanocrystals may be prepared with a wide variety of surface chemistries by exchanging the capping groups (see above). For example, they may be specifically attached to particular biological molecules [19, 21].

Studies of individual nanocrystals have given useful insights into their fluorescence properties [34], in many ways analogous to the unexpected results obtained from single molecule fluorescence [35]. The **emission spectra** of individual nanocrystals are significantly narrower than for bulk preparations of nanocrystals [19, 32, 34], since the bulk spectral width is broadened by the variation in particle diameter. A surprising observation was the occurrence of "**blinking**" in the fluorescence from single nanocrystals [36]. A similar effect had previously been seen in single fluorophores [35] but the mechanism appears to be different in nanocrystals [37].

Another aspect of nanocrystals that has been investigated at the single crystal level is their fluorescence **polarisation**. This has been characterised by using non-polarised excitation light and placing a rotating analyser in the emission light path. There seems to be some discrepancy in the literature regarding the degree of polarisation observed. In one study [34], CdSe nanocrystals with an aspect ratio of 1.1-1.2 were studied, and

were found to show a wide variation in the degree of polarisation, from 0 to 100%, with an average of about 50%. This was believed to be due to different orientations of the nanocrystals. Indeed, this was suggested to be a useful method for determining nanocrystal orientation in three dimensions [38]. Another study evaluated the variation in the level of polarisation in relation to the aspect ratio of nearly spherical and rod shaped nanocrystals. Here, they found a polarisation ranging from nearly 0 at an aspect ratio of 1 ranging up to 86% for an aspect ratio of 10 [24]. The reasons for this discrepancy are not clear.

A number of studies have examined light-induced changes (increases and decreases) in the fluorescence of nanocrystals, for example see [39-43].

2.3.3. Applications

Nanocrystals have a wide variety of potential applications, and indeed a wide variety of products are now available. In addition to the more specific applications listed below, there are many ideas for potential applications in nanotechnology in the broadest sense. Unsurprisingly, many of these applications are related to photonics; however, biological research is also expected to be a key area.

Light emitting diodes. The electroluminescent properties of semiconductor nanocrystals make them suitable for applications in LEDs, usually in combination with a semiconducting polymer. This type of device was first reported in 1994 [44] using CdSe nanocrystals in combination with poly(*p*-phenylenevinylene). Nanocrystals have many advantages for this type of device. Their wavelength tunability makes it easy to produce devices that emit in a particular wavelength region; they also have narrow emission spectra similar to those obtained by fluorescence. Furthermore, like organic polymers they are easy to process, whilst having much improved stability. Recent developments have improved the technology by the use of core/shell nanocrystals (in this case CdSe/CdS) which give improved quantum efficiency, stability to photo-oxidation (resulting in improved device lifetime), and electronic characteristics [45].

Lasers are an area where nanocrystals show many similar advantages, particularly their tunability. In 2000, Klimov *et al.* [46] demonstrated lasing in close packed films of CdSe nanocrystals, and another group obtained similar results with silicon [47]. This area is a high profile application of nanocrystals and refinements are still being made (see, for example, [48]).

Solar cells are another example of photonics applications. Huynh and colleagues [49] demonstrated hybrid solar cells consisting of CdSe nanorods embedded in a polymer matrix. These devices could potentially be much cheaper to produce than conventional inorganic solar cells, which are produced under clean room conditions. However, typically inorganic cells are 10% efficient, whereas the reported solar conversion efficiency of the hybrid arrays was only 1.7%. However, this is likely to be improved as the manufacturing process is refined. The absorption spectrum of the cells can be tuned by changing the diameter of the nanorods. Rods work better than lower aspect ratios because they provide a conduction path for charge carriers (electrons and holes). One route to increasing efficiency might be to orient the rods perpendicular to the electrodes.

Electronic applications have also been investigated. For example, CdSe nanocrystals have been used to build single-electron transistors [44].

Biological applications which have been proposed for fluorescent nanocrystals are many and varied, but all make use of their unique fluorescence characteristics. A key feature for biological applications is **multiplexing**, the ability to read out many signals simultaneously through the use of multiple, non-overlapping, emission spectra, with a single excitation wavelength (see section 2.3.2). Other key features are their photostability, intensity and adaptable surface chemistries. For example, it is possible to conjugate the nanocrystals to molecules such as streptavidin, biotin, antibodies or oligonucleotides, which are widely used in biological assays for their specific molecular recognition properties.

Many of these applications simply use nanocrystals as a replacement for organic fluorophores. One of the first applications demonstrated [19, 21, 50] was **immunocytochemistry**, the specific labelling of cellular components, which is conventionally performed using fluorescent antibodies or avidin. As with other applications, nanocrystals should allow the localisation of more cellular components simultaneously and with less photobleaching. Furthermore, the difference in fluorescent lifetimes (see section 2.3.2) enables nanocrystals to be distinguished from cellular autofluorescence by fluorescence lifetime imaging (FLIM) [33]. Recent papers have demonstrated the feasibility of this approach in practice [51-53], which is helped by the commercial availability of quantum dots with suitable surface attachment chemistries.

A related application is **blotting**, where antibodies and avidin molecules conjugated to enzymes are used to detect minute amounts of protein, via colorimetry or chemiluminescence. **Western blotting** is used to detect proteins that have been separated by electrophoresis whilst **dot blotting** is used to analyse unresolved mixtures of proteins. The methods currently used do not permit multiplexing; a blot can be probed for only one molecule at a time, which necessitates the use of replicate blots. Colorimetric methods in particular are poor for quantitative measurements. The use of nanocrystal-conjugated antibodies has been suggested as a method for obtaining multiplexed quantitative detection of proteins on blots [54]. Similarly, the conjugated nanocrystals could be used in multi-well plate **immunoassays**.

The preceding applications relate to nanocrystals conjugated to protein molecules, but there is also a huge range of potential applications for nanocrystals attached to oligonucleotides or other nucleic acids. For example, they have been proposed as a way of increasing the number of channels which can be used in the analysis of DNA microarrays [55, 56] (typically two channels only are used at present). Their bright fluorescence should also increase the sensitivity of the technique, and their stability to photobleaching would also be an advantage. Similar advantages would apply to other types of array, such as protein arrays.

Other applications of DNA-linked nanocrystals might include **fluorescence *in situ* hybridisation**, (FISH) which is used to “paint” chromosomes in different colours (among other applications). Again, nanocrystals would provide the ability to screen with more probes in a single assay. Oligonucleotides have been attached directly to hydroxylated quantum dots [57] or indirectly, using biotinylated oligonucleotides and streptavidin nanocrystals [58].

One attractive concept is the use of nanocrystals to produce “**barcodes**” by combining nanocrystals of different emission wavelengths [59]. These combinations can be embedded in latex spheres [60, 61], or even in cells [62]. This is the logical extremity of the multiplexing concept. For example, it has been suggested that it would be

possible to use six colours of nanocrystals with six intensity levels to give tens of thousands of usable codes [60]. Hydrophobic nanocrystals can readily be incorporated into porous polystyrene beads in a quantitative fashion, and the fluorescence spectrum can be read out from individual beads with sufficient accuracy to give reliabilities of detection well in excess of 99%. The surface of the bead can bear the target molecule of interest; for example an oligonucleotide, peptide, or antibody. There are a wide variety of applications for this technique in molecular biology, proteomics, and high throughput screening to support drug discovery; it has many advantages over the competing solid state array methods.

Most strikingly, perhaps, there has been an explosion of interest in the use of quantum dots to label **living cells** and tissues. They have also been used in living animals and have even been suggested as **therapeutic agents**.

Jaiswal *et al.* [63] showed that quantum dots could be used to label living cells. They showed that quantum dots could be taken up by endocytosis, or used to label membrane proteins. Labelled cells were followed for long periods without loss of the label or deleterious effects. Dahan *et al.* [64] tracked the diffusion of individual glycine receptors labelled by antibody conjugated quantum dots on the membranes of living neurons.

Dubertret *et al.* [53] showed that micelle-encapsulated quantum dots could be injected into *Xenopus* **embryos**, and that the quantum dots remained stable and were able to be followed throughout development to the tadpole stage. This permits cell lineages to be tracked during development. There appeared to be no dramatic toxicity or developmental abnormalities caused by the dots.

The logical next step was taken by Larson *et al.*, [65], who used quantum dots to trace blood flow in living mice. They imaged the dots by **multiphoton fluorescence microscopy**, which allows visualisation in thick specimens, for example in the skin of living animals. They also showed that quantum dots are uniquely suitable for this technique, as they have exceptionally high two-photon action cross-sections. They also noted that the mice appeared to suffer no ill effects from the procedure.

Similarly, near-infrared quantum dots have been shown to have potential applications to **medicine**; Kim *et al.* used them to identify sentinel lymph nodes [66]; Åkerman *et al.* showed that they could be **targeted** to specific tissues [67] by coating them with appropriate peptides.

A key issue in such medical applications, and indeed any *in vivo* or living-cell research, is the potential **toxicity** of the quantum dots. Their constituents contain many toxic components, cadmium usually being the most significant. However, it is not clear how toxic the quantum dots themselves are. One study addressed this issue [68] and found that cadmium selenide dots could indeed be highly cytotoxic, particularly if oxidation of the nanocrystal surface occurred, liberating free cadmium. However, they also showed that toxicity could be greatly reduced by capping of the quantum dots with ZnS, though such measures may not be adequate *in vivo*. This study is mirrored by recent concerns about the possible health effects of nanotechnologies, as addressed in the recent report by the Royal Society and the Royal Academy of Engineering [69].

2.3.4. Quantum Dots as Fluorescence Standards

Fluorescent nanocrystals would appear to be ideal candidates for fluorescence standards. Their surface passivation and coatings should make them relatively resistant to environmental changes; their simple inorganic structure should also make them relatively stable. It might therefore be imagined that they could act as a stable source of fluorescence to provide a fluorescence intensity standard; they ought to prove even more suitable as fluorescence wavelength standards due to the ability to mix multiple emission wavelengths to produce a calibration scale. This latter application would exploit their tunability, narrow emission spectra and lack of cross-talk.

Although in most cases the readout from fluorescence assays is in the form of an intensity measurement, fluorescence wavelength standards are likely to be particularly valuable. (In many assay protocols, controls are used to provide an internal, relative intensity calibration). Where full spectra are collected, wavelength standards would be useful for ensuring that the wavelength scale is correct. If the experimental measurement is in the form of the intensity at a particular wavelength or over a particular wavelength band, whether extracted from the full spectrum or measured directly, the Gaussian nature of most emission spectra means that small errors in wavelength can lead to large errors in the intensity measured. This would become a particular problem for the comparability of measurements between different instruments and different laboratories.

One could imagine a number of different types of application for nanocrystal-based fluorescence standards. If they could be incorporated into a stable, solid-state format, they would be ideal for calibrating instruments such as fluorimeters. For example, they could be made into solid cuvettes or microtitre plates. Such a standard could be provided with an instrument by a manufacturer, for periodic calibrations. The standards could also be used in liquid form, but here care would be necessary to avoid the effects of evaporation and other losses. The long-term stability of the crystals in solution would also be a concern, particularly for the water-soluble form of the nanocrystals. However, water-soluble nanocrystals could, for example, be added to an experiment to act as internal controls. They might also find application in microscopy applications for wavelength calibration or the measurement of point spread functions at a defined wavelength. They would also be very useful for the calibration of instrumentation for reading microarrays.

Clearly, nanocrystals have many properties which would render them highly promising as candidates for fluorescence standards. However, a careful evaluation of the actual fluorescence characteristics and stability is needed for each type. Furthermore, the issues of packaging the nanocrystals in suitable formats to act as standards need investigation.

2.4. Project Outline

The aim of this project was to investigate the suitability of quantum dots as fluorescence standards materials. The bulk of quantum dot samples used in the study were synthesised at York, and this work is described in section 3. The team at York also undertook extensive studies of the factors affecting the stability of quantum dots, and these are presented in section 4. Nanoco, a company specialising in the bulk

commercial production of nanocrystals, also kindly provided samples of nanoparticles for the project². A large number of batches of nanocrystals were tested for their suitability as standards materials at NPL, and these results are presented in section 5. NPL and Optiglass then worked together on the production and characterisation of the prototype standards materials. The liquid standards are discussed in section 6, and solid artefacts in section 7. Experimental protocols are given in section 9.

² For more information see the company's website at <http://www.nanoco.biz/>

3. Nanoparticle Synthesis and Characterisation

Several different types of semiconductor nanoparticle were prepared during the course of the project. Initially, optimisation of cadmium selenide particle synthesis was attempted in order to obtain different emission colours at appropriately high quantum yields.

When an appropriate range of colours was obtained, stabilisation of the particles by coating with either zinc selenide or zinc sulphide was attempted. A BSc project student, Marcus Turner, also carried out a number of syntheses of mixed cadmium selenide/sulphide particles [70]. This was in response to a recent Chemical Communications publication [71] suggesting that a wide range of emission colours could be obtained by varying the ratio of sulphur to selenium used in their preparation. Synthetic details are included in the experimental section (9).

The nanoparticles prepared in this study were characterized by UV/Vis and fluorescence spectroscopy. The sizes of the CdSe, CdSe/ZnSe and CdSe/ZnS particles were calculated using UV/Vis calibration curves published by Murray *et al.* [16] A sample was compared to values obtained by TEM showing close agreement. However, TEM results often displayed low contrast making good size determination difficult using this method. TEM results could not be obtained for the CdSeS particles and because the absorbance and emission spectra are related to both size and composition, rather than size alone, the size of these particles were not determined.

3.1. CdSe Nanoparticles

3.1.1.1. Synthesis

Syntheses were carried out by the methods outlined by Qu and Peng [72]. All syntheses were carried out under strictly air and water free conditions utilising Schlenk techniques to control an argon atmosphere. Cadmium stearate was prepared by the reaction of cadmium acetate and stearic acid at high temperature. The cadmium stearate was dissolved in molten trioctylphosphine oxide (TOPO) and hexadecylamine (HDA) and heated to approximately 300°C, depending on the necessary reaction conditions. A stock solution, also prepared under argon, of selenium in trioctylphosphine was added rapidly to the heated cadmium solution using a syringe.

The size and brightness (PLQY) of the resulting nanoparticles were controlled by the ratio of TOPO to HDA, ratio of cadmium to selenium and the reaction time and temperature. Since the number of variables that could influence the nanoparticle product was so large, early experiments were confined to studying the effect of reaction time. In order to do this, reactions were followed by the removal of the small samples from the reaction mixture at known time intervals. It can be seen from Figure 3.1 that fluorescence, emission wavelength (and therefore size) increases as the reaction progresses. Quantum yield also changes with reaction time.

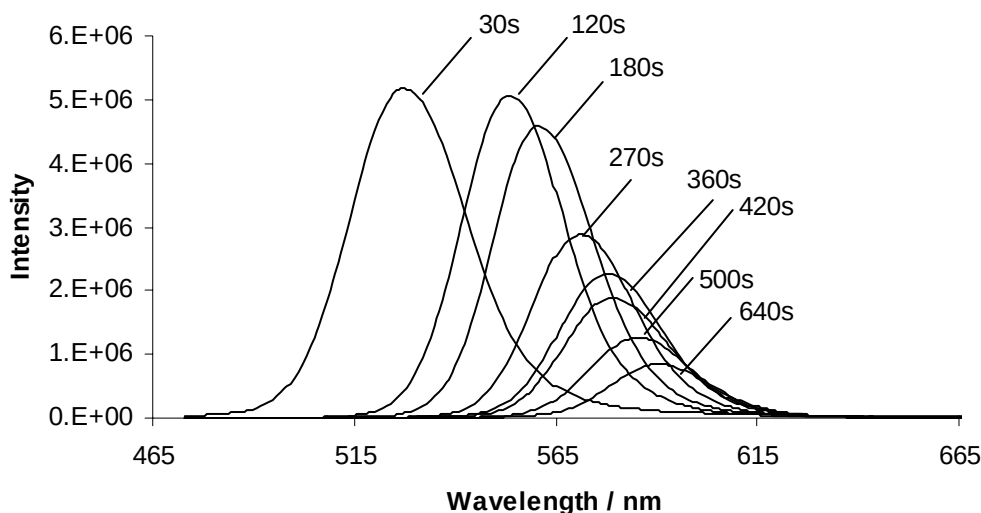


Figure 3.1 Fluorescence spectra of samples taken from the reaction of cadmium stearate with TOPSe over time.

Labels represent reaction time at which each sample was taken.

Despite these reactions clearly showing the influence of reaction time on particle size, the studies bore little relevance to carrying out larger scale synthesis of different sized particles, since small samples removed as described above cooled very quickly in a manner that is difficult to replicate when carrying out larger syntheses. Different methods of cooling were used in the preparation of different sized particles. Larger particles were allowed to cool slowly, whereas, the shorter reaction times required to produce smaller particles required the injection of toluene into the reaction mixture to quench the reaction rapidly.

The ratio of selenium to cadmium was also investigated. In order to produce larger nanoparticles that have an acceptable PLQY, a selenium:cadmium ratio of 5:1 was shown to be optimum. This was based upon observations by Qu and Peng [72] who showed that larger Se:Cd ratios result in reactions whereby a high quantum yield can more easily be obtained. In the same paper it was shown that lower Se:Cd ratios produced reactions in which the optimum quantum yield was achieved at a very early stage in the reaction followed by a rapid drop. Particles produced using lower Se:Cd ratios were smaller. Therefore, in order to produce smaller nanoparticles, these lower ratios were explored.

Further optimisation in the production of different sizes of nanoparticles was also carried out by variation of reaction temperature. The optimum temperature for the production of larger nanoparticles (*ca.* 4.3 nm) was shown to be 270°C. Reaction temperatures of 310°C allowed the smallest (*ca.* 2.3 nm) CdSe nanoparticles to be prepared. This temperature was also shown to be useful in the preparation of high quality mid-sized (*ca.* 3.8 nm) nanoparticles by the use of longer reaction times.

Purification was carried out using dissolution in a good solvent for TOPO, HDA and the nanoparticles, followed by precipitation of the particles by addition an equal volume of counter solvent. The resulting precipitate was separated by centrifuge followed by decanting of the supernatant. The best combination of solvents was found to be chloroform for initial dissolution and acetone as the counter solvent. Use of GPC chromatography was also shown to be effective for further purification.

However, purification using this method tended to reduce the PLQY of the sample due to separation of any labile, passivating ligand from the sample.

3.1.1.2. Characterisation

The particle sizes mentioned above are those that can be reproduced with reasonable accuracy with good PLQYs (5-20 %) and reasonable polydispersity. Size was routinely determined by UV/Vis spectroscopy. UV/Vis and fluorescence spectra of the different sizes of particle are shown in Figure 3.2.

The UV/Vis spectra of the uncoated particles display well-defined exciton peaks confirming low polydispersity [16]. The relatively narrow full width at half maximum (FWHM) of the fluorescence spectra emission band (32 nm for 4.3 nm and 2.3 nm particles) confirmed good monodispersity.

