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Fluorescently Labelled Nanomaterials in Nanosafety Research: Practical Advice to Avoid Artefacts and Trace Unbound Dye

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Abstract

Fluorescence labelling has become a fundamental tool in nanotoxicological research. There are, however, certain drawbacks when dealing with the labelling of nanomaterials. Very often the leaching of dye from the nanomaterial or the presence of unbound dyes in solution leads to the incorrect quantification and localisation of nanomaterials in cells. In this feature article we will discuss possible situations, which may give rise to incorrect quantification of the fluorescence associated with nanomaterials and their consequences in the evaluation of the fate of the nanomaterial and its intracellular dose.

Issues related to the labelling strategies, dye photostability, impact of the dye on the properties of the nanomaterial surface, and the presence of unbound dye will be discussed. We will also show how Fluorescence Correlation Spectroscopy can be used to trace the presence of free label in solution. In addition, we will discuss the interaction of fluorescence molecules with metallic nanoparticles which can lead to an enhancement or quenching of fluorescence depending on the distance between the dye and the nanoparticle surface. Finally, we will compare the fluorescence emission originating from quantum dots and organic molecules localisation

Keywords: Fluorescent labelling, Nanosafety, Nanomaterials, Metal Nanoparticles, Quantum Dots

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1. Introduction

The visualisation of nanomaterials and the quantification of nanoparticle dosage in biological environments are issues of capital importance for the toxicological evaluation of nanomaterials.¹ Nanoparticle visualisation is usually related to fate studies motivated by the need to understand intracellular trafficking of nanomaterial, at the organ, sub-organ, and cell levels.^{2,3} The ability to localise and track how nanomaterial translocate can yield invaluable information that describes the interaction⁴ mechanisms of nanomaterials with biological matter, and eventually their toxicity.^{5,6} Simultaneously, dose quantification of nanomaterial is fundamental for the toxicological evaluation in order to establish a relationship between dose and toxicological end-points, and for the development of predictive toxicology models.⁷

For both nanomaterial visualisation and dose quantification, there are several approaches and techniques available. One approach that is valid for both nanomaterial tracking and quantification is the use of a label, i.e., a fluorescent or radio label that can be incorporated, or attached to the nanomaterial, and as such provides a fluorescence signal or radioactivity suitable for tracking and quantification.^{8,4}

In vivo, radiolabelling and the techniques used for activity quantification/imaging have clear advantages over fluorescence labelling, since radioactivity in vivo is much more sensitive than fluorescence, without attenuation and it is more easily localised. Additionally, organ harvesting allows the precise determination of activity per organ, which is otherwise difficult to achieve by fluorescence techniques, due to autofluorescence in biological systems.

On the contrary, in vitro, fluorescence labelling is more suitable for cell studies as fluorescence microscopy provides sufficient resolution to allow visualisation of the labelled nanomaterial at the subcellular scale. Consequently, there are many effective protocols for the labelling of cellular compartments and organelles.¹⁰ The correct selection of cell markers and labels for the nanomaterials should ideally minimise spectral overlap of the labelled material, thus allowing for the intracellular localisation of the nanomaterials once uptaken by the cells.¹¹⁻¹³ Frequently, experiments are performed with living cells. For these reasons, confocal laser microscopy has been a preferred technique for intracellular visualisation of nanomaterial over other techniques, which do not require labelling such as confocal Raman microscopy¹⁴⁻¹⁶ or transmission electron microscopy.¹⁷ Confocal microscopy is a common technique within most laboratories working with cells. The rich availability of fluorescently labelled nanomaterials opens up the possibility of applying more advanced fluorescence-based techniques such as Fluorescence Correlation Spectroscopy (FCS),¹⁸ Fluorescence Lifetime Imaging (FLIM)¹⁹, Förster Resonance Energy Transfer (FRET), and Total Internal Reflection Fluorescence (TIRF).²⁰ These advanced techniques can yield valuable information regarding the state of the nanomaterial, the local intracellular environment, and translocation through the cell membrane.

Most importantly, fluorescence labelling provides a means to quantify the amount of nanomaterial per cell via Flow Cytometry, or by Fluorescence Cell Sorted FACS. Flow Cytometry techniques permit the determination of the fluorescence per cell in a cell suspension. After accounting for the cell autofluorescence it is then possible to relate the fluorescence per cell to the amount of uptaken

nanomaterial associated with each cell over a large population of cells, typically tens of thousands of cells.²¹

Indeed, fluorescence labelling has become a fundamental tool in nanotoxicological research. There are, however, certain drawbacks when dealing with the labelling of nanomaterials which are often the source of common mistakes. One is the proper choice of the label. Very often if the label is not photostable it will be bleached. This is a major concern in confocal microscopy or FCS. Secondly, the label may detach from the nanomaterial as the link to the nanomaterial may not be stable in biological media or can be exchanged for biomolecules, as is the case for thiol molecules. Thirdly, and probably the most important source of artefacts with labelled nanomaterials, results from the surface labelling of nanomaterials. This results from excess fluorescent label remaining in solution, even after purification. Normally, in order to maximise the yield of a labelling reaction there is an excess of label used. Once the labelling reaction is over this excess label must be removed, i.e. the nanomaterial washed leaving only the attached label. The removal of the excess label can be performed by centrifugation, dialysis, solvent extraction, or a combination of some or all of these techniques. This is often tedious work, sometimes requiring a long time, e.g. days or weeks for dialysis. Moreover, fluorescent dyes typically carry a charge and depending on the charge of the nanomaterial may remain trapped on the surface by electrostatic interactions or even hydrogen bonding, making it very difficult to completely remove them.

