Chemical synthesis and surface modification of ZnO nanocrystals for bio-imaging

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CHEMICAL SYNTHESIS AND SURFACE MODIFICATION OF ZNO NANOCRYSTALS FOR BIO-IMAGING

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School of Materials Science and Engineering

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Summary

Semiconductor quantum dots (QDs) offer significant advantages over organic fluorophores; however, the cytotoxicity, complex and toxic synthesis processes, and missing colours in blue-violet range ($< 490\text{nm}$) are major issues for their extended *in vivo* bio-applications. While ZnO is a good candidate because of its wide band-gap, large exciton binding energy, bio-compatible core material, and bio-friendly synthesis processing, chemically pure ZnO colloidal nanocrystals in quantum size, with excellent optical properties, and bio-compatible surface functionalities are not yet available.

In this research, colloidal ZnO nanocrystals were synthesized by a bio-friendly soft chemical method. The controlled particle size and size stability were obtained by proper precursor concentration, reaction temperature and time, and unique surface capping agents. Bandgap modifications were achieved by doping the ZnO nanocrystals with Co^{2+} , Ni^{2+} and Cu^{2+} cations respectively. The nanoparticle shape, size, and lattice structures were characterized by Zetasizer, FESEM, XRD and HRTEM. The optical properties were measured by UV-Vis spectrophotometer, single photon counting spectrofluorometer, and photoluminescence (PL) mapping. Modelling of the PL spectra was performed using the Multimode Brownian Oscillator model (MBO). Quantum yields were measured by comparing to standard fluorophores. Bio-imaging was carried out on two human tumour cell lines, a mouse tissue and mung bean plant cells. Cytotoxicities of the nanocrystals were examined by standard protocol and compared to commercial CdSe-ZnS QDs.

Single crystal ZnO nanoparticles in spherical shape and in size below 10-15 nm were obtained. All the colloidal solutions were stable for 30-45 days. The lattice structures were Wurtzite P6₃mc hexagonal structure with some growth in preferred planes within the doped ZnO. The surface capping was more effective in controlling the nanoparticle size, while dopants were effective in modifying the bandgap and optical properties. The double amino groups contained in the Z60 and Z61 capping agents proved more effective in capping the nanoparticles than the single amino- group in APTES. The PL and optical absorption spectra showed excellent optical properties and red shifted bandgap, which are required for bio-imaging applications. Quantum yields of the synthesized nanocrystals were the highest among the reported values of QDs. The PL spectra of pure ZnO and doped ZnO were successfully modelled by the MBO model.

Bio-imaging tests showed unique dual colour images with blue colour in nucleus and turquoise colour in cytoplasm using either pure ZnO or Co-doped ZnO capped by Z60 and Z61. All the nanocrystals were also well absorbed by the roots of the plant and transported to the sprouts, showing bright green and yellow emission colours at the vascular region and on the plant cell walls. Co-doped ZnO showed the best labelling quality. The cytotoxicity study proved no cell proliferation by the nanoparticles up to the concentration of 1000 $\mu\text{g/ml}$, which is the highest concentration reported so far.

The ZnO bio-markers obtained are the first ZnO based bio-friendly markers with bright blue-violet emission and unique dual colour feature. This has demonstrated the potentials of the new bio-friendly ZnO QDs for many other applications, such as drug delivery, optoelectronics and energy devices.

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Nomenclature

λ	wavelength of photon
h	Planck's constant (6.626×10^{-34} J-s)
c	speed of light in vacuum (3×10^8 m/s)
E_g	band gap energy
Φ_F	fluorescence quantum yield
E_g^*	bandgap of particle
E_g^{bulk}	bulk energy gap
r	particle radius
r^*	critical nucleus radius
e	charge of the electrons
m_e	effective mass of the electrons,
m_h	effective mass of the holes
ϵ	relative permittivity
ϵ_o	permittivity of free space
$\hbar$	Planck's constant divided by 2π .
ΔG_v	Change of Gibb's free energy per unit volume of the solid phase
$\Delta\mu_v$	Change of volume energy
γ	surface tension between solid and liquid phase
C	concentration of the solute
C_0	equilibrium concentration
k	Boltzmann constant (1.38×10^{-23} J/atom-K)
T	temperature
Ω	atomic volume
σ	supersaturation
K	coarsening rate
t	time

m	mass (kg)
n_p	number of particles
N_A	Avogadro's number (6.023×10^{23} molecules /mol)
p	pressure (Pa)
T_b	Boiling point (K)
T_m	Melting point (K)
u, v, w	Velocity components in x, y and z directions, respectively (m/s)
V	Volume (m^3)
$\bar{A}$	Mean fluorescence intensity of plant bio-images
δ	standard deviation of florescence intensity value of plant bio-image
H	hydrolysis ratio (molar ratio of H_2O/Zn^{2+})

Abbreviation

QD	quantum dot
NC	nanocrystal
NP	nanoparticle
AAO	anodic aluminium oxides
PL	photoluminescence
V_o	oxygen vacancies
MBO	multimode Brownian oscillator model
ZPL	zero-phonon line
LO-phonon	longitudinal optical phonon
FESEM	field emission scanning electron microscope
XRD	X-ray diffraction
HRTEM	high resolution transmission electron microscope
Zetasizer	size and zeta potential measuring device, Malvern Zetasizer Nano ZS

Chapter One: Introduction

1.1 Background

The integration of nanotechnology and biology is expected to produce major advancements in medical diagnostics, therapeutics and bioengineering [1-3]. Advancements in molecular medicines require optimum detection of individual biomolecules, cell components, and other biological entities. For these purposes, bio-markers made of organic fluorophores or semiconductor nanocrystals have been developed.

Organic fluorophores, such as, genetically encoded fluorescent proteins or chemically synthesized organic fluorescent dyes (e.g. fluorescein, ethidium, methyl coumain, rhodamine, etc.) [4] are the most commonly used fluorophores. However, they have a number of physical and chemical limitations such as (1) low photostability, which is not suitable for long term imaging, (2) narrow absorption bands, which makes it necessary to use as many excitation sources as types of fluorophore, (3) broad emission spectra, which often causes overlapping of different fluorophores, and (4) differences in chemical properties of different dyes, which make multiple, parallel assays impractical.

The discovery of fluorescent semiconductor (Group II-VI) nanoparticles (NPs), often called quantum dots or QDs, has provided potential means to overcome these limitations. These inorganic fluorophores are chalcogenide (S, Se, Te) nanocrystals such as cadmium selenide (CdSe), offer significant advantages over organic fluorophores: (1) size- and composition-tuneable fluorescence emission from visible to infrared wavelengths, (2) large absorption coefficients across a wide spectral range and high level of brightness, (3)

resistance to photobleaching, (4) being suitable for combinational optical encoding, in which multiple colours and intensities are combined to encode thousands of genes, proteins or small-molecule compounds [5-7].

Although QDs have been used in several “real” applications, some problems still have to be overcome [8-10]. Firstly, the “blinking” problem [11,12] limits the quantitative single QD based sensor application. Secondly, the potential cytotoxicity of Cd-containing materials [13,14] demands new biocompatible QDs that do not contain Cadmium, such as doped zinc selenide particles [15]. Lastly, specificity of the conjugation scheme for different species of biological molecules to different QDs requires more efforts in the surface modifications of QDs. This research aims to investigate these global issues of semiconductor QDs, and test the properties of the new QDs on bio-imaging of human cell lines and plant systems.

Alternative nanomaterials for bioanalysis are metal nanoparticles (NPs), such as Au, Ag, Pd, Pt, Co, Ni, Fe, Cu, Si and Ge. Au is the most widely used nanomaterial in the bioanalysis field [11] due to the presence of a plasmon absorbance band, and the shape and size-dependence optical properties making Au NPs suitable as colorimetric probes [16,17]. Therefore, ultrasensitive analysis of oligonucleotide, proteins, and other biomolecules has been achieved using Au NPs as biomarkers [18,19]. However, metal particles have much less available colours than QDs. Thus for luminescence, they are often coupled with organic dyes [11], and need specific equipment (Fluorescence Resonance Energy Transfer, or FRET) for visualization [20,21]. Compared to quantum confinement effects of semiconductor QDs, which depend on size up to 10nm, metal particles are far less flexible. The quantum size effect is only noticeable when size is below 2nm [22,23]. As such, its available emission spectra are not as wide nor as well

tuned as semiconductor QDs.

Besides metal NPs, metal oxides such as silica, iron oxide and ZnO NPs are also good alternatives to QDs. Silica NPs need proper incorporation of dye molecules inside a silica particle for luminescence [8,24]. Iron oxide NPs possess magnetism properties, therefore, have been used in gene therapy, magnetic resonance imaging, and other medical applications. However, they were found to be toxic to nerve cells as reported recently [25]. ZnO is a versatile semiconductor with a wide bandgap (~ 3.37 eV) and an extremely large exciton binding energy (60 meV), which makes the exciton state stable at room temperature or above. ZnO has great potential for bio-applications due to its excellent optical properties and environmentally friendly nature. This PhD thesis will focus on the synthesis methods, surface modification, particle and material properties, and bio-imaging applications of the ZnO nanocrystals (NCs).

1.2 Motivation

The initial motivation of this research is the global demand for early cancer detection. Since the specific antibody-antigen conjugation mechanism has already been developed for several types of cancers [22,26], it is now realized that the major bottle neck for early cancer detection is the bio-marker core material. Despite recent advancement in nanomaterials in bioanalysis [10,27,28], the required conditions for *in vivo* bio-applications have not been simultaneously satisfied. The cytotoxicity of currently used QDs (Cd-containing), the toxic solvent and ligands used in the synthesis of the QDs, and the large particle size (30-50 nm) after multilayer surface modification (a shell layer, a ligand layer and a polymer coat) require the urgent development of new QDs. This research aims to overcome the above-mentioned problems by providing: bio-compatible,

non-toxic quantum dots in quantum confinement size range ($<5\text{nm}$) with stable particle size in aqueous solution, unique surface chemistry for bio-conjugation, and high quantum efficiency for high quality bio-imaging. For this purpose, ZnO is a better candidate than metals and chalcogenide (S, Se, Te) nanoparticles due to its unique optical properties and biocompatibility. However, research on ZnO for bio-applications considerably lags behind the other candidates because the synthesis is not as well developed for obtaining controlled size, and the doping and surface modification of ZnO are less well understood. A few methods for obtaining ZnO nanocrystals in aqueous solution at low temperature have been reported, but they all involve strong alkaline media [29,30] or annealing [31,32], and are therefore not suitable for bio-applications. In order to make ZnO QDs for *in vivo* and *in vitro* bio-applications, the synthesis process has to meet several requirements. It must (1) only contain bio-compatible materials, (2) use suitable surface capping agent to ensure nano-sized ZnO particles in a stable colloidal solution, (3) provide chemical functional groups on ZnO surface that will eventually bind to biomolecules, and (4) ensure photostability and efficient fluorescence of the ZnO nanocrystals.

Since ZnO intrinsically emits light in the UV wavelength, doping it with suitable elements is an effective method to adjust their electrical, optical, and magnetic properties, which are crucial for its practical applications. After bandgap modification, ZnO could provide both UV and blue-violet emissions, which fill up the missing emission spectra range of current QDs (available from wavelength of 490 nm up to infrared). Therefore, an additional target of this research is to reduce the surface defects of ZnO nanoparticles and improve the surface physical properties through surface modification in order to ensure excellent and stable optical properties with controlled bandgap and high PL emissions. It is an added value if one marker can label both the nucleus and the cytoplasm of a cell

simultaneously. This eliminates the complexity of two-step labelling using two different markers and the need of two excitation sources in the case of fluorescent dyes.

1.3 Objectives

The major objectives of this research are to investigate the chemical synthesis methods to obtain pure, nano-sized, non-toxic, water-soluble and stable ZnO quantum dots with excellent photoluminescence property by using a simple process (low temperature and atmosphere environment), and to demonstrate the bio-imaging capabilities of the synthesized nanocrystals.

The detailed objectives are as follows:

- (i) To study the ZnO synthesis process parameters in order to achieve pure, nano-sized ZnO particles stabilized in a colloidal solution; and to characterize the size stability and crystal structures of the synthesized nanocrystals.
- (ii) To investigate surface modification methods using different capping agents to control particle size and provide bio-compatible functional groups for further conjugation to bio-cells in order to provide high quality bio-images.
- (iii) To understand the mechanism of bandgap modification of ZnO nanoparticles by different dopants in order to shift the emission peaks to visible wavelength range and provide high PL emission intensity for bio-detection. For this purpose, optical emission and absorption properties will be studied.
- (iv) To model the photoluminescent spectra of pure ZnO and doped ZnO nanocrystals in order to ensure high PL emission properties and high quantum yield of the synthesized nanoparticles.
- (v) To demonstrate the bio-imaging capabilities and bio-conjugation properties of the synthesized ZnO nanocrystals on selected human tumour cells as well as plant

system, and to study the cytotoxicity of nanoparticles to human cells and confirm the non-toxic properties of the ZnO nanocrystals.

1.4 Novelties

- (1) ZnO nanocrystals have been synthesized by many different processes, but the required conditions of ZnO nanocrystals for bio-imaging are not simultaneously satisfied. Common problems of the existing processes are: high temperature or too fast chemical reaction leading to surface defects (oxygen or zinc vacancies) resulting in poor optical properties, no suitable surface capping leading to particle agglomerations in water or uncontrolled particle shape, toxic ligands used in the process leading to post treatment and potential contamination/toxicity of the final colloidal solution, and no suitable doping leading to only UV emission, not detectable by common confocal microscopy. As stated in the citations [34,35] of our published papers [36-38], our work is the first real bio-application of ZnO nanocrystals. The successful application on cells is due to the well controlled particle size (between 1 nm to 5 nm) from our synthesis process, good crystal structure, excellent optical properties, and unique surface modifications.
- (2) The unique surface capping agents (Z60 and Z61) containing double amino- groups in the side chain have been proven to be better in capping capability than the single amino- group agent and most of the other capping agents. This ensures both the achievement of 1-2nm size ZnO nanoparticles and the covalent bio-conjugation of the nanocrystals to biological cells. These capping agents have not been reported by others.
- (3) Although ZnO has been doped by some elements, most of the reported results indicate a bandgap shift to shorter wavelengths, which is not suitable for bioimaging

application. The doping cations in this research are carefully selected and strategically incorporated into the synthesis process. Therefore, they are well sited into the ZnO lattice structure, providing well controlled optical properties. This is the first systematic study on the PL property and bandgap modification of ZnO for visible emissions.

- (4) The non-toxic nature of the ZnO nanocrystals is a major breakthrough in the field of semiconductor nanocrystals for bioimaging applications. No publication has been found on the cytotoxicity study of ZnO nanocrystals on cells. The nanoparticle concentration of 1000 $\mu\text{g/mL}$ that does not cause cell proliferation is the highest concentration reported so far, which is the advantage of our nanocrystals.
- (5) Another novelty of this research is the capability of dual colour imaging by the surface modified ZnO and Co-doped ZnO, where the nucleus and the cytoplasm of a cell are labelled simultaneously with two different emission colours. This is the combined effects of quantum size that can penetrate into the nucleus (below 2 nm), the unique surface chemical groups, the well controlled bandgap, the high quantum yield, and the non-toxicity of the nanocrystals. This work made contributions to the advancement of bio-imaging technology by inorganic nanocrystals, where significant multidisciplinary efforts are essential. Both materials fundamentals and the actual bio-applications benefit from this research work.

Chapter Two: Literature Review

In this chapter, the important properties of quantum dots and special properties of ZnO will be introduced. Then the published work on synthesis methods, the thermodynamic theory for size control, the doping of ZnO, and biomedical applications of QDs are reviewed.

2.1 Quantum dots and optical properties

2.1.1 Introduction to quantum dots

In the early 1980s, Dr. Alex Ekimov [39], also known as the Father of Quantum Dots, discovered quantum dots with his colleague, Dr. Efros [44], bringing along with his discovery a new era of science. Since then this has spurred on an avalanche of research to analyze and understand the fundamental workings of these novel tiny dots.

Quantum dots (QDs) are derived from semiconductor materials, whose crystals are composed of periodic group pairings of II-VI, III-V, or IV-VI elements. QDs are also known as nano-crystals (NC) or nano-particles (NP), and are considered a unique class of semiconductor because of their small sizes, which range from 2 to 10 nanometres (10-50 atoms) in diameter. At these small sizes, materials behave differently from their usual bulk sizes (much bigger than 10nm), and it is this attribute of quantum dots that contributes to one of its most fascinating features, in particular, the wavelength of fluorescence emissions. By altering the size of the QDs (within its Exciton Bohr Radius) or its chemical composition it is possible to tune the fluorescence emission to different wavelengths – from the near ultraviolet right up to the near-infrared spectrum, spanning a broad wavelength range of 400-2000nm [41]. This phenomenon is due to the confinement

of material's electrons in all three geometrical directions, giving rise to full quantization of motion, with discrete atom-like states, otherwise known as the Quantum Confinement Effect. Figure 2.1 shows the photoluminescence spectra of size and composition tuneable QDs currently available. It is seen that emission in UV and blue-violet range (less than 490 nm) are not available [3].

Fig. 2.1 Photoluminescence spectra of size and composition tuneable QDs. Emission in UV and blue-violet range (less than 490 nm) are not available [3].

The most widely studied QD is CdSe-ZnS core-shell nanoparticles. Figure 2.2 shows the schematic structure of a core-shell QD [42]. CdSe semiconductor quantum dots with diameters ranging between 1.5 and 8 nm exhibited strong and tuneable luminescence [4, 43-45]. They have been widely investigated because of their potential use in sensors [46], laser materials [47], thin film light-emitting devices (LEDs) [48], and biological labels [1, 24, 49, 50].

Fig. 2.2 Schematic diagram showing the structure of a single CdSe-ZnS core-shell QD [42].

Figure 2.3 shows a comparison between organic dyes and QDs on cell imaging and photo-stability. The bio-image labelled by organic dyes (Rhodamine Green), as illustrated in (a), fades within 30 minutes, while the bio-image labelled by QDs is still bright at 80 min. The photostability spectra (Fig.2.3(b)) shows that the QDs are several thousands times more photostable than organic dyes (Texas red).

When used as biological probes, QDs must be soluble in aqueous buffers. However, while most QDs with core-shell structures, such as CdSe-ZnS and CdTe-ZnS, are synthesized in organic solvents with hydrophobic surfactant, it is challenging to make plain QDs water-soluble and also achieve colloidal stability, photostability, efficient fluorescence, and low non-specific adsorption under aqueous biological conditions. Two main approaches exist in the literature for the design of water-soluble QDs: by using organic polymers [26], micelles [50,51], or thiol groups such as mercaptoacetic acid (MAA) and mercaptoundecanoic acid [49,52] coatings, and by using silica coatings [53]. However, the complex chemical processes and the increased particle size by these surface treatments are major issues.

Fig. 2.3 Photostability comparison between QDs and organic markers.
 (a) *Xenopus* animal pole blastomeres labelled by RG-D (Rhodamine Green Dextran) molecular probe and QD respectively, showing long lasting imaging by QDs, (b) photostability curves showing that QDs are several thousand times more photostable than organic dyes (Texas red) [7].

The following issues of QDs are related to this research and will be addressed in the next chapters. (1) First, the synthesis process of current QDs involves the use of toxic ligand and solvents, inert gas, hydrophobic ligand (which needs to be replaced later), and high temperatures (250-350°C), which are not environmentally friendly and not suitable for bio-applications [43,44]; (2) the surface defects of these QDs, especially after being

dispersed in water solution and exposed to UV light, make the quantum yield very low (3-20%) [54-56]; (3) the particle size of QDs after coating with a shell layer and an encapsulating copolymer layer is normally in the range of 20 to 50 nm [57,58], which is too large for labelling of small molecules and nuclei of cells (the preferred size for these applications and *in vivo* injection into body is below 5 nm and as small as possible); (4) the confirmed cytotoxicity of Cd-containing QDs at low concentration of 0.0625 mg/ml [13] requires a new bio-friendly core material, which should have intrinsically excellent optical properties; and (5) as the quantum yield of the core-shell QDs varies in a wide range (from 20% to 70%) [59,60] due to the sensitivity to surface defects and different surface modifications, higher and consistent quantum yield is needed for high quality bio-imaging.

2.1.2 Zinc oxide and its novel properties

Zinc oxide (ZnO) is a versatile material that has achieved applications in photo-catalysis [61], solar cells [62,63], transparent electrodes [64], sensors [65], varistor and transducers [66], electroluminescent devices [67] and ultraviolet laser diodes [68]. Compared to other II-VI compound semiconductors, ZnO has a wide band-gap of 3.37eV (as shown in Table 2.1) and a rather large exciton binding energy of 60 meV (compared to the thermal energy of 26 meV at room temperature). This makes it an exceptional efficient room temperature exciton-based emitter, and therefore, the brightest emitter, which requires only low excitation energy [69]. Furthermore, ZnO is an environmentally friendly material, which is desirable especially for bio-applications such as bio-imaging and cancer detection. Although ZnO is relatively easy to be fabricated by wet chemical methods, some researchers attribute the residual background emission to intrinsic defects of oxygen vacancies (V_o) and intrinsic zinc atoms, while others associate it to the incorporation of uncontrollable hydrogen impurities introduced during crystal growth. The green band in

ZnO luminescence spectra around 500-530nm region (as shown in Fig. 2.4) was found in almost all samples regardless of growth conditions. This green emission band is due to the surface defects of the ZnO particles, therefore, has caused much debate and research [70-72].