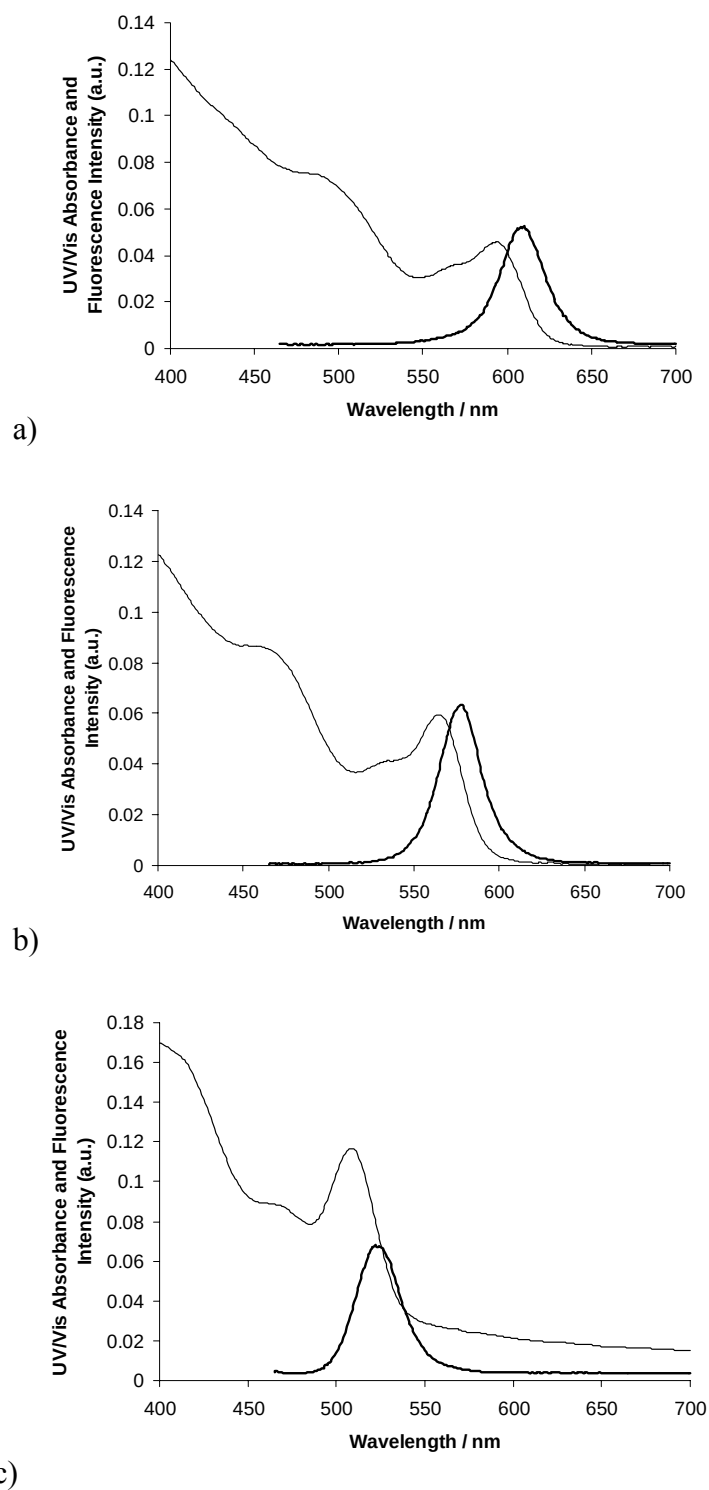


Figure 3.2 UV/Vis and fluorescence spectra of a) 4.3 nm CdSe particles; b) 3.8 nm CdSe particles; c) 2.3 nm CdSe particles.

3.2. CdSe/ZnSe and CdSe/ZnS Nanoparticles

3.2.1.1. Synthesis

A large number of different methods for the coating of CdSe nanoparticles with zinc chalcogenides exist in the literature. Many of these involve the use of diethyl zinc and volatile sulphur or selenium precursors added dropwise to a hot solution of CdSe particles in a coordinating solvent. Several of these methods were attempted with limited success. Reports of significant increases in PLQY are common for these coated particles compared to those of CdSe particles, for reasons discussed above. However, the best result obtained here using these methods was to produce particles of equal PLQY to the starting CdSe particles.

Recently Reiss *et al.* [73] reported the use of zinc stearate and TOPSe as good precursors in the thermolytic coating of CdSe nanoparticles. Minor modification of this method was used successfully to produce coated nanoparticles with PLQY values of up to *ca.* 33 %. This involves the dissolution of CdSe nanoparticles in a mixture of TOPO and HDA at 190°C, followed by the slow dropwise addition of the zinc and selenium precursors in a warm (*ca.* 50°C) solution of toluene and TOP. After the 1 h addition period, the resulting particles were annealed at 190°C for a further 75 min. Identical methods were used in the preparation of CdSe/ZnS particles with the use of sulphur in place of selenium for the synthesis. In all cases water and air were rigorously excluded from the reaction by the use of a Schlenk line to control vacuum and an argon atmosphere.

In early attempts of these coating reactions, a great deal of care was taken to calculate the appropriate amounts of the zinc and selenium/sulphur precursors in order to achieve the optimum number of monolayers. However, for the range of sizes of particles explored in this study, particles with very similar quantum yield and stability properties (*vide infra*) were prepared using the same quantity of precursor for a given 40 mg batch of CdSe particles, irrespective of size. It is possible to calculate the approximate number of resulting monolayers using the bulk property parameters of CdSe, ZnSe and ZnS. The thickness of the ZnSe overcoat was calculated to be 5.2 monolayers for the 4.3 nm core particles and 4.6 monolayers for the 3.8 nm core particles.

Successful ZnSe coatings of 4.3, 3.8 and 2.3 nm CdSe Particles were carried out. ZnS coating was only carried out on samples of 4.3 nm particles giving a shell thickness of 5.3 monolayers.

Purification was carried out by dissolution of the entire reaction mixture in chloroform, followed by the addition of an equal volume of methanol resulting in precipitation of the particles.

3.2.1.2. Characterisation

Again the polydispersity of the samples resulted in a FWHM of fluorescence emission of *ca.* 30 nm. UV/Vis and fluorescence spectra of the different sizes of particle are shown in Figure 3.3.

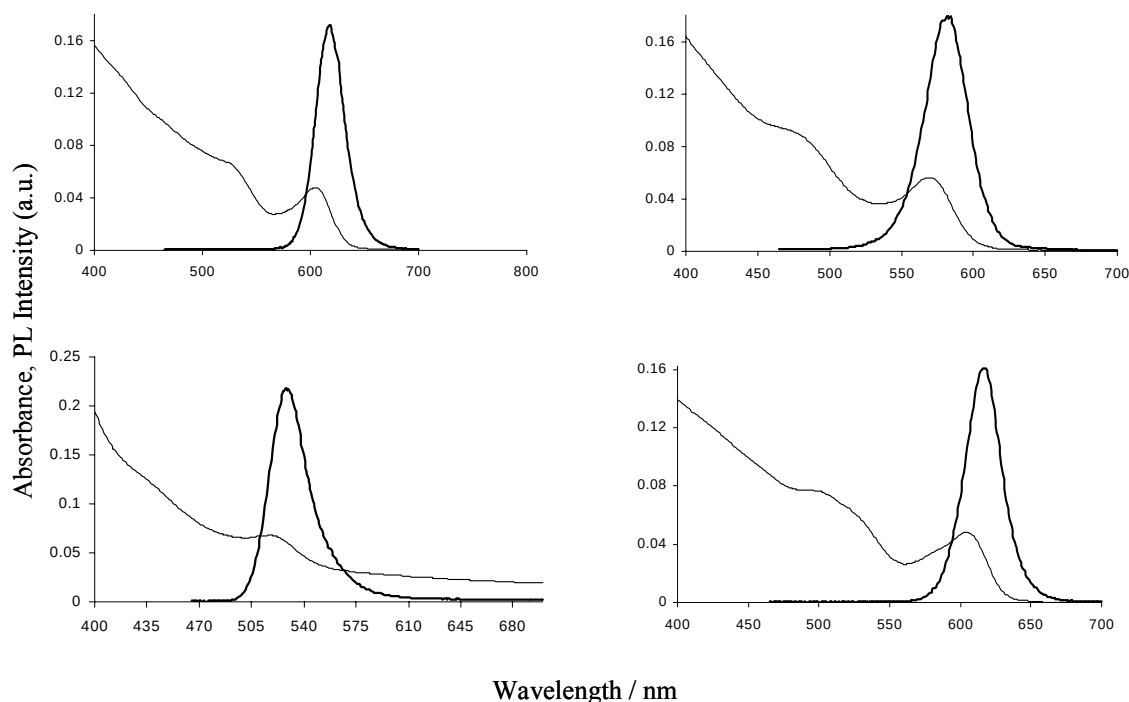


Figure 3.3 UV/Vis and fluorescence spectra of a) 4.3 nm core CdSe/ZnSe particles; b) 3.8 nm core CdSe/ZnSe particles; c) 2.3 nm core CdSe/ZnSe particles; d) 4.3 nm core CdSe/ZnS particles.

3.3. CdSeS Nanoparticles

Full synthetic details of CdSeS preparation are given in Marcus Turner's 3rd Year Project Report [70]. Syntheses were either carried out using the methods of Jang *et al.* [71] or by minor modifications thereof. Cadmium oxide was heated to 300°C in the presence of oleic acid and trioctylamine (TOA). At high temperature, colourless solutions of cadmium oleate in TOA were formed. Solutions containing varying proportions of TOPSe and TOPS in excess TOP were quickly injected into the cadmium solutions. The ratio of sulphur to selenium was strongly influential upon the colour of the resulting nanoparticles. Reaction times were controlled by quenching the mixture with ethanol.

A number of different colour samples were prepared with emission λ_{max} ranging from 460-580 nm.

Purification was carried out by the addition of excess ethanol to the reaction mixture resulting in precipitation of the particles. This method was not as effective at removing excess ligand (TOA) as the solvent/counter solvent methods mentioned above, although it is thought that using the same methods of purification as for CdSe particles would be more effective.

3.3.1.1. Characterisation

Characterisation of these particles is, at best, vague. Good size determination of the particles was not possible, since TEM images could not be obtained and no previous studies have been carried out for comparison. FWHM of fluorescence emission peaks were slightly larger than the particles mentioned in Sections 3.1 and 3.2. (40-50 nm). UV/Vis spectra also displayed less defined exciton peaks suggesting that the samples

were less monodisperse. However, these effects may be due to the mixed nature of the material from which the particles are composed. UV/Vis and fluorescence spectra of the different sizes of particle are included in Marcus Turner's 3rd Year Project Report [70]. PLQY values for the "as prepared" particles range from 1-13 %.

3.4. Conclusions

Several different types of particles have been prepared using thermolytic preparation techniques based upon those in the literature. A wide range of colours can be prepared reproducibly with appropriately high PLQYs for the intended application. Fluorescence emission FWHM are generally *ca.* 30 nm except for CdSeS nanoparticles which tend to be larger (40- 50 nm).

4. Stability Studies

Since the fluorescence properties of semiconductor nanoparticles are essential for many of their applications, the stability of the particles was measured in terms of emission λ_{max} and quantum yield. Preliminary studies showed that the best conditions for nanoparticle storage was in the dark, under argon. It is recognised that these conditions are not always available and so stability studies were carried out in light and air using a variety of additives to investigate potential as stabilising agents.

Distinct differences in the behaviour of both types and sizes were observed when solutions were treated with additives and exposed to air and high intensity visible light for equal periods of time. To ensure consistency of the experimental conditions, the samples were placed on a rotating platform in a specially constructed light box.

The additives used in the study were hexadecylamine (HDA), trioctylphosphine oxide (TOPO) and 2,6-di-tert-butyl-4-methylphenol (BHT). HDA and TOPO were chosen due to their use in the synthesis of the nanoparticles. Some of these surface-passivating ligands may be lost during purification and this was to be investigated along with the ligands ability to stabilize the particles. The additives were used individually and in combination to see whether a cooperative effect could be observed. BHT was also investigated as an additive since it is accepted that degradation of semiconductor nanoparticles proceeds via oxidative pathways and it was hoped that the antioxidant properties of BHT would slow these processes. Several other additives (such as triphenyl phosphine, shorter chain primary amines, 1,1'-bis(diphenylphosphino)ferrocene, molecular sieves, stearic acid, tetraoctylammonium bromide, octadecane thiol and zinc stearate) were also investigated, but were not pursued because of either a damaging or neutral effect on the nanoparticles.

4.1. *CdSe Particles in Chloroform.*

4.1.1. Initial Effect of Additive.

The initial PLQY values and those taken after one week from the experiments involving 4.3 nm and 2.3 nm particles are shown in Figure 4.1a and b respectively.

The initial effects of additives had a qualitatively comparable effect on the two different sizes of nanoparticle. It can be seen that in both cases, the samples containing HDA exhibit considerably higher PLQYs than the sample containing no additive i.e. "as synthesized". TOPO also has a small passivation effect reflected in the slightly elevated PLQYs of both samples of particles. The highest PLQYs were observed for samples containing both HDA and BHT. BHT alone seemed to have little effect on the PLQY of the samples.

The enhancing effect of amine and phosphine oxide effect has been observed by others [74-76] and has been attributed to higher surface passivation due to greater coverage of coordinatively unsaturated Cd at the surface by the excess ligand. It is thought that this effect is particularly strong for the amine because it is a better ligand for the surface cadmium.

The slightly higher quantum yield observed for the HDA/BHT sample compared to the sample containing only HDA may be due to the HDA sample undergoing oxidative degradation during the time (<60 min) between sample preparation and

fluorescence measurement. This oxidation would be suppressed by the presence of BHT (*vide infra*).

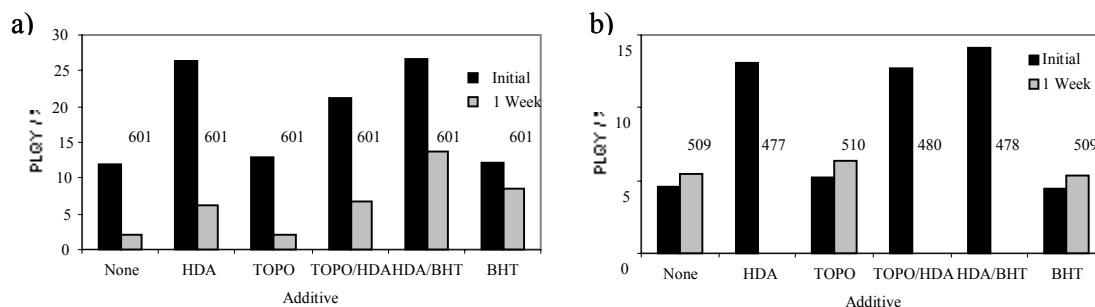


Figure 4.1 PLQY values for 4.3 nm CdSe particles (a) and 2.3 nm CdSe particles (b) in chloroform in the presence of various additives.

The numerical values above each bar are the λ_{max} / nm of the fluorescence spectra after 1 week of exposure to light/air. Values of λ_{max} immediately after sample preparation were 604 nm for the 4.3 nm particles and 520 nm for the 2.3 nm particles. Black bars are for initial quantum yields and grey for values after 1 week.

4.1.2. Photo-oxidation.

The stability of a given sample was measured by comparison, as a percentage, of PLQY after photo-oxidation to their initial PLQY (immediately after mixing with additives). After one week, all six samples of the 4.3 nm particles had substantially lower PLQYs than those observed initially. A small shift in the λ_{max} of the fluorescence emission band was also observed (3 nm towards lower wavelength). No broadening of the emission peak could be noticed. The greatest stability was conferred by the addition of BHT, giving a PLQY that was 68% of the initial value, after a week in the light-box. Despite losing almost 50% of the initial quantum yield, the sample that contained both HDA and BHT gave the highest final quantum yield due to the greater initial passivation afforded by the amine. Interestingly, the highest stability conferred by additives that were not antioxidants (giving 32% of initial PLQY) was a combination of TOPO and HDA, the two coordinating ligands used for synthesis. The samples that contained no additives or HDA displayed the lowest stability (16%).

The reduction of quantum yields seen for the 4.3 nm particles is slowed in the presence of an antioxidant. This is likely due to the inhibition of oxidative damage to the nanoparticle surface which leads to the production of defect sites at which non-radiative emission can occur [77]. The spectral diffusion effect (also termed “bluing”) has also been observed previously [78, 79]. However, this experiment involved the use of relatively high intensity lasers, and the use of ambient light has not been reported to cause the effect. Bluing is caused by an oxidative etching of the nanoparticle surface which results in shrinkage. Smaller particles have a higher band gap and hence fluoresce at lower wavelength.

2.3 nm particles were significantly more sensitive to the type of additive used. For samples containing either no additive, TOPO or BHT, the PLQY *increased* by ca. 20% upon exposure to light and air, resulting in very similar spectra, an example of

which (no additive (b)) is seen in Figure 4.2. The improvement in PLQY for these three samples is in sharp contrast with the loss of PLQY observed for the 4.3 nm particles. It is known however that irradiation of CdSe particles in air free conditions by above band gap wavelength light results in dramatic increases in quantum yield (photo annealing) [39, 80]. We believe that the observed modest increase in PLQY is due to the combination of this photo annealing and surface oxidation (confirmed by spectral diffusion).

The downward shift in emission wavelength was 10 nm for the three samples (with no observable broadening), significantly higher than for the larger particles. However, in the presence of HDA (whether alone or in combination with another additive), a remarkable degree of bluing and peak broadening was observed (Figure 4.2). This spectral diffusion and the associated broadening were so prominent that quantum yields for these samples could not be measured.

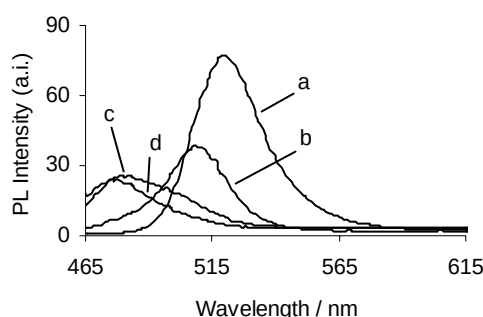


Figure 4.2 Fluorescence spectra recorded for samples with HDA prior to exposure to light and air (a), with no additive after 1 week (b), with HDA/TOPO after 1 week (c), HDA after 1 week (d).

The destructive effect of the amine could be due to HDA being a good ligand, not only for the particle-surface cadmium but also for Cd^{2+} in solution. Therefore, when oxidation of surface selenium occurs, leaving coordinatively unsaturated cadmium, the ions can be lost to solution more easily than in cases where the additive has poorer affinity for cadmium. The fact that HDA alone causes a greater degree of spectral diffusion than HDA and BHT suggests that the effect is related to surface oxidation.

The difference in behaviour of the two sizes of particle is due to several factors. The conditions required to make the two types of particle with sufficiently high quantum yields are significantly different. Namely, the ratio of Se to Cd in the reaction mixture to make the larger particles was 5:1 as opposed to 1.2:1 for the smaller particles. Also, the period of heating the reaction mixture was 30 s followed by slow cooling in ambient conditions for the large particles, as opposed to rapid quenching by the injection of toluene for the smaller particles. These two factors result in significant differences. It has been shown that the ratio of precursors results in differences in surface stoichiometry, [81] where different crystal faces can favor either Cd or Se. Therefore, the smaller particles have a bias toward surface cadmium, qualified by the greater relative degree of initial surface passivation by HDA (2.8 times greater than without additive) compared to the larger particles (2.2 times). This results in the greater sensitivity of the smaller particles to the effect of amine damage, along with the fact that smaller particles have a higher specific surface area and will change diameter to a greater degree from the loss of the same number of surface atoms.

Secondly, because the larger particles remain at higher temperature for significantly longer during synthesis, the particles have the opportunity to anneal thermally. Therefore, photo annealing will not be as effective for these particles as for the small particles that had been cooled very quickly.

4.2. CdSe/ZnSe Particles in Chloroform.

4.2.1. Initial Effect of Additive.

The values of PLQY for experiments involving CdSe/ZnSe are displayed in Figure 4.3a and b. From these charts, it can be seen that, as well as having a similar “as prepared” PLQY, the two different types of particles behave in a similar manner to one another in the presence of additives. The only obvious exception is that the smaller particles are slightly more sensitive to the quenching effect of HDA. In both cases it can be seen that the initial effect of additive is far less pronounced than in the previous two cases, giving PLQYs similar to those of the samples containing no additive (“as prepared”). This is to be expected, because the surface of the CdSe core in these particles is already passivated by the ZnSe shell. Therefore, differences in the passivating ability of a given additive is no longer as significant.

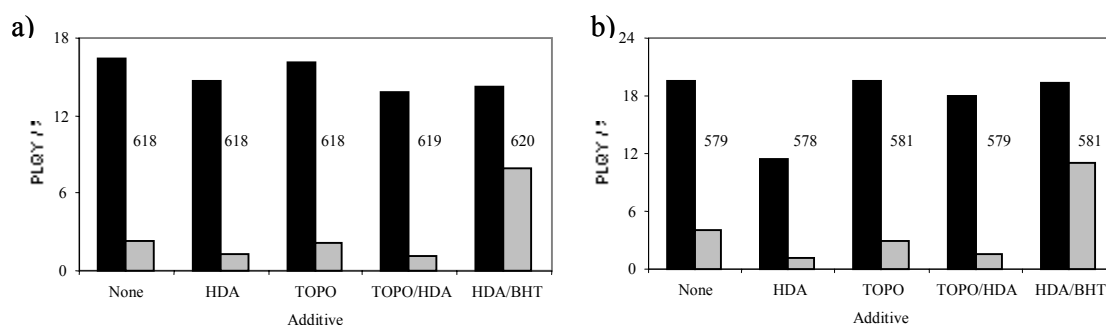


Figure 4.3 PLQY values for 4.3 nm CdSe core ZnSe coated particles (a); 3.8 nm CdSe core ZnSe coated particles (b) in chloroform.

The numerical values above each bar are the λ_{max} / nm of the fluorescence spectra after 1 week of exposure to light/air. Values of λ_{max} immediately after sample preparation were 618 nm for the 4.3 nm cored particles and 583 nm for the 3.8 nm cored particles. Black bars are for initial quantum yields and grey for values after 1 week.