As previously mentioned, labels are first located on the surface of the nanomaterial rendering it fluorescent, however, the label may detach in biological media, in the presence of salts, due to intracellular pH, or by biological action. Both the eventual detachment of the label from the nanomaterial or the presence of unbound dye are additional sources of artefacts. They may give a false intracellular localisation of the nanomaterial as the detected fluorescence is not truly associated with the nanomaterial. For flow cytometry studies the presence of free dye in solution can also result in an incorrect quantification of nanomaterial uptake if the free dyes are incorporated in the cells. Again, fluorescence measured per cell would not be solely associated with an uptake of nanomaterial. The presence of free dye in a nanomaterial solution is a very common situation that is, in general, ignored in most cases.⁸ Nonetheless, it is a source of artefacts and a cause of the lack of reproducibility in many experiments.

The main scope of this mini review article is to highlight potential sources of artefacts when dealing with labelled nanomaterials and draws on knowledge and experience gained as a result of the EU project QualityNano. The aim is to provide helpful insights to the community working with labelled nanomaterials in nanosafety, and also nanomedical issues. We will also show how FCS can be used to calculate the degree of labelling of nanomaterials and to trace the presence of free label in solution. Additionally, we will discuss the interaction of fluorescence molecules with metallic nanoparticles and how this impacts on the labelling of the nanoparticles and the fluorescence intensity of labelled nanoparticles. Aspects related to the impact of linkers as a mean to control the distance between the surface of the metallic NP and dye, to reduce the influence of the NP on the dye fluorescence, and the interaction between dyes, will be discussed as sources of quenching or enhancement of fluorescence, which must be taken into account during labelling and for quantitative assays involving fluorescence and specially for comparison among different labelled metal NPs. Finally, we will compare quantum dots fluorescence vs fluorescence of organic molecules.

2. Labelling of Nanomaterials

When deciding to label a nanomaterial there are certain factors to consider before proceeding, primarily, the choice of the label, where to label, and the labelling strategy, i.e.: core labelling, surface labelling, or the adsorption of fluorescently labelled polyelectrolytes, etc. Here we will discuss some factors to consider when choosing a fluorescent label and some of the different possibilities for fluorescent labelling of nanomaterial. The concentration of the attached label is also of importance, too much and one runs the risk of self quenching, too little and there may be insufficient emission intensity for confocal imaging, or detection via flow cytometry.

2.1. Choice of Fluorescent Label

Before performing any experiment one must select a fluorescent label that matches the requirements of the measurement to be undertaken, e.g.: high photostability, pH sensitivity, ion sensitive fluorescence lifetime, sufficient spectral overall, excitation wavelengths available, etc. For example, FITC which is commonly used in confocal microscopy for cell labelling and for flow cytometry is not suitable in FCS measurements due to its low photostability. However, it is

perfectly suited to pH sensing measurements.^{22,23} Another factor to consider in the choice of label is the labelling mechanism, i.e covalent bonding or electrostatic interaction for example. This may lead to leakage of the label under certain conditions.

2.2. Core Labelling

Examples of nanomaterials that are labelled or can be labelled in the core, mostly during fabrication: silica particles,²⁴ organic nanoparticles with labelled polymers,²⁵ core-shell nanoparticles²⁵ (core quantum dots for example²⁶), nanoparticle doping. For example, core labelling of Polystyrene (PS) particles can be performed using an organic solvent such as toluene or chloroform. The majority of fluorescent dyes must first be dissolved in an organic solvent, and then co-incubated with the PS particles. The organic solvents cause the PS particles to swell, allowing the dye to penetrate the particle. However, this can also lead to the complete dissolution of the PS particles if allowed to incubate for too long. The use of ethanol provides an excellent alternative to organic solvents as it induces a lower degree of swelling and thus has a reduced impact on the particle shape. Nonetheless, this requires good solubility of the fluorescent dye in ethanol rather than in an organic solvent. The solubility of the dye in ethanol does, however, present a disadvantage, namely during the particle rinsing phase a large amount of dye will simply be washed away. Solvent is also often difficult to completely remove, leading in many case to particles with variable physical characteristics.

2.3. Surface Labelling

The surface labelling of nanomaterials usually requires the surface functionalisation of the material in question unless there are functional groups already present on the surface.²⁸ The most common means of functionalising the surface of nanomaterials employs the use of carboxylates, amines, thiols, or other groups.²⁹ The interaction of the dye and the functional group also needs to be considered. That is for example, if the attachment is via covalent bonding or through electrostatic interaction. There are other alternatives for labelling. For example, it is possible to prepare a fluorescently labelled polyelectrolyte and then coat the nanomaterial using the Layer by Layer method.^{32,33} In addition to using fluorescently labelled polyelectrolytes, quantum dots may also be adsorbed onto the surface of a nanoparticle when this has a larger size than the quantum dots. This

can be done in a layer by layer fashion and can also allow for multiplexing, i.e. quantum dots with different emission wavelengths. This technique has already been used by Mulvaney et al. to produce a fluorescently labelled whispering gallery mode microcavity.³² A more exotic option for achieving fluorescent labelling of a nanoparticle surface is the use of J aggregates. These are typically non-fluorescent under normal conditions however, when the concentration is sufficiently high, J aggregates are formed which fluoresce. There is, however, a very small Stokes shift in J aggregates, making their use less common and more specialised.³³

In the following table we have compared the advantages and disadvantages of core vs surface labelling. We will develop the content of the table throughout the paper.

