

## TOPICAL REVIEW

# Luminescent nanomaterials for biological labelling

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### Abstract

The use of labelling or staining agents has greatly assisted the study of complex biological interactions in the field of biology. In particular, fluorescent labelling of biomolecules has been demonstrated as an indispensable tool in many biological studies. Types of fluorescent labelling agents that are commonly used include conventional classes of organic fluorophores such as fluorescein and cyanine dyes, as well as newer types of inorganic nanoparticles such as QDs, and novel fluorescent latex/silica nanobeads. The newer classes of fluorescent labels are gaining increasing popularity in place of their predecessors due to their better optical properties such as possessing an enhanced photostability and a larger Stokes shift over conventional organic fluorophores, for example. This paper gives an overview of the recent advances on these luminescent nanomaterials with emphases on their optical characteristics that are crucial in fluorescence microscopy, both advantages and limitations in their usage as well as challenges they face, and puts forward the future direction of fluorescent labels in the area of biolabelling.

## 1. Introduction

The rapid development of biological science has fuelled growing demands to analyse biomolecules such as polypeptides, proteins, and nucleic acids during the past decades, resulting in the development of various biological analytical systems like biosensors. The introduction of labelling or staining agents into biological systems is usually required as an organism lacks sensitive detectable signals. Exploiting exogenous labels for biological analysis was first introduced by American scientists Yalow and Berson in the form of radioimmunoassay (RIA) [1]. Although there are many advantages in using RIA, owing to its high sensitivity ( $10^{-9}$ – $10^{-12}$ ) and wide application, it does have a number of drawbacks such as its radioactivity and inherently short half-life [2]. Therefore, various non-radioactive labelling techniques based on enzyme-catalysed

reactions, bio/chemiluminescence, and fluorescence, for example, have emerged, amongst which fluorescent labelling is the most widely used [3, 4].

Fluorescence is a luminescence phenomenon that occurs in fluorophores. It is a process by which a fluorophore absorbs light of a particular wavelength and then re-emits a quantum of light with an energy corresponding to the energy difference between the excited state and the ground state [5, 6]. Fluorescent labels represent one of the most widely developing fields in biology and medicine, with enormous applications. All fluorescence parameters such as fluorescence intensity, emission spectrum, excitation spectrum, and fluorescence lifetime can be used to encode what is happening in the close neighbourhood of the monitoring molecule. For example, labels which are environmentally sensitive can be used as molecular reporters. Information on what is happening in

their molecular environment can thus be derived from their fluorescence signals and their exact locations can be monitored using fluorescence microscopy.

Conventionally, the earlier classes of fluorescent labels include organic dyes, fluorescent proteins, and lanthanide chelates, which are still the most commonly used fluorescent labels because they can be very small and highly water soluble (at high salt concentrations), as well as the ease of their usage and the existence of standard protocols for their bioconjugation. However, these conventional labelling agents have several limitations caused by their intrinsic properties such as, for example, broad spectrum profiles, low photobleaching thresholds, and poor photochemical stability. In recent years, the rapid advancement of material science has brought us some newer and exciting classes of labelling agents, which have the potential and ability to overcome a number of problems associated with the earlier classes of fluorophores. Members of these newly evolved classes of labelling agents include semiconductor quantum dots, lanthanide-doped inorganic nanoparticles, and fluorophore-tagged latex/silica nanobeads. A discussion on both the conventional and newer classes of labelling agents will be dealt with in the following sections, with emphasis on the latter.

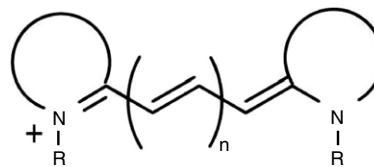
## 2. Organic dyes

Organic dyes are amongst the earliest types of classical fluorescent labels used in biology. Despite their inherent drawbacks such as having a short Stokes shift, poor photochemical stability, susceptibility to photobleaching and decomposition under repeated excitation, organic dyes are still popular due to their low cost, availability, and ease of usage. Although single organic dyes suffer from intermittent on/off emission (blinking) [7–9], the blinking can be used for single-molecule detection. In addition, recent research has produced organic dyes that possess better chemical and optical properties. Examples of commonly used organic dyes include fluorescein, rhodamine, cyanine, and Alexa dyes.

### 2.1. Fluoresceins and rhodamines

Fluoresceins are amine-reactive organic fluorophores widely used to label proteins [10]. A variety of fluorescein dyes are available and a popular choice is fluorescein-5-isothiocyanate, FITC, molecular formula  $C_{21}H_{11}NO_5S$ . Generally, FITC possesses relatively high absorptivity, high fluorescence quantum yield and aqueous solubility. Additionally, due to the fact that its excitation maximum at 494 nm closely matches the 488 nm spectral line of the argon-ion laser, FITC is the predominant fluorophore for confocal laser-scanning microscopy and flow cytometry applications.

However, FITC fluorophores suffer from quite a few major drawbacks. Perhaps two of the most important are the problems of photobleaching and its pH sensitivity. FITC is susceptible to serious photobleaching, and as such its fluorescence rarely stays beyond a few minutes under constant illumination. Photobleaching also limits the sensitivity that can be obtained in studies using FITC. Furthermore, its fluorescence is also affected by the pH of the environment and is significantly reduced below the pH value of 7. At the



**Figure 1.** General molecular structure of cyanine dyes. Their common names depend on the number of methine groups in the polyene chain. For example, the compounds with  $n = 0$  and 3 are referred to as monomethine and heptamethine cyanines, respectively. (Reprinted from [16], with permission.)

same time, it has also been reported that antibody conjugates prepared from FITC deteriorate over time [11]. As a result, these problems greatly hinder and limit the use of FITC dyes in ultra-sensitive biological studies.