4.2.2. Photo-oxidation

The trends in stability conferred by each of the additives on the two sizes of particle were very similar. The samples of 4.3 nm core particles that did not contain BHT all suffered large reduction in PLQY. The sample containing no additives suffered the least reduction in quantum yield, to 14% of initial PLQY. The samples containing a mixture of TOPO and HDA were the least stable (9% of initial PLQY), however, the variation in stability when exposed to HDA versus TOPO was much less marked than for the uncoated particles. When BHT was used in combination with HDA the final PLQYs were 55% of the initial value. The stabilizing effect of BHT/HDA on the coated particles was thus similar to that observed for the 4.3 nm uncoated particles.

Similarly for the particles with a 3.8 nm core, the sample containing no additives was the most stable of those without antioxidant, to 21% of initial PLQY. The samples containing a mixture of TOPO and HDA suffered the greatest loss of PLQY (8% of initial PLQY). BHT and HDA gave a final PLQY that was 55% of the initial value.

The poor quantum yield stability values for the coated nanoparticles were quite surprising since they had been hailed by many [82, 83] to be significantly more stable than uncoated particles. Interestingly, however, it was noted that although the loss of PLQY was substantial, the emission λ_{max} for the coated particles was much less sensitive to photo oxidation as compared to the uncoated ones. For instance, the maximum shift for the coated particles was 5 nm when exposed to HDA; the corresponding shift for the uncoated particles was 43 nm.

These results suggest that while the coated nanoparticles are less sensitive to their environment due to what might be seen as a sacrificial outer layer, they are still susceptible to significant photo degradation.

4.3. CdSe/ZnS Particles in Chloroform.

4.3.1. Initial Effect of Additive

The values of PLQY for experiments involving CdSe/ZnS are shown in Figure 4.4. In a similar manner to the zinc selenide coated nanoparticles, these CdSe/ZnS particles show only a little sensitivity to the additive in use. The maximum improvement when compared to the “as synthesized” particles was conferred by the mixture of TOPO and HDA (20% enhancement).

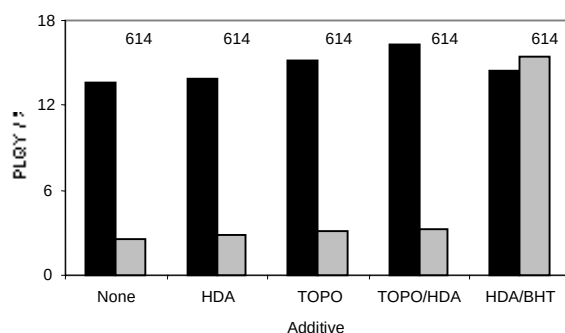


Figure 4.4 PLQY values for 4.3 nm CdSe core ZnS coated particles in chloroform.

The numerical values above each bar are the λ_{max} / nm of the fluorescence spectra after 1 week of exposure to light/air. The value of λ_{max} immediately after sample preparation was 616 nm.

4.3.2. Photo-oxidation.

Surprisingly, the phosphine oxide or amine did not confer any added or reduced stability to the particles when compared to the sample with no additive. All four samples had PLQY values of 20% when compared to their initial value. It appears that unlike CdSe/ZnSe particles, degradation of the CdSe/ZnS particles occurs without ligand involvement. When the antioxidant BHT, and HDA were added to the particles their stability was significantly improved giving a final PLQY 7% higher than the initial value. The bluing effect was consistent (2 nm) for all samples.

4.4. CdSeS Particles in Chloroform.

Full details of this study are to be found in Marcus Turner's 3rd Year Project report [70].

4.5. Solvent Effects.

The stabilizing effect of BHT suggested that the pathway for photo-oxidative degradation of the nanoparticles might be free radical in nature. Therefore, using chloroform as a solvent could lead to potentially more rapid degradation due to the ready formation of free radicals. To test if the mechanism of photo degradation is solvent-dependent, we studied solvent effect on the nanoparticle stability. Toluene was used as a solvent for 2.3 nm uncoated particles and ZnSe coated particles of core size 2.3 nm using HDA and a mixture of HDA and BHT as additives. Figure 4.5 shows the results obtained from these experiments.

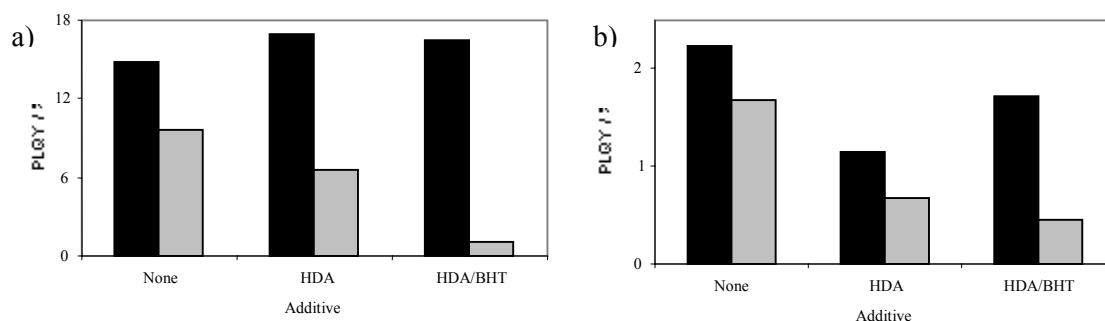


Figure 4.5 PLQY values for 2.3 nm CdSe particles (a); 2.3 nm core CdSe/ZnSe particles (b) in toluene.

Initially, the behaviour of the samples was not qualitatively dissimilar to that observed in chloroform. Surprisingly, however, the samples containing HDA and BHT showed a significantly lower stability than the samples containing HDA alone and the samples that contained no additives were the most stable. It is thought that this is due to a non-radical degradation pathway being dominant in toluene, thus BHT does not retard the process. However, it is not clear why the samples containing BHT and amine are less stable than those with just amine.

4.6. Conclusions

The results described above lead to several interesting observations. The effect of light and air over time results in the balance of two effects: photo annealing and surface degradation. When particles are prepared in a manner resulting in higher surface energy (short reaction periods) the annealing effect is more pronounced resulting in improvements in PLQY. Particles that have been prepared using longer periods of exposure to high temperatures experience surface degradation but do not benefit as much from annealing, resulting in severely reduced PLQY.

The amine HDA exhibits three effects over the nanoparticles used in this study: passivation of surface defects resulting in elevated PLQYs, fluorescence quenching when maximum passivation has been attained and finally surface degradation over time. This has strong implications for the applications of CdSe nanoparticles since the presence of excess passivating agent is often required in order to achieve high

quantum yields. However, prolong storage of nanoparticles in the presence of excess amine lead to more rapid degradation. Therefore, for long-term use in ambient conditions, alternative passivating agents must be sought that do not result in degradation.

The use of TOPO in solutions of nanoparticles does not have as strong a passivating effect, with the only significant improvements in initial PLQY being seen for the ZnS coated particles. However, TOPO is significantly less damaging to the particles over time, probably due to weaker binding of the phosphine oxide to cadmium.

The use of antioxidants has been shown to temper the effect of amine related degradation in CHCl_3 . However, this effect is solvent sensitive and so other antioxidants need to be examined for use in different solvents if ambient conditions are required in a given application.

5. Characterisation of Nanoparticles

All batches of nanocrystals received at NPL were subjected to thorough characterisation to determine their suitability as standards materials. The protocols used for these investigations are presented in section 9. Here, a selection of the results is presented, so as to illustrate the issues involved and the criteria for selecting nanocrystal batches for standards development.

5.1. Spectral Characteristics

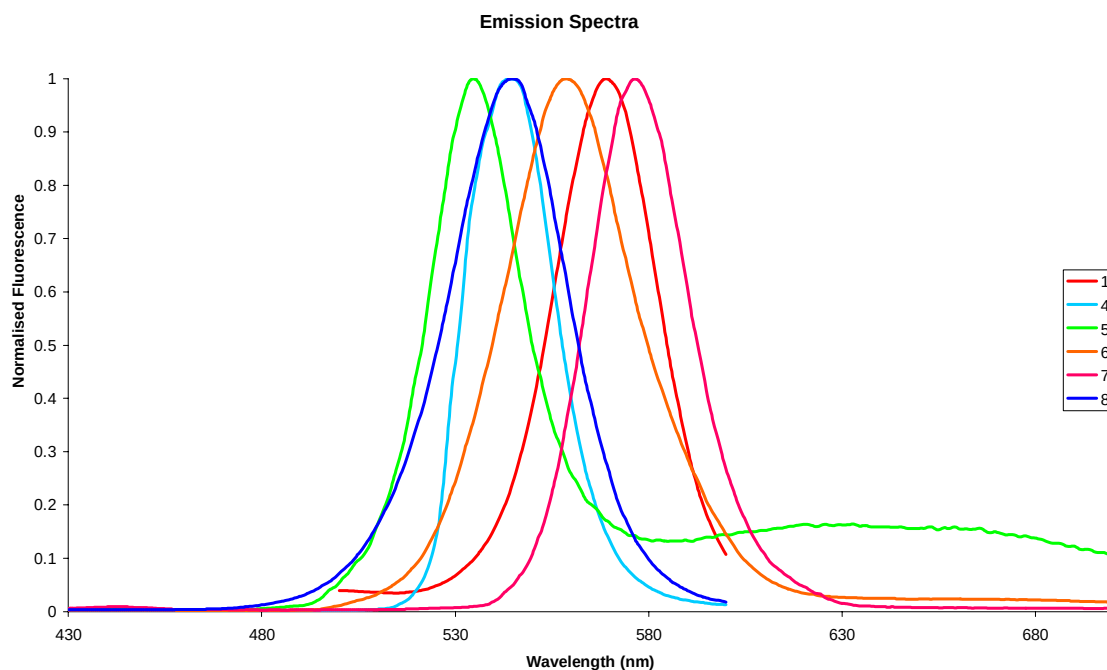


Figure 5.1 Representative emission spectra of quantum dot batches.

Note that for all batches except 8 there are narrow, symmetrical, Gaussian emission peaks. Batch 8 exhibits an additional “red tail”. Spectra have been normalised for comparison.

The accompanying plots show representative spectra from batches received at NPL. Figure 5.1 shows typical emission spectra. In most cases, the desired spectral shape is apparent, except for batch 5, which also proved to be unstable (see Figure 5.6). The broad peak in the red may be indicative of the aggregation of the quantum dots, increasing their effective size. The emission spectrum is one of the key characteristics in this application; if the spectrum is not narrow and Gaussian, it is not suitable for further consideration.

Also measured for each batch were the absorption (Figure 5.2) and excitation (Figure 5.3) spectra. The main function of the absorption spectra is to ensure that the absorbance of the quantum dot preparation is 0.1 or less. This is necessary to prevent inner filter effects, which would cause errors in the measurement of fluorescence. However, the spectra shown here are normalised for comparative purposes. The excitation spectra have a complex shape, but the most important feature is that the excitation profile is relatively broad. This means that a relatively free choice of excitation wavelength is possible, and that one wavelength will effectively excite

different batches of nanocrystal. Again, the spectra shown here have been normalised for comparison.

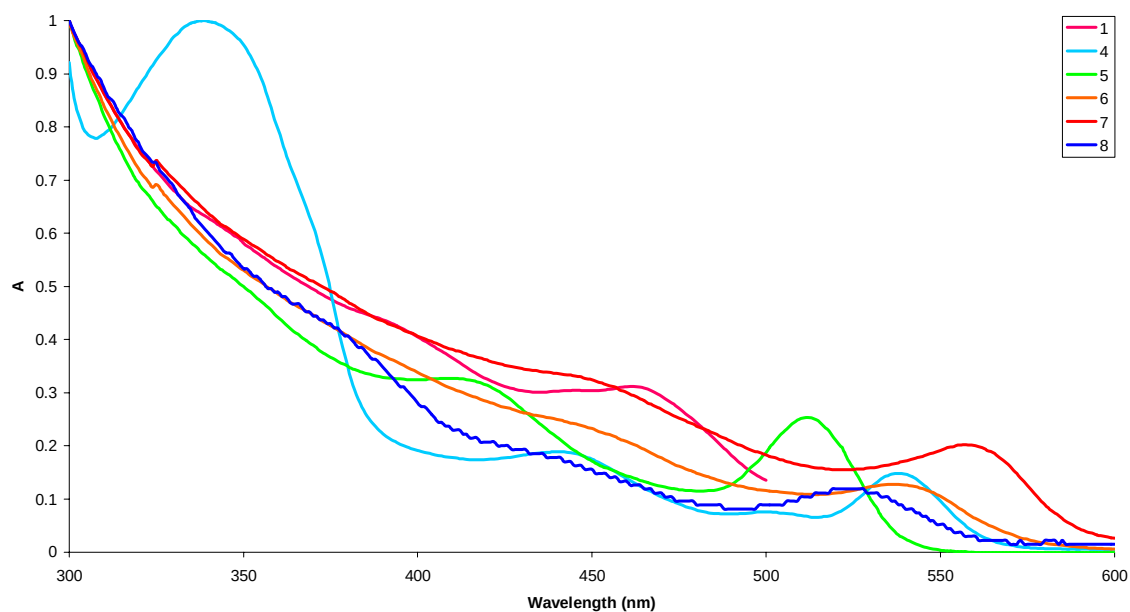


Figure 5.2 Representative absorption spectra of quantum dot batches. Spectra have been normalised for comparison.

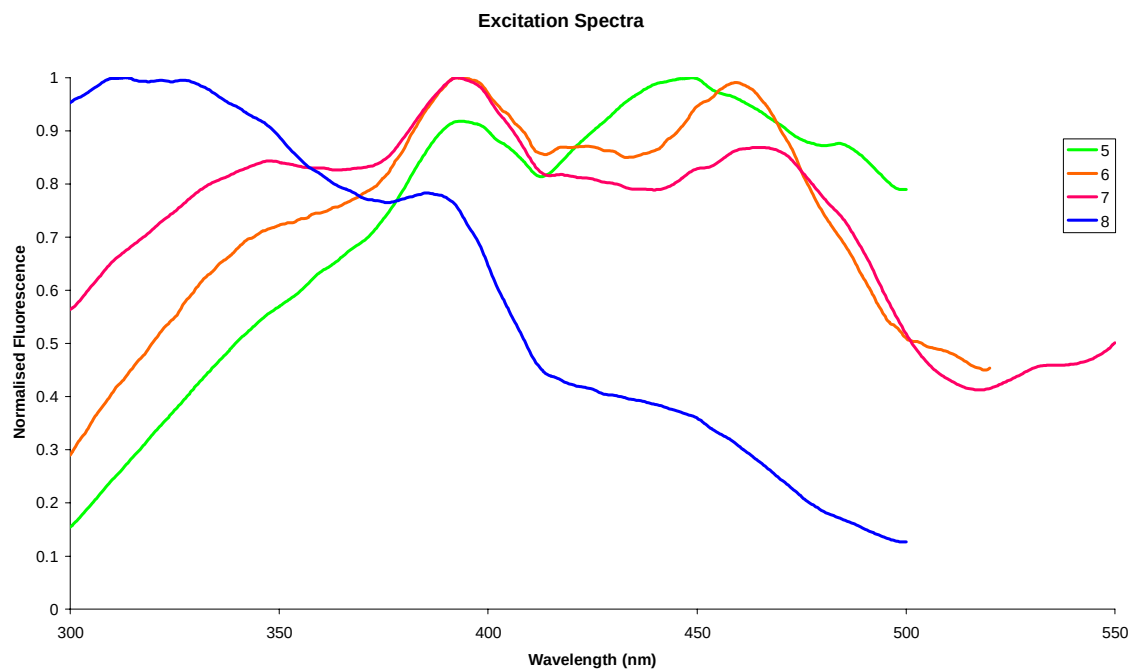


Figure 5.3 Representative excitation spectra of quantum dot batches. Spectra have been normalised for comparison.

5.2. Stability

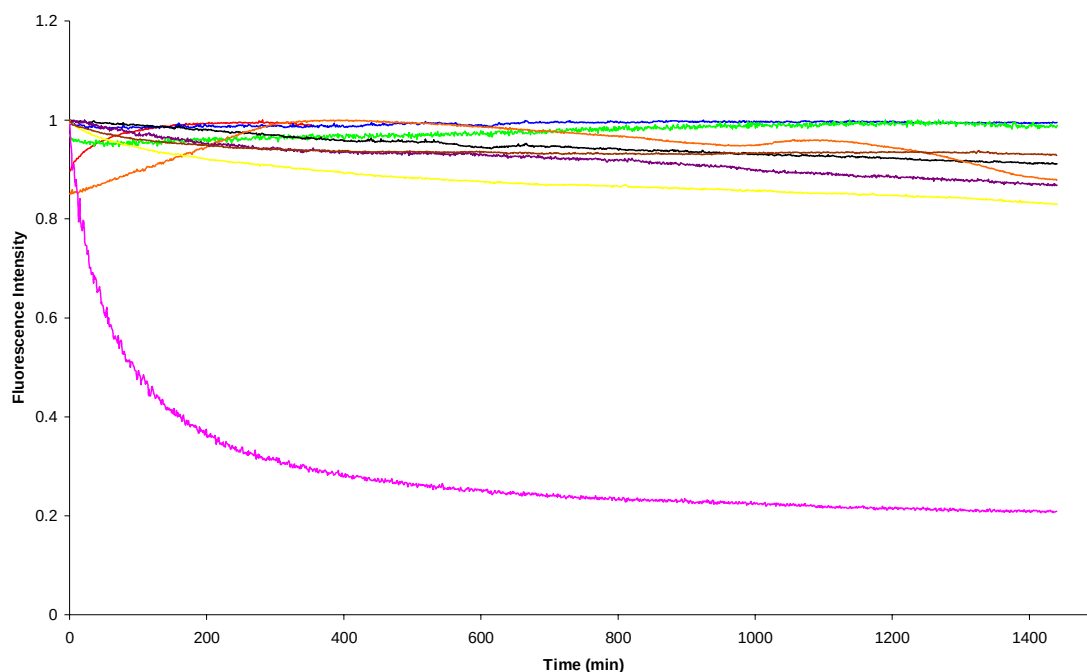


Figure 5.4 Representative plots of intensity stability of nanocrystal batches.
Note that in many cases the lines reach a plateau or decline slowly. In others, a more rapid intensity decay is apparent.

All batches were characterised for their stability over approximately a day, with a 10% illumination duty ratio. Typical results are presented in Figure 5.4. One batch shows a very rapid decline in intensity – this is due to precipitation of the nanocrystals. Other batches show smaller changes, with many showing extremely good stability. Some batches show an initial increase in intensity; this may be due to a photoannealing process. While stability over 24 hours does not necessarily indicate performance over a long time period, this experiment does give an indication of the response of the particles to illumination at an accelerated rate.

Since the intended application in this project was standards for fluorescence wavelength, it was also important to evaluate whether the emission spectrum of the nanocrystals showed any shifts over time. Example results are seen in Figure 5.5 and Figure 5.6. Broadly speaking, the wavelength stability of most batches was excellent. The chief stability problem encountered was the tendency of some batches to precipitate.

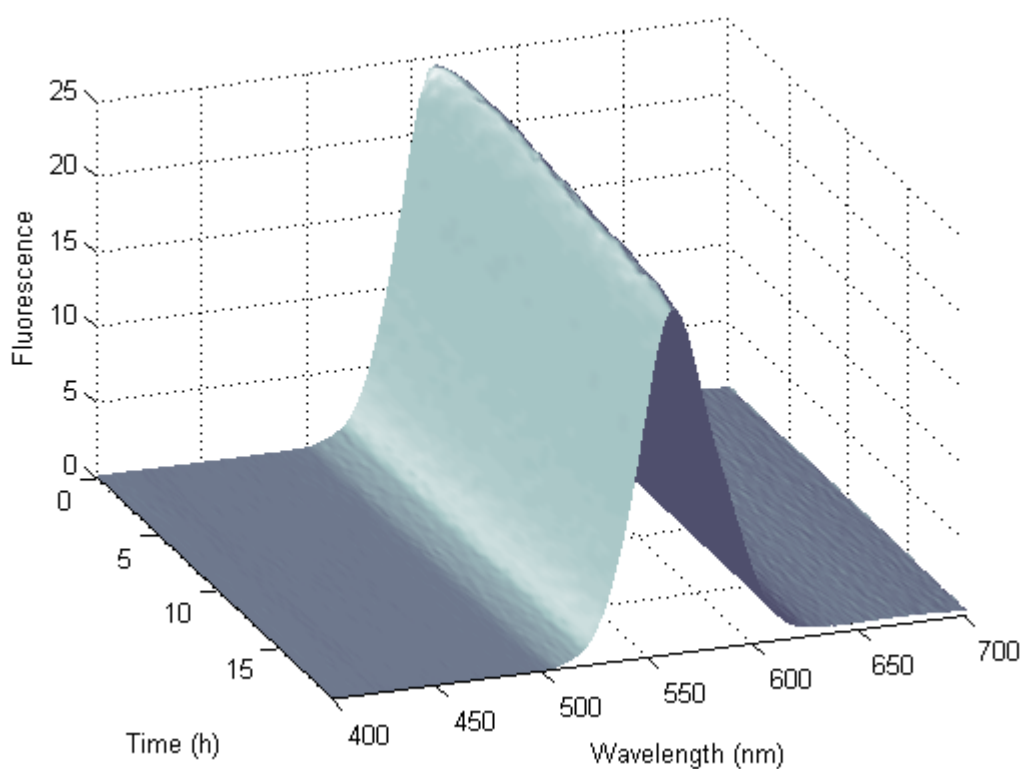


Figure 5.5 Spectral stability surface plot of a typical stable batch of nanocrystals. A small decline in peak intensity is apparent, but the spectral shape remains consistently Gaussian, and there are no significant wavelength shifts.