Type of labelling	Advantages	Disadvantages	Labelling Quality	Unbound dye
Core labelling	Limited influence of the dye on the surface characteristics of the NPs. Limited influence of the media on the photophysical properties of the dye	Limited to the cases where i) dyes can be entrapped in the NP during fabrication; ii) NP that can be doped with luminescent ions; iii) use of semiconductors; iv) NPs with intrinsic fluorescence (QDs).	UV Fluorescence Spectroscopy Fluorescence life time	The amount of unbound dye is limited as the NP are intrinsically fluorescence or the dyes are trapped in the core
Surface labelling	Larger number of NPs can be labelled as involves either covalent bounds or other interactions i.e self assembly, electrostatic adsorption, physisorption. There are multiple routes as well for NP functionalization that render the surface of NPs with groups that can be labelled, i.e. silanizations, thiol binding, etc	Usually unbound dyes remain in NP dispersions. Labelling affects the surface characteristics of the NPs i.e. charge or hydrophobicity and can consequently alter NP translocation and fate.	UV Fluorescence Spectroscopy Fluorescence life time FCS	Unbound dyes are often present and require significant efforts to remove them: Centrifugation, HPLC, solvent extraction, etc. Often dyes to be bound covalently remain physisorbed on the NPs and are particularly difficult to remove

Table 1. Comparison between core and surface labelling of Nanomaterials.**2.4. Sample Purification**

The purification of the labelled nanomaterials has a major influence on the success of a fluorescence measurement. The purification method of the labelled nanomaterial depends on the molecular weight of the unlabelled nanomaterial. If the nanomaterial to be labelled has a molecular weight of less than 10 kD, then purification should ideally be done by means of HPLC. Alternatively, tangential flow filtration or centrifugal washing can be used. The former is relatively quick, and there are a large number of membranes available today. If the molecular weight of the nanomaterial to be labelled is higher than 10 kD, the product can be separated from excess dye by gel filtration e.g. using a Sephadex G25 column. An alternative in both cases is extensive dialysis using the correct molecular cut-off size of dialysis membrane. In the case of nanoparticles extensive centrifugation can be performed, however, it should be noted that if the molecular weight of the label is sufficiently large then it may also precipitate during the centrifugation process. Therefore, sample purification via centrifugation should be repeated several times in order to remove the maximum quantity of unbound label.

For the attachment of a dye to the surface of a nanomaterial, it is normally necessary that there are amine or carboxylate groups present on the nanomaterial surface. However, other surface functionalities can also be used for covalent chemistry such as thiols, alcohols, ketones, or esters. During the labelling of nanomaterial the conditions are normally such that the functional group is not charged. As most dyes are charged it can often be found that some dye remains bound to the nanomaterial via electrostatic interactions when the dye and nanomaterial bear opposite charges. As such, it can be very difficult to remove by dialysis or centrifugation. In cases such as this, it is possible to re-disperse the nanomaterial in a 1 M NaCl solution, for example, that will weaken the nanomaterial dye interaction, thus allowing for an easier removal by centrifugation.

2.5. Cell Autofluorescence

Cell autofluorescence is often undesirable in confocal imaging or flow cytometry measurements. However, the careful selection of the fluorescence label and a suitable excitation wavelength can easily remove this problem. The cell cytoplasm often shows autofluorescence, which can be ascribed to the presence of NAD(P)H and flavins, amongst others, which tend emit in the range

400 nm to 600 nm, as can be seen in **Figure 1** below.^{34,35} Thus, using a label that can be excited at 633 nm with emission in the red should eliminate many of these problems. Time resolved fluorescence measurements provide another means of differentiating between cellular autofluorescence and that emanating from the chosen fluorescent label via fluorescence lifetime imaging microscopy (FLIM).³⁶ For example, FLIM has previously been successfully used to distinguish between GFP and cellular autofluorescence.³⁷

Figure 1. Typical autofluorescence emission from some of the single endogeneous fluorophores present in the intracellular environment. Excitation wavelength: 366 nm. Reproduced from Croce et al.³⁶

2.6. Non-Specific Binding

Having chosen a suitable label that meets the needs of the experimental conditions it is often advisable to check for non-specific binding of the label. This non-specific binding may result from the dye binding to buffer components or interacting with other molecules. This can lead to an effective reduction in the concentration of the labelled nanomaterial. In a study involving the use of fluorescently labelled gold (Au) NPs, we showed that binding of the labelled NPs to biomolecules present in the cell culture media produced an effective reduction in the concentration of the Au NPs.³⁸ The particular Au NPs used had been prepared with a Glycan in order to minimise non-specific binding. This measurement was performed by means of FCS. The effective reduction in Au NPs concentration manifests itself as a reduction in the correlation value measured which is inversely proportional to sample concentration. The FCS autocorrelation curves relating to this

experiment are shown in **Figure 2**, in which it can be clearly observed that there are significant differences in both diffusion time and correlation value for Au NPs in distilled water and cell culture media. The change in correlation value indicates the possible formation of Glc-Au-Hi NP aggregates in cell culture media. However, it does not completely rule out protein corona formation or non-specific binding, thus highlighting the need to characterise or have a priori knowledge of the labelling dye.

Figure 2. FCS autocorrelation data for Glc-Au-NPs in distilled water and cell culture media.

2.7. Impact on Surface Properties

The presence of dye on the surface of a nanomaterial may have a significant impact on the surface properties of the nanomaterial, in some cases the dye may be comparable in size to the material being labelled. Dyes are most often organic molecules with several organic rings and carboxylate, phenolic and amine groups. The organic characteristics of the dye can increase the hydrophobicity of the nanomaterial, which can, in turn, affect the cellular uptake and the penetration of the nanomaterial through cell membranes. It will alter the charge density of the surface and this can lead to aggregation, and to a different interaction with proteins. A way to avoid this, though not always possible, is to shield the dye within a suitable surface coating. In a recent study performed in our laboratory we showed that it was possible to sparsely attach a fluorescent label to the surface of CeO₂ NPs and following this synthesise polymer brushes on the NP surface.³⁹ In this work it was shown that the dye did not alter the NP surface characteristics and at the same time the NPs were labelled with a fluorescent dye that allowed for their intracellular detection (see

Scheme 1 below). CeO₂ were first silanized with an amine silane. Dyes were attached to the amines groups taking care that only a small percentage of all amines are labelled and the rest of the amines were modified with an initiator for Atomic Transfer Radical Polymerization (ATRP). From the initiator brushes were synthesized resulting in a polymer shell that cover the NPs and shields the fluorescence dye from the environment. In this way the influence of the dye on the surface properties of the NPs is minimized and the outer chemistry around the NPs corresponds to the polymer chosen for the brush synthesis.