Just like fluoresceins, rhodamine dyes have also been long considered as a popular choice of fluorophores in the labelling of biomolecules. One major reason is due to the location of their excitation peak, which at 520 nm is close to the 514 nm spectral line of the argon-ion laser, making it another popular and important fluorophore in studies involving confocal laser-scanning microscopy and flow cytometry. Compared to FITC, red-fluorescing rhodamine dyes, such as the succinimidyl ester of 5-carboxyrhodamine 6G [12], molecular formula  $C_{31}H_{29}N_3O_7$ , exhibit better photostability as well as longer wavelength emission maxima. This opens up opportunities for multicolour labelling or staining and offers better spectral separation from green fluorescence dyes such as FITC in multicolour experiments. However, on the other hand, its photostability still pales in comparison to the newer classes of staining agents and the nature of its broad emission spectrum may actually makes multiplexing detection difficult.

### 2.2. Cyanine dyes

Cyanine dyes belong in a large class of organic compounds which has been used extensively in applications ranging from photography [13] to lasers [14] over a long period of time. Recently, their applications in biological labelling [15] have attracted growing interests due to their high degree of photostability, relatively efficient quantum yield and good water solubility. As represented (figure 1 [16]), cyanine dyes are cationic molecules in which two heterocyclic units are joined by a polyene chain [17–19]. According to their optical properties, cyanine dyes can be generally classified into two subclasses: monomethine cyanines, which include the asymmetric cyanines thiazole orange (TO), oxazole yellow (YO), or a dimer of both TO and YO (TOTO, YOYO); and polymethine cyanines, which include the more commonly used dyes such as Cy3 and Cy5.

Monomethine cyanines like TO and YO do not fluoresce in solution but possess intense fluorescence when bounded to nucleic acids [20]. The increase in fluorescence is believed to arise when the rotation around the bond between the aromatic systems is restricted, which closes a channel for non-radioactive decay. For example, TO with a molecular formula of  $C_{19}H_{16}N_2S$  has an impressive fluorescence enhancement of about 3000-fold upon binding to nucleic acids [21]. Similarly, the thiazole orange dimer (TOTO) and the oxazole yellow

dimer (YOYO; an analogue of TOTO) are also found to experience an enhancement of fluorescence upon binding to nucleic acids (1100-fold for TOTO and 3200-fold for YOYO [22, 23]). As such, this class of cyanines is useful for labelling DNA. However, as these dyes interact noncovalently with nucleic acids, their affinity for nucleic acids is relatively low.

Similar to monomethine cyanine dyes, polymethine dyes like Cy3 and Cy5 have also been widely and routinely used in the labelling of biological compounds such as antibodies, nucleic acids, lipids and other amino-group-containing specimens [24, 25]. In addition, they are also a popular choice of fluorescent probes in microarray technology [26]. The Cy3 dye is an orange-fluorescing cyanine that produces a signal that can be easily detected using a fluorescein filter set while the Cy5 dye produces an intense signal in the far red region of the spectrum. Cy3 can be maximally excited near 550 nm. However, unlike both the FITC and 5-carboxyrhodamine 6G dyes, Cy3 can only be excited to about 50% of its maximum with the 514 nm or 528 nm lines of an argon-ion laser, or to about 75% of its maximum with the 543 nm spectral line of the helium–neon laser. Cy5, on the other hand, has an excitation peak at 650 nm and can be excited optimally with the 647 nm spectral line of a krypton/argon-ion laser.

The spectrum properties of cyanine dyes greatly depend on the number of methine groups in the polyene chain. Each extension of the chromophore by one vinylene moiety (CH=CH) causes a bathochromic shift of about 100 nm [15], producing cyanine dyes that emit in the near-infrared (NIR) region [27–29]. As there is no autofluorescence from the biological sample in this region of the spectrum, high detection sensitivity could be achieved. NIR dyes are also suitable for selective excitation with commercially available laser diodes, which can further enhance the observed fluorescence signal. However, it is known that an initial elongation of the polymethine chain will increase the fluorescence quantum yield of the dye, while any further increase tends to cause a drop [30]. As a result, NIR cyanine dyes usually exhibit low emission intensity. In addition, as with all other types of conventional organic dyes discussed, cyanine dyes also suffer from problems such as photobleaching. Similarly, another major drawback associated with this class of dyes is that these fluorophores generally have narrow excitation spectra and each member can only be excited optimally within a very limited range of wavelength, making multiplexing detection tedious and laborious. Despite these drawbacks, cyanine dyes are still promising candidates as labels for various biological studies, and efforts have been made to synthesis newer variants of cyanine dyes with more desirable and better fluorescence properties [31, 32].

### 2.3. Alexa dyes

Alexa dyes are a series of relatively new fluorescent molecules that are obtained by sulfonating aminocoumarin, rhodamine, or carbocyanine dyes [33, 34]. The introduction of sulfonic acid groups bestows negative charges on the dye that make the water-insoluble zwitterionic rhodamine or neutral coumarin molecules much more hydrophilic, allowing bioconjugation to

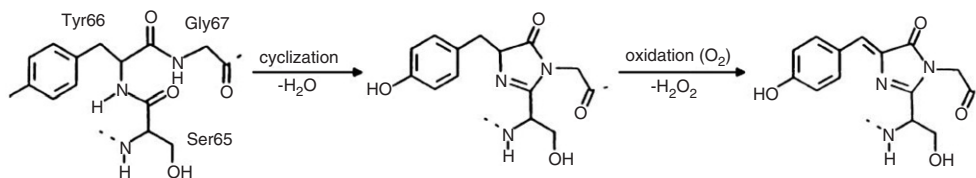
be performed in the absence of organic solvents. Furthermore, sulfonation also decreases the inherent tendency of molecules to form aggregates, presumably due to the increased polarity imparted by the sulfonic acid moiety [24], and thus decreases the interaction between molecules that strongly hinders their fluorescence yields.