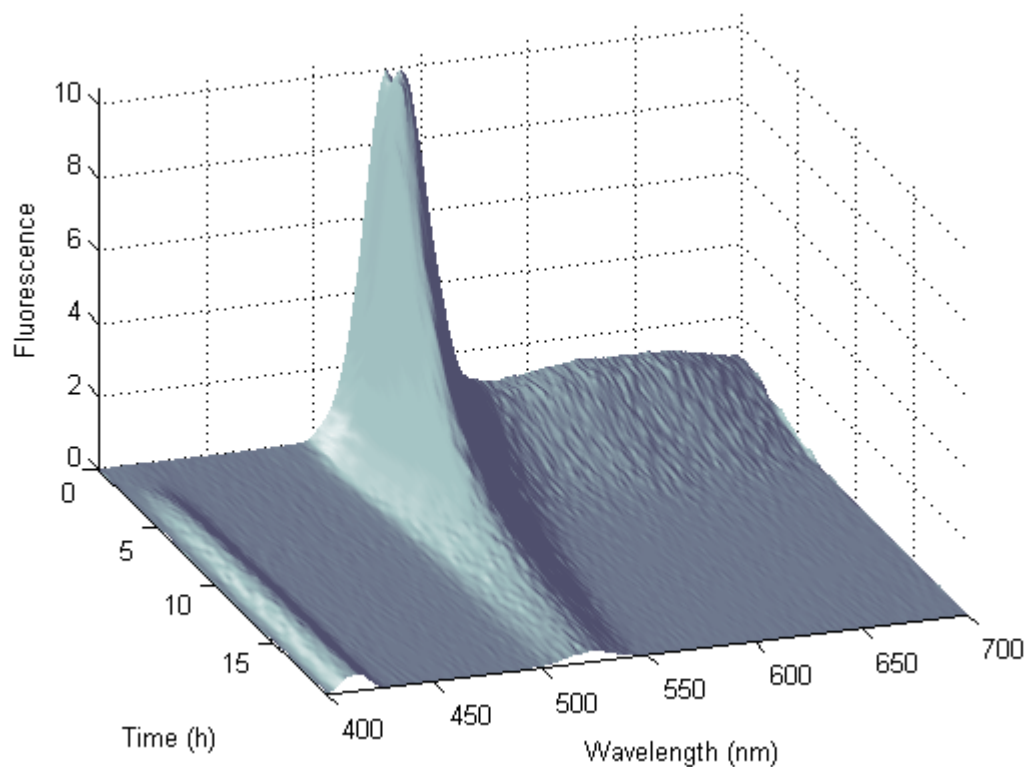


Figure 5.6 Spectral stability surface plot of an unstable batch of nanocrystals. A rapid decline in peak intensity is apparent, together with changes in spectral shape. Note that this is batch 5 in Figure 5.1.

5.3. Temperature Effects

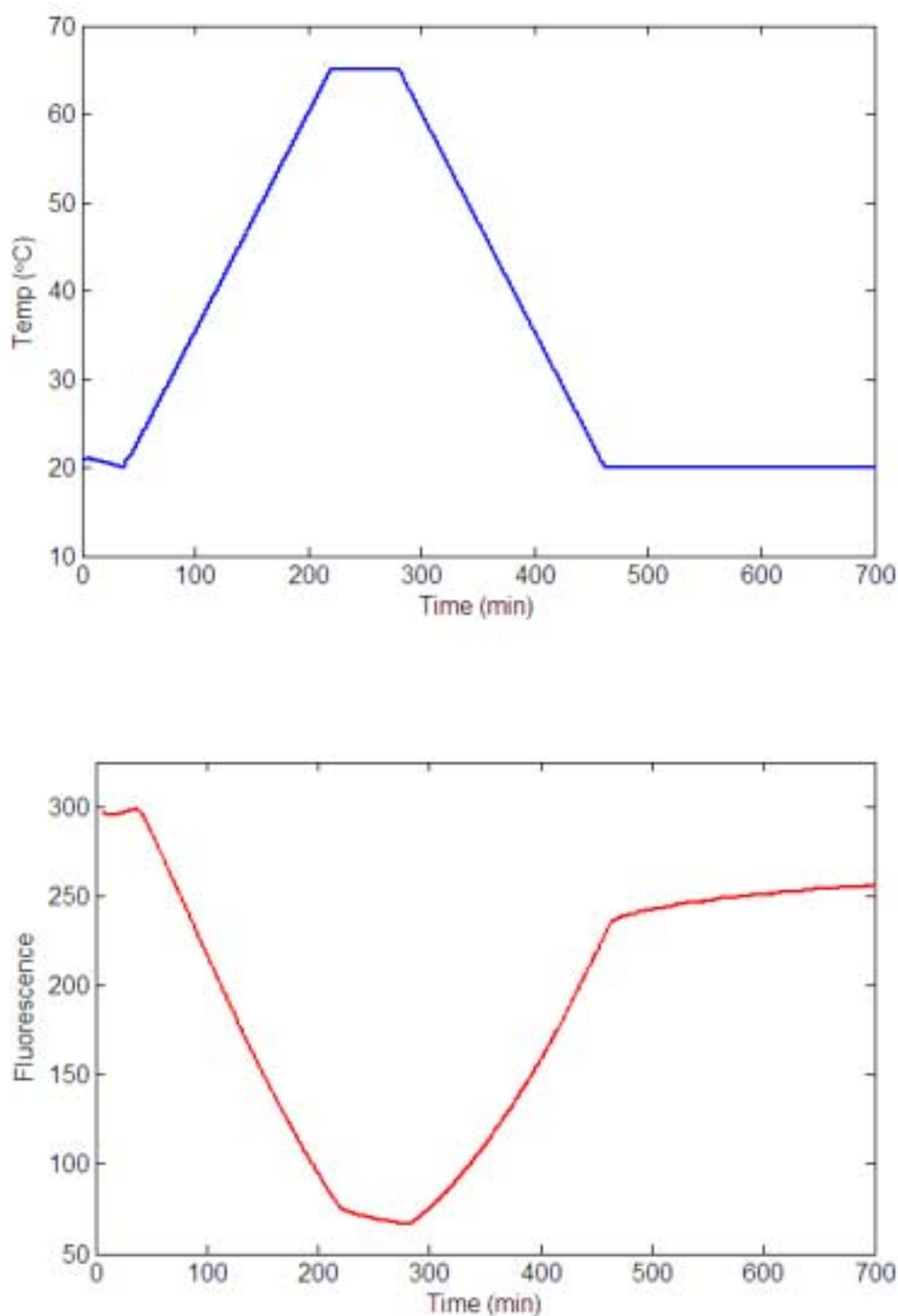


Figure 5.7 Temperature and fluorescence time series from hysteresis experiment.

The response of the quantum dots to temperature was investigated as described in section 9.2.2. The instrument parameters used are presented in Table 9.6, and the temperature cycle is given in Table 9.7 and illustrated in Figure 9.1. The response of a typical batch of quantum dots is shown in Figure 5.7, above. It will be seen that there

is a decrease in fluorescence with increasing temperature, and a recovery on cooling. However, the recovery is not complete; in other words, there is a hysteresis. The data are re-plotted as temperature vs. fluorescence in Figure 5.8. The hysteresis effect is very clear. It is also apparent that changes continue to occur at constant temperature, albeit at a slower rate. Previous studies have shown that almost complete recovery can be achieved with continuing illumination.

All batches that were characterised exhibited this hysteresis effect, as did the liquid standards. However, very little hysteresis was apparent in the solid standards materials. Previous work at NPL has shown that, although there is a small shift on peak wavelength with temperature, no hysteresis effect is observed. Therefore this phenomenon does not cause problems for wavelength standards materials, as long as they are measured at a constant temperature.

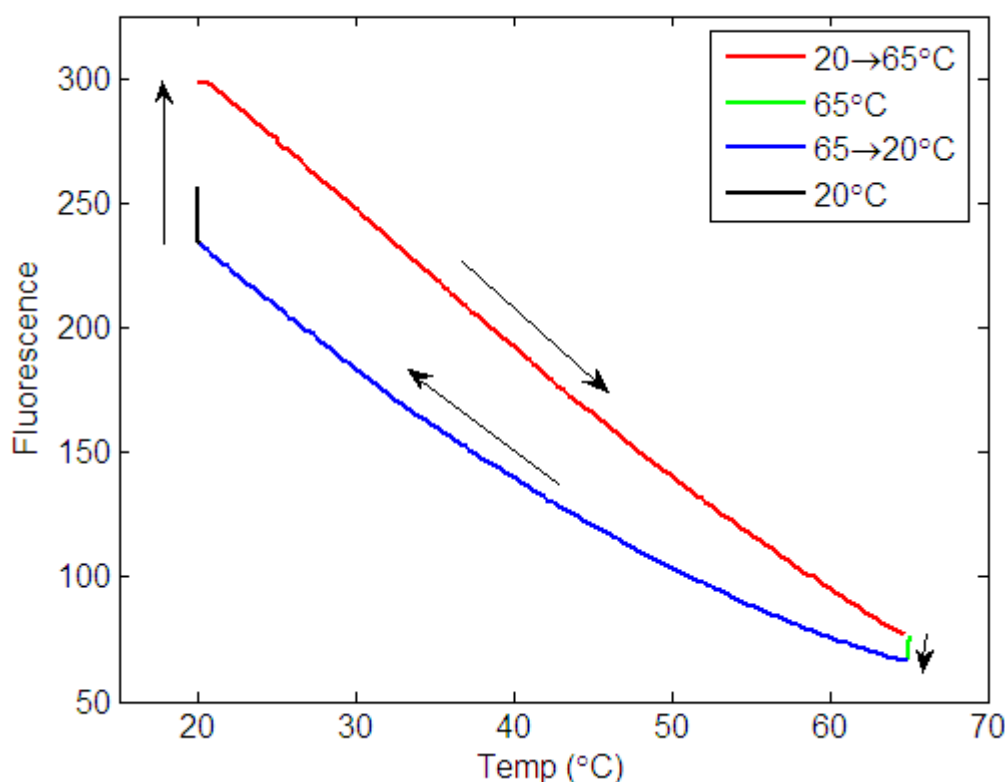


Figure 5.8 Typical temperature hysteresis curve of a batch of nanocrystals. Arrows indicate the pathway through the diagram. Fluorescence drops during heating from 20 to 65°C (red) and continues to drop slowly when held at 65°C. Fluorescence recovers during the cooling phase (blue) but reaches only 91% of the initial value. Further exposure to light at 20°C causes a slow recovery (black).

6. Production of Liquid Standards

6.1. *Introduction*

The simplest route to the production of standards based on quantum dots is to produce sealed cells. Here, the cells are simply flame sealed under argon or nitrogen. The advantages of this method are readily apparent:

- ✓ no solvent evaporation
- ✓ removal of oxygen, preventing photo-oxidation
- ✓ no need for sample preparation before use
- ✓ no safety issues as solvent and quantum dots are contained

However, there are potential problems also:

- ✗ the sealed cells are relatively fragile and must be shipped and handled with care (as with conventional cuvettes)
- ✗ the production process may change the characteristics of the quantum dots
- ✗ the quantum dots are not protected against aggregation

6.2. *Formulation of mixed samples of quantum dots*

The properties of single batches of quantum dots in toluene solution are described in section 5. However, in the production of mixed-wavelength standards, it is necessary to produce mixtures of different wavelengths of quantum dots. This potentially raises new issues. When mixing batches, one would assume that the emission spectrum of the mixture would simply be the sum of the spectra of the individual components. However, one might also speculate that additional phenomena would come into play. For example, a phenomenon commonly observed with organic fluorophores is fluorescence resonance energy transfer (FRET). Here, two fluorophores interact such that energy is transferred from a dye whose emission spectrum overlaps with the excitation spectrum of a second dye. The net result is a quenching of the emission from the shorter wavelength dye, and increased emission from the longer wavelength dye. However, it is not clear whether we would expect to see this phenomenon with a mixture of quantum dots, as the distances involved must be extremely short for FRET to occur.

In fact, the results first obtained when mixing quantum dots were different from either of the above possibilities (Figure 6.2). Two batches were mixed, one (Y6) with a peak emission wavelength of 615 nm and capped with TOPO, the other (N3) with a peak of 546 nm and capped with HDA. The plot shows the emission spectra of these two batches in green and red, respectively. The orange line on the plot indicates the sum of the two emission spectra – which would be the expected spectrum of the mixture. The blue line shows the observed emission spectrum of a mixture, with both batches of quantum dots at the same concentration as in the separate samples. It is immediately apparent that there is a very significant difference in the expected and observed spectra. Although the red peak is similar in intensity, the intensity of the orange peak is very much greater than would be expected (approximately ten times). This cannot be explained by FRET, as one would expect the intensity of the orange peak to decrease and the red peak to increase.

What actually appears to be happening is that the Y6 batch has an excess of capping ligand (TOPO), which is enhancing the quantum yield of the N3 batch.

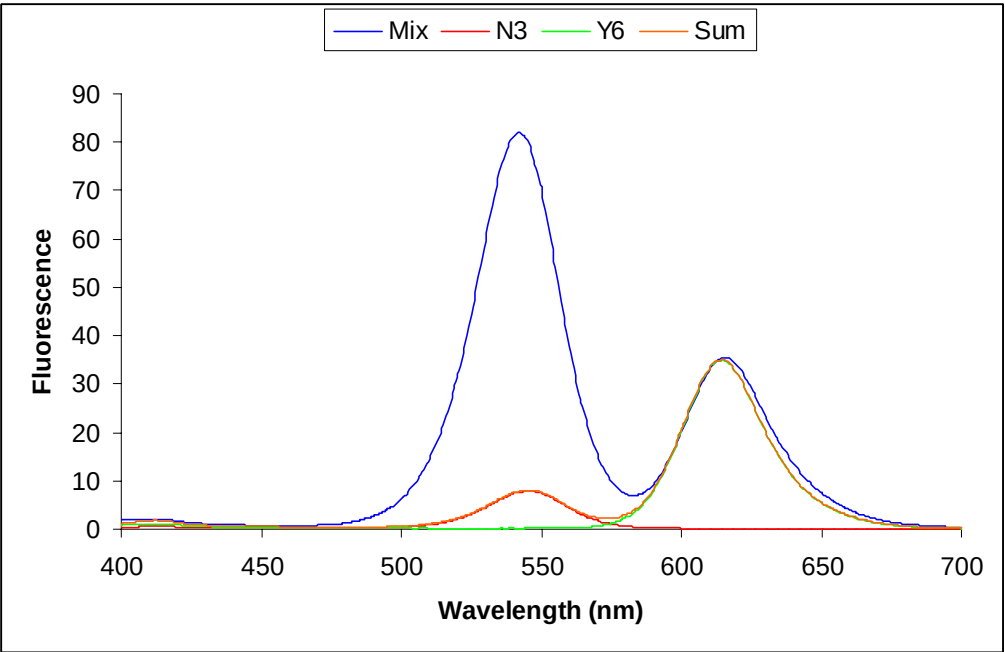


Figure 6.1 Spectra obtained on mixing samples of quantum dots

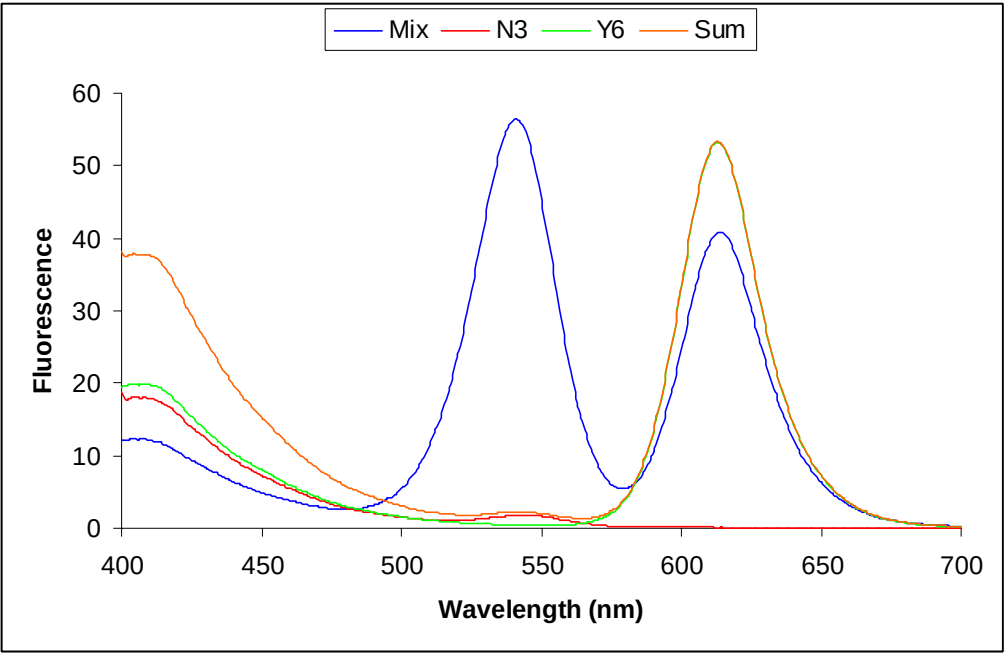


Figure 6.2 Spectra from mixtures of quantum dots after storage

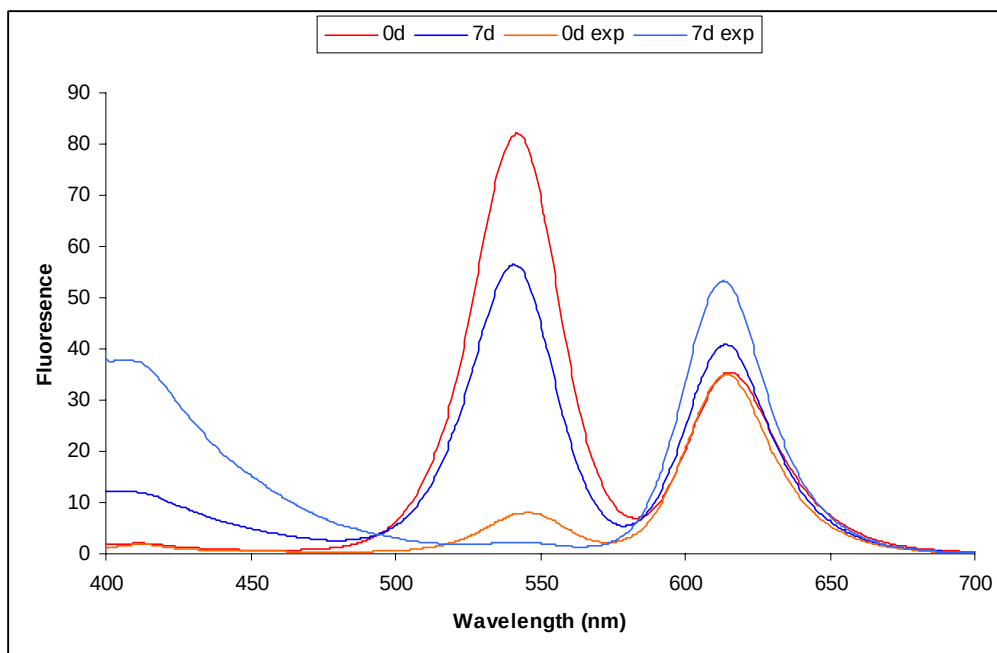


Figure 6.3 Changes in quantum dot spectra on storage.

6.3. Sealed-cell standards

6.3.1. Production of Sealed-cell standards

Two batches of cells were produced by Optiglass using a proprietary method, in which the quantum dot samples, dissolved in toluene, were flame-sealed into quartz cuvettes. These cells were characterised and the results are presented in the following sections.

6.3.2. Single wavelength standards

6.3.2.1. Characterisation



Figure 6.4 Prototype single-wavelength sealed cell standards artefacts.