Table 2.1 A list of the band gap energies of different semiconductors

Semiconductor	E _g (eV)
InAs	0.35
Ge	0.66
Si	1.12
InP	1.34
GaAs	1.42
CdSe	1.8
AlAs	2.15
GaP	2.27
CdS	2.5
ZnSe	2.8
ZnO	3.37
GaN	3.44

Zinc is a very important trace element in humans [74]. The average adult body contains $3.0 - 4.5 \times 10^{-2}$ mmol (2 to 3g) of zinc, of which 63% is found in muscle and bone, 20% in the skin, and 17% in plasma. Significant amounts occur in the liver, kidney, prostate, semen, eyes, and hair. Zinc does not appear to be in any specific storage but it complexes widely with amino acids, proteins and plasma albumin.

Fig. 2.4 Photoluminescence spectrum of ZnO nanoparticles measured at room temperature (as-prepared with high green emission, and annealed at 50 °C for 4 hours without green emission) [73].

Deficiency in zinc can lead to anaemia, acrodermatitis, enteropathica, impotence, loss of sense of taste and smell, impairment of the immune system, delayed wound healing, and a general retarding of physical, sexual and mental growth, especially in children. It can also result in an increase in the absorption of trace element copper and toxic metal cadmium. In the later, zinc salts are used to treat cadmium poisoning.

Zinc has been found to play an important part in many biological systems. Some of its functions are: (1) it is found in several liver enzymes, one of which is concerned with the conversion of ethanol to less toxic compounds; (2) it is required for the release of insulin from the pancreas, a low zinc level has been found in many diabetics; (3) it is involved in cell division; (4) it plays a part in the release of vitamin A from the liver; (5) it aids wound healing; (6) zinc sulphate, zinc oxide, zinc gluconate, zinc orotate are used in pharmaceutical products, such as astringent and supplement.

Finally, the Wurtzite hexagonal lattice structure of ZnO enables it to grow into different shapes easily [75-76], making the control of spherical shape and particle size within quantum range (<10nm) more difficult.

2.1.3 Photoluminescence properties and quantum confinement effect

Photoluminescence is considered to be any process in which a material absorbs electromagnetic energy at a certain wavelength and then emits part of it at a different wavelength. It is triggered by electromagnetic absorption in the Ultraviolet – Visible Light – Near Infrared spectral regions. Only a part of the absorbed energy is transformed into luminescent light. The rest of it ends up as molecular vibrations, or simply as heat. Photoluminescence is one of the most well received types of luminescence techniques because of an easily available selection of reliable and inexpensive excitation sources.. However, the higher excited state is unstable, and the electron quickly loses its energy and falls back down to one of its discrete stable or meta-stable ground states. The lost energy is then released as a photon of equivalent energy. The amount of energy that the photon possesses is associated with a corresponding wavelength, given by Planck's famous equation:

$$E = \frac{hc}{\lambda} \quad (1)$$

where, h = Planck's constant (6.626×10^{-34} Js)

c = speed of light in vacuum

λ = wavelength of photon

Figure 2.5 shows a generic energy diagram of a semiconductor. When a semiconductor bulk material is illuminated with UV light, electrons are excited from the valance band to the conduction band leaving a positively charged hole in the valance band. This positively

charged hole is attracted to the negatively charged electron in the conduction band via the Coulomb interaction. The electron and the hole bind together to form a quasi-particle called an exciton, which has a radius called exciton Bohr radius [77]. The electron eventually recombines with the hole and emits a photon (the photons form the fluorescence or photoluminescence emissions). The minimum energy that an electron must acquire in order to move to the conduction band is called the band gap energy, E_g , and the region between the valence band and conduction band is called the band gap. In bulk CdSe, an exciton has a Bohr radius of 5.6nm and emits red light ($\lambda \sim 680\text{nm}$). However, a NC often has a radius smaller than the bulk exciton Bohr radius, and this changes its optical properties through quantum confinement. In a NC, the quantum mechanical wave functions of the electron and hole are compressed by the confining walls of the core. Because of this compression, extra energy (quantum confinement energy) is required to generate an exciton in a NC versus in bulk. As the radius of a NC decreases, addition or subtraction of just a few atoms to the QDs has the effect of altering the boundaries of the bandgap. Changing the geometry of the surface of the QD also changes the bandgap energy, owing to the small size and the quantum confinement effects. Therefore, the bandgap of the QD can be controlled simply by adjusting the size of the dot and surface modification. Consequently the emission wavelength can be controlled with extreme precision (as shown in Fig. 2.1). Figure 2.6 schematically shows the correlation between QDs' size and the PL emission colour.

In the quantum size region, the band gap of the semiconductor increases when the size of the particles decreases, resulting in a blue shift of absorption bands. The quantum confinement effect was described by Brus [78] 20 years ago. The relationship between bandgap and size of the nanoparticle can be presented by the effective mass

Fig. 2.5 Generic energy diagram for a semiconductor. Electrons can be excited to the conduction band, leaving positively charged holes in the valence band.

Fig. 2.6 Schematic diagram showing the correlation between QDs' size and the emission colour.

model for spherical particles with a coulomb interaction term:

$$E_g^* \approx E_g^{bulk} + \frac{\hbar^2 \pi^2}{2er^2} \left(\frac{1}{m_e} + \frac{1}{m_h} \right) - \frac{1.8e^2}{4\pi\epsilon\epsilon_0 r} \quad (2)$$

where E_g^* is the bandgap of the measured particle, E_g^{bulk} is the bulk energy gap

r is the particle radius, e is the charge of the electrons

m_e is the effective mass of the electrons, m_h is the effective mass of the holes

ϵ is the relative permittivity; ϵ_0 is the permittivity of free space

$\hbar$ is Planck's constant divided by 2π .

From this equation, it could be deduced that in quantum size region, the band gap of the semiconductor increases when the size of the particles decreases. This results in a blue-shift of the absorption bands. For ZnO semiconductor, $E_g^{bulk} = 3.35\text{eV}$, $m_e = 0.24m_0$, $m_h = 0.45m_0$, and $\epsilon = 3.7$, where m_0 is the free electron mass. By measuring the bandgap of the particle (E_g^*), the particle radius could be determined. However, many assumptions were made when using this equation that could limit the particle size calculation. For instance, the effective masses of electrons and holes were based on bulk ZnO, but if the crystal structure of ZnO changes, the effective mass would also change.

The model is still well accepted by most as a guideline for study the size effect on optical properties of semiconductor nanocrystals, although there are new theories being proposed recently [79]. According to Brus' model, neglecting any surface atom rearrangement, ZnO particles must be smaller than 50 Å before a bandgap shift becomes obvious [80]. TiO₂ crystallites still show the same emission as the bulk material at 36 Å, and CdS particles exhibit size effect till diameter reached 80 Å [80]. Other studies [81-83] showed quantum confinement effects of ZnO particles from 6nm to 10nm. It was found that not only particle size but also surface polarizability and coating layer are important factors that determine the quantum size regime of a material. Figure 2.7 shows the correlation between bandgap and particle size of ZnO synthesized by a colloidal plus aggregation method [83].

Fig. 2.7 Optical bandgap in relation to particle size of ZnO nanocrystals. Aggregated particles were prepared by aging of the ZnO Colloids [83].

In the larger size regime (10 nm to 50nm), the luminescence intensity of ZnO particles does not depend on the nanocrystal size, but on volume defect arising from preparation conditions [84] and surface defects. An emission peak at green wavelength (510 nm) from ZnO particles has been frequently reported and identified as oxygen vacancies on the particle surface, which are related to the reduction of ZnO by high temperature treatment or extrinsic impurities (Cu-containing) [70,71,83]. A red and a yellow (Li-doped ZnO) luminescence bands were also reported [82]. Evidences showed that by effectively controlling the sizes of ZnO nanoparticles and by suitable surface passivation technologies, a variety of emission peaks can be produced.

2.1.4 Quantum yield

As mentioned above, when a fluorophore absorbs a photon of light, an energetically excited state is formed. The fate of this species is varied, depending upon the exact nature

of the fluorophore and its surroundings, but the end result is deactivation (loss of energy) and return to the ground state. The main deactivation processes are fluorescence (loss of energy by emission of a photon), internal conversion and vibrational relaxation (non-radiative loss of energy as heat to the surroundings), and intersystem crossing to the triplet manifold and subsequent non-radiative deactivation.

The fluorescence quantum yield (QY) is defined as the ratio of emitted photons to absorbed photons, which can be expressed by the equation:

$$\text{QY} = \text{photons emitted} / \text{photons absorbed}.$$

Quantum yield is essentially the emission efficiency of a given fluorochrome. In other words, the quantum yield gives the probability of the excited state being deactivated by fluorescence rather than by another, non-radiative mechanism. Quantum yield is a measure of the efficiency with which absorbed light produces some effects. A reliable method for recording QY is the comparative method of Williams *et al.* [85], which involves the use of well-characterized standard samples with known QY values. Essentially, solutions of the standard and test samples with identical absorbance at the same excitation wavelength can be assumed to be absorbing the same number of photons. Hence, a simple ratio of the integrated fluorescence intensities of the two solutions (recorded under identical conditions) will yield the ratio of the QY values. In practice, several steps are involved to ensure the accuracy of the measurement. They are: (1) cross-calibrating two standard samples to ensure that their QY values can be used with confidence, (2) working with carefully chosen concentration range to ensure linearity, and (3) including the refractive indices of the solvents in the ratio calculation. QYs of CdSe base QDs varies from 3 to 25% without coating layer, but after coating with silica or ZnS shell, QYs could reach up to 85% [43,84]. Recently, QYs of chemically synthesized ZnO

QDs have been reported in the range of 76-79% [33,38]. Table 2.2 lists some quantum yields values of QDs with various ligands and coatings in relation to the organic dye Atto 610. It is seen that quantum yield decreases with thicker coatings made of larger molecules such as PEG and diblock copolymer.

Table 2.2 Quantum yields of QDs with various ligands and coatings relative to the organic dye Atto 610 [55]

Quantum dot coating	Quantum yield (%)
Octadecylamine (in hexane)	79.3
Mercaptopropionic acid	63.4
Polyethylenimine	43.0
Alkylated polycarboxylic acid	64.1
Lipid-PEG	52.8
Diblock copolymer	20.9
Organic dye Atto 610	70.0

2.1.5 Modelling of photoluminescence spectra

It is well known that smaller particles have larger surface-to-volume ratio and surface defects [83]. The photoluminescence (PL) spectra of most reported ZnO nanoparticles showed a near-band-edge (NBE) UV line (also called zero-phonon line, or ZPL) which was accompanied by a visible luminescence (also called longitudinal optical, LO, phonon side band) which was caused by impurities and surface defects, thus resulting in a decrease in carrier/exciton lifetime and emission efficiency in the UV light devices. The green emission band in ZnO has been discussed intensively, and very different defect origins have been suggested to be responsible for the green band of ZnO. Shi *et al.* [86]

studied the PL emissions using a high quality ZnO bulk crystal at various temperatures, and modelled the observed two adjacent emission fine lines in each PL spectrum by employing the multimode Brownian oscillator (MBO) model. Although a few other models have been reported for modelling optical materials, such as the Gaussian model [87], Hartree–Fock (HF) and density functional theory (DFT) methods [88], and a simple Poission distribution model [89], the most recognized model is the MBO as it is a more comprehensive model that takes into account both the electron-LO phonon coupling and the dissipative effect of phonon-bath. Excellent agreement between this theory and experimental results was achieved on bulk ZnO. A detailed description of the MBO theory and its parameters are attached in Appendix I [90]. The software built on this model is used in this research to model the PL excitation and emission spectra of ZnO nanocrystals.

2.2 Synthesis methods of ZnO colloidal nanocrystals

2.2.1 Physical and chemical methods reported

In general, pure, crystalline, nano-sized ZnO with a narrow size distribution is highly desirable. Numerous physical and chemical techniques have been reported for the fabrication of ZnO nanostructures and nanoparticles, such as high-temperature thermal evaporation [91], vapour-phase oxidation of Zn power [92], thermal decomposition [93], metal organic chemical vapour deposition (MOCVD) [94], pulse laser deposition [95], chemical vapour transport and condensation (CVTC) [96], vapour-liquid-solid process [97], ultrasonic or infrared irradiation [98], and air oxidation of ZnS [99]. Other methods include hydrothermal [100], direct precipitation [32], solochemical or 2-stage method [101], sol-gel synthesis [102], and surfactant-controlled growth in a hot organic solvent

[103,104]. Despite these developments, the synthesis of pure ZnO nanocrystals within several nanometers size range at relatively low temperature, which avoids unwanted by-products and pollution to both the products and the environment, still remains an extremely sophisticated challenge. Also there is an increasing emphasis on the topic of 'green' chemistry and chemical processes [105,106], which demand a total elimination of generated waste, therefore, providing sustainable processes. This "green" chemical process is especially more important for bio-applications.

The physical and thermal processes mentioned above involve either high temperatures (140° to 800°C), expensive raw materials, complex process control, or sophisticated equipment, therefore, are not a good choice for this research. In addition, most of the processes produce larger particles with surface defects, which are not acceptable for our application. Conversely, soft chemical methods (sol-gel and surfactant-assisted syntheses) had proven to be a better alternative because of their low synthesis temperature, precisely controllable composition, surface modification possibilities, controllable growth rate, and consequently controlled particle size and size distribution.

A lot of researches on soft chemical methods are based on preparations under basic solution conditions [29,31,33,107]. Zinc salts such as $\text{Zn}(\text{ClO}_4)_2$, $\text{Zn}(\text{NO}_3)_2$, $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$, or $\text{Zn}(\text{CH}_3\text{COCHCOCH}_3)_2 \cdot 2\text{H}_2\text{O}$ (Zinc acetylacetonate) are dissolved in alcoholic or other organic solvents to which basic solutions containing NaOH, LiOH or NH_4OH are added. In bio-applications, chemically pure or compositionally well-defined ZnO is required as the alkali metal cations (Li^+ and Na^+) contaminate the ZnO and affect the ohmic conductivity and possibly cause harmful side effects to the human body. Although efforts have been made to remove these alkali ions

by washing in order to obtain pure ZnO powder [83], development of the process without adding a base is a primary requirement for our colloidal solution of pure ZnO. Recently, the chemical synthesis of ZnO nanoparticles through hydrolysis of zinc salts in polyol media (diethylene glycol or ethylene glycol) and monool solvents, i.e. methanol, ethanol, and 2-methoxyethanol has been suggested [103,108]. Since these processes are close to our synthesis process, their major results on the relationship between process parameters and the particle size, shapes, and properties are reviewed in detail.

An example of chemical synthesis of monodispersed ZnO powder was reported [109] using: (i) zinc acetate dihydrate and diethyleneglycol as the precursor and solvent, respectively, (ii) the concentration of the precursor that varied from 0.09 to 0.27 mol/L, and (iii) the reaction temperature at 180 °C. When the mixture was heated up to 180 °C, zinc acetate dihydrate dissolved completely at 130 °C. A milky-white zinc oxide was then precipitated in a few minutes when the temperature reached 180 °C.

Effect of precursor concentration:

It was found that the precursor concentration played an important role in controlling the particle size and shape. Figure 2.8 shows SEM micrographs of the particles synthesized by different precursor concentration. Spherical particles with a submicrometer size are obtained when the concentration is less than 0.18 mol/L (Fig. 2.8(a)).

Fig. 2.8 SEM micrographs showing the effect of concentration of zinc acetate dihydrate in diethyleneglycol on the particle shape and size. (a) concentration less than 0.18 mol/L (b) concentration ranging from 0.18 to 0.27 mol/L, and (c) concentration higher than 0.27 mol/L [109].

For reactant concentration in the range of 0.18 to 0.27 mol/L, agglomeration occurs, thus leading to an irregular shape and an increase in particle size (Fig. 2.8(b)). At higher concentration, aggregation does not occur and a gelatinous precipitate was obtained; therefore, rod shape particles can be seen in Fig. 2.8(c). The dimensions of the rods (60nm x 20nm) are smaller than the diameter of the spherical particles (200-400 nm). It was found that the anisotropic character of growth is strongly marked when the reactant concentration increases, and the growth along [001] increases more rapidly than [100] with increasing concentration [109]. The correlation between particle size and reactant concentration in terms of thermodynamic theory will be further discussed in Section 2.2.2.

Effect of polyol

Using zinc acetate dihydrate as precursor salt, several polyols have been tested as solvent, ethyleneglycol (EG), diethyleneglycol (DEG), tetraethyleneglycol (TTEG), poly(ethyleneglycol) molecular weight-300 (PEG $M_r = 300$), and glycerol. Zinc oxide could not be obtained as a single phase in pure ethyleneglycol; instead, an unknown compound was observed even when the temperature was raised to 200 °C. All the other polyols led to the formation of zinc oxide as a single phase.

Effect of water

Precipitation of zinc oxide, at a given temperature, is preceded by a complete dissolution of the precursor salt. This precipitation occurs probably through several chemical reactions for which hydrolysis appears to play a decisive role since no oxide can be obtained by heating a mixture of dehydrated zinc acetate and anhydrous DEG. Whichever polyol is used, it is clear that water has to be present in the reaction medium in order to obtain zinc oxide from zinc acetate. Hydrolysis occurs at 100 °C. However, by heating the solution to the boiling point of polyol, the reaction is greatly accelerated. This procedure can be called “forced hydrolysis” in analogy with the same procedure performed by aging dilute acidified solutions at temperatures ranging from 75 °C to 180 °C. These procedures allow a great variety of colloidal transition metal oxides to be synthesized, such as iron oxide, chromium oxide and cerium (IV) oxide [110].

Effect of anions

Various zinc salts have been tested instead of zinc acetate dihydrate: zinc chloride with added water, zinc carbonate hydroxide, $\text{Zn}_5(\text{CO}_3)_2(\text{OH})_6$, zinc sulfate heptahydrate, and zinc nitrate hexahydrate. All these salts were at least partly dissolved in DEG. The

carbonate hydroxide salt gave rise to an incomplete precipitation and the nitrate salt to a brown zinc oxide, indicating some degree of contamination arising from the reduction of nitrate anions. No precipitation was observed when, under otherwise identical conditions, sulfate or chloride zinc salts were used. These results clearly show that anions play a decisive role in the synthesis process. The dissolution of the salt may lead to the formation of dissolved zinc complexes that act as precursors to nucleation.

It is noticed from Fig. 2.8 that the particle sizes of the sphere particles are about 300-400 nm, which are not suitable for bio-imaging applications, although the monodispersed size is very useful for other applications such as templating and surface texturing. The larger size may be related to the higher temperature, polar solvent, and the absence of capping agent in these polyol synthesis processes.

Another example of chemical synthesis of nano-sized ZnO powder was reported [108] using monool solvents, i.e. methanol, ethanol, and 2-methoxyethanol, without using basic solution. In this paper, $\text{Zn}(\text{Ac})_2 \cdot 2\text{H}_2\text{O}$ are used as the precursor, and methanol (MeOH), ethanol (EtOH), and 2-methoxyethanol (2-ME) were used as the solvent respectively. It was found that layered hydroxide zinc acetate (LHZA) was formed as an intermediate for all the three solvents and the transformation of LHZA into ZnO was a key reaction step in any of the solutions. The reaction time necessary for the precipitation of ZnO was greatly influenced by the solvents used. Figure 2.9 shows the XRD patterns obtained from MeOH, EtOH, and 2-ME respectively, after different refluxing durations. For 2-ME, reflux for 72 hours was required to obtain pure ZnO, and for EtOH, 48 h reflux was needed. For methanol, refluxing time varied from 6 hours to 24 hours. Washing by methanol was required to obtain pure ZnO powder. HRTEM images (Fig. 2.10) shows that the particles

are spherical and have crystal sizes of 5 and 10 nm respectively after being refluxed for 6 to 24 hours.

Fig. 2.9 XRD patterns of particles obtained from colloids in different solvents after different processes.

(i) MeOH solution after refluxing for 12 h: (a) as-prepared, (b) washed; (ii) 2-ME solution after refluxing for 48 or 72 h, (iii) EtOH solution after refluxing for 10, 12, 24, or 48 h. ZnO, LHZA and a-LHZA peaks are labelled (•), (▽), and (▼), respectively [108].

Fig. 2.10 HRTEM images of particles obtained from the MeOH solution after refluxing for (a) 6 and (b) 24 h, particle size is 5 to 10 nm [108].