The excitation and emission wavelength ranges of Alexa dyes cover the entire spectrum from ultraviolet to red, comprising wavelengths that match conventional light sources. All these Alexa dyes exhibit high photostability as well as pH insensitivity over a very broad range, and those that absorb at wavelengths greater than 480 nm have the high molar extinction coefficient typical of fluoresceins or rhodamines. The performance of Alexa dyes has also been demonstrated to be superior to that of the commonly used dyes in various bio-applications, especially at high degrees of labelling [33–35]. As such, Alexa dyes are frequently used in biology and considered as competitive alternatives to conventional dyes. However, like fluorescein, Cy3, and Cy5, Alexa dyes carry a net negative charge. In some cases the negative charge of fluorophores may cause a nonspecific electrostatic interaction with positively charged cell structures [36]. In these few cases, conventional neutral dyes, such as rhodamine, may be the proper choice, notwithstanding their lower fluorescence output.

## 3. Fluorescent proteins

The most popular member of the fluorescent protein family is green fluorescent protein (GFP), which was originally isolated from the light-emitting organ of the jellyfish *Aequorea victoria* by Shimomura *et al* in 1962 [37]. GFP emits green fluorescence under light excitation, requiring no exogenously added proteins, substrates or co-factors, in contrast to other bioluminescence systems. The absorbance and fluorescence of GFP originate from a *p*-hydroxybenzylidene-imidazolidinone chromophore, which is generated by the cyclization and oxidation of the protein's own Ser–Tyr–Gly sequence at positions 65–67 [38, 39]. Heim and co-workers [40] have proposed the biosynthetic scheme for the chromophore (figure 2) based upon the following observations:

- (1) expression of recombinant GFP (e.g. in *E. coli*) is possible, i.e. fluorophore formation does not require any specific enzymes from *A. victoria*;
- (2) if soluble GFP is expressed in *E. coli* under anaerobic conditions, it is non-fluorescent but otherwise indistinguishable from native GFP on a denaturing SDS gel;
- (3) fluorescence of anaerobically expressed GFP gradually develops after the admission of air; even in very dilute cell lysates (i.e. oxidation is not enzymatically catalysed);
- (4) imidazolin-5-ones are known to undergo autoxidative formation of double bonds at the 4-position, which in this case would complete the fluorophore;
- (5) after readmission of atmospheric oxygen to anaerobically expressed, non-fluorescent GFP, development of fluorescence exhibits pseudo-first-order kinetics with a rate constant of  $0.24 \pm 0.06 \text{ h}^{-1}$ .



**Figure 2.** Mechanism proposed by Heim for the intramolecular biosynthesis of the GFP chromophore. The first step is a nucleophilic attack of the amino group of Gly67 onto the carbonyl group of Ser65 (left). Subsequent elimination of water results in the formation of an imidazolidinone ring (middle). In a second step the C $\alpha$ -C $\beta$  bond of Tyr66 is oxidized to give a large delocalized pi-system (right). (Adapted from [40], with permission. ©1994) National Academy of Sciences, USA).

Mutagenesis studies of GFP yielded variants with improved folding and expression properties that stimulated GFP's widespread use as a fluorescent protein tag [41, 42]. Certain mutations in the chromophore of wild type GFP (wtGFP) have been observed to affect the rate of chromophore formation *in vivo* [43, 44] and to alter the spectral characteristics of the native fluorescent or mature protein [40]. The currently known GFP variants may be divided into seven classes based on the distinctive component of their chromophores [45]: (1) wild-type mixture of neutral phenol and anionic phenolate; (2) phenolate anion; (3) neutral phenol; (4) phenolate anion with stacked  $\pi$ -electron system; (5) indole; (6) imidazole; and (7) phenyl. Each class has a distinct set of excitation and emission wavelengths. Classes (1)–(4) are derived from polypeptides with Tyr at position 66, whereas classes (5)–(7) result from having Trp, His, and Phe at that position, respectively. Glycine is by far the best nucleophile in the formation of chromophores because of its minimal steric hindrance, and as a result Gly67 is conserved in all known mutants of GFP that retain fluorescence.

GFPs have been used as tools in numerous applications, including as biological labels to track and quantify individual or multiple protein species [46], as probes to monitor protein–protein interactions [45, 47, 48], and as biosensors to describe biological events and signals [45, 47]. In particular, GFP fluorescence can be modulated post-translationally by its chemical environment and protein–protein interactions [45], facilitating the detection of gene expression *in vivo* [49, 50]. Using genetic engineering, GFP and its variants can be fused as tags to any protein of interest, often without altering the function of the protein, to analyse protein geography, movement, and chemistry in living cells [51]. Chimeric GFP fusions have the advantage that they can be expressed *in situ* by gene transfection. The resulting chimera often retains parent-protein targeting and function when expressed in cells [45]. However, the molecular mass of GFP (~26 kDa) may interfere with the expression and folding of the labelled protein and thus affect its interaction with other molecules. Furthermore, there is also the problem of potential aggregation of the fluorescent proteins inside the cells, that may lead to cellular toxicity [52]. Moreover, in addition to suffering from short time blinking, single fluorescent proteins were also observed to turn on and off on timescales of seconds [53, 54], creating a challenge in single-molecule detection.