Four sealed-cell standards were received from Optiglass (Figure 6.4) and were further characterized for their spectral properties and stability. Figure 6.5 shows a comparison of the emission spectra of the source quantum dot sample and the standard. There are clear changes in the shape and position of the peak in the standard. The peak wavelength shows a blue shift (from 545 to 524 nm) and a distinct broadening (FWHM changes from 35 to 62 nm). Closer examination also shows that the spectrum of the standard is less Gaussian than the source sample. These results suggest that the production process has resulted in increased polydispersity and reduced size of the quantum dots. The mechanism of this change requires further investigation, although it is most likely due to the heating during the flame-sealing process. However, the properties of the standard, although slightly degraded compared to the source material, are still suitable for use as a wavelength standard.

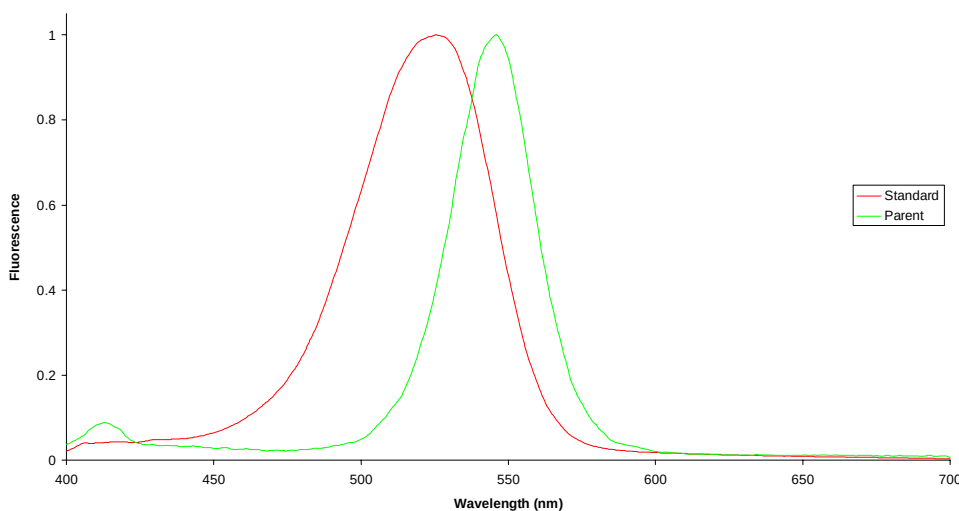


Figure 6.5 Spectrum of sealed cell standard compared to source quantum dot sample.

6.3.2.2. Short-term Stability

The stability of the liquid standard was evaluated over a short time period (approximately 20 hours). As Figure 6.6 shows, spectral changes were observed over this time period: a blue shift (Figure 6.7) a broadening of the peak (Figure 6.8) and an increase in intensity. These phenomena may be due to a photo-annealing process.

The magnitude of the wavelength shift is of particular concern, particularly as a linear trend is apparent with no signs of saturation. The magnitude of this effect is surprising. The characteristics of the sample appear to have been altered by the flame sealing process (Figure 6.5) and it may be that these effects would not be a concern over longer time periods.

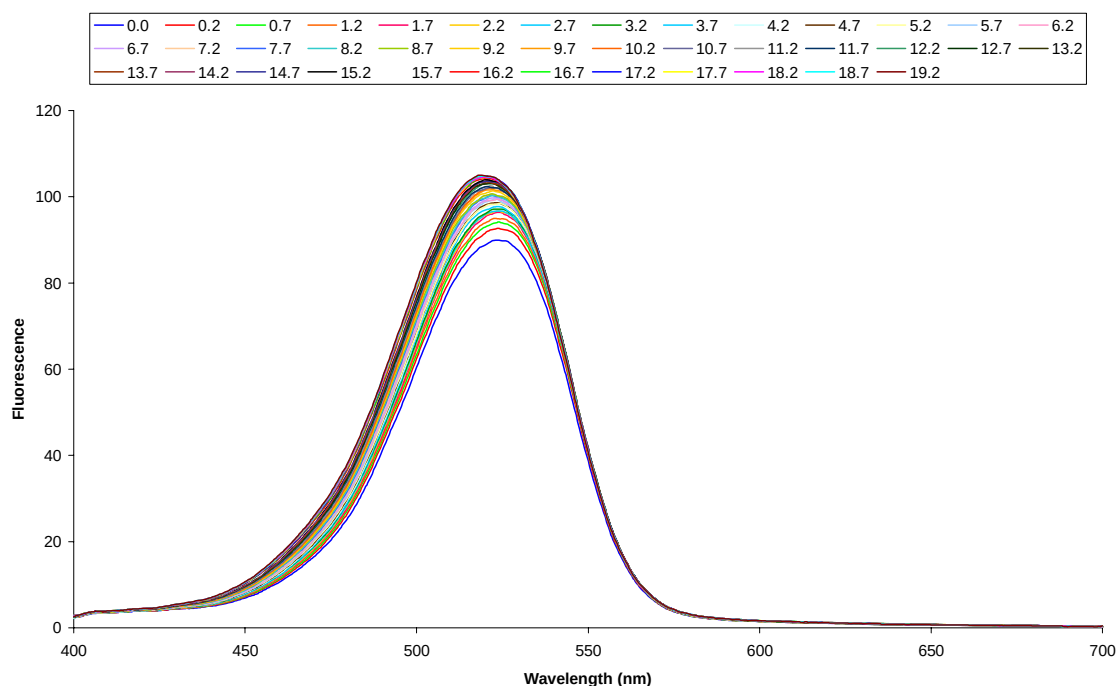


Figure 6.6 Spectra obtained in short-term stability study of sealed-cell standard. Legend indicates time in hours. Note the apparent increase in intensity and peak shift.

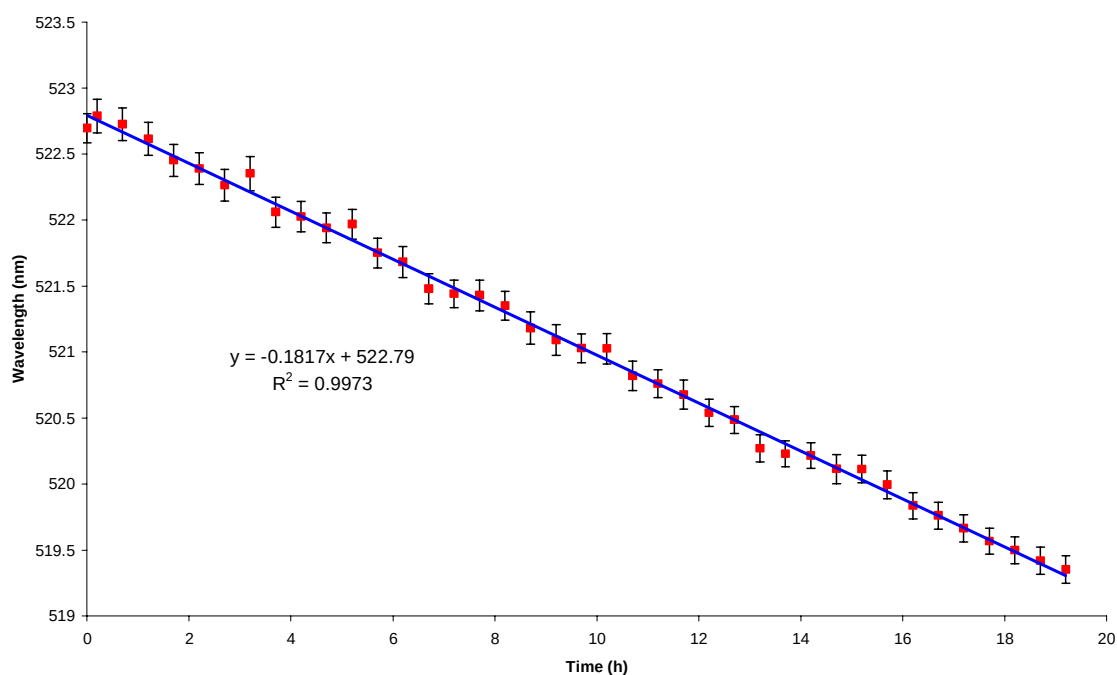


Figure 6.7 Peak Wavelength in short term stability study of sealed-cell standard. Note the steady shift towards the blue ($\sim 0.2 \text{ nm}\cdot\text{h}^{-1}$), giving a total shift of -3.5 nm over the 19.2 h experiment. Data are from Gaussian fits to spectra in Figure 6.6; error bars indicate 95% confidence limits of fit. Blue line is a linear fit with the equation displayed.

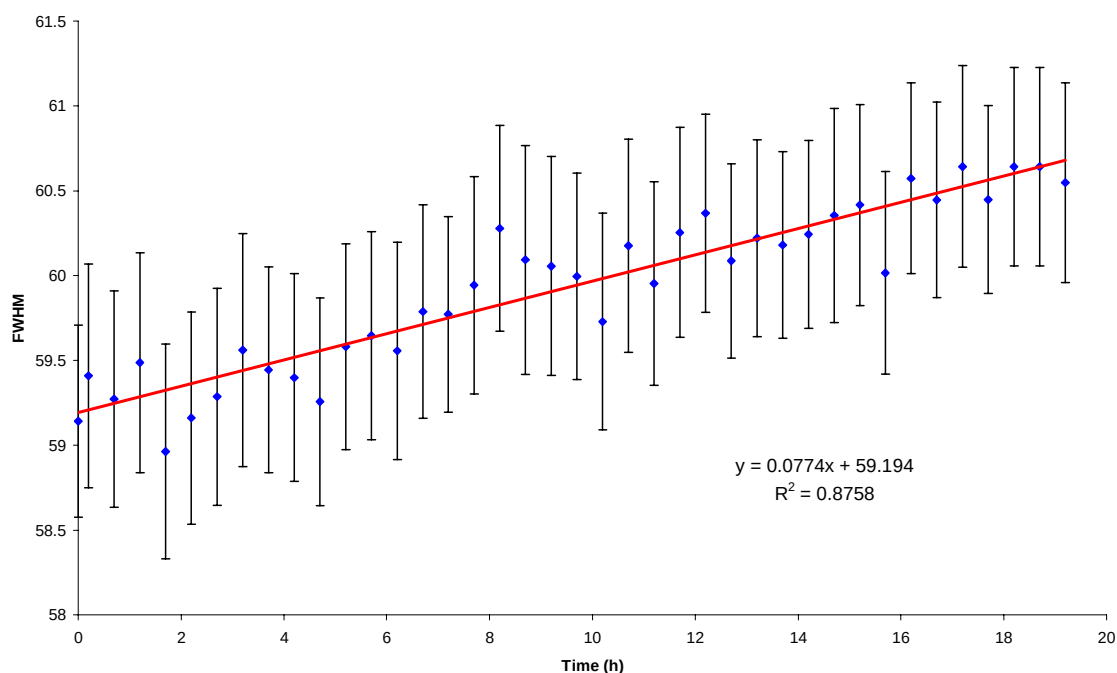


Figure 6.8 Peak width (FWHM) in short-term stability study of sealed-cell standard.

As before, this data is derived from Gaussian fits to the spectra; error bars indicate 95% confidence limits of fit; and the red line is a linear fit to the data. There is a slight trend to increasing peak width during observation.

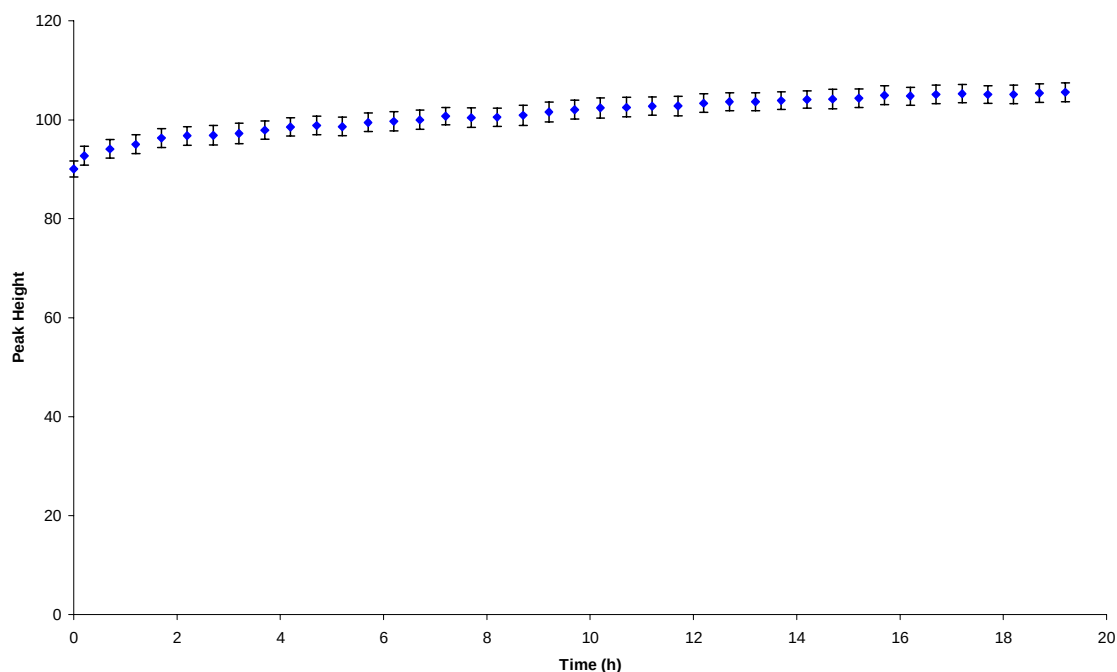


Figure 6.9 Peak Height (Intensity) in short term stability study of sealed-cell standard.

As before, this data is derived from Gaussian fits to the spectra and error bars indicate 95% confidence limits of fit. The intensity of the fluorescence appears to increase steadily throughout the experiment.

6.3.2.3. Long-term Stability

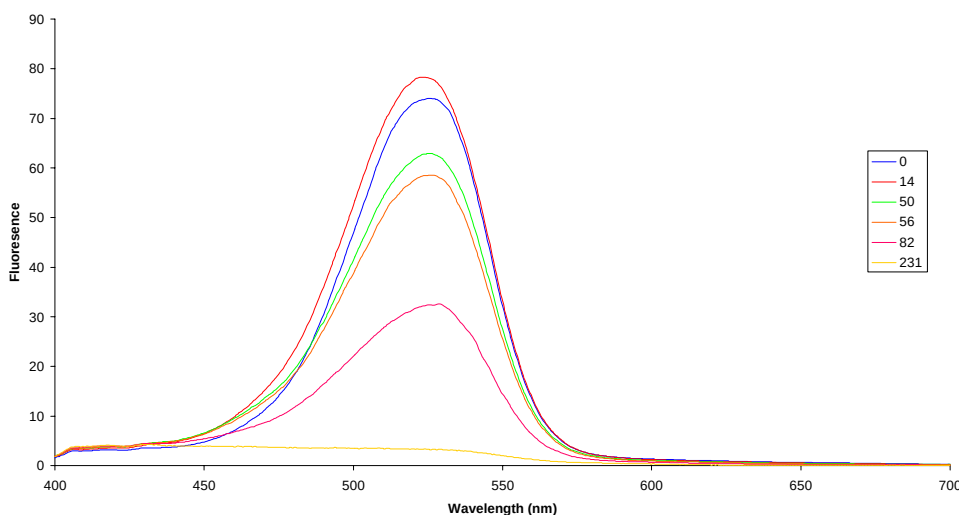


Figure 6.10 Long-term stability of sealed-cell standard.

The legend indicates the time in days when the spectrum was acquired.

The stability of the standard was characterized over a period of seven months. During this period, the artefact was stored in the dark at room temperature (21°C) between observations. The spectra obtained are shown in Figure 6.10. The spectra remain reasonably Gaussian in profile until at least 82 days, although they develop an increasing “tail” towards blue wavelengths. The intensity declines markedly over the course of time, until at 231 days the intensity is extremely low, and the Gaussian shape is lost. A more detailed analysis of the first 82 days of the experiment is presented in Figure 6.11. It can be seen that there is a small (5%) increase in the peak intensity after two weeks; this may be due to photoannealing or a similar process (see above). Thereafter the intensity declines until 83 days where it is down to 43% of the initial value. As is apparent from Figure 6.10, this decline continues until barely any fluorescence remains. In contrast, the wavelength stability over the course of 82 days is excellent, with a less than 3 nm range of observed values. It is not clear from the data whether there is a slight trend towards increasing wavelength, or whether the observed variation is due to measurement noise. A 15% increase in the spectral width is observed over the same time scale.

The mechanism of the observed changes in the properties of the quantum dots is not clear. The most striking change is in the intensity of the fluorescence. This decrease seems unlikely to be due to photo-oxidation, since there is no apparent blue shift in the spectra. This shows that the process of sealing the cells under an inert atmosphere has been successful at removing oxygen. A more likely cause is simple precipitation of the quantum dots, due perhaps to degradation of the capping ligands. However, no precipitate was visible in the cells, although such a small quantity of material might not be apparent.

It is therefore clear that although the long-term stability of the samples is much improved, the “shelf life” of the standard is probably limited to around 3 months. Further improvement could no doubt be obtained by the use of suitable additives, as described in section 4.

The contrast with the results obtained in the short-term stability trials (above) is very striking, and most likely indicates that different processes are at work in each case.

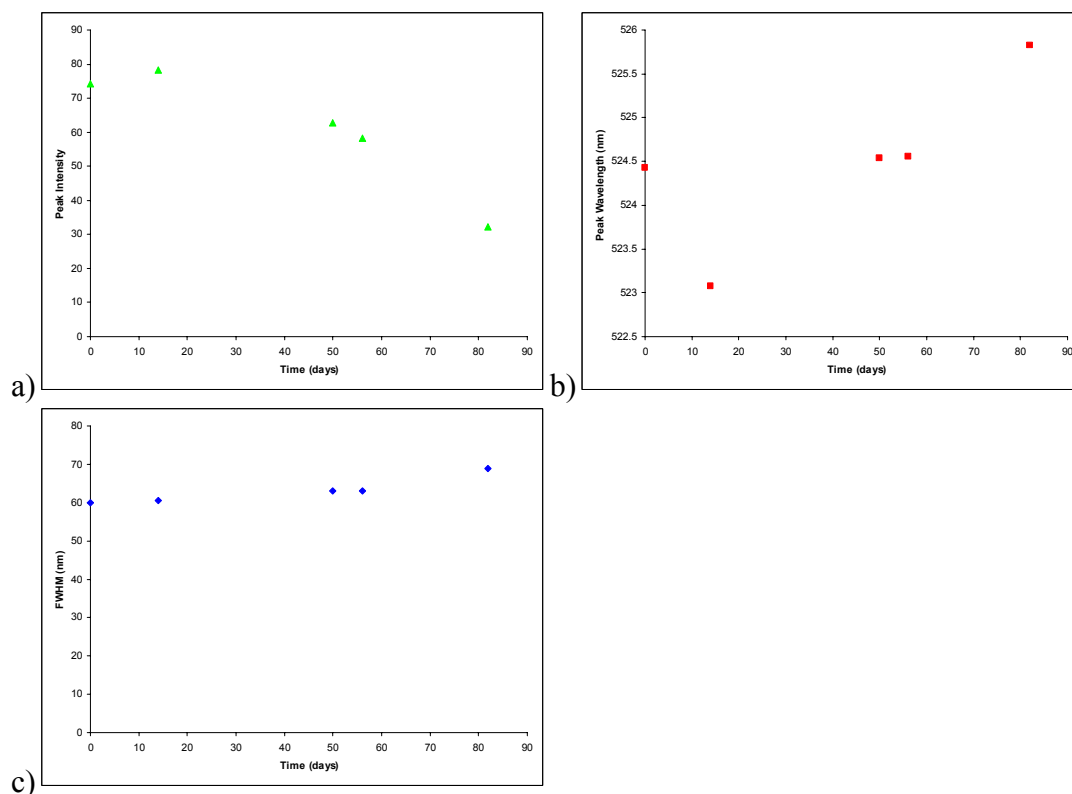


Figure 6.11 Long-term changes in characteristics of sealed cell standard.

a) Peak intensity; b) Peak wavelength; c) FWHM.

6.3.3. Mixed wavelength standards

A batch of mixed-wavelength standards (Figure 6.12) was produced as described above, using mixtures of the same batches characterised in section 6.2.



Figure 6.12 Prototype multi-wavelength sealed cell standards artefacts

The spectra of the four standard artefacts are compared in Figure 6.13. It is immediately apparent that there is a significant degree of variation in the intensity of the fluorescence from the four cells. This is somewhat surprising, since they were produced at the same time from the same starting material. It seems likely that the variation may be due to the degree of heating of the samples at the time of the flame sealing of the cells. Alternatively, it may be due to different light exposure of the samples, resulting in photoannealing or other effects.