Formation of ZnO in the absence of base

It has been found that LHZA or the other compound related to LHZA plays an important role as the intermediate product of ZnO. The formation mechanism of LHZA in the methanolic solution is briefly described as: $\text{Zn}(\text{Ac})_2 \cdot 2\text{H}_2\text{O}$ dissolved in MeOH undergoing a sequence of reactions such as solvation by MeOH, formation of methoxyacetate complexes, hydrolysis by water originating from $\text{Zn}(\text{Ac})_2 \cdot 2\text{H}_2\text{O}$ itself, polymerization of the complexes, and crystallization into LHZA. The kinetics of hydrolysis and polymerization was greatly influenced by water concentrations in the solutions. The overall chemical reaction to form LHZA is:

The pertinent reaction of the conversion of LHZA into ZnO is expressed by:

Zinc hydroxyl complexes, namely $[\text{Zn}(\text{OH})^{2-n}_n]_x$, are produced from the brucite-related “ $\text{Zn}_5(\text{OH})_8$ ” layers in the LHZA structure. The water concentration in the solution does not exceed double moles of the zinc concentration because water was supplied only from the starting material, $\text{Zn}(\text{Ac})_2 \cdot 2\text{H}_2\text{O}$. Therefore, the hydrolysis reaction is expected to proceed very slowly. In the absence of base (OH^-) in the solution, the n value should be less than 2 and the $[\text{Zn}(\text{OH})^{2-n}_n]_x$ complexes should have a positive charge. Such complexes correspond to those evolved in general sol-gel processing through a polymerization reaction between mononuclear hydroxyl complexes. That is, “ $\text{Zn}(\text{OH})\text{-Zn}$ ” bridges are formed by an ololation reaction resulting in polynuclear zinc hydroxide clusters. There exist the Ac^- ions in the methanol solution, the total amount of which is double of that of zinc. The Ac^- ions can act as a strong base because of the smaller dissociation constant, K_a , of acetic acid (K_a value is as small as $10^{-9.7}$ in methanol). At $\text{pH} > 9.0$, $[\text{Zn}(\text{OH})^{2-n}_n]_x$ is converted into ZnO because of a higher chemical potential of OH^- in equilibrium,

The dehydration reaction of $[\text{Zn}(\text{OH})^{2-n}_n]_x$ is fundamentally described as

If a higher chemical potential of basic species is derived from the Ac^- ions, the following equilibrium instead of (5) is conceivable,

The deprotonation reaction of $[\text{Zn}(\text{OH})^{2-n}_n]_x$ can be described as

Thus the “ Zn-O-Zn ” bonds are constructed and the complexes are finally transformed into solid ZnO .

Solvent effects

The most important differences among the solvents are their dielectric constants, which are 32.6, 24.3, and 16.9 for MeOH, EtOH, and 2-ME, respectively. The dielectric constant primarily determines solubility of electrolytic salts. In the present work, $\text{Zn}(\text{Ac})_2 \cdot 2\text{H}_2\text{O}$ was more soluble in MeOH than in EtOH or 2-ME. Therefore the dissolution of $\text{Zn}(\text{Ac})_2 \cdot 2\text{H}_2\text{O}$ had to be assisted by the addition of water, which has a higher dielectric constant of 78.5, for the synthesis in EtOH and 2-ME. In the MeOH solution, the dissolution of $\text{Zn}(\text{Ac})_2 \cdot 2\text{H}_2\text{O}$ accompanies dissolution of zinc by MeOH molecules. The zinc ion has a coordination number of six and forms an octahedral inner coordination sphere, $[\text{Zn}(\text{MeOH})_6]^{2+}$ [111]. In contrast, the EtOH or the 2-ME solution containing water has zinc coordination in which the water molecule also participates as a solvate. In any case, the positively (+2) charged zinc coordination sphere encounters the negatively (-1) charged Ac ions in the diffusion-controlled manner and forms an ion pair. If the bonding between Zn^{2+} and Ac^- is strong and covalent, the resultant sphere can be regarded as an inner-sphere complex containing the hydroxyl and acetate groups. The formation of the hydroxyl group is due to the enhanced acidity of the solvate water molecule causing the deprotonation by free solvent molecules in the solutions. The complexes undergo further hydrolysis by water, polymerization, and crystallization into LHZA. The formation of acetate-rich composition of α -LHZA in the EtOH or the 2-ME solution is explained by the lower dielectric constant of the solvents. LHZA is recognized as the reaction product between the α -form of $\text{Zn}(\text{OH})_2$ and Ac. The lower dielectric constant gives stronger Coulomb interaction between them resulting in the acetate-rich composition. Hydrolysis of α -LHZA producing the charged species is retarded also by the lower dielectric constant. Then the reaction time necessary for the formation of ZnO is considerably longer in the EtOH and the 2-ME solution.

From the above analyses, it was concluded that methanol is the most useful solvent for preparing ZnO nanoparticles. It must be highlighted that for all the solvents studied in this paper the synthesis temperature was kept at 60°C, which is below the boiling point of the solvents. This led to the longer refluxing time needed. Also there was no capping agent used, and powder was collected directly after the synthesis. Therefore, particle agglomeration is inevitable as seen in the HRTEM images. The photoluminescence spectra of the synthesized ZnO powders in methanol are shown in Figure 2.11. Very high green emission can be seen at 530 nm wavelength; the intensity is even higher than the intrinsic UV emission, indicating that a lot of surface defects have formed. The longer reflux time (12 h) created more surface defects as the intensity of green emission was higher than that of shorter reflux time.

Figure 2.11 PL spectra of ZnO colloid solutions in methanol refluxed for 6, 8, or 12 hours [108].

A bio-friendly synthesis method for ZnO using buffer tris(hydroxymethyl)aminomethane was reported [112], but particle size varied from 20 to 300nm, in addition, a heating at 180°C was required to remove the protons trapped in the interstitial sites of the lattice. The major challenges for all the above methods are the difficulties in achieving

simultaneous high purity, controlled particle shape and size, no surface defects, and optimal optical properties.

2.2.2 Thermodynamics of nucleation and growth

From the thermodynamic point of view, the synthesis of nanoparticles usually involves nucleation, growth and agglomeration. Nucleation is the key step for precipitation methods as it controls the number of nuclei present. This influences the secondary processes needed to manage size, morphology and properties of the products. For nucleation to occur, supersaturation condition has to be present. This happens when the concentration of the solute exceeds the equilibrium in the solution. Therefore, when either the concentration of the solute exceeds the equilibrium or the temperature of the solution decreases, a new phase is formed.

2.2.2.1 Theory of Gibbs free energy

In homogeneous nucleation, with no foreign species as nucleating aids, nanoparticles with a narrow size distribution will start to form. This will also allow the initial high Gibbs free energy in the supersaturated solution to decrease because of the segregation of nanoparticles. This reduction of Gibbs free energy (ΔG_v) allows nucleation and growth to happen in the solution. Assuming that the nanoparticles are spherical, the volume energy, $\Delta \mu_v$, can be described as [113]:

$$\Delta \mu_v = \frac{4}{3} \pi r^3 \Delta G_v \quad (9)$$

However, this volume energy reduction is counter balanced by the introduction of surface energy which is due to the formation of a new phase. The surface term can then be described as:

$$\Delta\mu_s = 4\pi r^2 \gamma \quad (10)$$

where γ is the surface tension between solid and liquid phases. Hence, combining the two equations, we can get the total free energy change equation as:

$$\Delta G = \Delta\mu_v + \Delta\mu_s = \frac{4}{3}\pi r^3 \Delta G_v + 4\pi r^2 \gamma \quad (11)$$

Figure 2.12 illustrates the changes in volume free energy ($\Delta\mu_v$), surface free energy ($\Delta\mu_s$), and total free energy (ΔG), as functions of the nucleus' radius. From this figure, we can see that for the nucleus of nanoparticle to be stable, its radius has to exceed the critical radius, r^* .

Fig. 2.12 Schematic illustration of the change in volume free energy, $\Delta\mu_v$, surface free energy, $\Delta\mu_s$, and total free energy, ΔG , as a function of nucleus' radius [113].

The nucleus with a radius smaller than r^* will not be able to grow bigger; instead, it will dissolve back into the solution. The opposite is also true. Thus, to find out what the minimum stable size for the nucleus, r^* should be, the activation energy of cluster

formation, ΔG^* is used. It is defined by:

$$r^* = -2 \frac{\gamma}{\Delta G_v} \quad (12)$$

$$\Delta G^* = \frac{16\pi\gamma}{(3\Delta G_v)^2} \quad (13)$$

where ΔG^* is the critical energy barrier that the nucleation process needs to overcome, and ΔG_v is the change of Gibbs free energy per unit volume of the solid phase. ΔG_v is also dependent on the concentration of the solute as shown in the equation:

$$\Delta G_v = -\frac{kT}{\Omega} \ln(C / C_0) = -\frac{kT}{\Omega} \ln(1 + \sigma) \quad (14)$$

where C is the concentration of the solute, C_0 is the equilibrium concentration, k is the Boltzmann constant, T is the temperature, Ω is the atomic volume, and σ is the supersaturation defined by $(C-C_0)/C_0$. As mentioned above, without supersaturation (i.e. $\sigma = 0$), ΔG_v is zero, and no nucleation will occur. When $C > C_0$, ΔG_v is negative nucleation happens almost immediately.

Thus, in order to increase the possibility of nucleus formation in the supersaturated solution, we can change several factors in the synthesis and preparation techniques. To reduce the Gibbs free energy and critical size, one needs to increase the Gibbs free energy per unit volume, ΔG_v . Equation 12 and 13 demonstrates that by increasing ΔG_v , there will be a decrease in the critical energy barrier and critical size, ΔG^* and r^* respectively. Equation 14 also shows that ΔG_v can be reduced by decreasing the temperature and supersaturation, σ .

2.2.2.2 Growth of nuclei

The nuclei growth process can be either diffusion-limited or growth-limited. A diffusion controlled growth mechanism is required for the synthesis of monosized nanoparticles by homogeneous nucleation. When the supply of growth species is very slow due to slow chemical reactions, the growth would most likely be predominantly by the diffusion-limited process. Growth is related to Ostwald Ripening (OR) where the smaller particles are consumed by larger particles. This mechanism and model is today referred to as LSW theory [114]. For a diffusion controlled process, the average radius of the particles, r , as a function of time, t is [115]:

$$r(t) = \sqrt[3]{Kt} \quad (15)$$

where K is the coarsening rate related to concentration and surface energy. Hence the particle size is proportional to the cube-root of time. This theory assumes a steady change in solution concentration with time, which has been found not suitable for describing ensembles of particles smaller than ~50nm [116]. However, this theory is still widely used for control of homogeneous nucleation and growth such as the soft chemical synthesis process used in this research.

2.2.3 Mechanisms of particle size control

As described above, the formation of nanocrystals involves two steps: nucleation and growth. The growth process can occur in two different modes, ‘focusing’ and ‘defocusing’, depending upon the concentration of the monomer present as shown in Fig. 2.13 [117]. A critical size exists at any given monomer concentration. At a high monomer concentration, the critical size is small and so all the particles grow. In this situation, smaller particles grow faster than the larger ones. As a result, the size distribution can be

focused down to one that is nearly monodisperse. If the monomer concentration is below a critical threshold, small nanocrystals are depleted as larger ones grow and the size distribution broadens, or defocuses. The preparation of nearly monodispersed spherical particles can be achieved by arresting the reaction while it is still in the focusing regime, with a large concentration of monomer still present. Howard Reiss [118] suggested that size focusing is optimal if the monomer concentration is kept such that the average nanocrystal size present is always slightly larger than the critical size. When the monomer concentration is depleted owing to growth, the critical size becomes larger than the average size present, and the distribution broadens as a result of Ostwald Ripening (OR). Therefore, judicious replenishment of the monomer can thus be an important feature of the synthesis strategy. Rogach *et al.* suggested [116] that the narrowest size distribution could be achieved if particle growth rates are limited by the diffusion of monomer from the bulk of the solution towards the particle surface. The above theory has already been utilized in controlling the CdSe core-shell QDs [119]. From an experimental point of view, OR provides a simple and precise way to achieve the desired particle size through control

Fig. 2.13 Particle growth rate in relation to particle size and monomer concentration [117].

of the duration of nanocrystal growth and the proper choice of capping agents passivating the surface of the growing nanocrystals.

2.2.4 Doping of ZnO

Doping of ZnO by Mg, Ni, Cu, In and Al has been reported to improve electrical conductivity [120,121]. Doping of ZnO by Mn, Ni, Cu, and Co has been reported to improve ferromagnetic properties [122-124]. Cong *et al.* [123] reported that 3% Ni-doped ZnO nanoparticles showed ferromagnetic behaviours in room temperature, but no change in XRD spectrum and HRTEM images. Only Raman spectra showed some differences (Fig. 2.14). Compared with the vibration mode of pure ZnO at 332, 383, and 437 cm^{-1} of ZnO nanoparticles, the Raman modes of $\text{Zn}_{0.97}\text{Ni}_{0.03}\text{O}$ nanoparticles showed red shift of 348, 385, 439 cm^{-1} , respectively. Furthermore, two additional modes were observed at about 813 and 891 cm^{-1} in the Raman spectra of the $\text{Zn}_{0.97}\text{Ni}_{0.03}\text{O}$ nanoparticles, indicating the lattice changes caused by Ni-doping.

Figure 2.14 Micro-Raman spectra of $\text{Zn}_{0.97}\text{Ni}_{0.03}\text{O}$ nanoparticles [123].

In a separate study [121], ZnO thin films were doped with 9 at.% of Ni, 13.7 at.% of Cu, or 12 at.% of Cd. Figure 2.15 shows the XRD results. Differences were found only in the relative peak intensities. The crystallites were very strongly oriented along the *c* axis

direction and perpendicular to the plane of the substrate. The impurification with Ni seemed to occur through substitution of Zn cations by Ni ions, because there was no distortion of the crystal structure of ZnO. In this study, it was found that Ni promoted the grain orientation of ZnO and maintained it even after the annealing at 450°C. This had positive consequences on the electrical conductivity of ZnO film. However, Cu and Cd had less effects on the grain orientation and lost the effect upon annealing, indicating less effective doping by Cu and Cd.

Fig. 2.15 X-ray diffraction patterns of as-prepared ZnO thin films (thickness, 750 Å) impurified with (a) Ni, (b) Cu, and (c) Cd [121].

Co-doped ZnO nanoparticles were prepared by thermal hydrolysis for improving ferromagnetic properties of ZnO [124]. It was found that the Zn-ferrite phase could only be seen after annealing at 400°C. The particle size increased from 9.3 to 10.7 nm after the Co-doping and annealing at 400°C.

Some other dopants such as Li, Na, K were also reported [29,31], but due to their small atomic radii, these elements occupied the interstitial sites, rather than substitution sites, thus inducing strain and increasing the formation of native defects (vacancies). For the bio-imaging applications in this research, the preferred dopants should have similar atomic radii with Zn, and should reduce the bandgap of ZnO to enhance photoluminescence (PL) emission in visible wavelengths. Therefore, Co, Ni and Cu were selected as the dopants for the current research.

2.3 Biomedical applications of quantum dots

The first biological applications of quantum dots were reported in 1998 [1,49]. Bruchez *et al.* [1] and Chan *et al.* [49] used CdSe QDs coated with silica and mercaptoacetic acid layers, respectively, and both groups showed specific labelling by covalent coupling of ligand to these surfaces. Subsequently, several authors have reported the labelling of whole cells and tissue sections using several different surface modifications of QDs [26, 124,125].

2.3.1 Surface modifications of QDs for bio-applications

Highly luminescent CdSe nanocrystals are usually synthesized with hydrophobic organic capping agents, such as trioctylphosphine/trioctylphosphine oxide (TOP/TOPO), which benefit particle size and shape control, but limit their applications in biology. To overcome this limitation, surface modifications are necessary to make them water soluble with active surface groups that can be directly coupled with biological molecules or through a bioconjugation step to bind to cells. Two approaches have been adopted for surface modification: first, by surface ligands exchange with amphiphilic polymers [51, 126] (Fig. 2.16), mercaptoalkanoic acid ligands [49,127,128] or other surfactants with

reactive groups of $-\text{COOH}$ or $-\text{NH}_2$ [129], second, by encapsulation of QDs in water miscible shell such as liposome [50,130], silica microcells [131], and glyconanospheres [132]. Figure 2.17 shows the schematic illustration of some surface modifications of QDs and how they are linked to biomolecules.

Fig. 2.16 Structure of an amphiphilic polymer, polydimethylaminoethyl methacrylate (PDMAEMA), and its ligand exchange with TOPO ligand on CdSe-ZnS core-shell surface [126].

Fig. 2.17 Schematic illustration of some surface modifications of QDs, and how they are linked to biomolecules [125].

(a) Use of a bifunctional ligand such as mercaptoacetic acid for linking QDs to biomolecules, (b) TOPO-capped QDs bound to a modified acrylic acid polymer by hydrophobic forces, (c) QD solubilization and bioconjugation using a mercaptosilane compound, (d) Positively charged biomolecules are linked to negatively charged QDs by electrostatic attraction, (e) Incorporation of QDs in microbeads and nanobeads.

A systematic examination of different surface coatings on CdSe/CdS/ZnS QDs on their particle sizes and optical properties was reported [55]. QDs of diameter 4.5 nm were synthesized by the well known solvent synthesis methods. QDs were transferred to water

using five different ligands or polymers, namely mercaptopropionic acid (MPA), polyethylenimine (PEI), amphiphilic diblock copolymer (PMMA-PEO), lipid-PEG, and alkylated polycarboxylate. Each phase-transfer protocol was independently optimized to maximize colloidal stability and quantum yield. The capping methods are described in detail below.

Mercaptopropionic acid-coated QDs: QDs were first reacted with MPA and washed twice with chloroform to remove the solvent, then resuspended in a 1.1 mM aqueous solution of MPA (pH 10) and incubated at room temperature for 24 hours to finalize the ligand exchange. The QDs were centrifuged at 14000 rpm for 15 min, dialyzed repeatedly against 50 mM borate buffer (pH 8.5), and then stored at 4 °C in the dark.

Polyethylenimine-coated QDs: PEI was mixed with a nanocrystal dispersion in chloroform and the solvent was slowly evaporated. The resulting dried film was dissolved in deionized water and the solution was centrifuged to yield a clear supernatant containing a white precipitate. PEI was removed via repeated dialysis against deionized water, and then 500 mM borate buffer was added to a final concentration of 50 mM.

Amphiphilic diblock copolymer-encapsulated QDs: QDs and PMMA-PEO were dissolved in tetrahydrofuran, mixed, and then dialyzed repeatedly against deionized water using a low molecular weight cut-off membrane (2000 Da). The resulting aqueous solution was then subjected to several cycles of centrifugation (14 000 rpm, 15 min), and then ultracentrifugation (540 000 rpm, 1 hour) to remove aggregates and excess polymer. Finally, the QDs were resuspended in borate buffer.

Lipid-encapsulated QDs: Lipid-PEG and QDs were mixed in a 5000 : 1 molar ratio in chloroform, and the solvent was slowly evaporated under a slight vacuum. The QDs were resuspended in deionized water and purified via ultracentrifugation as described above.

Alkylated polycarboxylate-encapsulated QDs. Poly(maleic anhydride-alt-1-tetradecene) was hydrolyzed at 80 °C in water (5% w/v) for 24 hours and then lyophilized, yielding an amphiphilic polycarboxylate. This protonated polymer was dissolved in chloroform and mixed with QDs at a polymer chain : QD ratio of 500 : 1. The solvent was slowly evaporated under a slight vacuum. The dried film was resuspended in borate buffer, centrifuged (14 000 g, 15 min) to remove aggregates, and purified from excess polymer via ultracentrifugation.

PEG-conjugated QDs. QDs encapsulated in alkylated polycarboxylate were mixed with amino-PEG at a PEG : QD ratio of 3000 : 1, and then excess EDC was added (EDC : QD = 5000 : 1). After 24 hours at room temperature, excess EDC was quenched with the addition of 1-thioglycerol, and the QDs were purified via ultracentrifugation.

The dynamic light scattering data, zeta potential analysis, transmission electron micrographs, and proposed self-assembly schematics are depicted in Fig. 2.18 to compare the five different encapsulation and coating strategies. The following conclusions can be drawn from the results:

QDs coated with MPA were the smallest overall (6–8 nm hydrodynamic diameter) due to a single monolayer of hydrophilic ligand. They were also negatively charged, and were highly clustered when spread on a TEM grid. QDs coated with PEI were larger from DLS measurements (10–12 nm), suggesting that the multidentate polymeric coating contributes

significantly to its size. PEI-coated QDs were highly positively charged, even in basic media. PEI contains primary, secondary, and tertiary amines capable of buffering over a wide pH range. QDs coated with the alkylated polycarboxylate were roughly 18 nm in diameter by DLS measurements, and negatively charged. QDs coated with lipid-PEG were significantly larger (30 nm) and nearly neutral.

Fig. 2.18 Colloidal properties of QDs, comparing five different encapsulations and coating strategies.