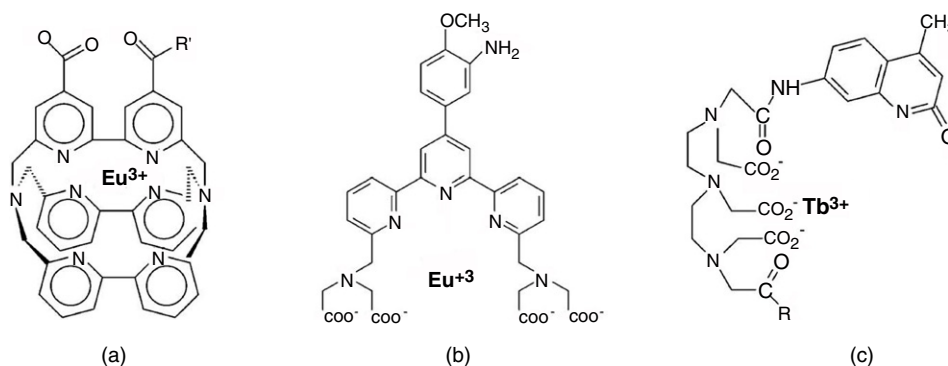
#### 4. Lanthanide chelates

Lanthanide chelates are an important group of luminescent materials that have been receiving much interest. In particular,

intense research has focused on the synthesis of photoactive lanthanide complexes for biological applications [55–57]. The lanthanides are a special group of elements within the periodic table that usually exist as trivalent cations. The electronic configuration of trivalent lanthanides is 4f<sup>*n*</sup>, with *n* varying from 1 (Ce<sup>3+</sup>) to 14 (Lu<sup>3+</sup>), and the electronic transitions within the 4f shell are responsible for their interesting photophysical properties. The f electrons are shielded from external perturbations by filled 5s and 5p orbitals, thus giving rise to line-like spectra. In general, excellent luminescence properties of lanthanide chelates are attributed to the intramolecular energy transfer between the ligands and chelated lanthanide ions [58].

Typically, all lanthanide chelate labels in biological studies contain an organic chromophore, which serves as an antenna or sensitizer to absorb the excitation light and transfer this energy to the lanthanide ions. An antenna is necessary because of the inherently weak absorbance of the free lanthanide ions (1 M<sup>-1</sup> cm<sup>-1</sup>, or 10<sup>4</sup>–10<sup>5</sup> smaller than conventional organic dyes). Consequently, lanthanide chelates exhibit broad excitation spectra owing to the organic ligands and narrow emission spectra resulting from the lanthanide ions. Thus, by varying the lanthanide ions in the chelates, different emission wavelengths can be achieved. The complexes also contain a chelate that serves several purposes, including binding the lanthanide tightly, shielding the lanthanide ion from the quenching effects of water, and acting as a scaffold for attachment of the antenna and a reactive group, the latter for coupling the chelate complex to biomolecules [59]. For most complexes the antenna is involved in binding the lanthanide, hence logically there is no clear separation between the chelate and antenna. However in some other cases, the chelate and antenna may be distinct entities (see figure 3 for example).

The spectral characteristics of lanthanide chelates include long fluorescence lifetime (sub-microsecond to millisecond range), sharply spiked emission spectra (<10 nm full width at half-maximum, FWHM), large Stokes shifts (>150 nm), and high quantum yield (~1) [55]. These properties make them useful alternatives to radioactive probes and organic dyes, particularly where there are problems of background autofluorescence [56, 60, 61] and as donors in luminescence resonance energy transfer experiments [59, 62]. In addition, lanthanide chelates with aromatic carboxylic acids are frequently used as structural and functional probes in biological systems [63, 64]. The sensitized luminescence of lanthanide ions by salicylic acid plays an important role in the analyses of trace salicylic acid and its derivatives in biological systems, and in fluorimetric immunoassay [65–67]. However, the fluorescence properties of lanthanides are dependent on



**Figure 3.** Structures of representative lanthanide chelates. For (a) cryptates and (b) terpyridine, the chelate and antenna are logically the same entity. For (c) DTPA-cs124, the complex contains a DTPA chelate and a cs124 antenna. (Adapted from [59]. Reprinted, with permission, from the Annual Review of Biophysics and Biomolecular Structure, Volume 31 ©2002 by Annual Reviews [www.annualreviews.org](http://www.annualreviews.org)).

their ability to form complexes with the chelating agents. Commonly used chelating agents are restricted to EDTA, DTPA,  $\beta$ -diketonates, and their derivatives [68]. As a result, the lanthanide ions involved are usually restricted to Eu(III), Tb(III), Sm(III), and Dy(III) as energy transfer between only these two groups takes place efficiently. Therefore, the development of novel lanthanide chelates with improved photochemical stability will remain a major challenge in the future.

## 5. Inorganic nanoparticles

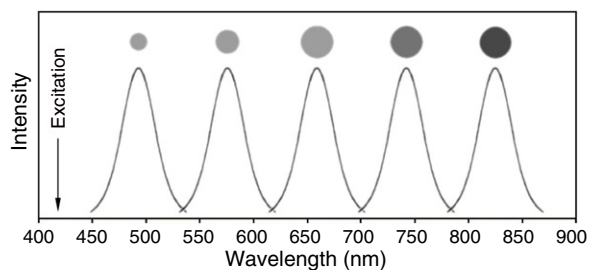
### 5.1. Semiconductor quantum dots

Quantum dots (QDs) are semiconductor nanocrystals composed of atoms from groups II–VI or III–V of the periodic table. They are generally defined as particles having physical dimensions smaller than the exciton Bohr radius, typically 1–5 nm. Due to this small size of only a few nanometres, which is of the same order of magnitude as the de Broglie wavelength of electrons and holes at room temperature, the states of the free charge carriers in QDs are quantized and the movement of these carriers is entirely determined by quantum mechanics [69]. As such, the small size of these QDs leads to a quantum confinement effect, which endows the nanocrystals with unique optical and electronic properties.