Scheme 1. Scheme of 'grafting from' polymer brush synthesis at the surface of cerium oxide NPs.

2.8. Dye Leaching or the Presence of Unbound Dye

As previously mentioned, one of the most common sources of artefacts is the presence of unbound dye. This unbound dye may result from insufficient sample purification but may also arise due to leaching or detachment of bound dye once the nanomaterial is exposed to the experimental

conditions. The experimental conditions may expose the labelled nanomaterial to changes in pH, enzymatic action, solvents of different polarity, etc. If the attachment is via electrostatic interaction, then the likelihood of the dye becoming detached, due to environmental conditions, from the nanomaterial surface increases.

Some bonds can easily hydrolyse such as ester bonds which can subsequently lead to leaching of dye from the nanomaterial surface. An interesting case of dye leaching is that of gold or silver nanomaterials that are coated with thiols. The labelling of such nanomaterials proceeds through the attachment of a thiol followed by the pre-thiolated dye.⁴⁰ Thiols form a dynamic bound and are non-locally bound to the gold surface. They can be easily exchanged by other thiols or replaced for example by proteins that are present in cell media or intracellularly. It is also worth noting that FITC, which is used extensively, is also widely known to have particularly weak bond stability at low pH.

A simple means of tracking the presence of unbound dye after labelling a nanoparticle is to perform a dialysis or a centrifugation of the nanoparticles and measure the UV absorption spectra of the dialysis media or the supernatant, respectively. However, the UV signal may be very weak and often undetectable if the concentration of dyes is low, which is typically the case after exchanging the dialysis solution a few times. After a certain time the free dye will be released to the dialysis media very slowly and might become hard to detect it. The same argument is valid for centrifugation. Fluorescence Correlation Spectroscopy (FCS) provides a valuable means to detect the presence of unbound dye in the supernatant or dialysis water with a sensitivity in the nanomolar range. To illustrate this, we have labelled SiO₂ NPs (G. Kisker GbR, 500 nm) with Atto-488 dye and dialyse the nanoparticles in a dialysis bag for 200 hours to remove unattached dye. The external dialysis solution was exchanged twice a day and it was measured by FCS at different time points. **Figure 3** shows the autocorrelation curves of these measurements. The amplitude of the autocorrelation curve determined from the maxima of the autocorrelation curve is inversely proportional to the number of fluorescent molecules in the solution. The lowest value for the amplitude in the external dialysis solution is observed 4 h after dialysis. The lowest amplitude means the highest concentration. Amplitude increases as the dialysis time reaches 200 hours, following several exchanges of the external dialysis solution. This means that the number of fluorescent molecules in the external dialysis solution decreases as the number of exchanges of the

dialysis solutions increases. Insert in Figure 3 shows the amplitude of the autocorrelation curve for each dialysis solution removed (Figure 3.a).

Figure 3. Autocorrelation curves measured with Fluorescence Correlation Spectroscopy. Measurements of a) the external dialysis solution at different time points for 200 hours, Insert showing the $G(0)$ values of the same samples at different time points corresponding each to a

different external dialysis solution and b) the inner volume of the dialysis bag after 200 hours dialysis.

When the amplitude became high and the fluorescent molecules could not be detected in the external dialysis solution, FCS measurements were conducted in the inner solution of the dialysis bag. The autocorrelation curve is shown in Figure 3, showing an amplitude close to 1, which correspond to the SiO₂ labelled nanoparticles. This proves that while the dye outside the dialysis bag is absent labelled nanoparticles remain inside the dialysis bag.

Figure 4. A) Sketch of the washing procedure to remove unbound fluorescent dye from labelled NP solution. In a general procedure after the labelling procedure (dye place exchange reaction), the excess of fluorescent dye is removed by washing/centrifuge cycles. The supernatants (a-c) and dye-labelled NPs (d-f) pellet are incubated with RBC and the fluorescence signal is quantified by CLSM and FACS. B) displays the minimum amount of free dye per NP that it is possible to detect by CLSM and FACS. In the case of NPs at 0.2 mM it is possible to detect 1 molecule of dye per NP. Reproduced from Andreozzi et al.⁴¹

There are different approaches reported in literature to determine the presence of free dye in a nanoparticle dispersion. An interesting method for the detection of free dye in a sample of fluorescently labelled nanoparticles has been reported by Andreozzi et al.⁴¹ The approach is sketched in **Figure 4**. Fluorescently labelled gold nanoparticles with an excess of dye in solution are exposed to erythrocytes (RBC). Erythrocytes are non endocytic cells and therefore will not eat the nanoparticles. The free dye in solution, however, will diffuse in the cellular membrane of

erythrocytes resulting in their labelling, hence fluorescence signal is detected. Confocal Microscopy and flow cytometry were used to evaluate the fluorescence of the erythrocytes. The confocal images in Figure 4A show that the RBCs exposed to the washed nanoparticles show no fluorescence while those with the dye in solution are fully red. Experiments were performed for different nanoparticle and dye concentrations. Figure 4B shows the minimum number of free dye molecules per nanoparticle that can be detected by confocal microscopy. The maxima of the intensity distribution is shown for different ratio of dyes and nanoparticles and it can be observed that the intensity increases as the free dye concentration increases. This methodology provides a simple means to determine free dye in nanoparticle dispersion using RBC and confocal microscope or flow cytometry.