(A) Model of QDs passivated by a monolayer of ODA and dispersed in a non-polar solvent (left), and a transmission electron micrograph (right, 200 nm x 200 nm). (B) Schematic representations of surface modification techniques used in this study. Drawings are not to scale and are only intended to demonstrate possible mechanisms of self-assembly, surface interactions, and the resulting nanoparticle size. (C) Transmission electron micrographs of QDs as dried films from aqueous solutions, counterstained with PTA. The dimensions of each image are 200 nm x 200 nm. (D) Dynamic light scattering data, plotted in arbitrary units of intensity (AU). (E) Zeta potential measurements for each QD type [55].

Compared to QDs coated with monodentate and polydentate ligands, all the QDs encapsulated in amphiphilic polymers were measured to be significantly larger via DLS and were surrounded by visible shells of low electron density in TEM micrographs. Coating QDs with the amphiphilic diblock copolymer generated even larger particles (35 nm), encapsulating clusters of 2–10 QDs within single nanoparticles, with thick polymeric shells in electron micrographs. These QDs had a slight negative charge, presumably due to a terminal hydroxyl group on the PEG, unlike the terminal methoxy group of the lipid-PEG QDs.

In a separate report, surface modification of nanocrystalline ZnO was performed by introducing chemically reactive functionality for subsequent bio-functionalization. A heterobifunctional organosilane crosslinking agent that contains an amine-reactive aldehyde group was attached to the ZnO surface, and then a fluorophore was attached to the reactive aldehyde to confirm that the modified nano-ZnO surface was available for subsequent biomolecular covalent attachment [133].

Organosilane crosslinker, 11-triethoxysilylundecanal (Gelest, Inc, catalogue # SIT8194.0) was added (2.6g, without purification) to the sonicated nano-ZnO solution. The solution was then placed on a stirring hot-plate and allowed to stir at $70^{\circ}\text{C} \pm 3^{\circ}\text{C}$ for 1 hour. The material was then vacuum filtered through a 40M fritted glass filter fitted with filter paper. The collected powder was washed with 200mL of 100% ethanol. The powder was allowed to dry in air for 5 minutes before adding it to a tared vial, and then cured in oven for 10 minutes at 110°C .

In the above surface silylation process, the silanol monomers underwent oligimerization and associated, via hydrogen bonding, with the hydroxyl groups on the surface of the nano-ZnO. With the addition of heat to the system, or over an extended period of time at room temperature, the hydrogen bonds were converted to covalent bonds with the concomitant loss of water as shown in Figure 2.19.

Fig. 2.19 Covalent attachment of the organosilane cross-linker, 11-triethoxysilylundecanal, to nano-ZnO [133].

The introduction of a chemically reactive modifier to the surface of the nano-ZnO presents a template for the design of new, optically responsive bio-sensing platforms. After the surface modifications, QDs can be further linked to bioaffinity ligands such as monoclonal antibodies [26,134], peptides [135], proteins [22,136], oligonucleotides [50,137] or small-molecule inhibitors. Linking to polyethylene glycols (PEG) [2] or similar ligands can also lead to improved biocompatibility and reduced non-specific binding. Figure 2.20 is a schematic illustration of the structure of a multifunctional QD probe, showing the capping ligand TOPO, an encapsulating copolymer layer, tumour-targeting ligands (such as peptides, antibodies or small-molecule inhibitors), and polyethylene glycol (PEG). For highly specific labelling of specific cells, subcellular targets, or intracellular delivery of QDs to living cells, avidin-biotin [1,26,134], antibody-antigen and ligand-receptor [137] interactions have been reported, but more effort is needed to meet the requirements on overall size (<5nm) of QD and the

interaction chemistry.

Fig. 2.20 Schematic illustration of the structure of a multifunctional QD probe. It shows the capping ligand TOPO, an encapsulating copolymer layer, tumour-targeting ligands (such as peptides, antibodies or small-molecule inhibitors), and polyethylene glycol (PEG) [7].

A few authors have reported the surface modifications of ZnO nano-particles using different capping agents for the purpose of improving the photoluminescence efficiency and controlling the size of the nanoparticles. Typical polymer capping agents are polyvinyl pyrrolidone (PVP) [138,139] and polyvinyl butyral (PVB) [140]. Photoluminescence efficiency is highly dependent on surface texture and defects. Surface defects such as dangling bonds of zinc and oxygen at the grain boundaries lead to the formation of non-radiative centres [138,141]. These centres allow photogenerated electrons in the conduction band to be trapped and recombined with the surface-trapped hole. Capping of ZnO nanoparticles can effectively passivate the surface, thus increasing the intensity of UV photoluminescence by reducing the surface related visible emission

[142]. Silica based materials are good capping agents for ZnO as they are chemically inert, suspend readily in water, and are relatively easy to synthesize in a controlled way. Moreover, they can be further improvised with a variety of organic or inorganic molecules that allow them to be suitable for biological systems [82]. But so far, there is no report on bio-imaging or cell detection using ZnO nano-particles.

2.3.2 Examples of biomedical applications of QDs

Great efforts have been made by a few groups to use CdSe-ZnS based QDs for *in vitro* and *in vivo* bioimaging, cancer detection and drug delivery applications. The following types of biomedical applications can be summarized:

1. Bioimaging on different cells to study luminescent properties and cell viability. Both *in vitro* and *in vivo* tests have been reported. Two-colour imaging was also conducted by using two types of QDs or combine with organic dyes. Details are listed in Table 2.3.

Figure 2.21 shows a typical two colour imaging, where the cytoskeleton fibres in 3T3 mouse fibroblast cells are labelled with biotinylated phalloidin and QD535-streptavidin (green), while the nuclei were stained with Hoechst 33342 blue dye [26]. As shown in the Fig.2.3 that the organic dye has much shorter photostability than QDs, this two colour imaging does not serve the purpose for long term imaging and cancer detection. It is desired that both colour markers have the same high emission efficiency and labelling capability.

Table 2.3. QDs for bioimaging applications and cell viability studies.

Target cell or animal	QDs used	Surface modification /bioconjugation	Figure No.	Ref. No.
Breast cancer cell 3T3 mouse fibroblast cells, human epithelial cells	CdSe-ZnS + organic dye (2-color imaging)	QD-IgG / Her2 antibody QD-streptavidin/antibody IgG-biotin/anti-human	Fig. 2.21	[26]
Live mouse bearing C4-2 human prostate tumours	CdSe-ZnS	QD-COOH/PEG/ antibody-antigen QD-peptides	Fig. 2.22	[22, 143]
Neoplastic cells Breast tissues	CdTe-CdS CdS/Cd(OH) ₂	QD-glutaraldehyde / direct	Fig. 2.23	[144]
Human epithelia cells	Commercial QDs	QD-polymer/biotinylated phalloidin/antibody	Fig.2.24	[129]
Human hepatocellular carcinoma (QGY)cells, Human breast cancer (MCF7) cells, in vitro	CdTe	QD-peptide/direct	NA	[145]
Vero cell (African green monkey kidney cell)	CdSe-TOPO	CdSe-mercaptoglycerol	NA	[146]
Insect cell line (Drosophila Schneider2) 293T human kidney cell	CdSe-ZnS	QD-amine/antibody	NA	[147]
<i>Euglena gracilis</i> EG277 Human embryonic kidney HEK 293 cells	CdTe-thiol	QD-thiol/protein	NA	[148]
Human THP-1 monocyte cell	CdTe-TGA	QD-thiolglycolic acid (TGA)/ direct labelling	NA	[149]
Plant protein	CdSe-ZnS (6.3nm)	QD-COOH/EDA(ethylene carbodiimide)/protein (SCA)	Fig.2.25	[150]

Fig. 2.21 Two colour imaging. Cytoskeleton fibres in 3T3 mouse fibroblast cells are labelled with biotinylated phalloidin and QD535-streptavidin (green), nuclei were stained with Hoechst 33342 blue dye [26].

Fig. 2.22 *In vivo* bio-imaging in mouse. (a) Molecular targeting and *in vivo* imaging of a prostate tumour in mouse using a QD-antibody conjugate (red). (b) *In vivo* simultaneous imaging of multicolour QD-encoded microbeads injected into a live mouse. (c) Live mouse models bearing C4-2 human prostate tumours labelled by QD-COOH, QD-PEG and QD-PSMA probes [22].

Figure 2.22 shows *in vivo* imaging of a prostate tumour in a mouse using a QD–antibody conjugate (red), a multicolour QD-encoded microbeads injected into a live mouse, and live mouse models bearing C4-2 human prostate tumours labelled by QD-COOH, QD-PEG and QD-PSMA probes. These are the latest developments in bio-imaging using QDs. This demonstrated that QDs are either coated with surface functional group of –COOH, with polymers (PEG, PSMA), or SiO₂ layer (microbeads), or grafted with antibodies, and then they can be injected into live animals for *in vivo* imaging. However, as analyzed earlier, the overall particle sizes are different from different surface treatments, and for *in vivo* imaging, the particle size is more critical as they need to be transported by body fluid or blood, as such, the overall size should be as small as possible (1-5 nm), while, in the meantime, the optical emission must be high even from a single dot. This is the target of this research.

Figure 2.23 shows the Neoplastic glial (glioblastoma) cells directly labelled by QD-glutaraldehyde, which is a yellow organic dye. Figure 2.24 shows human epithelial cells labelled with QDs and monoclonal anti-histone antibodies (two colours), and with a commercial polymer-coated QD conjugated with streptavidin. Figure 2.25 shows the only reported images of plant protein labelled with QDs, showing the well labelled pollen tube by the QDs.

Fig. 2.23 Confocal Microscopy image of Neoplastic glial (glioblastoma) cells incubated with QDs-Glut [144].

Fig. 2.24 Labelling of human epithelia cells by commercial QDs.
(a) Labelling of fixed human epithelial cells with QDs and monoclonal anti-histone antibodies, (b) Detection of human epidermal growth factor receptor 2 (Her2) with commercial polymer-coated QD conjugated with streptavidin [129].

Fig. 2.25 Labelling of plant protein by commercial QDs.
(d) Fluorescence image of a pollen tube culture with QDs added, showing the absence of non-specific QD labelling on the tubes. The pollen grains show autofluorescence.
(e) Bright field image of (d) [150].

2. QDs have been used to detect certain virus, blood groups, or stress response on cancer cells. Also due to the long emission capability of semiconductor QDs, they have been used for long term monitoring of progressive changes in the cells under certain conditions. Table 2.4 lists a few examples of such applications. The QDs used are the same as described above. These examples demonstrated that QDs can be further used for more biological functions.

Table 2.4 QDs for detection and progression monitoring in cells

Target cell/animal	QDs used	Surface modification /bioconjugation	Figure No.	Ref. No.
Respiratory syncytial virus (RSV) in HEp-2 (human epithelial larynx carcinoma) cells	CdSe-ZnS	QD-PEG-viral glycoprotein	Fig.2.26	[151]
Human erythrocytes of blood groups A, A ₂ , O	CdS	CdS-glutaraldehyde /anti-A	NA	[152]
Heat stress induced breast tumour cells	Commercial QDs 605	QD- streptavidin/biotin	Fig. 2.27	[137]
Cancer marker Her2 on breast cancer SK-BR-3 cells	Commercial QD-630, QD-535	QD-IgG/anti-Her2 antibody and ANA (anti-nuclear antigen)	NA	[26]

Fig. 2.26 Labelling of double proteins in RSV infected cells. Confocal microscopic image shows co-localization of the F and G proteins in RSV infected HEp-2 cell monolayer cultures 4 days post infection. The composite image shows orthogonal slices XZ and YZ suggesting that the F and G proteins are predominately located on the surface of the infected cells [151].

Fig. 2.27 Fluorescence microscopy of MX1 cancer cells labelled with quantum dots. After a 30 min incubation period at room temperature the cells exhibited vacuoles.

3. QDs have been used for delivery of medicines. The research in this area has just started; therefore, the list is short as shown in Table 2.5. The major challenges include how to attach the medicine onto QD surface and release it only on target cells, and the safety of the QDs. Currently, the medicine is either coated onto the QDs first, and attached to the target cell later, or embedded inside a hydrogel and released by salt removal.

Table 2.5 QDs for medicine delivery

Target cell/animal	QDs used	Surface modification /bioconjugation	Figure No.	Ref. No.
Vero cell, HeLa (DNA) cell, Stroke-prone spontaneously hypertensive rats	CdSe-ZnS-T OPO	QD-cap as medicine carrier, QD-COOH, QD-OH, Cap is a anti-hypertension drug	NA	[134, 153]
HT-29 colon cancer cell	ND (Nano-Diamond)	ND-COOH, ND-OH/anti cancer drug	NA	[154]

Chapter Three: Experimental Details

In this chapter, the materials and their properties, the synthesis process parameters, and the characterization methods are introduced and described in detail.

3.1 Materials and properties

3.1.1 Raw materials for ZnO synthesis and surface capping

All the chemicals employed, otherwise mentioned were purchased and used without further purification. Zinc acetate dihydrate, $\text{Zn}(\text{Ac})_2 \cdot 2\text{H}_2\text{O}$, 99.5%, Copper acetate monohydrate, Nickel (II) acetate tetrahydrate and Cobalt acetate tetrahydrate were all obtained from Sigma ALDRICH Chemical. Methanol, 99.9% was from Labscan Ltd. The capping agents 3-Aminopropyl-triethoxysilane (APTES or Am), 99.5% and Mercaptosuccinic acid (MS), 97% were from ALDRICH Chemical. Polyvinylpyrrolidone (PVP) was from ISP Technologies Inc., and 3-Mercaptopropyl-trimethoxysilane (MP), 97% was from Fluka. To make the silica (SiO_2) capping layer, Tetraethyl orthosilicate (TEOS) was hydrolyzed in ethanol and water with a very small amount of hydrochloric acid (HCl) acting as the catalyst. The TiO_2 capping agent was also prepared by sol-gel method, in which Titanium (IV) Isopropoxide $\text{Ti}(\text{OPr})_4$ was hydrolyzed in ethanol and water with HCl as the catalyst. In addition, the two reagents containing double amino-groups, i.e., Aminoethyl aminopropyl trimethoxysilane (Z60), Aminoethylaminopropylsilane triol homopolymer water solution (Z61), were from Dow Corning Co. Lithium hydroxide was used to induce precipitation for the collection of powders for XRD analysis. The chemical formula, structures, molecular weights (M_r) and the abbreviations of the materials used in this report are summarized in Table 3.1.

Table 3.1 Materials details of the reagents

Chemical name and Molecular formula	Chemical structure	Molecular Weight (Mr)	Abb.
Zinc acetate dehydrate, $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$		219.51	$\text{Zn}(\text{Ac})_2 \cdot 2\text{H}_2\text{O}$
Copper Acetate Monohydrate $\text{Cu}(\text{CO}_2\text{CH}_3)_2 \cdot \text{H}_2\text{O}$		199.65	$\text{Cu}(\text{Ac})_2 \cdot \text{H}_2\text{O}$
Cobalt acetate tetrahydrate $\text{Co}(\text{CH}_3\text{COO})_2 \cdot 4\text{H}_2\text{O}$		249.08	$\text{Co}(\text{Ac})_2 \cdot 4\text{H}_2\text{O}$
Nickel acetate tetrahydrate $\text{Ni}(\text{OCOCH}_3)_2 \cdot 4\text{H}_2\text{O}$		248.84	$\text{Ni}(\text{Ac})_2 \cdot 4\text{H}_2\text{O}$
Tetraethyl orthosilicate $\text{Si}(\text{OC}_2\text{H}_5)_4$		208.33	TEOS, SiO_2
Titanium (IV) isopropoxide $\text{Ti}[\text{OCH}(\text{CH}_3)_2]_4$		284.22	TIP, $\text{Ti}(\text{OPr})_4$, TiO_2
3-Aminopropyl-triethoxysilane $\text{H}_2\text{N}(\text{CH}_2)_3\text{Si}(\text{OC}_2\text{H}_5)_3$		221.37	APTES, Aps
Mercaptosuccinic acid $\text{HOOCCH}(\text{SH})\text{CH}_2\text{COOH}$		150.15	MS
Polyvinylpyrrolidone $(\text{C}_6\text{H}_9\text{NO})_n$		500	PVP
3-Mercaptopropyl-trimethoxysilane $\text{HS}(\text{CH}_2)_3\text{Si}(\text{OCH}_3)_3$		196.34	MP
Aminoethyl aminopropyl trimethoxysilane		222	Z60
Aminoethylaminopropylsilane triol homopolymer		180.085	Z61
Lithium hydroxide monohydrate $\text{LiOH} \cdot \text{H}_2\text{O}$	$\text{LiOH} \cdot \text{H}_2\text{O}$	41.96	LiOH
Methanol CH_3OH	CH_3OH	32.04	MeOH

3.1.2 Sol-gel formulation of SiO₂ and TiO₂ capping agents

To make the TiO₂ capping layer on ZnO particles, a coating solution was formulated by sol-gel chemistry. The Titanium (IV) isopropoxide precursor was hydrolyzed in Ethanol and DI water with a small amount of hydrochloric acid (HCl) as the catalyst. The molar ratios of these components are: Ti(OPr)₄:H₂O:EtOH: HCl = 1:4:217:0.5. The chemical reactions can be expressed as follow:

(1) Hydrolysis of Ti(OPr)₄ with water in ethanol and HCl (sol):

(2) Condense to form cross-linked network (gel)

The overall reaction leading to TiO₂ is:

The reason for adding a lot of ethanol into this reaction process was to reduce the reaction speed of both hydrolysis and condensation step, in order to prevent the formation of particles during the preparation, and to slow down the condensation within the solution before it was used as capping agent. The Ti-OH would condense with the –OH groups on ZnO surface to form the covalent bond, which will be discussed in Chapter 5. To prepare the capping agent solution, 87.83g of ethanol was mixed with 1.77g of deionized water and 0.87g of HCl solution (37% weight percent) for 10 min., then 5 g of Ti (OC₃H₇)₄ were added into the mixture. After stirring for 4 hours, an additional 87.83g of ethanol was added into the mixture and stirred overnight. This solution was then stored and used

as the capping agent.

The SiO₂ capping solution was prepared similarly. TEOS was hydrolyzed in DI water and ethanol with HCl as the catalyst. The molar ratios of the components were: TEOS: H₂O:EtOH:HCl = 1:3:23.1:0.04. The chemical reactions were:

(3) Hydrolyse of TEOS with water in ethanol and HCl (sol):

(4) Condensed to form cross-linked network (gel)

The overall reaction leads to SiO₂:

When used as a capping agent, the Si-OH would condense with the –OH groups on ZnO surface to form the covalent bond, which will be discussed in Chapter 5. In detail, 0.33 g of HCl was diluted into the mixture of 80.55 gram of ethanol and 12.28 gram of H₂O. Afterwards, 47.30 gram of TEOS was added dropwise. The solution was stirred for 2 hours before being diluted further with 161 gram of ethanol.

To prepare the mercaptosuccinic acid capping agent, 0.553g of the powdered acid were re-dispersed in 20g of methanol so that the agent could be added more easily. For the precipitation of ZnO powder, LiOH powder was dissolved in water under ultrasonic bath at a concentration of 3 mol/L. All other capping agents were used as received.

3.2 Synthesis and processing procedures

3.2.1 ZnO synthesis process

Zinc oxide, ZnO colloid was prepared with Zinc acetate dihydrate, $\text{Zn}(\text{Ac})_2 \cdot 2\text{H}_2\text{O}$ and methanol, MeOH, as the starting reagents. Zinc acetate was used because acetic acid which was one of the products of the hydrolysis reaction was very soluble in the solvent medium. The acetate groups that decompose under annealing produce readily removable volatile by-product [108]. Methanol was used as the solvent because it could readily dissolve Zinc acetate in comparison to other alcohols such as ethanol. The higher solubility was due to the higher dielectric constant of methanol (32.6) than ethanol (24.3). Dielectric constant primarily determines the solubility of electrolytic salts [155]. Moreover, its low boiling temperature was also attractive for reflux synthesis.

Figure 3.1 shows the setup of the synthesis process. A 3-neck flask containing the reactants was put on a heating mantle. A thermometer was attached to a side neck, and a refluxing tube was attached to the central neck. A magnetic stirrer was used to maintain the homogeneity of the reaction. A flowchart of the synthesis process was shown in Fig. 3.2. At room temperature, 0.015, 0.03 or 0.06 moles (equivalent concentration 0.075, 0.15 and 0.3 mol/L) of $\text{Zn}(\text{Ac})_2 \cdot 2\text{H}_2\text{O}$ were dissolved in 200ml of MeOH under vigorous stirring for 15 min. or more to obtain a colourless solution, and then ultrasonicated for 10 minutes. The solution was heated at a constant temperature of 65° to 68°C under reflux for 5 to 7 hrs. Careful attention was paid to prevent excessive loss of the solvent during heating. The solution was rapidly cooled down in ice water to nearly 5°C when the reflux time was reached. The final solution was weighed after cooling and found to be 160 ± 0.5 grams. The solution was further separated into 20g solution per bottle, so that different

types of capping agents could be added to it.