The spectra are normalised and superimposed in Figure 6.14. It is immediately apparent that while the longer wavelength peak superimposes almost exactly, there has been some change in the relative intensity of the two peaks. This may be attributed to the same effects as mentioned previously, although the differential effect on the two populations of quantum dots is surprising.

The normalised spectra of the sealed cell standards are compared with the source mixture in Figure 6.15. It is immediately apparent that the emission peaks show significant blue shifts, as in the single wavelength standards. There is also a significant broadening of the green peak, as seen previously (section 6.3.1); this is likely to be due to similar mechanisms.

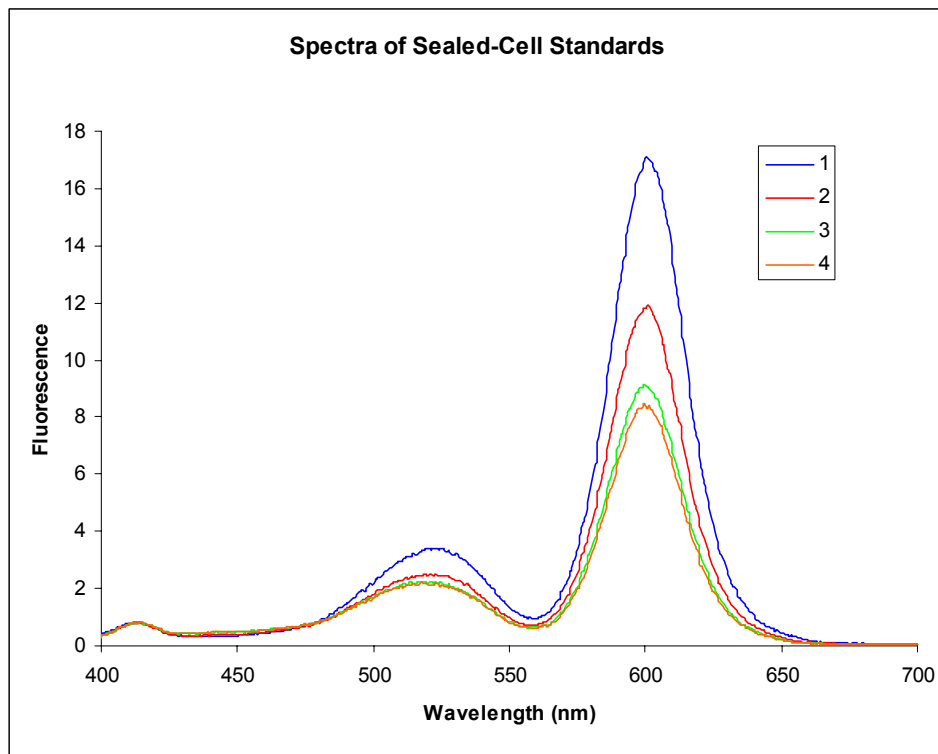


Figure 6.13 Spectra of multi-wavelength standard artefacts.

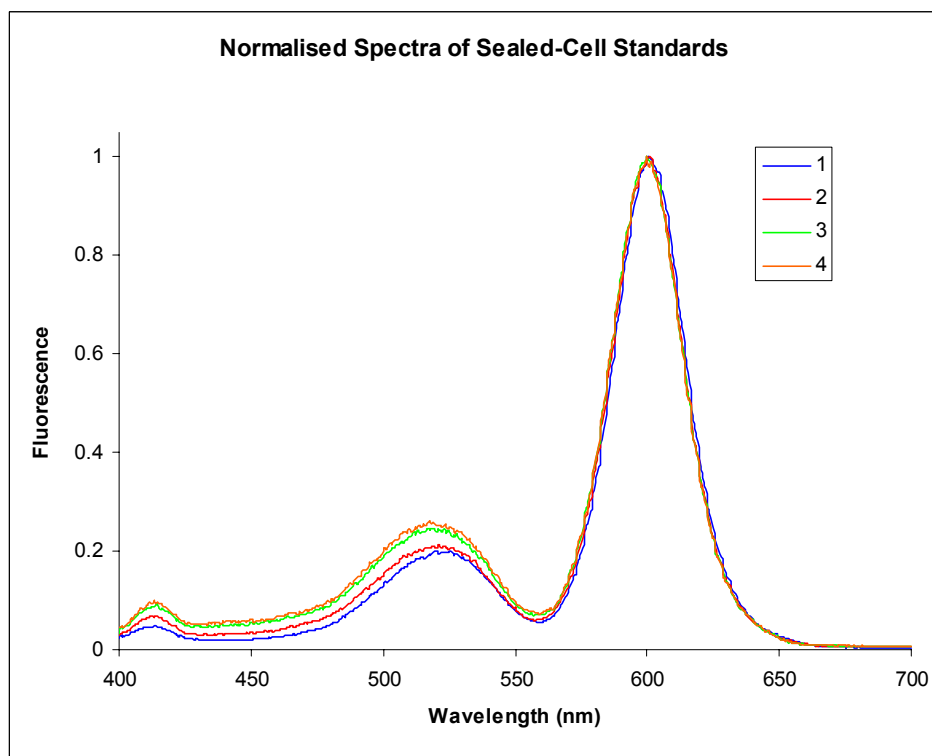


Figure 6.14 Normalised spectra of multi-wavelength standards artefacts.

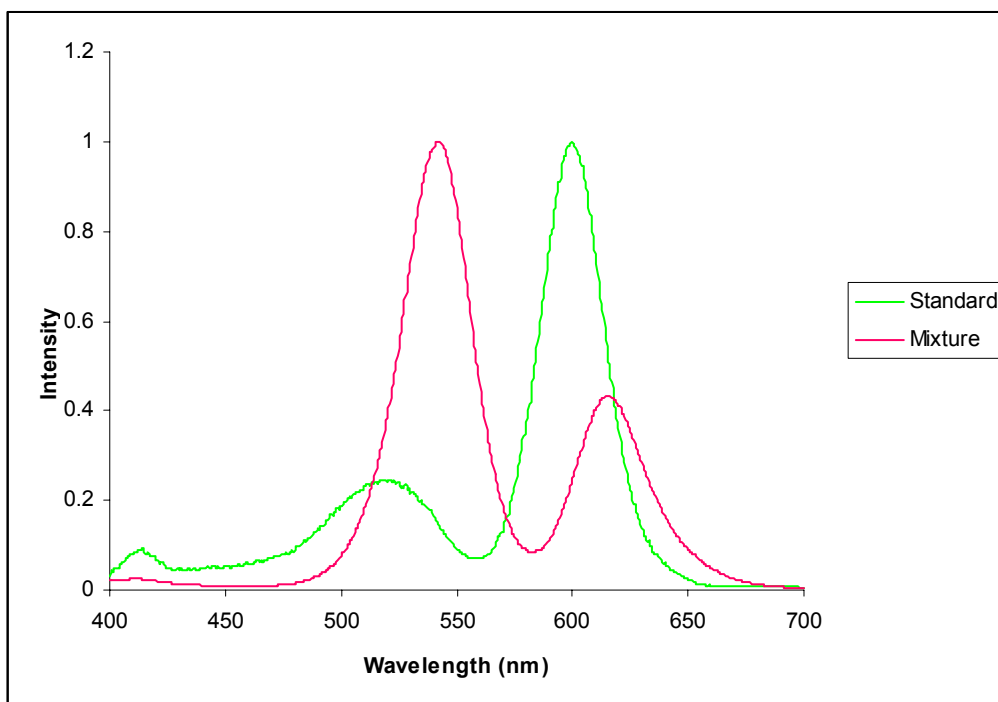


Figure 6.15 Comparison of standard spectra with source mixture.

6.4. Conclusion

The production of sealed cells seems a viable method of producing fluorescence wavelength or spectral standards based on quantum dots. The production process, although it may cause some wavelength shifts, does not significantly damage the fundamental characteristics of the quantum dot spectra. However, the long-term study included here shows that the shelf life of the standards is relatively short. We would suggest that the minimum practical shelf life for a commercial standard should be a year. However, given that a three-month life has been achieved here, this does not seem unattainable, and may well be achieved with the addition of suitable additives. Furthermore, the effects of the production process on the short-term stability of the standards need further investigation; for example, can these effects be eliminated by exposing the standard to light before further characterisation?

7. Production of Solid Standards

7.1. *Introduction*

To achieve the optimum stability of quantum dots for a standards application, it was decided to attempt the production of solid standards materials incorporating quantum dots. By embedding quantum dots in a matrix with suitable optical characteristics, a standard material with maximum stability and ease of use could be produced.

Embedding the quantum dots in a solid matrix should have many advantages:

- ✓ The quantum dots cannot diffuse in the matrix; therefore processes such as aggregation, which are a significant problem with liquid standards, cannot occur.
- ✓ Small molecules such as oxygen and radicals, which may damage the quantum dots, will also have zero, or very low, permeability through the matrix. This should eliminate damage pathways such as photo-oxidation.
- ✓ The standard can be machined into a physical format that is compatible with existing instrumentation (e.g. a cuvette, 96 well plate, etc.).
- ✓ No sample preparation by the user is required, maximising ease of use.
- ✓ No safety issues as there are no solvents and the toxic constituents of the quantum dots are sealed in the matrix.

Possible disadvantages include:

- ✗ Localised photobleaching may result in inhomogeneity of fluorescence.
- ✗ Polarisation effects may occur.
- ✗ The embedding process may change the characteristics of the quantum dots.

Solid standards materials have been available for several years, and usually incorporate organic dyes. Optiglass manufactures such products, typically as a set of six, including dyes with a range of spectral characteristics. Therefore it was decided to attempt to produce similar materials with quantum dots. The results of this work are described in the next section.

7.2. *PMMA standards*

7.2.1. **Production of Standards**

A suitable batch of quantum dots, which had been thoroughly characterised, was dried down from an aliquot containing 8.23 mg of quantum dots. This aliquot was dissolved up in a small volume of toluene, and then diluted into methylmethacrylate to make a final volume of 500 ml. The initiator, (2,2'-azobis(isobutyronitrile), or AIBN, was added and the solution polymerised. The polymerised material was cut into blocks and then optically polished to give standard cuvette dimensions (12.5 × 12.5 × 45 mm).

7.2.2. Characterisation of Standards



Figure 7.1 Prototype PMMA quantum dot standard artefacts.

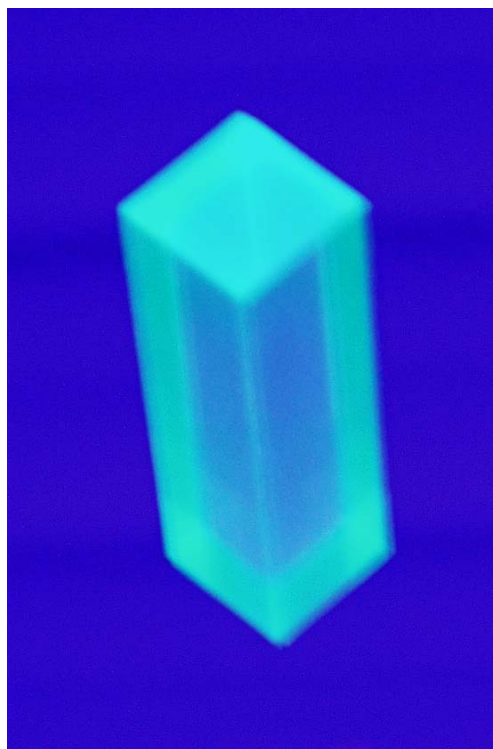


Figure 7.2 Fluorescence from a PMMA Quantum Dot cuvette format standard.

The PMMA standard blocks were shown in Figure 7.1. When placed on a UV light box, bright blue/green fluorescence was observed (Figure 7.2). The blocks were then placed in the spectrofluorimeter for further characterization.

It was immediately apparent that significant changes had occurred in the spectral characteristics of the quantum dots. The orange curve in Figure 7.3 is the emission spectrum of the parent nanocrystal sample, excited at 370 nm; the green curve is the spectrum of the PMMA block excited at the same wavelength. (The two curves have been normalized for comparison). It is immediately apparent that there has been a blue shift of the main emission peak from 547 to 526 nm, together with the

appearance of a broad peak further into the blue region of the spectrum. Furthermore, changing the excitation wavelength changes the relative height of the two peaks. This indicates that a mixture of species is now present. The green peak is maximally excited at approximately 393 nm, whereas the blue peak at ~431 nm is maximally excited at ~353 nm (Figure 7.4). Emission spectra for these excitation wavelengths are also shown in Figure 7.3.

The spectral characteristics of the PMMA standards are clearly not suitable for use in a fluorescence wavelength standard. Furthermore, on exposure to light, the spectrum changed over time (data not shown). Clearly, the polymerisation process has in some way damaged the nanocrystals. What could be the mechanism of this process?

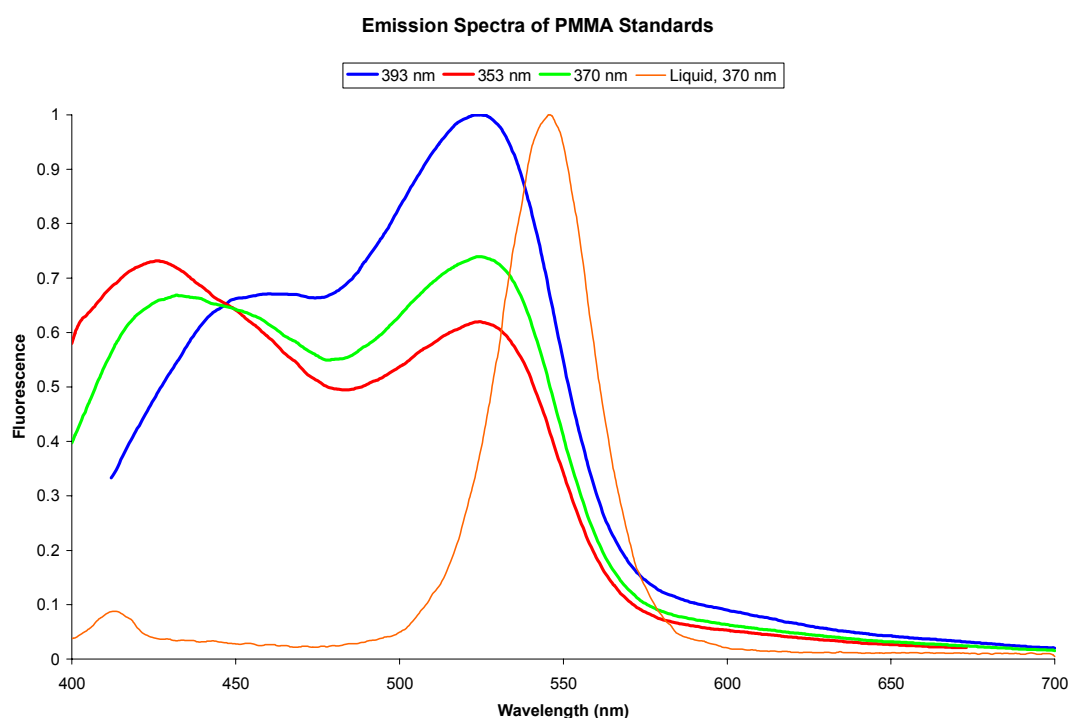


Figure 7.3 Emission spectra of PMMA standards.
The legend indicates the excitation wavelengths used.

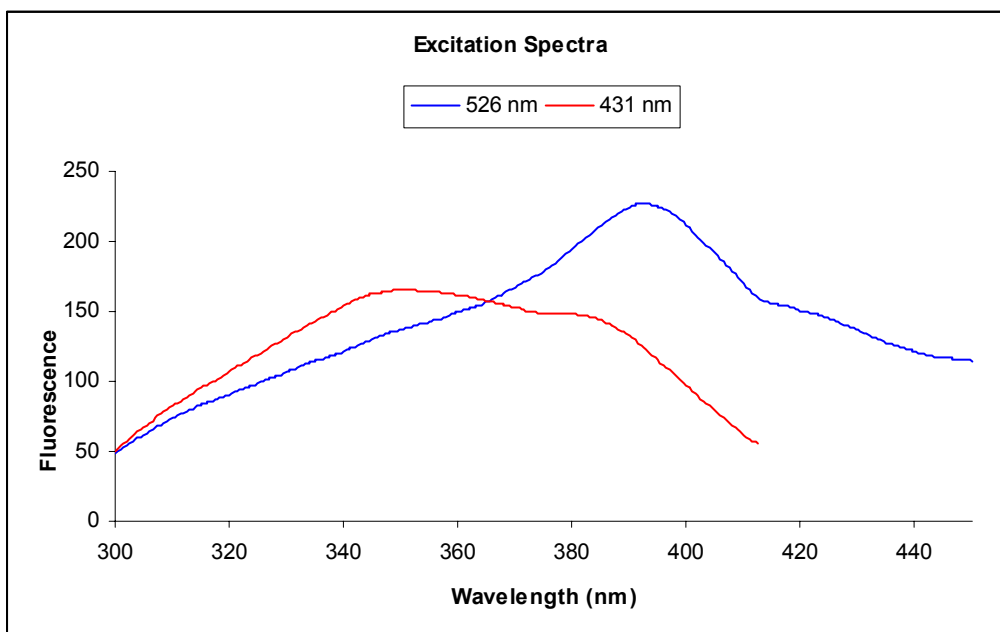


Figure 7.4 Excitation spectra of PMMA standards.
The legend indicates the emission wavelengths used.

Several possibilities suggested themselves:

- Thermal damage
- Oxidation by ambient O₂ during polymerisation
- Damage from radicals produced during the polymerisation reaction

Therefore it was decided to follow the fluorescence of the quantum dots under a variety of polymerisation conditions in an attempt to identify the main factors in the damage process and hopefully to find conditions under which polymerisation could proceed without damage. These efforts are described in the next section.

7.2.3. Investigation of Polymerisation procedure

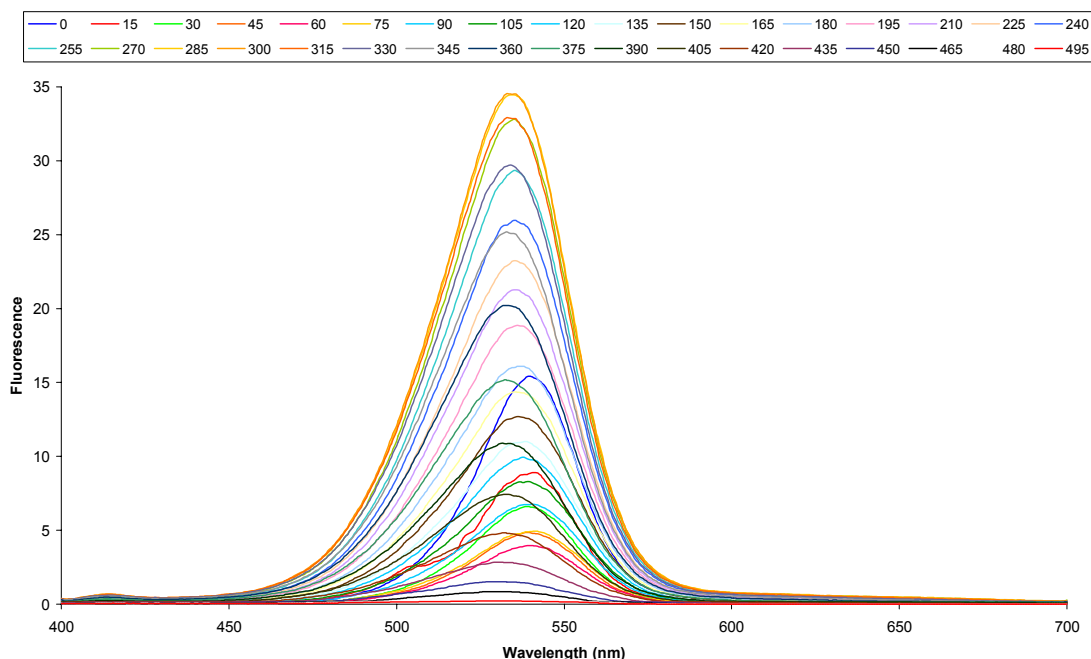


Figure 7.5 Spectral changes in quantum dot fluorescence during PMMA polymerization.

Legend indicates time of acquisition in minutes.