2.9. Photobleaching

The most obvious feature of photobleaching is a loss in fluorescence emission intensity. In some instances this may be advantageous; however, in the majority of scenarios it can be detrimental to the measurement. For example, if trying to image nanoparticles in cells, the task becomes next to impossible if photobleaching is seen. Photobleaching can be a cause of major loss of information, especially for confocal microscopy and Fluorescence Correlation Spectroscopy. To give an example, fluorescein is easily photobleached while rhodamine is not and therefore preferred for FCS measurements. Photobleaching is the result of exposing a dye to sufficient excitation energy that it alters their chemistry as a result of a redox process and the molecules lose their capability to fluorescence. The photostability of dyes is normally provided by the suppliers and available for most dyes. It is, however, an important issue to take into account when designing an experiment with fluorescence techniques and for labelled nanoparticles can lead to a loss of information regarding the intracellular localisation of the nanoparticles or their concentration.

3. Experimental Techniques

3.1. Flow Cytometry

Flow cytometry can yield a vast amount of information regarding the cellular uptake of fluorescently labelled nanomaterials over a large cell population in a statistically meaningful manner. It does this by surveying tens of thousands of cells in a relatively short time span, building up fluorescence intensity distributions and dot plots which can be used to distinguish between

different populations of fluorescently labelled material. Flow cytometry is a useful tool in quantifying the uptake kinetics of NPs with time as it allows tracking changes in fluorescence per cell in a cell population as a function of time following exposure of cells to labelled nanoparticles. However, flow cytometry is not capable of distinguishing between labelled NPs or free drug uptaken by cells. Sometimes, leaking of a fluorophore from NPs can also lead to a change in the fluorescence intensity per cell, which does not correspond with the actual fluorescence due to the uptake of the labelled nanoparticles. To give an example, we will discuss a study performed by our laboratory, where we have successfully shown that it possible to monitor the degradation kinetics of fluorescently labelled poly(lactide-Co-glycolide) (PLGA) particles in the intracellular environment.⁴² In this work, Rhodamine B labelled PLGA was co-incubated with cells in order to determine if it were possible to monitor the intracellular degradation of particles formed from PLGA. The degradation of PLGA occurs through autocatalytic hydrolysis of the ester bonds, with a degradation rate that depends on the copolymer composition.⁴³⁻⁴⁵ Decreases in the maximum emission intensity, as well as broadening of the fluorescence distributions, over time showed that it was possible to monitor the degradation of the PLGA particles in physiological conditions, see **Figure 5**. As can be clearly seen, the fluorescence intensity decreases in physiological media, indicating a loss of fluorescent material due to PLGA degradation. In the case of PLGA particles in the intracellular environment we see an increase in fluorescence as material is released from the degradation of the PLGA particles inside the cells. In this case fluorescence was measured per cell. The reason why the fluorescence increases is that, upon degradation, the rhodamine label moves from the interior of the nanoparticle to the endosomal environment. The change from an organic environment to an aqueous one has a significant impact on the fluorescence yield, resulting in the observed increase in fluorescence. These results are based on the change in fluorescence intensity upon liberation of the dye. Similar scenarios during uptake studies may result in an increase of the intensity, which does not reflect the actual amount of NPs taken up by the cells but the actual leakage of dye from the NPs. Again, the stability of the dye in the NPs must be ensured and it is extremely useful to determine how the dye emission will change with variations in the polarity/pH of the environment.

Figure 5. Fluorescence intensity distributions corresponding to PLGA. (a) In physiological conditions, (b) in intracellular conditions.

3.2. Fluorescence Correlation Spectroscopy (FCS)

Fluorescence correlation spectroscopy (FCS) provides a suitable methodology for measuring mobility, association, and ligand kinetics of biological molecules in the cellular environment.⁴⁶⁻⁴⁸ FCS measures the time dependent fluorescence signal within the focal volume (typically <1 fL) and analyses the recorded signal in order to yield information regarding a fluorescent probes diffusion time, concentration, chemical kinetics, and also allows the size of the probe molecule to be estimated.⁴⁸⁻⁵³ In FCS measurements photobleaching will manifest itself as a reduction in the average count rate data recorded and will also lead to shorter than expected diffusion times. These shorter than expected diffusion times results from the photobleaching having the effect of the NP of interest exciting the confocal detection volume, thus leading to erroneously short diffusion times. However, if we want to determine the diffusion time of labelled NPs or fluorescent proteins inside

the cells, we can use photobleaching to our advantage. Upon uptake, the majority of material uptaken by the cell is trafficked for processing by the cell, leading to the immobilisation of this material. This immobile fraction stills emits fluorescence which will not yield any meaningful FCS information. Nonetheless, by photobleaching this immobile fraction we can reveal if there is a mobile fraction of material still present and thus generate meaningful FCS data.

To investigate the intracellular dynamics of the Glc-Au-Hi NPs, HepG2 cells were incubated with Glc-Au-Hi NPs at a concentration of $0.05 \mu\text{g/mL}$ for a period of 30 minutes at 37°C .³⁸ Following this incubation FCS measurements were performed. FCS data were collected without prebleaching to display the lack of coherent correlation data that is recorded (see **Figure 6**). As a result, we cannot calculate the diffusion time, local concentration, particle size etc. However, contrary to this, while implementing prebleaching, fluorescence fluctuations can be recorded and fitted with a single component autocorrelation function (see **Figure 6**) thereby allowing FCS data to be collected. However, it should be noted that not all measurements following prebleaching yield meaningful results. This is possibly due to the immobile fraction not being completely photobleached or the absence of NPs in the selected measurement location.