Research on QDs started with the realization that the optical and electronic properties of small semiconductor particles were strongly dependent on particle size, as was first described by Éfros and Ekimov in 1982 [70, 71]. At the beginning, the synthesis of QDs was mainly carried out in aqueous phase, yielding low-quality nanocrystals with poor fluorescence efficiencies and large size variations. In 1993, Bawendi and co-workers [72] synthesized highly luminescent CdSe QDs via a high temperature organometallic process, which forms the basis of various methods to produce high-quality, monodispersed QDs today. This method basically entails the combination of an appropriate metallic or organometallic precursor (such as Zn, Cd, or Pb) with a corresponding chalcogen precursor (such as S, Se, or Te) in a solvent at high temperature. The commonly used solvents are tri-*N*-octylphosphine oxide (TOPO), in conjunction with tri-*N*-octylphosphine (TOP), hexadecylamine (HDA), or stearic

acid [73–75]. However, as the earliest QDs typically consisted of an uncapped semiconductor core only, their emission is typically weak and always unstable. It is generally understood that there are two major problems with uncapped QDs, the first of which is the presence of gap surface states arising from surface non-stoichiometry and unsaturated bonds, etc. Crystalline imperfections and defects that exist on the surface of QDs will capture excited state energy and provide non-productive and non-emissive pathways for deactivating the QD cores after they have been excited with light. Secondly, the surfaces of uncapped QDs tend to be very reactive due to their high surface-area-to-volume ratio, and consequently they become easily polluted by a variety of agents, which in turn provide additional trapping pathways that also result in quenched emissions. At the same time, uncapped QDs are so reactive that they are prone to spontaneous dissolution or photochemical degradation; even the simple act of diluting core samples often leads to irreversible decomposition of the nanocrystals. Therefore, control of the surface properties is the key for the formation of highly luminescent QDs and this has led to the development of core–shell QDs with higher quantum yields and improved photochemical stability [76, 77].

However, as QDs are mostly synthesized in organic solvents, they are insoluble in water, often non-biocompatible, and do not have any reactive functional groups for conjugation with biomolecules. Until recently, the research on QDs was primarily focused on the traditional applications in optoelectronic devices, quantum-dot lasers, and high density memory [78–80]. It was not until 1998 when Alivisatos [81] and Nie [82] simultaneously demonstrated that QDs could be made water soluble and conjugated with biological molecules that the applications of QDs were extended to the biological field. The advantages of using QDs as fluorescent labels are numerous. Firstly, they permit great assay sensitivity and stability over conventional organic fluorophores. For example, Chan and Nie reported that QDs are 20 times as bright, 100 times as stable against photobleaching, and one-third as wide in spectral linewidth compared to conventional organic dyes such as rhodamine [82]. Secondly, it has also been observed that the onset of absorbance and emission maxima of the QDs shift with a change in the size of the QDs or chemical composition [79, 83]. Thus, the wavelength of emission can be



**Figure 4.** The spectral properties of QDs can be tuned by changing the size of the particle or composition. In the visible spectrum region, the emission is usually adjusted by selecting the particle diameter of CdSe QDs, whereas at longer wavelength the emission is adjusted by selecting the particle composition. QDs with different emission colours can be excited simultaneously at a single wavelength.

tuned by altering their size and chemical composition, giving rise to a wide spectrum of emission colours. In addition, unlike conventional fluorophores, QDs exhibit continuous absorption profiles and can be excited efficiently at any wavelength shorter than the emission peak [81]. As a result, many QDs of different sizes and compositions can be excited simultaneously with a single wavelength of light (figure 4) and this has proved to be extremely useful in multiplexing detection studies [84]. Furthermore, since QDs' emission could be size-tuned to improve spectral overlap with a particular acceptor dye, they are also considered as efficient fluorescence resonance energy transfer (FRET) donors [85]. Thirdly, QDs also emit light at a rate slow enough to eliminate most of the autofluorescence in the background but fast enough to maintain a high photon turnover rate. Therefore, they are ideal probes for time-gated detection with enhanced selectivity and sensitivity [86]. Fourthly, it is possible to obtain polarized fluorescence by using shape-controlled QDs, which can be exploited to study conformational change and energy transfer in biological systems [87].

The unique optical properties of QDs make them appealing as fluorescent labels in biological investigations. A range of biomolecules, including deoxyribonucleic acid (DNA) and proteins, have been conjugated to QDs and used in diverse biomedical studies such as *in vitro* detection assays [88, 89], deep tissue imaging [90–92], and most recently in the selective and generalized imaging of live cells and organisms [93–98]. For example, Nie [96] has developed a multifunctional QD probe linked with tumour-targeting antibodies for cancer targeting and imaging. In his *in vivo* studies on mice expressing human cancer, it was shown that these QD probes accumulated specifically at the tumour sites. At the same time, he also demonstrated multicolour visualization of the cancer cells under *in vivo* conditions by spectral imaging. In addition, imaging tissue with QDs under NIR excitation has achieved much better results than using available dyes. By synthesizing NIR emitting QDs (840–860 nm) and using only  $5 \text{ mW cm}^{-2}$  excitation, Kim [91] demonstrated the imaging of lymph nodes 1 cm deep in the tissue, where lymphatic vessels were clearly visualized draining QD solutions into the sentinel nodes. More interestingly, green-emitting QDs have been excited in the NIR region at low intensity via a two-photon excitation

technique, which allowed the imaging of mouse capillaries hundreds of micrometres deep at subcellular resolution in a mouse brain [92, 99]. In addition to the extensive studies on their biological application as fluorescent labels, the emerging importance of QDs has been documented in a number of recent reviews [84, 100–105]. For example, Nie and co-workers [84] presented an excellent overview of the synthesis, optical properties and surface chemistry of QDs and provided some examples of QD-bioconjugation; Niemeyer [100] gave a comprehensive review covering the biomolecular interactions of all nanoparticles; while Medintz [104] looked at the current methods for preparing QD bioconjugates and presented an overview of their applications as well.