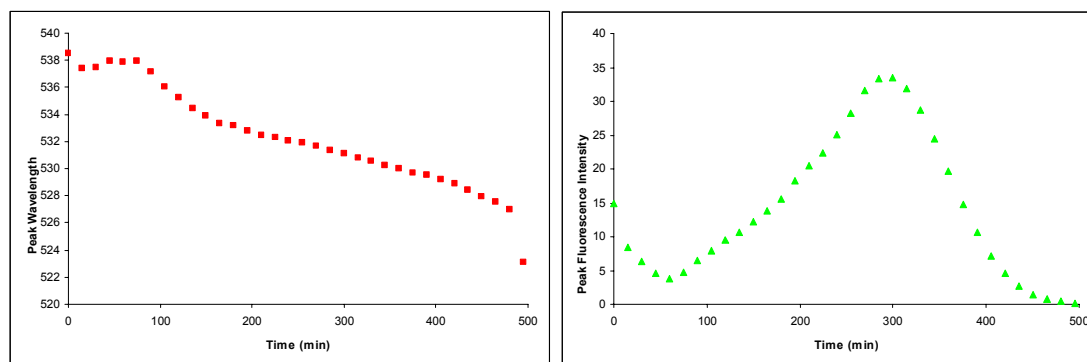


Figure 7.6 Changes in spectral characteristics of quantum dot fluorescence during PMMA polymerisation.

Left: peak wavelength Right: Peak intensity.

The spectral characteristics of the quantum dot fluorescence were observed to change markedly during the polymerisation process. The spectra from a typical run are shown in Figure 7.5, and an analysis is shown in Figure 7.6. It is apparent that during the run, the intensity of fluorescence at first decreases steeply, and then increases. This may be due to an initial decline in fluorescence in response to increased temperature, which has been observed previously, followed by a photo-annealing process. Finally, there is a rapid decrease in fluorescence down to zero. The peak wavelength shows a steady shift to the blue, up until the point where the spectrum ceases to be recognizably Gaussian in shape. This process most likely indicates a physical decrease in the size of the quantum dots.

Over the course of a number of trials, no conditions were found which reproducibly resulted in polymerisation but did not severely damage the quantum dots (data not

shown). It was hypothesized that the damage was most likely due to the radicals produced during the polymerisation process, and therefore, alternative polymerisation methods were sought.

7.3. Investigation of Alternative methods

The team at York carried out an investigation into possible alternative production methods for solid standards materials. Their conclusions are presented in this section.

7.3.1. Poly(methyl methacrylate)

Initial work using PMMA was carried out at Optiglass Ltd. and NPL. Initial attempts were thought to be promising due to the high solubility of nanoparticles in MMA. However, spectra of the encapsulated particles after polymerisation were shown to be significantly inferior to those of solution samples in toluene or chloroform.

It is thought that the reduced quality in these samples is due to a degradation process caused by the nature of the polymerisation process. Polymerisation of MMA in order to form bulk solids conventionally involves the use of a radical mechanism. This involves the initiation of a radical reaction by the thermal decomposition of AIBN at 65°C. It has been shown that if a solution of nanoparticles is heated in the presence AIBN, they are severely degraded. This degradation involves a significant drop in PLQY accompanied by a large shift in emission λ_{max} . These are effects that have been observed in the feasibility studies mentioned above and have been ascribed to photo-oxidation processes associated with the presence of radicals.

Other techniques can be used to produce PMMA, involving the use of anionic living polymerisation reactions. However, there are few examples in the literature that utilise this technique to produce bulk optically clear samples. An attempt was made to produce PMMA using n-butyl lithium to initiate the polymerisation of freshly distilled MMA. This attempt proved to be unsuccessful. However, it was thought that the general technique of anionic polymerisation may be useful and therefore another monomer was considered.

7.3.2. Poly (cyanoacrylate)

Cyanoacrylate polymerises readily in the presence of hydroxide (OH^-). The moisture present in air and on surfaces that are exposed to air provides a sufficient source of this anion to initiate the polymerisation of this monomer. This has led to the use of cyanoacrylates as adhesives in many different applications, since solidification of the monomer readily occurs in ambient conditions. This property makes the use of ethyl or methyl cyanoacrylate a promising candidate for encapsulation of particles due to the ease of polymerisation at room temperature. The optical properties of poly(cyanoacrylate)s are inferior to those of PMMA, having an absorbance cut off of *ca.* 400 nm. However, methods of minimising this problem are discussed later in the section.

7.3.2.1. Block Samples

Unlike MMA, nanoparticles cause the rapid polymerisation of cyanoacrylate if dissolution is attempted. The presence of excess amine in the nanoparticle samples was suggested to be a possible cause of this effect since addition of butylamine to cyanoacrylate initiates rapid polymerisation. It was necessary to prepare samples by dissolving samples of the particles in chloroform and adding the solution dropwise to

a stirred sample of cyanoacrylate. Initially the solutions were cured at elevated temperatures (*ca* 65°C) in order to evaporate the chloroform. However, samples prepared in this manner contained too many bubbles for use in the intended application. The use of vacuum to remove chloroform was also attempted, but the rapid increase of nanoparticle concentration resulted in spontaneous polymerisation and bubbling. In order to reduce the formation of bubbles, samples were allowed to cure at room temperature in the dark.

Samples prepared using this method were bubble free, optically clear, and brittle suggesting polishability. However, fluorescence of these samples was visibly reduced compared to the stock samples in chloroform used in their preparation.

Due to the late stage of the project, the preparation of samples that did not require polishing became important. Solid sample preparation in fluorimeter cuvettes was suggested. Since cuvettes made from PMMA and polystyrene are readily available, preparation of cyanoacrylate samples in these containers was considered. Several problems became apparent. PMMA and polystyrene are easily damaged by chloroform and the cyanoacrylates used. It also became apparent that shrinkage during polymerisation could cause cracking of glass or quartz cuvettes.

7.3.2.2. Thin Layer Samples

In order to overcome the problems mentioned in the above paragraphs the use of a laminate system was proposed. A routine method of obtaining fluorescence spectra of optically dense samples is to place the high optical density sample at an angle that minimises reflection and the emitted fluorescence can be detected in the normal fashion. Therefore layered samples with high concentrations of nanoparticles on glass or quartz can be used to obtain good fluorescence spectra. It is hoped that stabilisation of the nanoparticles can be achieved by a coating of polymer. Since the thickness of this polymer coating is much lower than the path lengths in block samples, the problem of poly(cyanoacrylate)'s higher absorbance at higher wavelengths would be minimised.

Initially, a chloroform solution of nanoparticles was dropped onto a clean glass slide and allowed to dry. Cyanoacrylate was used to cover the layer of nanoparticles. The sample prepared using this method showed good fluorescent properties when viewed by eye. Unfortunately, the nanoparticle layer acted as a barrier between the polymer and the glass, preventing adhesion and allowing separation of the layers. Therefore, samples were prepared by adding a layer of monomer to a glass slide and allowing this to cure. A nanoparticle solution was then dropped onto the layer of polymer and the solvent was allowed to evaporate. A third layer of monomer was spread on top of the nanoparticle layer and a second glass slide was positioned carefully upon the other layers. The device was allowed to cure for a further 18 h. The resulting structure is illustrated in Figure 7.7.

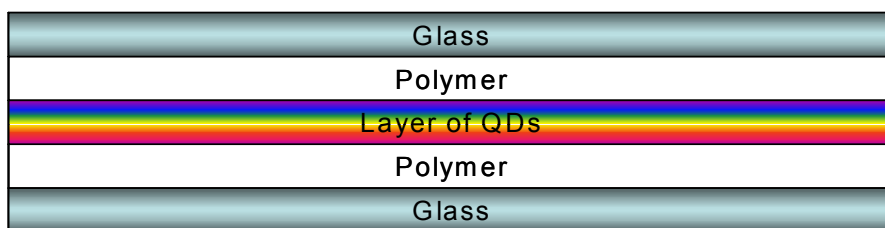


Figure 7.7. Scheme of layered device for use in fluorimeter solid sample holder.

Due to the concentration increases of solutions during evaporation, the nanoparticle layer was not homogeneous. It was shown that roughening of the polymer surface produced samples of improved appearance. Spin coating of the polymer layer followed by spraying of the nanoparticles solution may be a way to improve these systems further and allow for greater control of nanoparticle loading.

Several samples were prepared in the manner outlined above, containing 1-3 different sizes of nanoparticles. Spectra are shown in Figure 7.8.

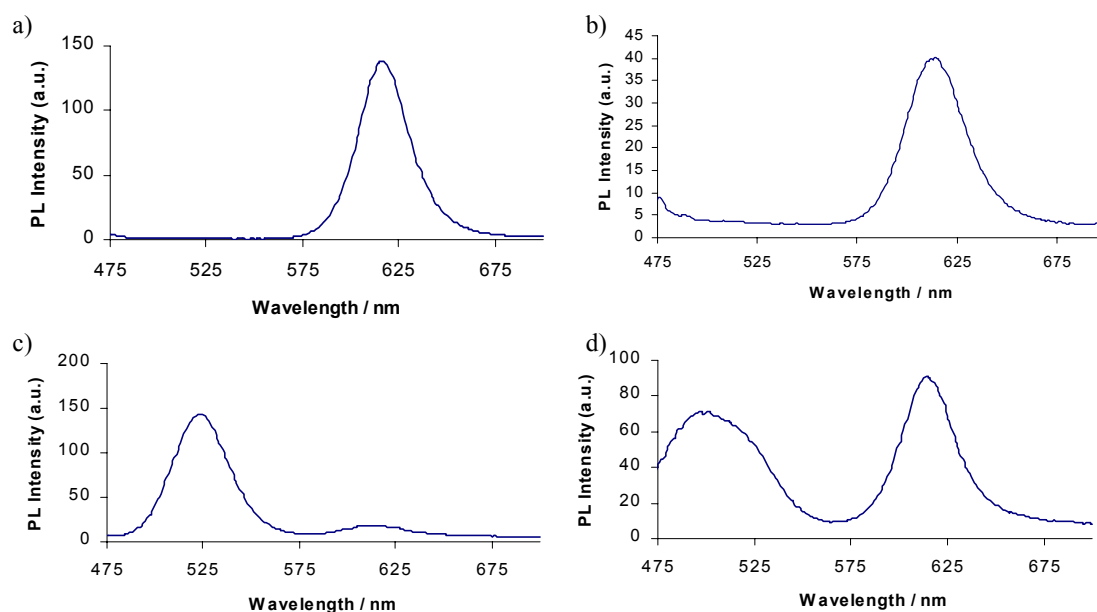


Figure 7.8. Fluorescence spectra of solid cyanoacrylate samples containing quantum dots.

a) 4.2 nm CdSe particles; b) 4.2 nm core CdSe/ZnSe; c) 4.2 nm core CdSe/ZnSe and "green" CdSeS particles; d) 4.2 nm CdSe particles with "violet" and "green" CdSeS particles.

It can be seen from Figure 7.8c and d that the CdSeS particles have a dominant emission in the spectra. Comparison with the single size nanoparticle samples and mixed solutions suggests that this effect is not just a loading or intrinsic PLQY effect. An experiment using the three component system (4.2 nm CdSe particles with "violet" and "green" CdSeS particles) showed that the PLQY of the CdSe particles reduced significantly with time as shown in Figure 7.9.

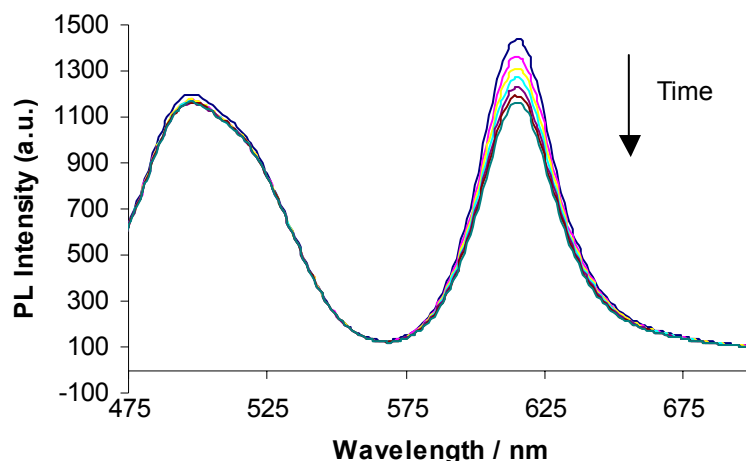


Figure 7.9 Spectra taken over a 10 minute period of a layered sample containing 4.2 nm CdSe particles with "violet" and "green" CdSeS particles.

Since the λ_{max} of the CdSe emission does not shift significantly during the course of the experiment, the degradation of PLQY is not attributed to photo-oxidation. It is thought that the CdSe or CdSe/ZnSe in the mixed samples is interacting with the CdSeS resulting in a reduction of their PLQY. Alternatively, the CdSe and CdSe/ZnSe have different ligands at their surface (TOPO and HDA) than the CdSeS (TOA). It is accepted that at least a proportion of these ligands are labile, [84] and the stability studies reported above have shown that all of these ligands can have significant and variable effects on the PLQY of different nanoparticles. Therefore it is possible that ligand exchange between different types of nanoparticles could cause the observed effect. Further study is required for this to be confirmed. However, if samples containing different colours of particles are to be prepared, the ligand ratio and type in the different samples should be similar.

7.3.3. Epoxy Resin

Epoxy resin was purchased from Intertronics. The epoxy component was of low viscosity and had a long curing time when mixed with the diamine component (16-20 h at room temperature, 1 h at 65°C). Upon curing, a polishable, colourless, optically clear solid was formed without significant shrinkage. These factors make the material ideal for use in encapsulation and optical applications.

Since amines have been shown to increase the PLQY of nanoparticles in the solution studies mentioned above, it was hoped that a positive effect would be observed in the samples of nanoparticles encapsulated using this method due to the presence of the diamine curing agent. The degrading effect of amines in solutions over extended periods of time are thought to be due to amine ligand mobility at the surface of nanoparticles and should not be problematic in solid samples. The optical properties of the epoxy resin are similar to those of poly(cyanoacrylate) and thin layer samples were prepared as well as block samples

7.3.3.1. Block Samples

Initial experiments showed that the epoxy resin did not damage either polystyrene or PMMA cuvettes. Therefore, due to the superior optical properties, disposable PMMA

fluorescence cuvettes were used in order to prepare block samples. The first examples of block epoxy samples involved the dissolution of the nanoparticles in the mixed epoxy diamine at the beginning of the curing process. Due to the relatively high viscosity of the mixture compared to that of organic solvents, dissolution was slow and inefficient. Heating and sonication followed by decanting and separation of undissolved solid by centrifuge was necessary to prepare samples of appropriately high quality.

The first samples prepared using this method contained “green” CdSeS. Unfortunately the cured samples were opaque. Because of these observations other methods of dissolution were attempted as well as other particle types being explored.

It has been shown that the nanoparticles used in these studies are soluble in amines (see section 3). Therefore dissolution of the nanoparticles was attempted using only the diamine component of the epoxy resin. Much higher concentrations of particles were achieved and precipitation did not occur when the epoxy component was subsequently added. This produced a much higher quality of sample and allowed variation of concentration to be explored. When samples were prepared using “Green” CdSeS with the new method opacity was greatly reduced allowing spectra to be obtained as shown in Figure 7.10.

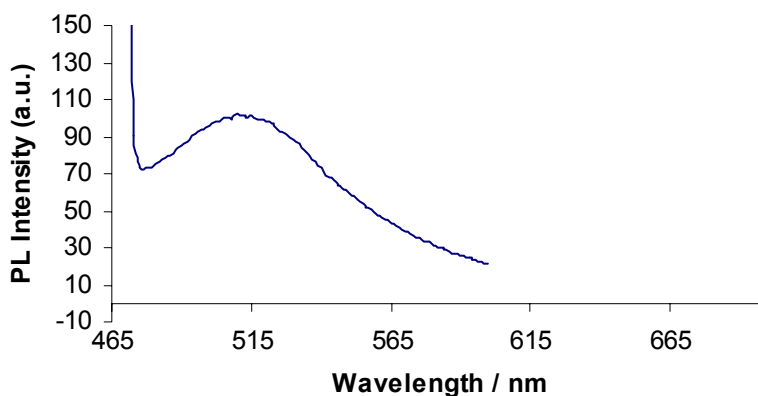


Figure 7.10 Spectrum of "green" CdSeS in solid epoxy resin.

If 4.2 nm core CdSe/ZnSe were used, instead of the CdSeS particles, the sample quality was higher still showing no signs of the opacity observed above. However, it became apparent upon analysis of the samples containing CdSe/ZnSe that the epoxy resin has intrinsic fluorescence ($\lambda_{\text{max}} = 515 \text{ nm}$), which dominates the spectrum at low concentrations of the particles. The spectrum of a low concentration sample is shown in Figure 7.11.

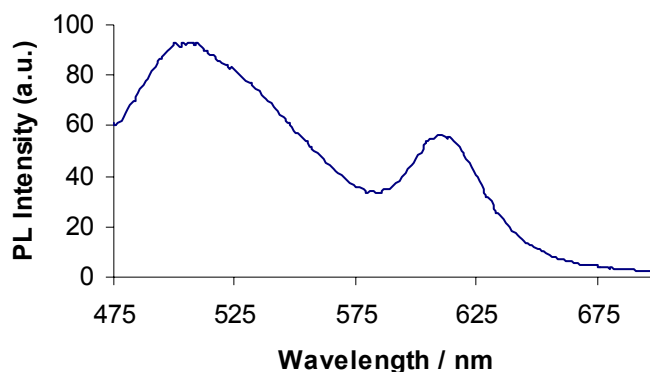


Figure 7.11 Spectrum of 4.2 nm CdSe/ZnSe particles in epoxy resin.

Samples were also prepared using a mixture of 4.2 nm CdSe/ZnSe and “green” CdSeS. In a similar manner to previous experiments the fluorescence of the “green” particles seemed to increase at the expense of the CdSe/ZnSe as shown in Figure 7.12.

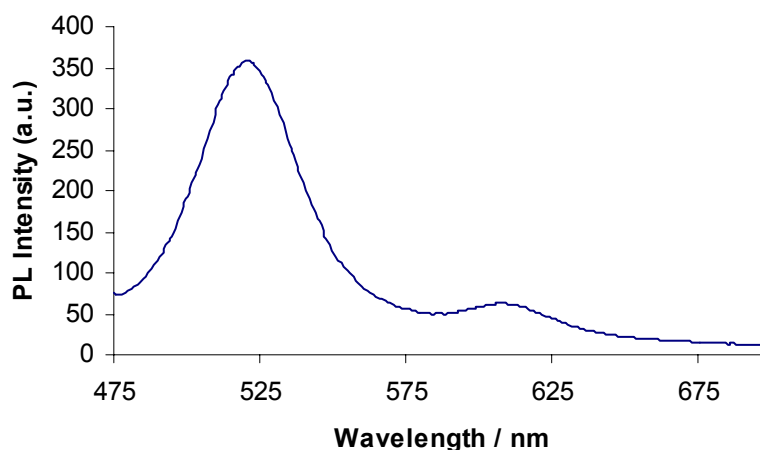


Figure 7.12 Spectra of mixed samples in epoxy resin.

7.3.3.2. Layered Samples

In order to improve the relatively poor optical properties of the epoxy resin, layered solid samples were prepared on cleaned glass slides. Due to the high concentrations of nanoparticles achievable in the epoxy resin, a different method was used to that of cyanoacrylate. A high concentration sample of “green” CdSeS was prepared and dropped onto the reverse of the glass slide and allowed to dry. The second sample was prepared in exactly the same manner, except that a second glass slide was laid on top of the epoxy resin mixture. The best spectrum was obtained for a sample which did not contain a second glass slide. It is thought that this is due to the thicker layer or resin allowing excitation of a larger number of nanoparticles. This spectrum is shown in Figure 7.13.

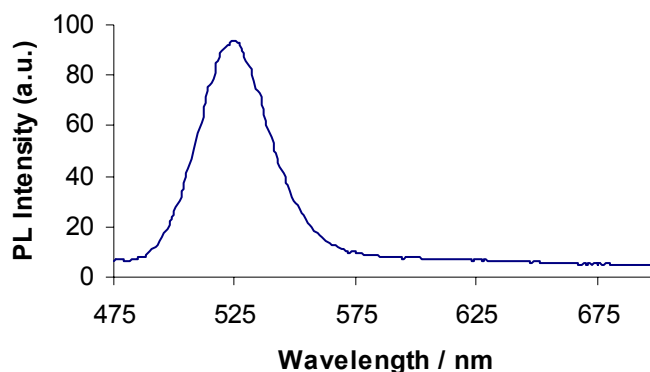


Figure 7.13 Fluorescence spectrum of layered epoxy resin samples containing "green" CdSeS.

7.4. Conclusion

The studies reported in this section have shown that it is possible to produce solid fluorescence spectral standards based on quantum dots. However, it is also clear that a number of technical issues remain to be addressed before this method can be used in the commercial production of standards materials.