Figure 6. a1) FCS autocorrelation data prior to prebleaching, b1) & c) FCS autocorrelation data after applying prebleaching. a2) CLSM image of Glc-Au-Hi NPs (shown in red) uptaken by cells. b2) Transmission light image of the cells. c2) HepG2 cells with the membrane stained with Cellmask Orange (shown in green). d2) Images (a2, b2, and c2) combined.

As the amplitude of the correlation function is inversely related to the concentration of the fluorophore, which are associated to the NPs, we can now use this as a measure of the homogeneity of intracellular concentration of Glc-Au- Hi NPs at the measurement sites. In Figure 6 we can see that the amplitude of the correlation curves for the two chosen sites are almost identical. Assuming that there is only one dye molecule per Glc-Au-Hi NP and that they are all labelled, we can estimate the concentration of NPs as being equal to the dye concentration which can be calculated from the amplitude of the correlation curves. We see that for the Glc-Au-Hi NPs we calculated relative local concentrations of approximately 9.40 nM for the selected measurements sites. As the concentration of Glc-Au-Hi NPs used during cell incubation is the same as that used for making FCS measurements in distilled water we can easily compare the concentrations. However, as we cannot easily say whether there has been 100% uptake of Glc-Au-Hi NPs, we cannot easily speculate about whether we are measuring data related to small NP aggregates or if there is protein corona formation around the NPs.

4. Fluorescence Enhancement and/or Quenching by Metal Nanoparticles

Organic fluorophores are of crucial importance in optical sensing and imaging due to their ability to re-emit light upon excitation. The signal generation in fluorophore sensing is via the excitation of the electrons of the fluorophore by optical energy. However, this fluorescence emission can be substantially altered when the fluorophore is placed near an entity possessing a strong electromagnetic (plasmon) field, such as in the case of noble metal NPs (e.g.:Au, Ag and Cu).

In noble metals, visible light excites localised surface plasmon resonances (LSPRs) which result in an intense peak in the absorption spectra in the UV to visible region of the spectrum. At this resonance wavelength, metal NPs both scatter and absorb incident light very efficiently. At the same time, the excitation of a LSPR results in an enhanced electromagnetic near field in close proximity to a NP, within a zone proportional to the particle radius.^{54,55} This enhanced field ‘focuses’ light on a length scale far below the wavelength of light and provides a means to use

metal NPs as energy transfer antennae in various applications. However, it substantially affects the emission of a fluorophore placed near to the NP surface.

When a fluorophore is placed at a relatively short distance from a metal NP possessing a strong plasmon field, the electrons of the fluorophore participating in the excitation/emission interact with the field. The nature and level of interaction depends on different parameters. These primarily include the plasmonic electric field created by the metal NPs, which is ultimately determined by the composition and morphology of the metal NP, the absorption and emission wavelength, and intrinsic quantum yield of the fluorophore. On the other hand the distance between the NP and the fluorophore and the relative difference between both wavelengths (fluorescence emission with respect to the SPR of the metal NP) are also contributing factors. Besides these contributions solely associated with the presence of the metal NPs, the fluorophore-fluorophore interactions can also significantly reduce the observed fluorescence intensities and lifetimes of the fluorophores.⁵⁶ The sum of these interactions results in a change in the fluorescence emission level that manifests as a quenching or enhancement of its fluorescence emission.

4.1. Quenching or Enhancement?

Although quenching⁵⁸⁻⁶¹ and enhancement⁶²⁻⁶⁴ phenomena have traditionally been studied separately, recent studies have indicated that both are caused by the presence of metal NPs in the vicinity of the fluorophore.⁶⁵⁻⁶⁷ In the instance where the electromagnetic field generated by the metal NP is efficiently transferring energy to the fluorophore, there is an enhancement of the level of fluorescence emission. In this situation, the fluorophores absorb more light than in the absence of the metal NP (leading to an increase of the excitation decay rate of the fluorophore) which is translated into a higher fluorescence quantum yield. Alternatively, when the energy is efficiently transferred from the fluorophore to the metal NP, the quantum yield of the fluorophore decreases (due to an alteration in the radiative and non-radiative decay rates) resulting in fluorescence quenching.^{68,69}

This enhanced emission or quenching of the fluorophore near the metal surface can be understood with regards to the ability of the metal surface to extinguish, by absorption or scattering processes, the incident light. For metal NPs, the induced plasmons radiate a fraction of the incident energy whenever the scattering cross section of the metal NP is dominant compared to its absorption cross section (**Figure 7**). Thus, small metal NPs are expected to quench fluorescence because absorption

is dominant over scattering while larger NPs are more likely to enhance the fluorescence because the radiative scattering component is dominant over absorption. Thus, for instance, the scattering efficiency of small (< 20 nm) metal (Au and Ag) NPs is negligible while it increases over 5 (for Ag NPs, > 30 nm,) or 2 (for Au NPs, > 40 nm).

Beyond the basic study and quantification of the modifications of the emission of a fluorophore when it is placed near an entity possessing a strong electromagnetic (plasmon) field, these aspects are of crucial importance for the design of metal NPs with a maximised fluorescence enhancement.⁶⁶ These NP based platforms could be beneficial in the development of highly efficacious optical contrast agents for bio-sensing/imaging, offering greater sensitivity and signal-to-noise ratio, especially for fluorophores with a low quantum yield. Besides, if both quenching and enhancement are implemented in a single fluorophore, then the resulting product can be a highly specific (e.g., FRET) and highly sensitive optical contrast agent.