To cater for the increasing use of QDs in biological applications, much effort has focused on the synthetic routes of QDs. Many publications have described the synthesis of highly luminescent mono-dispersed QDs [106–108] and especially noteworthy are those that involved the large-scale production of QDs whilst removing the need for hazardous and pyrophoric reagents [108–111]. Meanwhile, various schemes aimed at the improvement of QDs' biocompatibility and colloidal stability have also been presented [112–117]. Special attention should be paid to the strategies that involve phase transfer using amphiphilic molecules as detergents for the QDs coated with hydrophobic groups [93, 94, 96, 118]. These strategies are particularly advantageous as they allow for the retention of the original surfactant molecules, which seem to increase the stability and fluorescence efficiency of the QDs over those with the original surface completely changed or exchanged with a hetero-bifunctional linker molecule [83]. The original TOPO ligands on the QD surface are used to interact with an amphiphilic polymer like octylamine-modified polyacrylic acid and this leads to the formation of a stable hydrophilic shell around the QD [93]. Another example is to encapsulate TOPO-coated QDs with phospholipid molecules. The hydrocarbon tails of the phospholipids are interlocked with the alkyl chains on the QD surfaces, and the polar phosphate head groups are self-assembled to form micelles in solution [94].

However, despite QDs' potential and success so far in biological applications, there exist several limitations associated with their use. A typical problem in QDs is optical blinking [119–121], which makes the application of QDs in quantitative assays difficult. It was also observed that the size of the QDs may affect the function of attached ligand molecules [122]. Furthermore, QDs themselves are not biocompatible and have to be surface modified before they are used in live cell or animal experiments [123–125]. Therefore, QDs are only complementary to conventional organic fluorescent labels and will not take their place completely. They are better suited for applications where good photostability is required [126].

## 5.2. Lanthanide-doped compounds

Lanthanide-doped inorganic compounds generally consist of an inorganic matrix activated with trivalent lanthanide ions in the form of solid solution [127]. The inorganic matrix not only acts as a host crystal to hold the lanthanide ions tightly but also has the function of sensitizing their luminescence. Several kinds of inorganic materials such as oxide, complex oxide, and fluoride have been used as the host matrices for lanthanide

ions. Similar to lanthanide chelates, the luminescence of lanthanide-doped inorganic nanoparticles is characterized by narrow emission bandwidths determined by the lanthanide ions, which, when used in conjunction with different dopant ions and matrices, can generate different emission colours. At the same time, lanthanide-doped nanoparticles offer substantial advantages over lanthanide chelates such as high photochemical stability and long fluorescence lifetime (up to several milliseconds).

Like semiconductor-based luminescent materials, conventional lanthanide-doped bulk phosphors have played a vital role in numerous practical applications such as laser. However, research and studies on nanoparticles of these materials lagged behind QDs. Increasingly, efforts have been focused on lanthanide-doped luminescent nanoparticles and that yielded notable progress. Several research groups have reported the synthesis of well dispersed colloids of yttrium vanadate [128–130], lanthanum fluoride [131, 132], and lanthanum phosphate [133–137] nanoparticles doped with lanthanide (III) ions. Especially, the syntheses of core–shell nanoparticles, in which only the core has been doped with luminescent lanthanide ions, have solved the problem of low quantum yield that is inherent to lanthanide-doped nanoparticles [138, 139]. These materials, taking advantage of size-induced changes, are expected to create new or enhanced materials for advanced phosphor and photonic applications [140–142]. In particular, they are strongly fluorescent, low in toxicity, and readily synthesized in water, which greatly facilitates further biofunctionalization. Moreover, although blinking in single lanthanide-doped nanoparticles was observed previously [143], it has been demonstrated that the blinking can be avoided by doping a higher concentration of the lanthanide ions in the nanoparticles [144]. Owing to these properties, they also serve as promising candidates for fluorescent labels. As a result, their biological applications have been reported favourably in recent years [144–148].

Another unique feature of lanthanide-doped phosphors is their ability to emit photons in the visible range after being excited with infrared light, in a process known as up-conversion [149]. There is growing interest in the study of up-converting phosphors, as they are one of the most promising materials for the production of solid-state lasers, especially blue-light-emitting lasers. Meanwhile, the use of up-converting phosphors as fluorescent labels for sensitive biological assay and detection has attracted even more interest of late [150–154]. For example, by using 400 nm green-emitting  $Y_2O_2S:Yb, Er$  particles as reporters for nucleic acid microarray, a detection sensitivity augmentation of fourfold was detected when compared to the conventional Cy5 [154]. Up-converting fluorescence labels possess several distinct advantages compared to commonly used down-converting phosphors:

- (1) a low optical background is expected due to the absence of autofluorescence of biomolecules upon infrared radiation;
- (2) due to the large wavelength separation between excitation and emission, the optical train is very simple and so there is no need for time-resolved detection; and
- (3) simultaneous detection of multiple analytes can be realized since different colours of visible light can be obtained from different up-converting phosphors excited by the same IR laser [150].

However, previous up-converting phosphors used for biological labelling are somewhat too large in size, and thus cannot be used for the sensitive analysis of molecules such as DNA, RNA, or protein [154, 155]. Accordingly, nanosized up-converting phosphors have received considerable attention and much literature has described the preparation of strongly luminescent up-converting nanoparticles [155–158]. In particular, the syntheses of lanthanide-doped nanoparticles show that multicolour up-conversion emission in the liquid phase [156, 157] has made a big step towards the development of nanomaterials with a great potential for applications in tracing and determining biomolecules. In addition, the use of up-converting nanoparticles in the detection of biomolecular interactions has also been demonstrated [158].