To some of the bottles containing 20g synthesized solutions, a type of capping agent was added directly to each bottle and stirred overnight. Those solutions without capping agents added to them were observed for white precipitates appearing in them. Upon seeing the white precipitates, capping agents in precise amount were introduced. Some of the solutions became clear after the first capping. They were then capped for a second time when white precipitates appeared again, after which, the solution became very stable. The synthesis procedure and addition of capping agents were repeated for refluxing times of 5hrs, 5.5hrs, 6hrs and 6.5hrs, in order to study the influence of reflux time on particle size and colloidal stability.

Fig. 3.1 Setup of the ZnO synthesis process.

Fig. 3.2 Flow chart of the ZnO synthesis and capping processes.

The initial experiments were conducted by dissolving 0.06 moles of Zn(Ac)₂·2H₂O in 200 ml MeOH, to make the monomer concentration of 0.3 mol/L. Several capping agents including SiO₂ and TiO₂ sol-gel solutions were added to study the particle size and stability.

In the subsequent experiments, lower monomer concentration of 0.15 mol/L was used, in order to avoid too fast precipitation and to allow enough time for the capping agents to react and condense to the ZnO surface. TiO₂, Am, Z60 and Z61 were used as the capping agents as they showed higher potentials in capping capability. Meantime, dopants precursors containing Co, Cu and Ni cations were added in the beginning of the synthesis process, which were dissolved in methanol together with the Zn(Ac)₂·2H₂O. The molar ratio of dopant to Zn(Ac)₂·2H₂O was varied from 5%, 10%, 15% and 20%. When the reflux was completed, the solution was chilled down to about 5°C by ice water and the capping agent in a calculated amount was added to each of the synthesized solution and stirred for overnight. The measurements on particle sizes were started on the next day (day 1). To investigate the stability of the particle size in colloidal solution, the particle size was measured regularly with a few days' intervals, and plotted in a graph to show the growing trend. Meantime, the zeta potential values were also measured as a further indication of the particle size stability.

To add dopant into the ZnO, the proper amount of dopant precursor such as Cu(CO₂CH₃)₂ · H₂O, Co(CO₂CH₃)₂ · 4H₂O or Ni(CO₂CH₃)₂ · 4H₂O powder was weighed and added to the initial Zn(Ac)₂· 2H₂O+MeOH solution and they dissolved together. The molar ratio of the dopant to Zn(Ac)₂· 2H₂O was varied from 5%, 10%, 15% to 20%.

3.2.2 Collection of ZnO powder for XRD analyses

For XRD analyses, uncapped ZnO powders were collected. For higher concentration (0.06 mol in 200ml MeOH) ZnO solutions, the solutions were left in the room condition to age for 1 day after reflux was stopped. 50g of the solution was taken out and placed in another bottle. The remaining solution was left for further aging for a longer period of time at standstill condition. The excess amount of methanol was added to the 50 g of

solution to the uncapped precipitates and stirred. Centrifugation was carried out at 10000 rpm for 10 min. The precipitates were then further washed with ethanol by repeating centrifugation for 3 times. The collected precipitates were dried in the oven at 70°C for 1 day. The dry powder was ground before XRD analysis. For the lower concentration ZnO solutions (0.15 mol/L), a small amount of LiOH solution (2 ml of 3mol/L solution into a 50g ZnO solution) was added to induce the precipitation. The precipitates were washed with ethanol by repeating centrifugation at 10000 rpm for 20 minutes, for three times. The collected precipitate was then dried at 70°C in the oven overnight. Washing was performed to ensure the removal of the Li-containing by products and water from the ZnO solution.

3.2.3 Addition of capping agents

The amount of capping agent to be added to ZnO colloid is very crucial to ensure effective stabilization of the particles. With too little capping material, the particle surface will not be covered completely, and particle growth will continue and surface defects will not be eliminated. While with too much capping material added, the capping layer may be too thick, the optical property may be reduced, and the particle size may be increased. Meanwhile, some of the capping agents, such as TEOS, TiO₂, APTES and MPTES, have additional reactive groups (Si-OH or Ti-OH), which may form covalent bonds with two or more molecules of themselves, and result in aggregated larger particles. Therefore, the amount of capping agent must be carefully calculated before adding into the ZnO colloid. We created a spreadsheet using EXCEL software, to include the input of ZnO particle size, capping agent type, its molecular weight, and the amount of the synthesized ZnO solution. The amount of capping material needed was automatically calculated. The assumptions for our model included: spherical shape of ZnO particles, capping agent with the affinity

to attach to ZnO surface (join with –OH groups on ZnO surface) first before joining with other groups.

Calculation procedures

The amount of capping agent to be added to a 20g ZnO solution was calculated based on the concept that a monolayer of capping agent will grow on the whole surface of the ZnO particles. The thickness of the monolayer is about 0.5nm, which is the molecular size of the silane materials. Therefore, the desired capping material is about 2 molecules per 1 nm² area of ZnO surface. The calculation steps are as follows:

- (1) The particle size of the ZnO is input as a known parameter, this is known from the Zetasizer measurement and the TEM analysis.
- (2) The surface area and volume of a ZnO particle can be calculated assuming a spherical shape. The mass of one ZnO particle is calculated from the density of pure ZnO multiplied by the volume of a particle.
- (3) The total mass of ZnO material in 20g synthesized solution can be calculated from the divided moles of the original Zn acetate material added in the solution according to the ratio of 20g : 160g.
- (4) The total number of ZnO particles in the 20g synthesized solution is calculated by dividing the total mass of ZnO with the mass of one particle.
- (5) The total surface area of all the particles in the 20g solution is calculated using the value from steps (2) and (4).
- (6) Using the value in step (5) with unit of nm², the total number of molecules of capping agent is calculated by the value in (5) times 2 (because we want to attach 2 molecules per 1 nm² of surface area).
- (7) The total number of moles of capping agent is calculated by dividing the value in

-
- (6) with the Avogadro's number (6.023×10^{23} molecules /mol).
- (8) The quantity of the capping agent needed is obtained by multiplying the value in
- (7) with the molecular weight of the capping agent.

A detailed calculation procedure and an example of the spreadsheet are attached in Appendix II. It is clear that the capping agent was added based on an estimated particle size. If the actual particle size is larger than estimated, the total surface area would be less, and there would be some excess capping material that could coat onto the first capping layer to form the second layer, or join with other groups to form clusters.

For APTES, it was found that maximum capping capability was achieved when the amount of capping agent was added in 2 portions. It was noticed that after the first addition of capping agent, the precipitated particles disappeared, and the colloidal became a clear solution; after 1-2 days, some white precipitates appeared again, and this time, the second addition of capping agent was performed, after which, the colloidal could stay uniform for very long time (up to 2 months). For TEOS, if the amount of agent added was much more than the calculated amount, condensation and a gel-like solid would be formed instead of a capped colloidal solution. The typical amounts of capping agents used in our ZnO samples are listed in Table 3.2.

Table 3.2 Typical amounts of capping agents added to ZnO colloids

Capping agent	Amt added to 20g of ZnO colloidal (g) C=0.3 mol/L Particle size: 6 nm	Amt added to 20g of ZnO colloidal (g) C=0.15 mol/L Particle size: 1 nm
APTES	0.0405 + 0.0405	0.2429
MS	0.055	0.1648
MP	0.072	0.2155
PVP	0.07 - 0.18	1.0974
SiO ₂ sol	0.30 - 0.35	1.0464
TiO ₂ sol	2.18 – 3.74	11.207
Z60	0.081	0.2436
Z61	0.066	0.1976

3.3 Characterization

3.3.1 Particle size and Zeta-potential analyses by Zetasizer

Particle size in the solution and zeta potential were measured by a Malvern Zetasizer Nano ZS, which was equipped with a 4mW 633nm laser. The system measures the size by measuring the Brownian motion of the particle using Dynamic Light Scattering (DLS) and then interpreting the size of the particle [156]. The colloidal sample was injected into a dip cell (Fig. 3.3(a)) to the level of 10 mm from the bottom. For the comparison of size and size stability of different samples, at least four measurements on each sample were performed, and the average size of the four measurements was calculated.

Zeta potential is used to measure the velocity of the particle when an electrical field is applied. An electrical double layer exists around each particle due to the presence of surface charges, which originates from either one of these: (i) ionization of surface groups,

(ii) differential loss of ions or (iii) adsorption of charged species. The magnitude of the zeta potential provides us with a guideline to the potential stability of the colloidal system. If the particles have a large negative or positive zeta potential (the larger the better), they will repel one another and attain dispersion stability. To measure the zeta potential, a folded capillary cell (Fig. 3.3(b)) was used. The sample was slowly injected into the cell ensuring that no air bubbles were formed. Two stoppers were inserted at both sides and the sample was ready for measurement.

Fig. 3.3 Picture of (a) dip cell used for zeta size measurement and (b) folded capillary cell used for zeta potential measurement.

3.3.2 Particle shape and crystal structure analyses by FESEM

The Field Emission Scanning Electron Microscopy (FESEM) (JEOL JSM-6340F) was used to analyze particle shape and size using high magnifications of up to 100000 times. In the first part of this research, solid aluminium round disc (diameter 8 mm) was used as the sample holder. A drop of ZnO colloidal solution was applied on the disc surface, after drying in the oven for a few minutes; a second drop was applied and dried. It was found that samples prepared in this way were not good, as some showed overlapping of particles,

and others did not contain any particle (the droplet could easily roll off). Therefore, some experiments on the anodizing of aluminium foil were conducted to make porous Al_2O_3 films according to the reported methods [157]. These anodic aluminium oxides (AAO) have been extensively used as templates for preparing various nanostructures, owing to its self assembled monodispersed pores with high density (10^{11} pores/ cm^2), high aspect ratio (the ratio of the length of a pore to its diameter), and controllable pore size. Figure 3.4 shows the pore distributions of a reported AAO (a) [158]; the AAO sample was made in this research (b), and the Anodisc 13 was purchased from Whatman®. It was observed that pore size could vary a lot depending on the process parameters. For our nanoparticles, we used both the AAO fabricated in our laboratory and the purchased Anodisc 13 as the sample holders for FESEM analyses. The AAO template was either soaked in the colloidal solution for 1 hour, or used to scope a few times in the solution to catch the nanoparticles, and then left to dry in the oven for 4-8 hours at 60°C . After drying, they were mounted onto the FESEM sample holder for analyses.

3.3.3 Crystal structure analysis by XRD

The crystalline structures of ZnO nanoparticles were characterized by X-ray Diffraction (XRD) analysis in Bragg-Brentano geometry employing a Shimadzu 6000 diffractometer with $\text{Cu-K}\alpha$ radiation ($\lambda = 1.5418 \text{ \AA}$). The collected dry samples were ground into fine powders before analysis. The spectrum was compared to the ICSD (Inorganic Crystal Structure Database) database. To determine the lattice parameters as well as the quantitative phase composition, further evaluation of the diffraction patterns by means of the Rietveld refinement method was carried out using the TOPAS software.

Fig. 3.4 SEM micrographs of AAO templates fabricated by different methods. (a) AAO template fabricated by anodisation in 0.3 M oxalic acid at room temperature (the average pore diameter is 65 nm) [158], (b) AAO template produced in our laboratory, average pore size 48 nm, (c) AAO template purchased from Whatman®, average pore size 250 nm.

3.3.4 Crystal structure analyses by HRTEM

Further analyses of the crystal sizes were performed using High Resolution Transmission Electron Microscope (HRTEM, JEOL JEM2010) with LaB_6 as the filament. A copper grid was used to scope a few times in the ZnO colloidal solution, and was dried in the oven at 60°C for 1 to 2 hours before TEM analysis. The lattice parameters measured on the TEM diffraction pattern was analyzed by the Fast Fourier Transform Technique (FFT), and

converted from the reciprocal lattice back to real lattice by the GEMS software, and then compared to the ZnO Wurtzite (P63mc) hexagonal structure found in the ICSD standard database. In this way, the lattice structure of the materials was further confirmed in addition to the XRD analysis.

3.3.5 Photoluminescence and light absorption measurements

Photoluminescence (PL) spectra were measured at room temperature using a single photon counting spectrofluorometer (Fluorolog-3 Jobin Yvon Horiba), under excitations at 335 nm and 488 nm. The excitation source is a 450 W xenon lamp (excitation range: 230nm to 1000 nm), coupled to a double grating fast scanning monochromator. To study the PL spectra at low temperatures, the PL spectra were measured at room temperature and at 4.3K with an Accent PL mapping system (rpm 2000). The sample was excited with the 325 nm line of a He-Cd laser. The luminescence was dispersed with a monochromator and was recorded with a Charged Coupled Device (CCD).

The light absorption spectra of ZnO solutions were obtained by UV-310PC, UV-Vis-NIR Spectrophotometer (Shimadzu). A deuterium lamp was used with the wavelength ranging from 393 to 282nm. The spectrophotometer measures the amount of the light absorbed, transmitted or reflected at each wavelength of radiation. From the absorption spectra of the nanocrystals compared to that of larger ZnO particles, we can prove the quantum confinement effect. Both the absorption spectra and PL spectra were used to calculate the quantum yield (QY) of the synthesized ZnO nano-particles.

3.3.6 Quantum yield measurement

In order to measure quantum yield, four standard solutions were made according to the

literature [159] by Jobin Yvon Horiba, who is the manufacturer of the spectrofluorometer. Two commercial organic fluorescence materials, Flourescein and Anthracene, were selected as the standard materials and cross-calibrated between them to ensure the measurement systems and parameters are suitable for the QY measurement. Flourescein was prepared in 0.01 M Sodium Hydroxide (NaOH) [160] in concentrations of 3×10^{-5} and 7×10^{-5} mol/L. Anthracene was prepared in pure ethanol [161] in concentrations of 1×10^{-3} and 7×10^{-3} mol/L. Four tests solutions were also prepared: ZnO in methanol doped with 5% Cobalt in concentrations of 1.5×10^{-1} mol/L and 7.5×10^{-2} mol/L, and ZnO in methanol with 10%Co in concentrations of 1.5×10^{-1} mol/L and 7.5×10^{-2} mol/L. UV-Vis absorbance spectrum of the solvent background for the chosen sample was recorded, taking note of the excitation wavelength to be used. Next, the fluorescence spectrum of the same solution was taken from a 10mm cuvette containing the solution, and then the integrated fluorescence intensity, which was the area of the spectrum, were calculated. Repeat the two steps for five solutions with increasing concentrations for the chosen sample. The above steps were repeated for the remaining samples. A graph was plotted using the integrated fluorescence intensity against absorbance. The result should be a straight line with gradient m , and intercept = 0. To calculate the QY, a standard sample with known fluorescence QY value was used as the reference, and the QY of the unknown sample was calculated using the following equation:

$$\Phi_X = \Phi_{ST} \left(\frac{Grad_X}{Grad_{ST}} \right) \left(\frac{\eta_X^2}{\eta_{ST}^2} \right) \quad (24)$$

where the subscripts **ST** and **X** denote the standard and test respectively, Φ is the fluorescence quantum yield, **Grad** the gradient from the plot of the integrated fluorescence intensity vs absorbance, and η the refractive index of the solvent.

3.3.7 Cell culture for cytotoxicity test and bio-imaging

To test the bio-imaging behaviour of the synthesized ZnO nanocrystals, a human osteosarcoma (tumour) cell line (MG-63 from ATCC) derived from human bone was used. The Product Description of this cell line together with the cell culture protocol is attached in Appendix III. This is a fibroblast type of cell that adheres to the culture dish for growth. Cell viability or cytotoxicity of nanoparticles was evaluated by the well known MTT assay (methyl tetrazolium salt test)[162]. After the cells were cultivated in a culture dish for one day, nanoparticles in different concentrations were added to the media and incubated for another day. Then a CellTiter 96® AQueous one solution cell proliferation assay was added. After incubation for 3-4 hours, the absorbance at 490 nm was recorded using a 96-well plate reader (TECAN Infinite M200 Fluorescence / Absorbance multifunctional microplate reader). A control cell was always used on the same 96-well culture flask. The absorbance of the cell with nanoparticles added was compared to the absorbance of the control cell (no nanoparticle added), and cytotoxicity was defined as the nanoparticle concentration that reduced 50% cell viability [162].

For bio-imaging, a glass bottom culture dish was used for cell culture. After one or two days of cell growth, the medium was aspirated, and the concentrated nanoparticles were added. Imaging of these cells was conducted within 4 hours. Some cells were fixed by 2% paraformaldehyde after the nanoparticles were added, but it was found that the fixing material caused higher reflection, making the confocal bio-imaging less sensitive. To confirm the applicability of the nanoparticles for other cells, bio-imaging was also conducted on human histiocytic lymphoma cell line (U-937 from ATCC)). This is a monocyte type of cells, which grow in suspension. The Product Description is attached in Appendix IV. For bio-imaging, we just needed to withdraw a small amount of the cell suspension and put on a microscope glass, and then 2 μ L of nanoparticle colloid was

added. After the sample was dried in air for a few minutes, bio-imaging was performed. Bio-imaging of human cells was carried by the Nikon C1Si Spectral Imaging Confocal Laser Scanning Microscope System. This system is equipped with multiple excitation sources and the excitation at 408 nm was used for this research.

Another cell line, L929, which originated from mouse subcutaneous connective tissue, was used to check the cytotoxicity of ZnO nanoparticles. The Product Description is attached in Appendix V. To speed up the cell proliferation, nanoparticles were added just after the cells were cultivated on a 12-well culture flask. The cell growth status was observed by a fluorescence microscope on the 2nd day and 3rd day. Fluorescence images were taken and compared to the control cells.

3.3.8 Mung bean seedling growth for bio-imaging of plant cells

The plants used in this research are mung beans (*vigna radiata*). They are considered to be vascular plants, which are defined as “any plant containing food-conducting tissues (the phloem) and water-conducting tissues (the xylem).” [163]. These two tissues extend from the leaves to the roots, and are vital conduits for water and nutrient transport. Figure 3.5 shows a typical cross-section of a young *vigna radiatas* stem. Xylem tissue conducts water and mineral nutrients from the soil upward in plant roots and stems. Phloem tissue, on the other hand, conducts carbohydrates manufactured in the leaves downward in plant stems. It is composed of sieve tubes (sieve tube elements) and companion cells.

Fig. 3.5 Cross-section of a young *vigna radiata* stem [163].

In order to observe the effect of the uptake of quantum dots on mung bean plants, the samples were prepared by growing the seedlings in a control big Petri dish for two days before separating them into individual small Petri dishes and adding the quantum dots. The observation of the effects was done with a confocal microscope two days after the addition of quantum dots (four days growth), after careful cross-sectioning.

Growing the seedlings

A maximum of two seedlings were grown in each small Petri dish at one time. The rest of fifty seedlings were grown as control samples for comparison.

Firstly, the big Petri dish was washed with clean tap water and lined with a piece of wet filter paper. Then, 50 seedlings were sowed in the big Petri dish and separated at maximum distance from each other. Lastly, the big Petri dish was covered, leaving a small opening for air flow.

Each seedling was left to germinate for two days. After 2 days, the seedlings were taken out from the big Petri dish and transferred to small Petri dishes. Each small Petri dish

contained two seedlings and quantum dots were added (0.0705ml, two drops from a disposable plastic pipette) to the root area of each seedling. Only one type of quantum dot was allowed in each dish to prevent cross contamination. The seedlings were then allowed to grow for another 2 days from the addition of samples, till just before they were sectioned for imaging. Figure 3.6 shows the growing process of mung bean seedlings and the addition of nanoparticles.

Fig. 3.6 Growth of mung bean seedlings and addition of nanoparticles. (a) 0 day growth, (b) 2 days growth, (c) 4 days growth without nanoparticle added, (d) 2 days growth and 2 days nanoparticles added.

Plant Cross-Sectioning

A sharp Gillette razor blade was used to manually hand-section thin sample slices for imaging. DI water was used as the medium for imaging. As a Confocal Laser Scanning Microscope was used, it was not crucial to achieve single cell cross-sections. However, each cross-section was still made to be as thin as possible.

Imaging was performed by using a Leica True Confocal Scanner (TCS) SP2 confocal laser scanning microscope. One excitation sources were used during the imaging; an Ar

laser emitting at 488nm. For emission detection, simultaneous three-channel detection was used. Table 3.3 shows the wavelength ranges of the green, yellow and red channels.

Table 3.3 Wavelength range of each detection channel in the Leica laser scanning microscope

Channel No.	Detection Range (nm)	Region Colour
Channel 1	500 – 550	Green
Channel 2	550 – 600	Yellow
Channel 3	600 - 750	Red

The 10x objective lens were used during the imaging process; any additional magnification was performed with digital magnification. Twenty consecutive section scans were completed for each cross-sectioned sample, and the average intensity image was obtained. Five types of images were produced – Channel 1 (green image), Channel 2 (yellow image), Channel 3 (red image), Channel 4 (transmission image) and finally an overlay image of the above four images.