Figure 7. Extinction, Absorption and Scattering Efficiency of Au and Ag Spherical Nanoparticles. Absorption (blue curve), scattering (green curve) and extinction (black curve) spectra of Au NPs of diameter 10 nm, 50 nm and 100 nm. Spectra are shown in terms of efficiency, which is the ratio of the calculated optical cross-section of a NP to its actual geometrical cross-section.

4.2. Distance Dependence

There have been numerous studies where metal NPs induced a quenching or enhancement of fluorescence emission. Generally, fluorescence quenching is observed for NPs smaller than 20 nm. However, both fluorescence enhancement and quenching have been observed as a function of distance for strongly scattering large Au spheres. For instance, Feldman et al.⁵⁹ reported strong fluorescence quenching, even for the largest separation investigated (16 nm), for a fluorophore emitting at 670 nm in the presence of 12 nm Au NPs. Au NPs of similar size have also been found to quench the emission intensity of fluorophores which have an emission wavelength close to the LSPR wavelength of the Au NPs (530 nm).^{70,71} In the majority of experiments, organic molecules such as polymers or DNA are used as linkers and spacers to separate the metal NPs and the fluorophores. The use of these molecular spacers often limits the precise distance control, and the separations that can be achieved are restricted by the length of the linker molecule. Besides, as indicated above, if more than one dye molecule is attached to a NP, dye-dye interactions can cause pronounced fluorescence quenching. In order to overcome these problems, Mulvaney and coworkers⁶⁷ developed a Au@SiO₂ core/shell system that enabled the study of the interactions between Au NPs and fluorophores via electromagnetic coupling upon light excitation, determining experimentally the distance dependence (by the systematic variation of the silica shell thickness) and the wavelength dependence (using 4 different dye molecules) of this coupling. The obtained results reveal that fluorescence is quenched by more than 90 % at short distances (4.6 nm) and recovers to reference values above $d > 40$ nm. Between these extremes, the recovery of the fluorescence intensity with increasing distance was strongly wavelength dependent, with the most pronounced (> 95 % up to a distance of 10 nm and still significantly affected above 25 nm) when the absorption peak position of the fluorophore coincided with the SPR of the Au NPs. For Alexa 700 (emission wavelength 710 nm) fluorescence intensity recovers to 50 % at 10 nm, and is completely unaffected for separations above 25 nm.

It should be noted that FRET and the transfer discussed here are based on very different phenomena. FRET is a result of dipole dipole interaction with non radiative transfer between two dyes involved. Efficiency of FRET decreases with r^{-6} , where r is the distance between the donor and acceptor. Fluorescence enhancement by scattering is conceptually different as it is the result of the absorption or scattering, from a metal nanoparticle at a certain distance and involves radiative processes with a different distance dependence to FRET.

Scattering intensity follows a decay, which is inversely proportional to the square of the distance from the scattering particle, and therefore the enhancement should be appreciable at larger distances from the NPs than the quenching. Quenching by adsorption is effective at short distances and it will depend on the intensity of the plasmon, which in turn depends on the nanoparticle chemistry, size and shape. Adsorption will be more effective the closest the excitation wavelength of the dye is to the plasmon of the NPs. Scattering phenomena are highly wavelength dependent and also highly influenced by size and shape. The distance dependence of both phenomena will be very much defined by the characteristics of the NPs and dyes involved.

4.3. Wavelength Dependence

The spectral separation between the fluorophore emission and the absorption of the metal NP is of crucial importance when determining the electromagnetic coupling between the fluorophore and the metal NP. Thus, the strongest quenching is found for a fluorophore emitting exactly at the spectral position of the SPR, being 40% more efficient than a fluorophore with an emission wavelength red-shifted 200 nm towards the near-infrared.

In the case of scattering and enhancement it is interesting to observe that this effect can be modulated by selecting the shape of the metal NPs. Thus, for instance, Ag spherical NPs display a maximum scattering peak near 400 nm while it is considerably red-shifted, peaking near 660 nm, in ellipsoidal-shaped NPs of similar sizes. As a result, spherical 50 nm Ag NPs would enhance fluorescence near 400 nm, while ellipsoids would be more efficient at 700 nm.

4.4. Dye-dye and Dye-Surface Interactions.

Changing the local environment of a dye molecule, for example by binding it to a surface, and locating many dye molecules in close proximity to one another can significantly affect the dye luminescence. The confinement of several dye molecules to the surface of a NP leads to a significant local increase in dye concentration. This facilitates multimer formation between dyes, which can lead to significant fluorescence quenching.

In this context, the key question is how many dyes can be bound to one particle before dye-dye interactions dominate the observed fluorescence signals? This problem was nicely studied by Mulvaney and coworkers⁶⁶ in the case of silica NPs of 50 nm diameter. As a result, they obtained

that for average dye molecules separations above 15 nm (less than 30 dye molecules per particle) only the effect of the dye's surface confinement was seen to result in a 20 % decrease in fluorescence relative to the fluorescence of the free dye. A more pronounced decrease in fluorescence was observed for dye molecule separations below 10 nm (more than 75 dye molecules per particle). Finally, for the highest dye loading, the fluorescence decreases by more than 90 % compared to the free dye in solution.

5. Quantum Dots versus Fluorophores

As a promising alternative to organic dyes, luminescent quantum dots (QDs)—semiconductor nanocrystals—have emerged as unique fluorescent (biological) labels.^{47,48,72,73} Whereas organic fluorophores are restricted by their narrow excitation spectra, QDs can be excited by any wavelength from UV to red (**Figure 8A**). This enables efficient excitation and collection of fluorescent emission.^{74,75} Another limitation of organic fluorophores is their broad emission spectra, which limits the number of fluorescent probes that can be simultaneously resolved (**Figure 8B** and **8C**). In contrast, QDs have narrow, tuneable emission spectra and thus, emission from many QDs can be resolved over the same spectral range allowing 'multiplexing' (simultaneous detection of multiple signals). Moreover, in contrast to organic fluorophores, QDs are highly resistant to chemical and metabolic degradation and have a higher photobleaching threshold. Finally, whereas organic fluorophores require customised chemistry for conjugation of biomolecules to each fluorophore, a universal approach can be used for conjugating biomolecules to all QDs.