Despite the promising prospect of lanthanide-doped nanoparticles in biological labelling, their syntheses and biological applications are both in the early stages. Lanthanide-doped nanoparticles previously reported are mostly made via high temperature or bombarding experiments [159–161]. Particles are formed without any ligands on the surface and thus they have no dispersibility in solvents, which is a basic requirement for biological experiments. Therefore, further refinement in the synthetic process is required to eliminate the need for high-temperature annealing of as-prepared materials. In addition, it is noted that most lanthanide-doped nanoparticles involved in biological studies currently are typically prepared in an aqueous solution [144, 158]. The biofunctionalization of those synthesized in organic environments or using an organic surfactant, which present a large number of lanthanide-doped nanoparticles known to date, is sparingly done so far [147]. Consequently, the surface modification of lanthanide-doped nanoparticles and their conjugation with biomolecules is still an open area for investigation.

## 6. Fluorophore-tagged latex/silica nanobeads

### 6.1. Organic fluorophore-tagged nanobeads

It is well known that a major problem with traditional organic fluorophores lies with their poor photochemical stability, which would in turn result in low sensitivity in the biological studies. Organic fluorophore-tagged fluorescent latex nanobeads are a good alternative that have evolved to address the shortfall. Unlike single organic fluorophore such as FITC or rhodamine, each nanobead may contain up to thousands of molecules of an embedded fluorophore that are encapsulated in a polymer shell and protected from the outside environment. As such, these nanobeads emit more bright light (no blinking) than single fluorophore molecules owing to the large number of fluorophore molecules present in one bead. At the same time, since they are encapsulated, fluorophore molecules are now less affected by the external environment and are more stable against photobleaching as well [162].

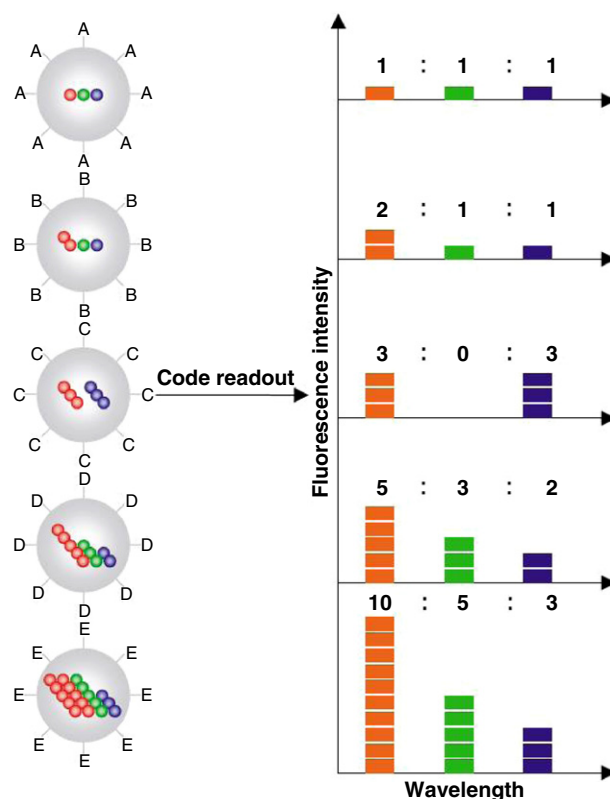
Currently, fluorescent latex nanobeads doped with organic fluorophores are commercially available. Their applications in biochemical assays with great flexibility and improved detection limit have been documented by several reports [162–165]. For example, Nie [162] has conjugated 20 nm fluorescent latex nanobeads with the enzyme EcoRI (MW = 31 kD), a site-specific endonuclease that can be

utilized with high specificity to tag sequence-specific sites of  $\lambda$ -DNA molecules under conditions which allow DNA binding and not DNA cleavage, and used the resulting nanoconjugates to probe specific sequences on  $\lambda$ -DNA molecules. He further demonstrated that the nanoconjugates bound at specific sites and could be directly visualized by multicolour fluorescence microscopy, opening up new possibilities in optical gene mapping that can help the fundamental study of DNA–protein interactions. In another work, performed by Härmä [164], streptavidin was covalently coated onto polystyrene nanobeads which had been previously tagged with lanthanide. Using these nanoconjugates, they are able to detect biotinylated PSA with a detection limit of 60 zeptomoles ( $60 \times 10^{-21}$  mol) in a microtitre plate using a standard time-resolved plate fluorometer. At the same time, individual PSA molecules could be visualized in the same microtitre wells with a time-resolved microscope.

However, a major shortfall associated with fluorescent latex nanobeads, for instance polystyrene nanobeads, lies with the encapsulating polymer itself. Problems often encountered with polymer-coated nanobeads include a larger particle size, swelling, particle agglutination, agglomeration and the leaking of fluorophores through tiny surface defects [166]. These problems have prevented latex fluorescent nanobeads from being used as ultra-sensitive fluorescent labels for effective biochemical analysis. A newer and more popular encapsulating material now being used is silica. It has been demonstrated that a silica shell can be used to encapsulate both inorganic [166–168] and organic [169, 170] dyes, as well as lanthanide chelates [171, 172]. The presence of a hydrophilic silica shell not only enhances the aqueous solubility of the dye molecules; it also acts to improve the stability of the fluorophores and protects them from photobleaching. Furthermore, silanol groups on the outer surface of the nanobeads also allow for the easy conjugation of desired biomolecules through well established silica surface chemistry. As a result, this newer class of fluorophores, which displays excellent photostability and sensitivity over the conventional classes of fluorophores, has been demonstrated as suitable candidates for a range of biological diagnostics and studies involving DNA [168, 170], antibodies [173], and cells [174]. Unfortunately, as both fluorophore-tagged latex and silica nanobeads inherently possess the spectral properties of the organic fluorophores, they will still have some limitations associated with the encapsulated fluorophores.