Chapter Four: Results

4.1 Influences of synthesis parameters on ZnO particle size, shape and crystal structure

4.1.1 Selection of ZnO synthesis parameters

The major process parameters that influence the nucleation and nuclei growth are the monomer concentration, refluxing temperature, refluxing duration/time, and dwell time before adding capping agent into the cooled solution. The adjustable range of temperature is rather limited, as the boiling point of methanol is 65°C. Due to the fast evaporation of solvent, the reaction temperature was only varied between 65° to 68°C. When 65°C was used, the XRD results showed more intermediate products, and longer refluxing time was needed to ensure the complete conversion of raw material to pure ZnO. On the other hand, it was difficult to maintain the temperature at 68°C or higher for several hours because the methanol was already in the boiling state at 65°C. Therefore, temperature was fixed at $66.5 \pm 0.5^\circ\text{C}$, and refluxing time was varied to study the particle precipitation behaviour. It was found that with well combined temperature and time, the resultant particle size is more determined by the reactant concentration. For the experiments using monomer concentration of 0.3 mol/L and refluxing temperature at 67°C, we studied the particle precipitation behaviour by varying the refluxing time. Figure 4.1 shows the graph of precipitation time of ZnO particles against refluxing time at constant temperature. An exponential decay curve is displayed. This observation show that synthesis time is a key factor that determines the ZnO particles nucleation and further growth. By slightly increasing the refluxing time, the precipitation could be significantly faster. Generally for reflux times greater than 7hrs, ZnO precipitated almost immediately after the reflux

condition had stopped. Conversely, for refluxing times that were below 6hrs, the precipitation of ZnO took at least several days (as shown in Table 4.1). The observed precipitation of ZnO could be the aggregations or clusters of nanoparticles after long term storage in stationed condition at room temperature.

Figure 4.1 Various precipitation time of ZnO against their respective reflux time

Table 4.1 Reflux time and precipitation time (in hrs)

Reflux time (hrs)	Precipitation time (hrs)
5.0	196
5.5	75
6.0	15.5
6.5	5.5
7.0	1.5

The decay curve could be explained by the nucleation and growth theory mentioned in Section 2.2.2.1 and 2.2.22. A longer refluxing time promotes the nucleation and nuclei growth, and results in a higher degree of supersaturation (σ), which causes a bigger

reduction in Gibbs free energy (ΔG_v). This higher driving force enables the nucleation to occur spontaneously. Also from Equation (12), this decreased critical energy barrier causes a smaller critical particle size (γ^*). Consequently, all particles are slightly larger than γ^* , and all could grow at a higher growth rate. Due to the excessive growth of particle size, we reduced the initial concentration of $\text{Zn}(\text{Ac})_2 \cdot 2\text{H}_2\text{O}$ to 0.15 mol/L in our subsequent experiments. We could slow down the precipitation and allow for the addition of capping agents and their reactions with the ZnO surface to form effective capping monolayer by refluxing at a temperature between 65° to 67°C, and refluxing time between 6.5 and 7 hours. With these experimental settings, the average particle size was about 10-30 nm at 0.3 mol/L monomer concentration, and 2-10 nm at 0.15 mol/L monomer concentration without capping. These results have confirmed that the main factors influencing the critical particle size are concentration, time and temperature as described in Equations (12) to (15). When temperature and time were fixed as above, the critical particle size was 10 nm at 0.3 mol/L monomer concentration, and 2 nm at 0.15 mol/L monomer concentration.

4.1.2 Effect of capping agents on particle size

At the preliminary stage of capping agents selection, the following capping agents were added to the 0.3 mol/L concentration ZnO colloidal immediately after the refluxing solution was cooled down to about 5°C: PVP, MS, MP, APTES(Am), and SiO_2 (TEOS) (refer to Table 3.1 for full names of the capping agents). The typical size distribution results in three different size ranges are shown in Figure 4.2 and the full results with statistical data on size distributions analysed by Zetasizer are attached in Appendix VI.

(a)

(b)

(c)

Figure 4.2 Typical particle size distributions of the synthesized ZnO nanocrystals colloids measured by Zetasizer.

For the average size of 1.6 nm, the size distribution is from 1.3 to 2.0 nm (standard deviation ± 0.31 nm, 19.4%), for the average size of 52 nm, the distribution is from 37.8 to 91.3 nm (standard deviation ± 15.4 nm, 29.6%), and for the average size of 104 nm, the

distribution is from 58.8 to 164.2 nm (standard deviation ± 34.1 nm, 32.8%).

It should be noted that the measured data for the particle sizes smaller than 15 nm may not be taken as absolute values due to the resolution of the Dynamic Light Scattering mechanism used in the Malvern Zetasizer instrument”.

To study the colloidal stability, the particle size changes after the colloids had been stored for different periods of time were used as the measure. Since the same instrument and setting were used, the relative changes in size have been plotted in the following paragraphs to indicate the colloidal stabilities of the synthesized nanoparticles.

Figure 4.3 shows the size growing curves of differently capped ZnO samples in comparison with the uncapped ZnO. It can be seen that the growth rate of uncapped ZnO particles was more significant than all the capped particles. The best capping agent was Am, which limited the growth of the particles more effectively. It was found that the size grew to 60 nm in 3 weeks and to 100 nm in 6 weeks. Ms and TEOS were also effective in limiting the particle size, but the particle growths were faster than Am capped ZnO. Mp capped particles had very fast precipitation and sedimentation. Therefore, no particle size could be measured. Pv has a large molecular size with the molecular weight of 10,000. This results in larger particle size as shown in the graph. The slightly larger size on day 1 than on day 7 of the TEOS and Pv capped particles may be due to the weaker affinity of the capping agents to the ZnO surface, and as such, the capping material just surrounded the ZnO particle, but were not yet strongly bonded. At this time, the laser light still sensed the thermodynamic vibration of the capping material giving the overall size of the particle. On day 7, the capping layer had condensed on the ZnO surface; therefore, the overall size was smaller than on day 1. Upon longer storage, two particles could join

together because the -OH groups were still available on the side chain of the capping material. Detailed mechanisms of the particle capping and growing will be discussed in Chapter 6. The size of TiO₂ capped ZnO particles remained constant although it was relatively larger than the uncapped ZnO. Based on the above results, Am and TiO₂ were selected as the suitable capping agents for subsequent experiments.

Figure 4.3 Particle size growth rates of uncapped and capped ZnO colloids.
(Raw: uncapped ZnO, Am=APTES, TEOS=SiO₂, Pv=PVP, Ms=MS, TiO₂)

To confirm the relative stability of the particle size, zeta potential values were measured. Table 4.2 shows the results of uncapped, Am, TiO₂ and SiO₂ capped ZnO colloidal solutions. Both Am and TiO₂ capped ZnO particles possess higher absolute potential values than the uncapped ZnO. SiO₂ capped ZnO is also higher than uncapped ZnO, but lower than Am and TiO₂ capped ZnO particles. Both TiO₂ and SiO₂ capped surfaces are negatively charged.

Table 4.2 Zeta potential values of differently capped ZnO particles in colloidal solutions

Capping agent	Zeta potential (mV) in 7 days
Uncapped	- 5.4 ± 0.5
Am	25.8 ± 1.0
SiO ₂	-11.1 ± 0.7
TiO ₂	- 24.6 ± 0.9

Using monomer concentration 0.15 mol/L, temperature 67°C, and refluxing time 6.5 hours, we studied the effects of dopants on the particles size and colloidal stability.

Cu-doped ZnO

Figure 4.4 shows the plots of particle size growing trends of 5%, 10% and 15%Cu-doped ZnO particles in the colloidal solutions capped with Z60, Z61, Am and TiO₂ respectively. The sizes of differently capped particles with the same dopant content were plotted in one graph so that the effects of capping agents on particle growth rates can be seen clearly. As seen from Fig. 4.4(a), Z60 capped 5%Cu-ZnO was most stable; the size remained at 1.5 nm for more than 45 days. APTES capped ZnO was also stable, as its size remained below 2 nm for 45 days. Z61 capped 5%Cu-ZnO was stable for up to 30 days, TiO₂ capped 5%Cu-ZnO had a relatively larger size from the beginning (3-4 nm), and was stable for 10 days, and then grew slowly. From Fig. 4.4(b), the trends of Z60, Z61 and TiO₂ capped 10%Cu-ZnO are similar to 5%Cu-ZnO, only APTES capped 10%Cu-ZnO have larger size after 30 days, and Z61 capped particles grow from 15 days. However, these changes are not observed in the Fig. 4.4(c) on 15%Cu-ZnO, where all the Z60, Z61 and APTES capped 15%Cu-ZnO particles are stable for 45 days, and TiO₂ has the same trend as the other Cu-doped ZnO.

(a)

(b)

(c)

Figure 4.4 Plots of particle size growing trends of 5%Cu (a), 10%Cu (b) and 15%Cu (c) doped ZnO particles in the colloidal solutions. The standard deviations of the size data are below 20%.

In summary, Z60 has the best capping capability, followed by APTES, Z61 and TiO₂. TiO₂ causes the larger particle size from the beginning and remains stable for 15 to 30 days, and then grows larger. In comparing the stabilities of 5%, 10% and 15%Cu doped particles, the 15%Cu-ZnO particles are relatively more stable than the lower dopants particles. This may be related to the change of lattice structure caused by doping (layered structure will be shown in XRD results in Section 4.1.4), which will be analysed further.

It was observed that the lowest size of the colloid may be in several days or up to 30 days after synthesis. The reason may be that in the initial stage, capping agent molecules were just absorbed on the ZnO surface making a hydrodynamic equilibrium. The particle size measured by the laser at this time contained the particle core and the loosely absorbed capping layer. After some days of storage in room condition in a bottle (no stirring), the

capping molecule condensed on the particle surface forming a covalent bond, thus the overall size became smaller.

To further study particle stability by different capping agents, the zeta potential values of all the Cu-doped ZnO colloids were measured within 30 days, and the average values of all the same types of capping agents were calculated. Table 4.3 shows the average zeta potential values in terms of the type of capping agents. The Z60 and APTES have higher absolute zeta potential values, indicating more stable colloids, Z61 is slightly lower, and TiO_2 is the lowest. All capped particles have higher absolute zeta potentials than the uncapped Ni-doped ZnO, thus showing the importance of capping. These results are very much in line with the stabilities concluded from Figure 4.4.

Table 4.3 Average Zeta potential values of the 5%-15%Cu-doped ZnO particles with different capping agents.

Surface capping	Zeta potential (mV)
Uncapped	-5.43 ± 0.5
-Z60	23.89 ± 1.0
-Z61	19.53 ± 0.7
- TiO_2	-16.01 ± 0.6
-APTES	25.98 ± 0.9

Ni-doped ZnO

Similar studies on particle size and size growing trend were conducted for 5%, 10% and 15%Ni-doped ZnO colloids. Figure 4.5 shows the size growing graphs of 5%, 10% and 15%Ni doped ZnO capped by Z60, Z61, Am and TiO_2 respectively. It is observed from the graphs that the APTES capped particles are stable for 30 days, and grow quickly after 30 days; TiO_2 capped particles have a size below 5 nm for 30 days, and then grow slowly; Z60 and Z61 capped particles are below 3 nm for 45 days, although a slight growing trend is seen from 30days to 45 days. In comparing the 5%, 10% and 15%Ni dopant, the

(a)

(b)

(c)

Figure 4.5 Plots of particle size growing trends of 5%Ni (a), 10%Ni (b) and 15%Ni (c) doped ZnO particles in the colloidal solutions. The standard deviations of the size data are below 20%.

15%Ni-ZnO particles are relatively smaller in size at the stable stage (capped by Z60 and Z61), and have less growth from 45 days onwards. This may be related to the smaller ionic radius of Ni^{2+} ($r=0.55$) compared to Cu^{2+} ($r=0.57$) and Co^{2+} ($r=0.58$), and the dopants are smaller than Zn^{2+} ($r=0.60$). The substitution of Zn^{2+} sites by the dopants may lead to lattice shrinkage resulting in smaller crystal size. Therefore, higher dopant level could result in smaller particle size. In summary, both Z60 and Z61 had good capping capability, followed by TiO_2 and APTES. TiO_2 caused larger particle size from beginning and stable for 15 to 30 days, and then slowly grew larger, while APTES capped particles grew quickly after 30 days. In comparing the stabilities of 5%, 10% and 15%Ni doped ZnO colloids, all the graphs with the same capping agent showed the same particle

stability. Therefore, it can be concluded that Ni dopant level has no direct influence on the colloidal stability. The stability of particle size in colloidal solution is mainly related to the surface capping condition.

Zeta potential values of all the Ni-doped ZnO colloids were measured within 30 days, and the average value of the same type of capping agent was calculated. Table 4.4 shows the average zeta potential values in terms of the type of capping agents. The Z60, Z61 and APTES have higher absolute zeta potential values, indicating more stable colloids, and TiO₂ is lower. Uncapped ZnO is slightly more stable than the uncapped Cu-doped particles. All capped particles have higher absolute zeta potentials than the uncapped Ni-doped ZnO, thus showing the importance of capping. These results are in line with the stabilities concluded from Figure 4.5.

Table 4.4 Average Zeta potential values of the 5%-15%Ni-doped ZnO particles with different capping agents.

Surface capping	Zeta potential (mV)
Uncapped	-11.38 ± 0.5
-Z60	23.32 ± 0.9
-Z61	22.47 ± 0.9
-TiO ₂	-16.87 ± 0.7
-APTES	28.15 ± 1.0

Co-doped ZnO

Similar studies on particle size and size growing trend were conducted for 5%, 10%, 15% and 20%Co-doped ZnO colloids. Figure 4.6 shows the size growing graphs of 5%, 10%, 15% and 20%Co doped ZnO capped by Z60, Z61, Am and TiO₂ respectively. The graphs show that the APTES capped particles are stable for 30 days, and grow quickly after 30 days; TiO₂ capped particles are below 6 nm for 45 days for 5%Co and 20%Co, and are only stable for 30 days for 10%Co and 15%Co. This indicates a slightly better stability

(a)

(b)

(c)

(d)

Figure 4.6 Plots of particle size growing trends of 5%Co (a), 10%Co (b), 15%Co (c) and 20%Co (d) doped ZnO particles in the colloidal solutions. The standard deviations of the size data are below 20%.

than Cu- and Ni-doped ZnO particles. Z61 and Z60 capped particles show stability for 30 days for the 5%, 10%, and 15%Co doped ZnO. 20%Co doped ZnO particles are less stable, therefore, is not preferred. As such, the doping level has been controlled at 15% and below in our next experiments.

Zeta potential values of all the Co-doped ZnO colloids were measured within 30 days, and the average value of the same type of capping agent was calculated. Table 4.5 shows the average zeta potential values in terms of the type of capping agents. The Z60, Z61 and APTES have higher absolute zeta potential values, indicating more stable colloids, and TiO₂ is lower. Uncapped ZnO has the same value as the Ni-doped particles, is also slightly more stable than the uncapped Cu-doped particles. All capped particles have higher absolute zeta potentials than the uncapped Ni-doped ZnO, thus showing the importance of capping. These results are in line with the stabilities concluded from Figure 4.6.

Table 4.5 Average Zeta potential values of the 5%-20%Co-doped ZnO particles with different capping agents.

Surface capping	Zeta potential (mV)
Uncapped	-11.38 ± 0.5
-Z60	21.45 ± 0.8
-Z61	20.04 ± 0.8
-TiO ₂	-16.44 ± 0.7
-APTES	28.20 ± 1.0

From the above size and zeta potential results, we can conclude that the best capping agent is Z60, which controls the size below 2-3 nm for at least 30 days, and in most cases, for more than 45 days. Z61 is the next preferred capping agent as it controls the size below 3 nm for 30 days in most cases (with only one exception which lasted for 15 days), and showed particle growth after 30 days in only some cases. APTES can control the

particle size at below 3 nm for 30 days only, for almost all the cases, after which they grow very quickly. TiO_2 is able to control the size below 6 nm for 30 days, and sometimes, for 45 days or more. Since we were interested in its optical properties, and bio-imaging applications, we continued with this capping agent for our further experiments. Since APTES has the same amino- side group as the Z60 and Z61, we did not continue with this capping agent for optical and bio-imaging studies.

4.1.3 Effect of capping agents on particle shape and structure

Particle shape and structures were first observed under FESEM and further analyzed by HRTEM. For the higher monomer concentration (0.3mol/L) experiments, within one week after the synthesis, particles were deposited on aluminium disc for FESEM analysis. For the lower concentration (0.15mol/L) experiments, we used AAO templates as the sample holder. Figure 4.7 shows the FESEM images of differently capped ZnO particles synthesized by higher monomer concentration process. An image of uncapped ZnO sample is also displayed as a control.

(a) Uncapped ZnO, 1 day after synthesis

(b) Uncapped ZnO, 1 week after synthesis

Figure 4.7 FESEM images of differently capped ZnO particles.

These images show particle shape and structures. (a) and (b): uncapped ZnO particles prepared 1 day, and 1 week after synthesis, (c): Am capped ZnO, (d): SiO₂ capped ZnO, (e): TiO₂ capped ZnO, (f): Ms capped ZnO, (g): Mp capped ZnO, (h): PVP capped ZnO particles.

These images show that uncapped ZnO particles are in different sizes ranging from a few nm to a few hundred nm. The particle shapes are irregular. The Am capped particles have uniform size of about 20 nm in close to spherical shape with a regular distribution pattern

on the metal substrate, thus indicating a well controlled shape and size. SiO_2 coated ZnO particles show regular shape and size (about 20nm), but unlike Am capped, they are slightly coagulated. This could be caused by the sample preparation process (a big droplet of colloidal solution was applied on a spot of the flat surface) and also possible condensation reaction (-OH groups) between particles. Similarly, the TiO_2 capped particles are about 50nm and also slightly coagulated. MS capped particles are about 70nm. The shapes are close to spherical indicating good capping function, but the size is relatively large. The Mp added colloids show a fast precipitation of white sediments, and the image displays uncontrolled particle shape and size. For the PVP capped ZnO, circular particles in 50nm consisting several smaller particles (about 10nm) could be seen. This means that PVP, as a large molecule, could not cap the surface of small particles; instead, it formed clusters with a few small particles. As such PVP is not an effective capping agent for the small individual ZnO particles. From these shape and structure analysis, the preferred capping agent is Am, while SiO_2 and TiO_2 may be selected for study on optical and other properties.

Further FESEM analyses were performed on the Cu, Ni, and Co doped ZnO particles. AAO templates were used as the sample holders. Both the small particles from Z60 and Z61 capped colloids and the grown up particles from uncapped Cu, Ni, or Co doped ZnO particles were observed. Figure 4.8 shows the FESEM images of the capped particles. The pure ZnO (undoped) in Fig.4.8(a) displays small round particles (20-30 nm), which are reasonably separated compared to the uncapped ZnO in Figure 4.7 (a) and (b). In Figure 4.8(b), Cu-doped ZnO particles capped with Z60 are shown. The particles are small in size (10-20 nm) with round shapes, and with a few larger particles (up to 50 nm). This could be due to insufficient capping agent, or aggregation during the drying of overlapping particles.

(a) Pure ZnO capped with Z60

(b) 5%Cu-ZnO capped with Z60

(c) 5%Ni-ZnO capped with Z60

(d) 10%Co-ZnO capped with Z60

Figure 4.8 FESEM images of ZnO particles doped with Cu, Ni and Co respectively deposited on AAO templates.

- (a) pure ZnO capped with Z60, (b) 5%Cu-ZnO capped with Z60,
(c) 5%Ni-ZnO capped with Z60, (d) 10%Co-ZnO capped with Z60.

The relative larger particle size on SEM images compared to Zetasizer is due to the fact that Zetasizer measures the particle size in the stable colloidal solution, where the particles repulse each other by the surface charges thereby remaining as single particles. During the deposition of the colloids onto sample holder for SEM analysis, the repulsion and the stability of the colloidal solution were disturbed, particles could have agglomerated and overlapped upon drying. Another factor is that the resolution of FESEM has reached its limit at 100,000 times, which is not enough to show 2 nm features. Therefore, TEM analyses were conducted.

Figure 4.8(c) shows the 5%Ni doped ZnO particles. The shapes of particles are spherical and sizes are very small and uniform (about 10nm). This indicates the Z60 is an effective capping agent for Ni-doped ZnO. Figure 4.8(d) shows the 10%Co-doped ZnO particles. The shape is not as regular as the Ni-doped ZnO, and the size is slightly larger (about 40-50nm). This means that 10%Co doped ZnO tends to grow faster with certain preferred facets. This will be further analyzed and verified by TEM and XRD.

In order to study the influence of dopant on the growing behaviour of ZnO crystal, we also took the FESEM images for grown-up particles of differently doped ZnO particles. Figure 4.9 shows the FESEM images of Ni, Cu, and Co doped ZnO particles without capping, which were taken after 2 months of synthesis. 5%Ni doped ZnO showed flower-like particle structure as illustrated in Fig. 4.9(a) and (b). The crystals tend to grow into layered structures surrounding the nuclei centres, resulting in perfect circular. The side view of the layered structures can be seen clearly on some of the particles in (a). The close-up view in (b) shows the top view and central structure of the flower-like particles. This is a novel discovery as no previous publication on this has been found.