QD properties of interest to biologists include high quantum yield, high molar extinction coefficients (approx 10–100 times that of organic dyes),^{76,77} broad absorption with narrow, symmetric photoluminescence (PL) spectra (full-width at half-maximum approx 25–40 nm) spanning the UV to near-infrared, large effective Stokes shifts (difference between positions of the band maxima of the absorption and emission spectra), high resistance to photobleaching and exceptional resistance to photo- and chemical degradation.^{78–81} The unparalleled ability to size-tune fluorescent emission as a function of core size (for binary semiconductor materials), and the broad excitation spectra, which allow excitation of mixed QD populations at a single wavelength far

removed (> 100 nm) from their respective emissions make them highly attractive for fluorescence based studies.

The main drawback of QDs is that they have poor aqueous solubility. Premium quality, monodisperse QDs with superior physico-chemical properties require synthesis in high boiling point, non-polar organic solvents⁵⁶ (mixture of trioctyl phosphine/trioctyl phosphine oxide, TOP/TOPO) incompatible with an aqueous environment. Rendering these hydrophobic NCs water-soluble requires surface functionalisation with hydrophilic ligands, either through ligand exchange,⁸³ or by encapsulating the original NCs in a thick heterofunctional organic coating, driven mainly by hydrophobic absorption onto the TOP/TOPO-capped QDs.^{84,85} These ligands mediate both the colloid's solubility and serve as a point of chemical attachment for biomolecules. Capping ligands serve another critical role in insulating/passivating/protecting the QD surface from deterioration in biological media.^{85,86} However, it is often the case that ligands do not form a stable bound with the QDs, which poses additional difficulties in handling QDs in aqueous media.

In this context, the question is whether QDs would replace fluorescent dyes. Rather than replace traditional dyes, QDs could complement dye deficiencies in particular applications, especially in cellular labelling,⁸⁷ where the elucidation of complex cellular processes (studied on the single biomolecule level) requires extensive multiplexing (6–10 colours), multicolour resolution and high photobleaching resistance. Other areas, such as medical imaging (near-infrared dots used as tissue probes and contrast agents), assay labelling (bar-coding to high-throughput or parallel assay formats -such as gene-expression-) and optical bar-coding (including magnetic properties for capture) are likely to follow suit.⁸⁶

Figure 8. Size-dependent fluorescence spectra of CdSe quantum dots and relative particle sizes (A and B). Emission and absorption of quantum dots (C) and ATTO565 dye (D) with a similar emission wavelength. Quantum dots show a higher quantum yield, higher molar extinction coefficients and a broad absorption with narrow, symmetric photoluminescence (PL) spectra.

6. Label toxicity

A final consideration in this work is the impact of the label on the toxicity of the NPs. Labels can be toxic themselves as they are organic molecules, often charged and bearing aromatic rings. Toxicity may result from the release of the labels from the nanoparticles, especially when the label is placed on the NP surface. Also, with regards toxicity, it is important that unattached dye be removed from the NP solution. Besides, the toxicity resulting from surface modification of a NP with an organic molecule can change the hydrophilicity, or alter the surface charge, which may have consequences in terms of cell and organ uptake and translocation of the NPs, which can indeed alter the fate of the NPs and consequently their impact on biological functions. Quantum dots are often composed of elements that are known to be toxic, such as Cd^{+2} that restrict their use in vivo

but in vitro have been shown to be not particularly toxic. However, there are several alternative quantum dots with components that display much lower toxicity.⁸⁸⁻⁹⁰

Fluorescence labelling is a tool for quantification and visualisation and as such provides fundamental information about the fate of the NPs that can be correlated with their biological end points. Care must be taken to avoid artefacts and that the labelling does not significantly change the physico-chemical properties of the NPs and their interaction with biological matrixes. As such, care must be taken when deciding between labelled and unlabeled NPs when evaluating their toxicity.

7. Conclusions

Fluorescence labelling is a fundamental tool for the visualisation of nanomaterials at cell level and provides relevant information about their trafficking and fate and can also be used to quantify nanoparticle dose at cell level. We have shown different situations throughout this paper that can lead to an erroneous determination of fluorescence or the fluorescence associated with nanomaterials. These situations can lead to the incorrect localisation of the nanomaterial in the cell or to an overestimation or an underestimation of the amount of the nanoparticles in cells. These situations can be a result of the leakage of the fluorophores from the nanomaterials, the presence of an excess of label from the labelling reaction as free dye in the nanomaterial suspension, to a limited photostability of the dyes used for the labelling or an enhancement or quenching of the dye fluorescence through the interaction with the surface of metal nanoparticles, etc. We have also shown some methodologies to visualize the free dye that could be present in the nanomaterial solution. On the whole, the data shown here, which is based on practical experience of the authors and a vast literature, can help guide those working on nanosafety research in the assessment of fluorescence data associated with nanoparticles and in the proper design of experiments, taking into account issues mentioned in this manuscript.

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ACCEPTED MANUSCRIPT

Graphical Abstract

Highlights

1. It is fundamental to assess the presence of free dyes detached from nanomaterials.
2. Free dyes can lead to wrong conclusions regarding nanomaterial localization in cells.
3. Fluorescence Correlation Spectroscopy can be used to visualize free dyes.
4. Fluorescence of metal nanoparticles depend on the distance of dye to surface.