At the start of the study, the best candidate material for embedding the quantum dots was felt to be polymethylmethacrylate (PMMA). This material is used in the production of existing fluorescence standards, due to its excellent optical properties. However, it was found that the polymerisation process resulted in severe damage to the quantum dots, and a number of trials failed to come up with a suitable process.

Alternative materials investigated included cyanoacrylates and epoxy resins. It was found that the production of thin layers of quantum dots in polymers was a valid alternative to solid blocks, although refinements to the production process are required. Epoxy resins were found to be a possible route to the production of block standards, although issues of background fluorescence and transmittance in the UV were significant here.

Further studies with these new types of material are required to improve materials and production methods and to investigate stability and related issues.

8. Conclusions

As set out in the introduction (section 2.3.4), quantum dots (or fluorescent semiconductor nanocrystals) have intriguing properties, which appeared to make them ideal candidates for fluorescence standards materials. In the course of this project, we set out test this idea, and to make and test prototype standards materials. The overarching conclusions of this project are summarised below.

Synthesis of quantum dots (section 3):

- A range of different sizes and compositions of semiconductor nanoparticles have been synthesized with appropriate physical properties for their application as fluorimetry wavelength standards.
- High PLQYs have been achieved for these particles, which have appropriately low polydispersity, resulting in high quality spectra.

Stability of quantum dots (section 4):

- In solution, the particles have been shown to undergo significant photo-oxidative degradation.
- However, this effect can be significantly reduced with the use of anti-oxidants or by sealing samples under argon and storage in the dark.

Characterisation of quantum dot samples (section 5):

- It is important to characterise the spectral properties and stability of quantum dot samples before using them in standards applications. Some batches show poor emission spectra and stabilities.
- Quantum dot samples show a significant reduction in fluorescence quantum yield on heating, and a hysteresis is apparent on cooling. The loss of fluorescence can be restored by irradiation.
- Heating causes small, reversible changes in emission wavelength. This property is not a significant problem for standards applications provided that spectra are measured at a specified temperature.

Production of liquid standards (section 6):

- Mixtures of samples with different ligand systems have been shown to produce unpredictable variation in the PLQY of samples. This is thought to be due to ligand exchange.
- The sealed-cell production process does not damage the fundamental characteristics of the quantum dot spectra. However, it may cause some changes to peak wavelength, shape and intensity.
- The useful life of the standards in storage is relatively short (three months). It should be possible to improve this result with the use of suitable additives.
- Short-term instability of the standards was observed on exposure to light. This may be related to photoannealing of the nanocrystals after heating during production of the samples, and requires further investigation.

Production of solid standards (section 7):

- Quantum dots were successfully incorporated into PMMA blocks, which exhibited bright fluorescence.
- The radical polymerisation process used in the polymerisation of MMA appears to damage the quantum dots, resulting in a disruption of their desirable spectral characteristics.
- Polymer encapsulated samples have been successfully produced in two formats, using both epoxy resin and poly(cyanoacrylate). Short-term studies suggest good stability. However, the long term stability of these samples has not been fully investigated.

Overall, one can conclude that quantum dots do indeed show great promise as fluorescence wavelength standards. However, further work is needed on their incorporation into practical standards materials, in solid and liquid formats. In both cases the issues are the same: the effects of the production process on the quantum dots' characteristics, and the stability of the artefacts on exposure to light and during storage.

9. Experimental Section

9.1. *Experimental Work at York*

9.1.1. Materials and Equipment

Cadmium acetate (98%), Cadmium oxide (99.5%), selenium (100 mesh, 99.999%), stearic acid (95%), oleic acid (90%), trioctylamine (98%), trioctylphosphine (90%), trioctylphosphine oxide (99%) and were purchased from Aldrich; hexadecylamine (>98%) from Lancaster Synthesis Ltd; sulfur from BDH Laboratory Reagents Ltd, zinc stearate from Riedel-de Haën and 2,6-Di-tert-butyl-4-methylphenol (>99%) was obtained from Sigma. All solvents used were of analytical grade and were purchased from Fisher Scientific Ltd. Cyanoacrylate adhesive was purchased from a variety of sources but the majority of experiments were performed using Bostik superglue. The epoxy resin (OPT5001-4G) was purchased from Intertronics. All syntheses were carried out under argon using standard Schlenk line techniques. UV/Vis spectra were recorded using a Hitachi U-3000 UV/Vis spectrophotometer and fluorescence spectra using Hitachi F-4500 fluorescence spectrophotometer using a solid sample holder where necessary. Light levels for stability studies were measured as photosynthetically active radiation (PAR) intensity and were carried out using a Skye Instruments SKP210. Stability study samples were rotated on a platform constructed from a circular biscuit lid (Danish Butter Style) of *ca.* 200 mm diameter obtained from the Physical Organic Coffee Club, with a piece of white card lining the bottom. This was bolted directly to the drive shaft a 2 rpm electrical motor and attached to a wooden base.

9.1.2. Synthesis of CdSe nanoparticles.

CdSe nanoparticles (4.3 nm diameter) were prepared using a modification of the procedure employed by Qu and Peng [72]. Cadmium acetate dihydrate (0.55 mmol, 142 mg) and stearic acid (2.13 mmol, 607 mg) were heated to 150°C in a 100 cm³ round bottomed flask until a colourless liquid was obtained. Excess H₂O and acetic acid were removed *in vacuo* and the flask cooled before hexadecylamine (3.8 g) and trioctylphosphine oxide (2.0 g) were added. The mixture was then degassed by pump/purge and heated to 120°C under vacuum for 20 minutes to dry and further degas the mixture. The reaction vessel was back filled with argon and heated to 300°C at which point, a solution of selenium (2.67 mmol, 211 mg) in trioctylphosphine (3.33 cm³) (prepared under argon by sonication) was rapidly injected into the vigorously stirred reaction mixture. The solution was allowed to heat for 30 s before the flask was removed from the heater mantle and allowed to cool to room temperature. Purification was carried out by dissolution of the reaction mixture in chloroform (50 cm³) followed by precipitation using an equal volume of acetone. Typical yield 105 mg.

“Green” particles (2.3 nm diameter) were prepared in the same manner except that 0.7 mmol (55.6 mg) of selenium were used and the reaction was heated to 310°C and quenched immediately after the introduction of the selenium solution, by an injection of 20 cm³ of argon-saturated toluene. “Yellow” particles (3.8 nm) were prepared in the same manner but with a reaction time of 10 s.

Synthetic tips: If these reactions are not stirred vigorously (with a 10 mm glass stirrer bar), or the selenium solution is not injected quickly enough, a red tail is observed in the emission spectrum. However, it is recommended that the mixture is not stirred constantly through the time taken to reach the injection temperature (occasional stirring is necessary for accurate monitoring of temperature) because the mercury thermometer can be broken in this way.

9.1.3. Synthesis of ZnSe and ZnS Coated nanoparticles.

ZnSe coated nanoparticles were prepared using a modification of the procedure employed by Reiss *et al.* [73] CdSe nanoparticles (*ca.* 40 mg) were dispersed in chloroform (5 cm³) and added to a degassed mixture of hexadecylamine (3 g) and trioctylphosphine oxide (5 g) at 190 °C. The mixture was placed under vacuum in order to remove the chloroform and the flask was back filled with argon and allowed to return to 190 °C. To the heated reaction mixture, a stock solution of zinc stearate and selenium in 1:1 trioctylphosphine/toluene (10 cm³) was added dropwise over 1 h by cannula. After this addition, the resulting solution was allowed to stir at 190°C for 75 min. Once the solution had cooled to room temperature, the particles were isolated by dissolution in chloroform (25 cm³) followed by precipitation using an equal volume of methanol. Typical yield 210 mg. The stock solution used above was prepared by adding zinc stearate (1 mmol, 632 mg) and selenium (1 mmol, 79 mg) to a Schlenk tube and degassing. Trioctylphosphine (5 cm³) was added to the solid under argon and the mixture was sonicated. After dissolution of the selenium, toluene (5 cm³) was added and heated gently to form an optically clear solution.

ZnS coated nanoparticles were prepared using the same method except sulfur (1 mmol, 32 mg) was used in place of selenium.

Synthetic tips: The cannula transfer of the zinc chalcogenide solution is not absolutely routine. Because of the high melting point of the coordinating solvents used and the long addition period, the use of a normal needle to act as a vent is not appropriate (it tends to block in a few minutes). The use of a Pasteur pipette as a vent allows the melting and removal of the solidified solvent from the vent with relative ease. The long addition period and the relatively small volume of the stock solution means that, in order to guarantee the argon atmosphere, a *slow* flow of argon must be maintained to the reaction vessel from the Schlenk line.

9.1.4. Synthesis of CdSeS Nanoparticles

An example of cadmium selenide sulfide particle synthesis is described below. The particles produced by this method had an emission λ_{max} of 525nm. A similar protocol to that employed by Jang, Jung and Pu [71] was employed. 0.0496g cadmium oxide (0.0386mmol), 0.46g oleic acid (1.63mmol) and 16g of trioctylamine (TOA) were heated to 160°C in a 100cm³ round bottomed flask. Excess water, air and water were removed *in vacuo* by heating to this temperature over 20 minutes after undergoing degassing by pump and purge of argon. A colourless solution was obtained. The reaction vessel was then heated to 300°C under argon and 1cm³ of the selenium/sulphur stock solution quickly injected. The vessel was allowed to react for four minutes and was quenched by addition of 10cm³ degassed absolute ethanol. The selenium/sulphur stock solution was prepared under argon by dissolving 19.9 mg of selenium and 61.9 mg sulphur in 10ml TOP.

Purification was carried out by the addition of 40 cm³ absolute ethanol to precipitate from the TOA. The resulting solid was separated by centrifuge.

Further synthetic details for the synthesis of particles with different ratios of chalcogenide and reaction time are found in Marcus Turner's 3rd Year Project Report [70].

9.1.5. Stability Studies

4 cm³ aliquots of nanoparticle solutions (1 mg cm⁻³) were added to trioctylphosphine oxide (20 mg), hexadecylamine (20 mg), 1:1 mixture of trioctylphosphine oxide and hexadecylamine (10 mg each) or 1:1 mixture of hexadecylamine and 2,6-di-tert-butyl-4-methylphenol (20 mg each) in a sample tube. The samples were then shaken for 30 s and a portion was added to chloroform (3 cm³) in order to make samples that had an optical density of 0.1 at 460 nm. These diluted samples were then used to obtain the quantum yield of the nanoparticles by taking photoluminescence emission spectra (excitation 460 nm). The peak areas were compared to that of a rhodamine standard of known quantum yield. Meanwhile the original samples were placed in a light box containing standard fluorescent strip lights (PAR intensity (400-700nm) 15.2 $\mu\text{mol m}^{-2}\text{s}^{-1}$) for 168 h at the edge of a rotating circular platform. After this period, samples were taken and diluted with chloroform to an optical density of 0.1 at 460 nm and analyzed by fluorimetry.

9.1.6. Polymer Encapsulation

9.1.6.1. Poly(cyanoacrylate)

The most effective method for the dissolution of nanoparticles in cyanoacrylate was to add a solution (*ca.* 4 mg cm⁻³ in chloroform) dropwise by pipette to stirred liquid cyanoacrylate until the desired concentration is achieved. Other methods generally provoke rapid polymerisation resulting in low quality samples.

Layered samples were best prepared by adding two drops of cyanoacrylate onto a glass slide cut to 19mm x 30mm and spreading quickly with a small glass rod. This was allowed to cure at room temperature and then lightly sanded with 500-grade glass paper. Chloroform or dichloromethane solutions were added dropwise and spread again with the glass rod. The solvent was allowed to evaporate and more solution was added, spread out and dried until the desired loading was achieved. Three drops of cyanoacrylate were dropped on top of the nanoparticle layer and a glass slide was placed onto the previous layers in a manner similar to the preparation of microscope samples. Again, curing was carried out at room temperature in order to produce the final device.

9.1.6.2. Epoxy Resin

The highest concentration of nanoparticles and the greatest degree of control of that concentration in solution is afforded by dissolution of the particles by gentle warming in the diamine component of the epoxy resin, prior to mixing with the epoxide. In this way, the desired concentration of particles can be achieved. The epoxide was then mixed with the solution of particles in a sample tube and agitated using a mechanical agitator. Block samples were made by pouring uncured mixtures into disposable PMMA fluorescence cuvettes and allowed to cure for 24 h at room temperature in the dark. Layered samples were prepared by dropping concentrated mixtures onto glass

slides cut to 19 mm x 30 mm. Samples of this type were either left to cure at room temperature in the dark or by laying a second slide on top of the resin in a similar manner to that described above for cyanoacrylate samples.

9.2. Experimental Work at NPL

The experimental work at NPL focussed on the characterisation of batches of nanocrystals, and of the solid and liquid prototype standards artefacts. The protocols used are detailed below.

9.2.1. Spectrophotometry

All spectrophotometry was performed in 1 cm path length quartz cuvettes. For accurate measurement, samples were diluted to give absorbances in the range 0.8-1.0 where possible. Measurements were made on a Helios Gamma spectrophotometer, and data were acquired on a PC using the Vision software provided by the manufacturer (Thermo Spectronic). Data were exported to Excel for further analysis.

9.2.2. Spectrofluorimetry

All fluorescence measurements were made using 1 cm path length quartz cuvettes (except where standards were being characterised). Due to the known volatility of the samples, cells were either stoppered or screw cap types to minimise solvent evaporation. For fluorescence work, samples were diluted so as to give absorbances between 0.05 and 0.1 where possible, to minimise inner filter effects [85].

All measurements were made on a PerkinElmer LS55 fluorimeter using FL WinLab 4.0 software. Method files were used to ensure consistent measurement conditions. The four-cell sample changer accessory was used together with a circulating water bath (Grant GR150 and a slaved chiller) to permit temperature control. For temperature cycle experiments, the Grant LabWise software was used to programme the water bath and log the temperature.

The majority of data analysis was performed in Excel; custom MATLAB routines were used for curve fitting and for the interpolation of temperatures onto fluorescence time series for temperature cycle experiments.

Typically, for each sample, a 3D excitation/emission scan (Table 9.1) was performed at low resolution in order to perform an overall characterisation of the fluorescence characteristics of the material. This was followed by higher resolution excitation (Table 9.3) and emission (Table 9.2) scans, using emission and excitation wavelengths determined from the initial scan. Further experiments were then performed to evaluate the stability characteristics of each batch, with respect to fluorescence intensity (Table 9.4) and spectral characteristics (Table 9.5).

The effects of temperature, including the temperature hysteresis effect, were determined for representative samples. This was achieved through simultaneous computer control of the fluorimeter and the water bath, with subsequent interpolation of temperature data onto the fluorescence time-points. The fluorimeter settings are shown in Table 9.6 and the temperature programme is shown in Table 9.7 and Figure 9.1.

Emission scan range (nm):	400-700
Excitation scan range (nm):	300-600
Excitation Increment (nm):	50
Number of Scans:	7
Scan speed (nm/min):	100
Excitation slit (nm):	10
Emission slit (nm):	10
Temperature (°C):	20

Table 9.1 Typical instrument parameters used in the acquisition of “3D” excitation/emission scans.

Emission scan range (nm):	400-700
Excitation wavelengths (nm):	370 Sample-specific
Scan speed (nm/min):	25
Excitation slit (nm):	10
Emission slit (nm):	2.5
Temperature (°C):	20

Table 9.2 Typical instrument parameters used in the acquisition of emission spectra.

The sample-specific excitation wavelength reflected the peak wavelength previously determined for the sample. Note the use of the minimum emission width to maximise spectral resolution. The scan speed has been increased to compensate for the lower signal level.

Excitation scan range (nm):	300-600
Emission wavelength (nm):	Sample specific
Excitation slit (nm):	10
Emission slit (nm):	10
Scan speed (nm/min):	100

Table 9.3 Typical instrument parameters used in the acquisition of excitation spectra.

Excitation spectra were not performed at high resolution, since they are not so critical for the application concerned. The emission wavelength was the peak wavelength determined from previous scans.

Excitation wavelength (nm):	370
Emission wavelength:	maximum
Excitation slit (nm):	10
Emission slit (nm):	10
Cuvette Number:	1-4
Duration (min):	1440
Data Interval (min):	1
Integration Time (s):	6
Auto lamp off:	Yes
Delay before measurement:	0

Table 9.4 Typical instrument parameters used in intensity stability studies.

This is done using the Wavelength Program application in FL WinLab, which permits 4 samples to be analysed in parallel. This program collects data over approximately 24 hours. A long integration time is used to collect precise measurements, and the lamp is switched off when readings are not being taken (this ensures that all four samples receive an equal light exposure, with a duty ratio of approximately 10%). No “settling time” was necessary before measurements were made.

Emission scan range (nm):	400-700
Excitation wavelength (nm):	370
Scan speed (nm/min):	25
Excitation slit (nm):	10
Emission slit (nm):	2.5
Number of scans:	40
Delay time (s):	1800
Auto lamp off:	Yes
Delay before measurement:	0

Table 9.5 Typical instrument parameters used in wavelength stability studies.

This is done using the kinetics mode of the Scan application in FL WinLab (only one sample can be analysed in a run). The choice of a narrow slit width for the emission spectrum gives a higher sensitivity to spectral changes. A full experiment lasts about 20 hours.

Excitation wavelength (nm):	Maximum
Emission wavelength:	Maximum
Excitation slit (nm):	5
Emission slit (nm):	5
Cuvette Number:	1-4
Duration (min):	1440
Data Interval (min):	1
Integration Time (s):	1
Auto lamp off:	Yes
Delay before measurement:	0

Table 9.6 Typical instrument parameters used in temperature hysteresis studies. This is done using the Wavelength Program application in FL WinLab, which permits 4 samples to be analysed in parallel. Excitation and emission wavelengths are set to the maxima for the specific sample. This fluorimeter method was run simultaneously with the temperature programme shown in Table 9.7.

Step	Temperature (°C)	Duration (min)	Chiller	Rate (°C/min)	Comment
1	20	30	On	0	Equilibrate
2	20→65	180	Off	0.25	Ramp up
3	65	60	Off	0	Maintain
4	65→20	180	On	-0.25	Ramp down
5	20	240	On	0	Recovery
6	20	Indefinite	On	0	Run-out

Table 9.7 Water bath programme for temperature hysteresis experiments This programme was used to control the Grant GR150 water bath via the supplied LabWise software. A Grant C1G chiller was slaved to the water bath via a relay to permit rapid cooling and temperature control close to ambient when required.

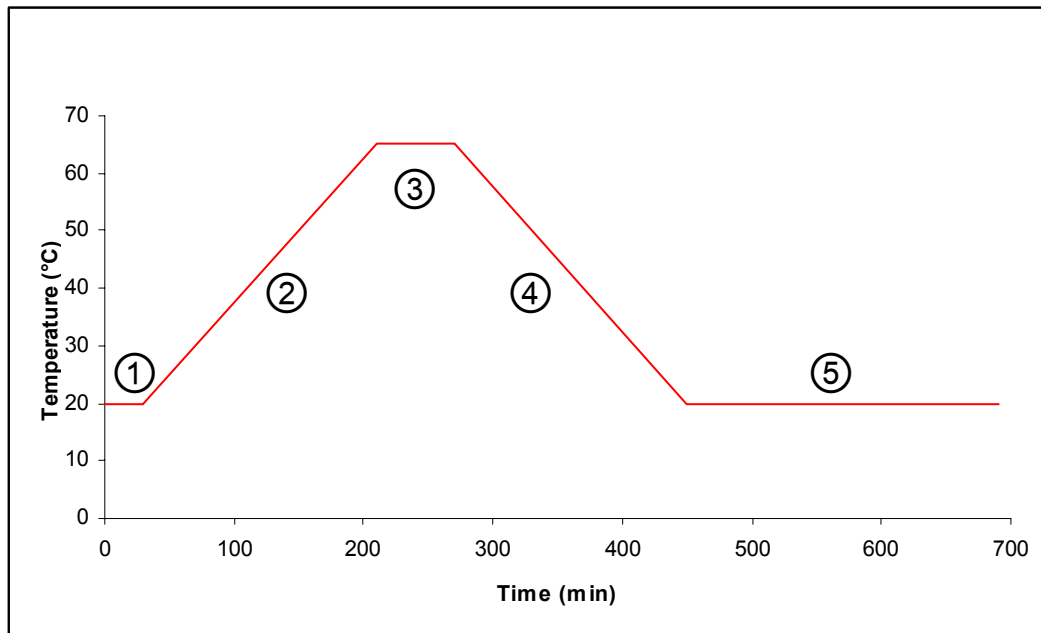


Figure 9.1 Temperature cycle used for hysteresis experiments.

Numbers correspond to program steps in Table 9.7: ① Equilibrate; ② Ramp up; ③ Maintain; ④ Ramp down; ⑤ Recovery.

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