(a) 5%Ni-doped ZnO without capping agent added, 2 months after synthesis

(b) 5%Ni-doped ZnO without capping agent added, 2 months after synthesis

(c) 5%Cu doped ZnO particles without capping agent, 2 months after synthesis, enlarged image

(d) 5%Co doped ZnO without capping agent, 2 months after synthesis

Figure 4.9 FESEM images of ZnO particles doped with Ni, Cu and Co respectively without capping agent added. Particles were deposited on AAO templates. (a) and (b): 5%Ni-doped ZnO, (c): 15%Cu doped ZnO, (d): 5%Co doped ZnO

The Cu doped ZnO in Figure 4.9(c) shows that particles are close to circular shape with smooth surfaces, but no other special structure is observed. This indicates that the Cu dopant does not have a strong preference towards a specific plane of the crystal. Although 5%Co doped ZnO in Fig. 4.9(d) displays some fine structures inside the circular particles, no clear layers can be seen.

The possible mechanism of formation of the flower-like particles could be similar to that of flake-like film formed from the layered hydroxide metal acetates (LHMAs) reported by Fujihara *et al.* [164]. The precursor $\text{Ni}(\text{Ac})_2 \cdot 4\text{H}_2\text{O}$ could form LHMA in the form of $\text{Ni}(\text{OH})_{2-x}(\text{Ac})_x \cdot n\text{H}_2\text{O}$ ($0 < x < 2$, $1 < n < 4$) when dissolved in methanol and refluxed. This Ni based LHMA and the Zn based LHMA undergo chemical reactions such as solvation, complex formation, hydrolysis, polymerization and crystallization. Then solid particles are formed through nucleation and crystal growth. The secondary nucleation is favoured more on the LHMA particle surface along the layer edges and growth proceeds in the direction of the bulk solution due to lower nuclei/particle interfacial energy. There is no growth hindrance until the growing edges come into contact with each other, or stopped by the capping material on the particle surface. Then the interlaced assembly of flower-like structure is formed. This layered structure has been confirmed by XRD analyses as shown in the later sections. Due to the strong capping force around the whole surface of the initial spherical particle, the growing of the layered structure is restricted within a circle forming circular structures layer by layer outwards. Co and Cu precursors may have less tendency in forming LHMA, and the particles did not grow as large as the Ni doped ZnO, therefore, the layered structures inside the particles were not obvious. Since these flower-like particles are large, have no benefit to the bio-imaging, we did not conduct further analysis on this structure. However, the analysis on these particles helped

us to enlarge and observe the ZnO structures by different dopants. This also confirmed the capping capability of the selected capping agents. This special structure may be useful for making porous semiconductor films having high specific surface areas for specific electrochemical applications, such as chemical sensors, catalysts, photonic crystals, and dye-sensitized solar cells [164].

Since the sizes of well capped particles are below 10 nm, only the resolution of HRTEM is high enough to observe the detailed crystal structures within a single particle. Figure 4.10 shows the TEM images of the APTES capped pure ZnO. Some individual particle in size of 6 to 10 nm can be seen. The shape is close to spherical; and the crystalline structure is seen clearly in (b). No capping layer can be seen clearly as the capping layer should only be a monolayer, and the molecular size of APTES should be about 0.5 nm (taken reference of the SiO₂ lattice unit cell parameter).

Figure 4.11 shows the TEM images of 10%Ni doped ZnO capped with Z60. A few circular crystals can be seen in (a), and their sizes are between 4 to 6 nm. In Figure 4.10(b), we can observe a uniform capping layer (as pointed by the arrows) on a few of the larger sized particles. The particles are about 20 nm and the capping layer is about 5 nm thick. This thick capping layer could be due to the excessive capping agent added or because the stirring was not well done, so that the capping materials were not uniformly distributed to all the particle surfaces. This capping layer is a proof of the affinity and absorption of Z60 capping agent to ZnO surfaces.

Figure 4.10 TEM images of APTES capped pure ZnO, showing crystal size about 6 nm.

Figure 4.11 TEM images of 10%Ni doped ZnO capped with Z60. These images show crystal size between 4 to 6 nm, and uniform capping layer on larger particles. Scale bars: (a) = 5nm, (b) = 20nm.

Figure 4.12 shows the TEM images of 5%Co (a) and 5%Cu (b) doped ZnO particles capped with Z60. In both images, similar crystals in size between 3 to 5 nm can be seen. This is a proof of the effectiveness of the capping agent.

From the above FESEM and TEM analyses, we have confirmed that capping agents can effectively control particle size and shape. The nanocrystal colloids are stable for at least 30 to 45 days. In the next section, we will analyse the crystal lattice structures by XRD and further confirm the structure by HRTEM in combination with the GEMS simulation software.

Figure 4.12 TEM images of 5%Co (a) and 5%Cu (b) doped ZnO particles capped with Z60. Scale bars: 5nm

4.1.4 Analyses on crystal structures

Figure 4.13 shows the XRD spectra of two pure ZnO powders prepared on day 1 and day 7 after synthesis, and a TiO₂ capped ZnO powder. Both (a) and (b) possess the characteristic peaks of ZnO Wurtzite P6₃mc hexagonal structure as given by the JCPDS data base No.36-1451 (data sheet is attached in Appendix VII), with cell parameters of $a = 3.249 \text{ \AA}$ and $c = 5.206 \text{ \AA}$. The low and wide peaks of (a) indicate a smaller crystal size, while the high and narrow peaks of (b) indicate a larger crystal size. Since no capping agent was added, the powders collected after 7 days of synthesis (b) had grown larger compared to (a) the powder collected on the next day after synthesis.

Rietveld simulation (software TOPAS) based on the Scherrer equation [139] was used to simulate and refine the lattice parameters of the pure ZnO powder (in Fig. 4.13(a)).

Figure 4.14 shows the simulation results, where the ZnO spectrum (blue line) was 100% matched with Zincite (Wurtzite P63mc) (red line), with lattice parameters $a = 3.2523 \text{ \AA}$, $c = 5.20837 \text{ \AA}$, and crystal size 15.38 nm. The lattice parameters of our powder are larger than the database values. The larger lattice parameters are expected due to the lattice relaxation in our single crystal nanoparticles. In the following XRD analyses for doped ZnO, we will use these measured values as the reference since the same synthesis process has been used.

Figure 4.13 XRD spectra of uncapped ZnO powders (a and b) and TiO₂ capped ZnO (c). Powders were collected 1 day (a) or 7 days (b and c) after the reflux reaction was stopped. The peaks marked with “•” are identified as the major peaks of TiO₂ orthorhombic (Brookite) structure, and the peaks marked with “▽” are identified as the major peaks of TiO₂ tetragonal (Rutile) structure.

As for the TiO₂ capped ZnO shown in Fig.4.13(c), several peaks have been identified as a combination of TiO₂ tetragonal (Rutile) and orthorhombic (Brookite) structures. This

indicates that TiO_2 had already condensed on ZnO particles and had not been washed away during centrifugation process. ZnO particles capped by the other capping agents (Z60 and Z61), were tested by XRD and found to be no different from pure ZnO spectra. This may be due to the too small amount of capping agent used, as the XRD is not able to detect crystal content lower than 5%wt. Therefore, in the powder collections for doped ZnO samples, we did not add any capping agent. Only the lattice structure changes after different dopants have been added are important to us.

Figure 4.14 Rietveld simulation of XRD spectrum of uncapped ZnO powders collected one day after the synthesis.

Cu-doped ZnO powder

Figure 4.15 displays the XRD spectra of 5%, 10% and 15%Cu doped ZnO powders compared to pure ZnO (precipitated by LiOH). Peaks at $2\theta = 31.7^\circ, 34.36^\circ, 36.2^\circ, 47.54^\circ,$

56.6°, 62.76° and 67.82°, seen for the pure ZnO curve, correspond to the (100), (002), (101), (102), (110), (103) and (112) directions of ZnO hexagonal structure. All the Cu-doped ZnO powders contain the complete characteristic peaks of pure ZnO Wurtzite P63mc hexagonal structure. In addition, there are three more pronounced peaks at $2\theta = 33^\circ$, 58.96° and 69.22°, and the peaks become sharper for higher dopant molar percentage.

Figure 4.15 XRD spectra of 5%, 10% and 15%Cu doped ZnO powders precipitated by LiOH, comparing to the corresponding pure ZnO. Peaks labelled with “▽” are assigned to layered ZnO (JCPDS No.21-1486).

After matching with JCPDS database, it was found that these excess peaks could be assigned to a layered ZnO structure (JCPDS No.21-1486 attached in Appendix VIII), which has the three highest peaks at $2\theta = 33^\circ$, 58.76° and 62.68°. Unfortunately, this data card does not contain a defined lattice parameter; therefore, no simulation could be performed on these XRD spectra. Only qualitative analyses on the influence of dopant on the lattice structure are made as shown below.

On closer examination of the peaks, it is noted that there is a slight right shift of the peaks with increasing dopant molar percentage. Table 4.6 shows the 2θ positions of the two maximum peaks for each curve in Figure 4.15. Scherrer's equation describes the correlation between θ and the crystal size (D). Scherrer's equation is described as:

$$D = 0.9\lambda / (\beta \cos\theta) \tag{25}$$

where D is the average crystal size, λ is the wavelength of the X-rays, β is the observed full width at half maximum (FWHM) and θ is the angle at FWHM. This equation indicates an increasing trend for crystal size with increasing 2θ .

Table 4.6 2θ values corresponding to the peaks in Figure 4.15

Solution	2θ values	
Pure ZnO	32.98	58.96
with 5% Cu	33.00	58.96
with 10% Cu	33.02	59.06
with 15% Cu	33.08	59.08

As the peak shifts to the right, θ increases, and $\cos\theta$ decreases. Since the major peaks also become sharper at higher dopant levels, indicating a smaller β , from Eq. (25), crystal size D increases. This increased D value means the lattice parameter in the preferential direction also increases, while the other directions remain unchanged; as a result, the lattice becomes stretched in one plane, leading to the layered structure. With higher dopant levels, peaks at 33° and 59° become sharper and are more right shifted, indicating increased d-spacing of the lattice plane resulting in larger crystal size, and more dominated layered lattice structure.

Comparing Fig.4.15 to Fig.4.13, it is noticed that all the peaks in pure ZnO Wurtzite structure have become relatively lower. While the peaks at 33° , 58.76° and 62.68° , which belong to the layered ZnO structure, became the major peaks. This indicates that the lattice structure has changed from a hexagonal structure dominated to a layered structure dominated due to the doping of ZnO by the dopant ions Cu^{2+} . This has proved that the dopant has indeed successfully doped into the ZnO lattice.

Another sample was synthesized by adding only 2%Cu into ZnO. The powder was collected by the same method and tested on its XRD spectrum. Since the lattice stretching is much less, the spectrum matched with ZnO datasheet, and Rietveld simulation was performed. Figure 4.16 shows the simulation results. The blue line stands for the XRD spectrum for 2%Cu-ZnO, the red line stands for the reference pure ZnO (P63mc), and the black line stands for the difference between the former two. The XRD pattern fits well with the reference pure ZnO plus 6 additional low intensity peaks, which are assigned to the layered ZnO (JCPDS No.21-1486). The resultant lattice parameters obtained by the simulation are: $a = 3.2546(3) \text{ \AA}$ and $c = 5.2133(6) \text{ \AA}$ (the number in brackets is the standard deviation in the last digit). Compared with the pure ZnO obtained earlier, the 2%Cu doped ZnO has a slightly larger a ($0.0023 \text{ \AA}$) and larger c ($0.0049 \text{ \AA}$). Since the ionic radius of Cu^{2+} ($r = 0.57$) is smaller than that of Zn^{2+} ($r = 0.60$), the 2%Cu-ZnO should have smaller lattice parameters if the Zn^{2+} site is substituted by Cu^{2+} . But we obtained larger lattice parameters, as well as peak position (2θ) right shifted. Based on these analyses, we can conclude that the doping mechanism by Cu is not a simple substitution of Zn^{2+} site by Cu^{2+} ; it is also dominated by preferential substitution along a plane leading to crystal growth into the layered structure. To further prove this mechanism, we continued to analyze the other doped ZnO powders.

Figure 4.16 XRD spectra of 2%Cu doped ZnO powder and the Rietveld simulation result.

Co-doped ZnO powder

Figure 4.17 depicts the XRD spectra of 5%, 10%, 15% and 20%Co doped ZnO in comparison with the pure ZnO. Additional peaks at $2\theta = 26.3^\circ$, 28.68° , 30.86° and 40.34° can be identified and assigned to the layered ZnO crystal. In addition, a broad peak between $2\theta\ 21^\circ - 24^\circ$ is also observed, which was found due to the glass holder for the XRD measurement [108]. The peak positions are found slightly right shifted as indicated in Table 4.7. It is also noticed that as the dopant concentration increases, the major peaks become sharper. Both phenomena are similar to Cu doped ZnO. As discussed by the Scherrer's Equation, the sharper peaks mean smaller FWHM or β , and the peak right shift means larger θ . Both phenomena lead to increased D spacing in the preferential plane, and promote the growth of layered crystal structure.

Figure 4.17 XRD spectra of 5%, 10%, 15% and 20%Co doped ZnO powders comparing to the pure ZnO. Peaks labelled with “▽” are assigned to layered ZnO (JCPDS No.21-1486).

Table 4.7 2θ values corresponding to the peaks in Figure 4.17

Solution	2θ values	
Pure ZnO	32.98	58.96
with 5% Co	33.00	59.12
with 10% Co	33.10	59.24
with 15% Co	33.20	59.32
with 20% Co	33.24	59.36

In order to quantify the result on the influence of Co dopant on the lattice parameters, we synthesized a 2%Co-doped ZnO powder, and analyzed by Rietveld simulation. Figure 4.18 shows the XRD spectrum (blue line) with the pure ZnO spectrum (red line). The

black curve shows the difference between the two spectra. As seen from the figure, 2%Co doped ZnO has much less preferential or additional peaks compared to the higher dopant ZnO. The spectrum is 100% matched with the pure ZnO datasheet (too small peaks are not considered by the model). The resulting lattice parameters from this simulation are: $a = 3.2514(3) \text{ \AA}$ and $c = 5.2096(5) \text{ \AA}$ (number in bracket is the standard deviation). Compared to the lattice parameters of 2%Cu doped ZnO: $a = 3.2546(3) \text{ \AA}$ and $c = 5.2133(6) \text{ \AA}$, the 2%Co doped ZnO has a smaller lattice size. The ionic radius of Co^{2+} ($r=0.58$) is smaller than that of Zn^{2+} ($r=0.60$), but slightly larger than Cu^{2+} ($r=0.57$), this means the lattice size change is not directly proportional to the dopant ionic radius. If the dopants only occupy the interstitial sites, the XRD spectra should not change so much. The dominant peaks should still be the major peaks of pure ZnO. Also from the photoluminescent spectra (Fig.4.27, 4.29 and 4.31), there is no second peak of green

Figure 4.18 XRD spectra of 2%Co doped ZnO powder and the Rietveld simulation result.

emission (at 550nm), which is originated from the lattice defects, such as interstitial defects. Therefore, we can conclude that the doping mechanism is the substitution of Zn^{2+} sites by the dopants.

Ni-doped ZnO powder

Figure 4.19 shows the XRD patterns of 5%, 10% and 15%Ni doped ZnO in comparison with the pure ZnO. Similar to Figure 4.15, which shows the Cu doped ZnO, this Ni doped ZnO also appears to have a slight right shift as dopant concentration increases, as well as sharper peaks at 33° and 59° . The shifts of the peak positions are listed in Table 4.8. Compared to the peak shifts by Co and Cu dopants, the shifts of the peaks by Ni dopant are slightly larger. Similar to the Co and Cu doped ZnO, the lattice structure has changed from a hexagonal dominated to a layered structure dominated, The ionic radius of Ni^{2+} ($r=0.55$) is the smallest compared to Cu^{2+} , Co^{2+} , and has the most difference from Zn^{2+} ($r=0.60$). This may be the reason that Ni doped ZnO has the most deficient structure from the original ZnO crystal structure. Therefore, we can almost confirm that the doping mechanism by Cu^{2+} , Co^{2+} and Ni^{2+} are the substitution of Zn^{2+} by the dopants, and the unit cell size is slightly larger than pure ZnO because of the layered structure.

The stretched lattice (layered structure) was also revealed in the Raman spectrum (refer to Fig.4.43), where the main peak is shifted from 437 cm^{-1} to 429 cm^{-1} . Two additional peaks at 813 cm^{-1} and 891 cm^{-1} were also detected, which are due to the Ni^{2+} occupation of Zn^{2+} sites, which introduced lattice defects as the ionic radius of Ni^{2+} (0.55) is smaller than that of Zn^{2+} (0.60).

Figure 4.19 XRD spectra of 5%, 10% and 15%Ni doped ZnO powders in comparison to pure ZnO powder.
Peaks labelled with “▽” are assigned to layered ZnO (JCPDS No.21-1486).

Table 4.8 2θ values corresponding to the peaks in Figure 4.19

Solution	2θ values	
Pure ZnO	32.98	58.96
with 5% Ni	33.10	59.18
with 10% Ni	33.20	59.30
with 15% Ni	33.30	59.32

TEM verification of the ZnO and doped ZnO crystal structures

In order to prove the above doping mechanism, HRTEM analyses were conducted to observe the crystal structures of pure ZnO and doped ZnO. A TEM sample was prepared using the pure ZnO nanocrystals capped by Z60, a few days after the synthesis. Figure 4.20 shows the image. Crystals in size of 2-3 nm are seen. By measuring the lattice parameters on the TEM image, using the Fast Fourier Transform Technique (FFT), the

diffraction pattern of crystal structures were obtained. Using GEMS software, the reciprocal lattice was transferred back to real lattice, which was then compared to the ICSD standard database. From the crystal diffractions patterns shown in the graph, it is confirmed that the lattice parameter matched with ZnO Wurtzite (P63mc) hexagonal structure, thus confirming the crystals are pure ZnO.

Figure 4.20 TEM image of pure ZnO capped with Z60, sample was prepared a few days after the synthesis.
The lattice parameters are analyzed by FFT and GEMS software, and matched with ZnO Wurtzite (P63mc) hexagonal structure.

As the layered crystal structure has been defined as the dominant mechanism of the doping of ZnO by Cu, Co and Ni, we intentionally allowed the growth of doped particles and analysed them by TEM. We chose a 10%Co doped ZnO colloid and a 10%Cu doped ZnO colloid, which were capped by Z60 and had been aged for 8 months. Carbon grid

was used to scoop a few times in the colloid, and then dried in oven at 60°C for 1 hr.

Figure 4.21 shows the TEM image of the 10%Co-ZnO. Within the large particle, layered structures are seen clearly.

Figure 4.21 TEM image of 10%Co doped ZnO capped with Z60.
Sample was prepared from a 8 month aged colloidal solution.

An interesting phenomenon is that although the crystals tend to grow in one direction, the whole particle still remained spherical, and the growing directions appeared bent by some force, which we believe is the capping force from the Z60 capping agent. Since this lattice structure is not well defined in the ICSDS database, we could not match it with any established crystal structure. But the long and layered lattice structure proved the conclusions drawn from the XRD analyses.

Another sample was prepared from an aged solution of 10%Cu doped ZnO capped with Z60. The TEM image is shown in Figure 4.22. In the thick area, layered structures within a large particle, which is similar to Fig. 4.21, can be seen. In addition, at the boundary of the larger particle, nanotube like structure is clearly seen. The lattice fringes of the wall of the nanotube are similar to the layered structure in Fig.4.21. This means that if the surface is not capped properly, the lattice structure would grow outward at the local surface resulting in nanotube like structure. If the surface is capped strongly, the crystal would grow within a confined spherical shape. These findings further confirmed the doping mechanism of the substitution of Zn^{2+} by the dopants, and induce a layered lattice structure. Depending on the capping force, the layered structure may grow straight in the preferred direction, or grow into a curved structure and is confined within a spherical shape. This also confirms the importance of using capping agent to control particle size and shape.

For Ni doped ZnO, the layered structures are already very obvious in the FESEM image (Figure 4.8 (a) and (b)), therefore, no TEM analysis was necessary.

Figure 4.22 TEM image of 10%Co doped ZnO capped with Z60.
Sample was prepared from a 8-month aged colloidal solution.

4.2 Optical properties of the synthesized and doped ZnO nanocrystals

One of the major objectives of this research is to achieve high quality optical properties, which will ensure the high quality of bio-imaging. In this section, the photoluminescence spectra, optical absorption, bandgap modifications by dopants and surface capping, and quantum yields results will be reported.

4.2.1 Photoluminescence properties

As mentioned in Chapter one ZnO is a wide bandgap ($\sim 3.37\text{eV}$) semiconductor with large exciton binding energy (60 meV), and is a good candidate material for various

applications, such as the short wavelength light-emitting diode and UV lasing diode, solar cell, UV-absorber, gas sensor etc. However, it has been shown that operating performances of ZnO are greatly influenced by its particle morphology and size as well as chemical composition and purity. Therefore, analyses on the correlation between synthesis parameters (including doping and surface modification) and optical properties will enable us to control the quality of the ZnO nanocrystal, and further fulfil the requirements in the final application.