## 6.2. Quantum dot-tagged nanobeads

As mentioned, since QDs themselves are not water soluble and are non-biocompatible, they cannot be used for biological labelling directly. Conventional methods to solve these problems include surface modifications on single QDs, such as conjugation of mercaptoacetic acid to ZnS-capped or uncapped CdSe and coating of silica on ZnS-capped CdSe QDs [175–177]. However, QDs capped with small molecules such as mercaptoacetic acid are easily degraded by hydrolysis or oxidation of the capping ligand [178]. In comparison, coating QDs with a silica layer can provide improved stability without affecting their optical properties [177, 179]. In addition to capping single QDs with



**Figure 5.** Schematic illustration of optical coding based on wavelength and intensity multiplexing. Large spheres represent polymer microbeads in which small coloured spheres (multicolour QDs) are embedded according to predetermined intensity ratios. Molecular probes (A–E) are attached to the bead surface for biological binding and recognition, such as DNA–DNA hybridization and antibody–antigen/ligand–receptor interactions. The number of coloured spheres (red, green, and blue) does not represent individual QDs, but is used to illustrate the fluorescence intensity levels. Optical readout is accomplished by measuring the fluorescence spectra of single beads. Both absolute intensities and relative intensity ratios at different wavelengths are used for coding purposes; for example, (1:1:1), (2:2:2), and (2:1:1) are distinguishable codes. (Adapted from [182], with permission. Copyright (2001) Nature Publishing Group (NPG) [www.nature.com](http://www.nature.com)).

a silica monolayer, multiple QDs were also encapsulated into silica nanobeads [179–181]. Unfortunately, because of the hydrophobic nature of QDs, it is difficult to encapsulate QDs directly in silica unless they are previously made hydrophilic or unless special silane precursors with hydrophobic tails and hydrophilic heads are used.

Yet another successful attempt in making water-soluble QDs is to encapsulate them in monodisperse latex nanobeads. Nie [182] and co-workers used the swelling method for tagging 1.2  $\mu\text{m}$  polystyrene beads with different combinations of QDs of various colours to create QD bar codes and demonstrated that the use of six colours and ten intensity levels can theoretically encode up to one million biomolecules (see figure 5). DNA hybridization studies demonstrate that the coding and target signals can be simultaneously read at the single-bead level, implying the potential of this coding technology in gene expression studies, high throughput screening, and medical diagnostics. Using a similar technique, Xu [183] developed



a method for preparing Qbead<sup>TM</sup> particles suitable for high-throughput genotyping of single-nucleotide polymorphisms (SNPs) using QD-doped carboxylated microspheres coupled with oligonucleotides and described their first practical biological application to SNP genotyping of the cytochrome P450 family. In addition, Wang [184] reported on obtaining QD-labelled microspheres constructed using layer-by-layer deposition of alternating polyelectrolyte and CdTe nanocrystal layers that are suitable for further bioconjugation. Recently, Nabiev and co-workers [185] studied the optical properties of QD-tagged carboxylated polystyrene beads and found that their level of brightness is comparable to standard polymeric nanobeads doped with organic fluorophores (Fluoresbrite<sup>TM</sup> yellow—orange nanobeads) but are more than 50 times more photostable than the latter. In our laboratory, the synthesis of polystyrene particles of controlled sizes on both micro- and nanoscales with incorporated QDs and intracellular uptake of the nanosized beads have also been investigated [186, 187]. However, despite these breakthrough advances in the synthesis of QD-tagged latex beads and their applications to multiplexed optical encoding, there remain many problems yet unresolved. The main challenges that must be addressed in the use of these nanobeads in biological applications are how to better control the incorporation of the QDs into the polymer particles, as well as the control of colloid stability and monodispersity.

More recently, work has also been done in the area of incorporating QDs into biocompatible polymer beads. For example, Wang and Gao [188, 189] have synthesized QD-tagged hydrogel spheres, and by using them multiplex optical encoding has been realized by loading differently sized QDs into a gel sphere. In addition, using chitosan, a natural biopolymer, water-soluble chitosan-encapsulated CdSe/ZnS–3MPA QDs nanoparticles have been synthesized in our laboratory [190]. The biocompatibility of these QD-tagged chitosan nanoparticles has been assessed in cell culture studies and it was revealed that chitosan-encapsulated QDs nanoparticles exhibited improved biocompatibility over their bare, non-encapsulated counterparts. Importantly, as both hydrogel and chitosan are water soluble, biocompatible and contain reactive functional groups, biomolecules can be further conjugated onto the surface of these beads. As such, these recent investigations certainly open up vast opportunities for creating a new generation of fluorescent markers with immense promise and potential in biological assay and detection.

## 7. Conclusion

In most biological analysis, the sensitivity of detection is primarily dependent on the exogenous labelling agent. Therefore, ideal fluorescent labels should have small molecular weight and high emission efficiency, be photochemically stable etc. Traditional organic fluorophores have some limitations for bioapplications. Although organic fluorophore-tagged latex or silica nanobeads can provide improved physical and chemical properties, they still have some limitations inherent to organic molecules. Inorganic nanoparticles, in comparison, exhibit many superior qualities over organic fluorophores and their derivatives, and are viewed as the direction of fluorescent labels in the future. In particular QDs, possessing a narrow, tunable, and symmetric emission spectrum, open up new possibilities

for many multicolour experiments and diagnostics. Recently developed nanobeads tagged with different-sized QDs at set ratios have been used as multicolour-encoded beads and have great potential in multiplexing studies.

In conclusion, it is felt that inorganic nanoparticles and their derivatives are ideal biological labels that have good potential in biological staining and diagnostics. In addition, there have been numerous well established synthetic routes that yield high quality QDs; and QDs emitting light in the visible to NIR region of spectra are also commercial available. As such, studies on QDs are now increasingly concentrated on their biological applications instead of preparation. As the synthesis and biological applications of lanthanide-doped inorganic nanoparticles are both in the early stage, much work still needs to be done before these nanoparticles become useful labels in practical application. At the same time, owing to their quick optical response and ease of usage, organic fluorophores will still be attractive choices for labelling of biomolecules, especially in short-term studies that do not require any multiplexing detection.

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