Figure 4.23 shows the PL spectra of pure ZnO capped by different capping agents. These samples were from the high monomer concentration synthesis process. The PL spectra were measured on dried powder. Two peaks can be seen clearly for all the samples. One peak is at the wavelength of 377nm, thus corresponding to the intrinsic UV emission of pure ZnO; another peak is at visible range (490 nm), which is the green emission caused by surface defects as detected by many other researchers. The lower intensity of green emission compared to the UV emission indicates the good quality of our nanocrystals because of their fewer surface defects. However, all the capped samples showed lower intensity than the uncapped ZnO, meaning that the capping layer has negative effects on the optical property of the ZnO. Since the drying of powder may generate defects and our final application is to use the colloids directly, we measured the PL of ZnO and TiO₂ capped ZnO on their colloidal solutions of two different concentrations (0.15 mol/L and 0.075 mol/L) as shown in Figure 4.24. It is seen that both low and high concentrations of TiO₂ capped ZnO have a higher intensity than the uncapped ZnO, which means TiO₂ is a better capping agent than Am, Pv and Ms as compared to Figure 4.23. More importantly, these spectra show only one peak at the UV wavelength (the small shoulder at the main peak could be due to the emission from the surface capping by TiO₂ or the water molecules on the surface), the visible emission is completely eliminated, indicating

Figure 4.23 PL spectra of pure ZnO capped by different capping agents. (synthesized by high monomer concentration process, measured on dried powder). (Raw = uncapped, Am=APTES capped, Pv= PVP capped, Ms=Ms capped ZnO). Excitation at 325 nm.

Figure 4.24 PL spectra of pure ZnO and TiO₂ capped ZnO in different concentration measured on colloidal solutions, excited at 325nm.

perfect nanocrystals without any surface defect. This also means that our synthesis parameters and the capping agents have been optimized, and the ultimate UV emission property of ZnO has been obtained.

In the following paragraphs, we report the complete PL spectra of 5%Cu, 10%Ni and 5%Co doped ZnO with Z60, Z61 and TiO₂ capping agents respectively, and in their original methanol medium as well as after their exchange with the water medium. All the colloidal solutions were exchanged with the water solution before bio-imaging. The selected doping concentrations and capping agents were the most promising ones based on the results of size stabilities, crystal structures and optical properties.

Pure ZnO

Figure 4.25 shows the PL spectra of undoped ZnO capped with Z60, Z61 and TiO₂ in methanol and water solutions respectively, and excited at 325 nm. It shows that the Z61 and Z60 capped ZnO nanocrystals in methanol have higher emission intensities than their corresponding water solutions, while the TiO₂ capped ZnO colloid has slightly lower intensities than its water solution. In terms of emission wavelengths, the Z61 and Z60 capped ZnO colloids emit at about 400 nm, which is a redshift of about 20nm from the intrinsic ZnO UV emission (377nm). But there is almost no shift of ZnO PL emission peak after being capped by TiO₂, as also seen in Figure 4.23. In terms of water effects, we notice that for Z60 and Z61 capped ZnO, the PL intensity of water solution dropped a little, while for TiO₂ capped ZnO, the PL of the water solution increased a bit and right shifted a little. Since all the intensity values are high enough for bio-imaging, we are more interested on the right shift of PL peak, as this could lead to PL emission in a visible colour in bio-imaging, which is preferred over UV emission. Based on these optical results, we selected ZnO-Z60 and ZnO-Z61 for further studies of their bio-imaging

properties.

Figure 4.25 PL spectra of undoped ZnO capped with Z60, Z61 and TiO₂ in methanol and water solutions respectively, excited at 325nm.

It is noticed that all the emission peaks in Fig.4.25 are very wide. This could be because that each wide peak consists of multiple sharp peaks. In a separate measurement, it was found that the Z60 and Z61 capping materials also emitted photoluminescence in visible wavelength. This indicates that the wide PL peaks could be the combined emission from the core ZnO and the capping layer. Although pure water did not show PL emission, the -OH sites on ZnO surface also induce emission at visible range as observed by Nishikawa *et al.* [165] and Zhao *et al.* [166].

Since our bio-imaging tests on plant cells are detected by the Leica confocal microscope which is equipped with an excitation source at 488nm, we also measured the PL spectra

excited at 488nm as shown in Figure 4.26.

(a)

(b)

(c)

Figure 4.26 PL spectra of undoped ZnO capped with Z60, Z61 and TiO₂ in methanol and water solutions respectively, excited at 488 nm.

Compared to Figure 4.25, all the emission intensities of these spectra are lower, because the excitation energy is lower (2.54 eV) than UV excitation (3.81 eV). The emission peaks are all shifted to longer wavelengths, because lower excitation leads to lower

emission (lower eV = longer wavelength according to Planck's equation). The emission peaks of Z60 and Z61 capped ZnO in MeOH are at 540 nm, and the others are at 570 nm. In this graph, the Z60 capped ZnO has a higher intensity than Z61 capped ZnO, and TiO₂ capped ZnO has a much lower intensity.

Cu doped ZnO

Figure 4.27 shows the PL spectra of 5%Cu doped ZnO capped with Z60, Z61 and TiO₂ in methanol and water solutions respectively. The excitation was at 325 nm. Since the same line colour is used for the same capping agent as in Figure 4.25, we could directly observe the relative intensities and peak positions. Similar to Figure 4.23, the Z60 and Z61 capped 5%Cu-ZnO methanol colloids have the highest PL intensity, followed by their water solutions, while TiO₂ capped ZnO colloids have lower PL intensities. In terms of PL peak positions, Z60, Z61 and TiO₂ capped ZnO water solutions emit fluorescence at about 400 nm, while others emit fluorescence at about 385 nm. In comparing the Y-axis scales of Figure 4.23 and 4.25, we notice that 5%Cu doped ZnO have lower PL intensity than the undoped ZnO. This means that the 5%Cu doping has a negative effect on the PL property of ZnO. Nevertheless, this lower intensity may be still high enough for bio-imaging. Therefore, based on these results, we selected 5%Cu-ZnO-Z60 and 5%Cu-ZnO-Z61 water solutions as the candidates for further bio-imaging studies.

Similarly to pure ZnO, we also measured the PL spectra under excitation at 488 nm. Figure 4.28 shows the spectra. It is seen from this graph that Z60 capped 5%Cu-ZnO in MeOH has much higher intensity than the others, and its emission peak is at 520 nm. All others emit at 570-580 nm with lower intensities. Among the colloids of water solutions, the Z60 and TiO₂ capped ZnO colloids showed higher intensities; therefore, we shall test the bio-imaging of these colloids.

(a)

(b)

Figure 4.27 PL spectra of 5%Cu doped ZnO capped with Z60, Z61 and TiO₂ in methanol and water solutions respectively, excited at 325nm (a). (b) shows the close up of the lower intensity samples.

Ni doped ZnO

Figure 4.29 shows the PL spectra of 10%Ni doped ZnO capped with Z60, Z61 and TiO₂ in methanol and water solutions respectively, and excited at 325 nm. In this figure, we

Figure 4.28 PL spectra of 5%Cu doped ZnO capped with Z60, Z61 and TiO₂ in methanol and water solutions respectively, excited at 488 nm.

notice that the water solutions of Z60 and Z61 capped 10%Ni-ZnO colloids have higher PL intensities than their methanol solutions. In the meantime, the emission peaks are at 420 nm. These larger red shifts could be beneficial for visible emission in bio-imaging, and in addition, the Y-axis scale of this figure is higher than 5%Cu doped ZnO. Therefore, the 10%Ni doped ZnO with Z60 and Z61 capping agents are selected for bio-imaging. As seen in Figure 4.30, no bandgap shift by TiO₂ capped ZnO. This is similar to the undoped ZnO (Figure 4.25). PL spectra under excitation at 488 nm are shown in Figure 4.30. Similar to the spectra excited at 325 nm, the Z60 and Z61 capped 10%Ni-ZnO colloids have higher intensities. All emission peaks are broad, ranging between 530 to 570nm, indicating that they could lead to images with more colours.

Figure 4.29 PL spectra of 10%Ni doped ZnO capped with Z60, Z61 and TiO₂ in methanol and water solutions respectively, excited at 325nm.

Figure 4.30 PL spectra of 10%Ni doped ZnO capped with Z60, Z61 and TiO₂ in methanol and water solutions respectively, excited at 488 nm.

Co doped ZnO

The PL spectra of 5%Co doped ZnO capped by Z60, Z61 and TiO₂ in water and methanol solutions are shown in Figure 4.31. Similar to the Ni doped ZnO, the Z61 and Z60 capped 10%Ni doped ZnO in water solutions have higher emission intensities and the peaks are red shifted to 430 nm. The corresponding MeOH solutions emit fluorescence at 410 nm

Figure 4.31 PL spectra of 5%Co doped ZnO capped with Z60, Z61 and TiO₂ in methanol and water solutions respectively, excited at 325 nm (a). (b) and (c) are the close up of the lower intensity range.

(red shifted 30 nm). No emission peak shift is observed by the TiO_2 capped 10%Ni-ZnO, although the PL intensity is increased.

These results show that Z60 and Z61 capped 5%Co doped ZnO have more red-shifts than Cu and Ni doped ZnO, and are therefore more promising for bio-imaging application. An interesting phenomenon was observed on Co doped ZnO without capping agent. Due to the very stable particle size in the colloids of these nanoparticles, we could use them for optical measurement and bio-imaging without using any capping agent.

In order to compare the PL and bio-images of uncapped Co doped ZnO with the capped ones, Figure 4.32 shows the PL spectra of the 5%Co-ZnO without capping and with Z60, Z61 and TiO_2 capping agents. It is seen that uncapped 5%Co-ZnO emits at about 390 nm, Z61 and Z60 capped 5%Co-ZnO emit at 410 and 420 nm respectively.

Figure 4.32 PL spectra of 5%Co doped ZnO, uncapped and capped with Z60, Z61 and TiO_2 respectively in water solutions, excited at 325 nm.

The PL spectra under excitation at 488 nm are shown in Figure 4.33. The Z61 capped 5%Co-ZnO has much higher intensity than the Z60 capped, and its emission peak is at

510 nm, while the Z60 capped ZnO emits fluorescence at normal range of 570 nm. The TiO₂ capped ZnO has lower intensity and emits fluorescence at 585 nm (red range).

Discussion on the mechanisms of PL spectrum changes by doping and surface

capping:

The influences of surface capping and doping of ZnO on the PL properties should be discussed from the mechanisms of photoexcitation and emission of semiconductor materials. Upon photoexcitation of a semiconductor particle, an electron-hole pair is created. This pair can exist as a Wannier exciton and when it recombines, emits a photon with an energy close to the bandgap of the material. This is referred to as exciton emission. The electrons and holes can also be trapped somewhere in the particle. The energetic position of a shallowly trapped charge carrier is related to the band structure, which is a function of particle size (so called quantum confinement effect). The energetic position of a deeply trapped charge carrier is independent of particle size and is determined by the chemical nature of the trap and by the local structure surrounding the trapped charge carrier. Emission of trapped charge carrier can occur via a radiative process (called trap emission) or a non-radiative process (called non-radiative recombination). Exciton emission, trap emission and non-radiative recombination are the three competing processes that determine the final PL spectrum [167].

(a)

(b)

Figure 4.33 PL spectra of 5%Co doped ZnO capped with Z60, Z61 and TiO₂ in methanol and water solutions respectively, excited at 488 nm (a). (b) is the same graph with smaller Y-axis scale to show the peaks of lower intensities samples.

The surface capping of ZnO influenced the PL spectrum in terms of emission intensity and the emission wavelength. If the surface is well capped (e.g. by Z60 and Z61), the surface defects are reduced, and the capping layer plays a role in confining the PL emission (less loss to the surroundings) resulting in higher PL intensity. A lot of publications on ZnO discussed the higher green emission than the intrinsic UV emission due to the surface defects (oxygen or Zinc vacancies) [70-72]. This indicates the importance of surface capping to reduce the non-radiative recombination from the trapped holes at the surface. If the capping material also emits photons at a longer wavelength, it provides a shallowly trapped charge carrier, which changes the bandgap of ZnO, and PL emission peak is red-shifted. From Fig.4.25, we found that Z60 and Z61 capped ZnO had a redshift in bandgap, but TiO₂ capped ZnO did not shift at all. Also ZnO water solution showed higher intensity compared to ZnO methanol solution. These indicate that Z60, Z61 and water enhance the PL emission through reduced energy loss or non-radiative emission.

The influence of dopant on the PL spectrum is more related to the deep trapped emission. This depends on the chemical nature and the surrounding structure of the trapped charge carrier. Since the dopant does not emit photons at the same wavelength with the host material (ZnO), the presence of dopant in the lattice provides competitive pathways for recombination. Therefore, in general, dopants will reduce the radiative emission of the ZnO, therefore, PL intensity is reduced to certain extent. Meantime, since the dopants absorb energy and emit in a different wavelength, the bandgap is changed resulting in a shift in emission peak. These mechanisms have been demonstrated by the Co, Cu and Ni dopants presented in this research.

To conclude the photoluminescence results and prepare for the comparison of bio-imaging colour of the PL emission, we summarized all the PL intensities and emission peaks positions of all the water solutions in Table 4.9.

Table 4.9 Summary of PL intensities and emission peak positions of all colloids in water solutions.

Capping	Z60 capped		Z61 capped		TiO ₂ capped	
Excitation	325 nm		325 nm		325 nm	
	PL intensity (x1000 cps)	Emission peak (nm)	PL intensity (x1000 cps)	Emission peak (nm)	PL intensity (x1000 cps)	Emission peak (nm)
Pure ZnO	15000	410	18000	405	9000	385
5%Cu-ZnO	1200	400	3200	400	300	400
10%Ni-ZnO	10000	410	9600	420	1600	385
5%Co-ZnO	1000	425	5500	425	270	370
Excitation	488 nm		488 nm		488 nm	
	PL intensity (x1000 cps)	Emission peak (nm)	PL intensity (x1000 cps)	Emission peak (nm)	PL intensity (x1000 cps)	Emission peak (nm)
Pure ZnO	700	565	310	560	80	570
5%Cu-ZnO	22	570	13	580	26	585
10%Ni-ZnO	200	540	150	530	63	520
5%Co-ZnO	180	570	710	520	23	580

4.2.2 Optical absorption properties

The two purposes of this absorption study are (1) for measuring quantum yield, (2) for studying quantum confinement effects.

- (1) The absorption intensity of a nanocrystal is directly related to the PL emission energy, in most cases, the emission energy is a portion of the absorbed energy. An excited ZnO particle can return to the ground state via three different processes:
- (a) exciton emission (the UV emission), (b) trap emission (the visible emission), and (c) non-radiative recombination (energy lose, no emission). The latter can

occur at the surface or at the quenching centres in the particle. By measuring the absorbed energy and total emitted energy, we can calculate the quantum yield (QY) of the nanocrystal, and the QY is a direct indication of the quality of the nanocrystal.

- (2) The onset of the absorption bandedge of nanocrystals relative to the bandedge of bulk ZnO provides a direct indication of any quantum confinement effect. If nanoparticle size is in the quantum confinement range, the absorption bandedge should be blue shift. In this research, this blue shift is not important as we want to focus on the red shift in order to achieve a controlled visible emission. The commonly observed green emission due to trapped holes is caused by surface defects, and is therefore, very much dependent on the synthesis process, which is not controllable. Therefore, in our study, we will use the absorption curves to show possible quantum confinement effects to further confirm the particle size effect only.

The optical absorption spectra of the nanocrystals were measured by UV-Vis spectrophotometer, which was equipped with the excitation source ranging from 393 to 282 nm. Figure 4.34 shows the absorption spectra of some of the capped ZnO particles compared to the bulk ZnO. It is seen from this graph that the uncapped ZnO has a band-edge at 380nm, while all other capped colloids showed the band-edge at about 350nm. The wavelength of 380nm corresponds to the bulk band-edge of 3.3 eV for ZnO. The absorbance at wavelength less than 380nm indicates smaller particles. Therefore, it can be concluded that all the capping agents have effectively controlled the growth of particles. The uncapped ZnO grew quickly during storage, which can also be observed from the colour of the solution and the sediment formed at the bottom of the container.

Figure 4.34 Optical absorption spectra of ZnO colloids of uncapped (Raw), Mp, Pv, TEOS, Am and Ms-capped. Samples were tested after stored in ambient condition for 7 weeks.

Figure 4.35 shows the absorption curves of the Z60, Z61 and TiO_2 capped pure ZnO in methanol solutions. These samples were synthesized by the lower concentration (0.075 mol/L) process, therefore, had smaller particle sizes. The TiO_2 capped ZnO has a bandedge at 350 nm (3.542 eV), while Z60 and Z61 capped ZnO have bandedges at 320 nm (3.875 eV). This shows that TiO_2 capped particles are relatively larger than Z60 and Z61 capped ZnO, as shown in the size analyses results in Chapter 4.1. Compared to the absorption curves in Figure 4.34, the bandedges shifted to shorter wavelengths, indicating their smaller crystal sizes.

Figure 4.35 Optical absorption spectra of ZnO colloids in methanol with Z60, Z61 and TiO_2 capping agents. Samples were tested after stored in ambient condition for 7 weeks.

Figure 4.36 shows the absorption spectra of 5%Cu doped ZnO with Z60, Z61 and TiO_2 capping agents. The bandedges of TiO_2 capped ZnO is at 380 nm (3.263 eV), while Z60 and Z61 are at about 350 nm (3.542 eV), giving about 30 nm red shift compared to the undoped ZnO. This could be due to the slightly larger size of Cu doped particles, but they are still at the shorter wavelengths than the bulk ZnO shown in Figure 4.34. There are visible absorptions for Z60 and Z61 capped Cu-ZnO, which indicate bandgap modification by Cu doping and the surface capping. Since there is no visible absorption in undoped ZnO (Fig. 4.35), the visible absorption is more likely due to the Cu doping. The absorption peaks in the visible range are at 610 nm for Z60 capped and 633 nm for Z61 capped Cu-ZnO. The corresponding bandgaps are 2.033 eV and 1.959 eV respectively, which are calculated by Planck's equation (Eq. (1)). The bandgaps of the undoped ZnO

capped by Z60 and Z61 were both 350nm (3.542 eV) shown in Fig.4.35. Therefore, the Cu doping introduced an intermediate bandedge at 2.033 eV (Z60 capped) or 1.959 eV (Z61 capped). This is because that Cu^{2+} dopant has smaller ionic radius ($r=0.57$) than Zn^{2+} ($r=0.60$), when it substituted the Zn^{2+} , there was a defect in each substituted site inducing trap emission and absorption at lower energy level. The slight difference between Z60 and Z61 capped ZnO may be due to the different surface chemical status resulting in slightly different light absorptions.

Figure 4.36 Optical absorption spectra of 5%Cu doped ZnO colloids in methanol with Z60, Z61 and TiO_2 capping agents. Samples were tested after stored in ambient condition for 7 weeks.

Figure 4.37 shows the absorption spectra of 5%Ni doped ZnO capped by different capping agents. In this graph, the absorption peaks in both the UV range and visible range are seen, indicating well defined bandgaps of Ni doped ZnO nanocrystal. The well defined absorption peaks may be related to the well defined sphere shapes of the Ni

doped ZnO in comparison to the Cu and Co doped ZnO particles, which are not perfectly sphere (see Figure 4.8 and 4.9). The absorption peaks in UV range are 315 nm (Z60 capped), 324 nm (Z61 capped) and 349 nm (TiO₂ capped), which are the absorptions by the ZnO main crystals. The corresponding bandgap values are 3.936 eV, 3.827 eV and 3.553 eV respectively. These blue shifted bandgaps are due to the quantum confinement effect by the smaller crystal sizes. The absorption peaks in visible range are 532 nm (Z60 capped) and 580 nm (Z61 capped), corresponding to 2.331 eV and 2.138 eV. Similar to the discussion for Cu doped ZnO, these additional bandedges are due to the Ni doping, which introduced lattice defect at the substituted sites, inducing trap emission and absorption at lower energy levels. The slight difference in the visible bandedges between Z60 and Z61 capped ZnO may be due to the different surface chemical status resulting in slightly different light absorptions.

Figure 4.38 shows the absorption spectra of 5%Co doped ZnO capped by different capping agents. In this graph, the absorptions bandedges of Z60 and Z61 capped crystals are more red shifted (450 and 440 nm), while absorption of TiO₂ capped ZnO is at 350 nm. The corresponding bandgap energies are 2.775, 2.818 and 3.542 eV respectively. The visible absorptions of Z60 and Z61 capped ZnO are almost connected with the UV absorption, which is different from the Cu and Ni doped ZnO. This indicates less substitutional defect and more shifted absorption bandgap of the main crystals to a longer wavelength, which is beneficial for visible bio-imaging. The visible absorption peaks are at 520 and 530 nm of Co doped ZnO capped by Z60 and Z61 respectively, corresponding to 2.384 and 2.339 eV. Since 10% and 15% dopants in ZnO behaved similarly to the respective 5% dopant in the absorption, those absorption curves of higher dopants are not listed here.

Figure 4.37 Optical absorption spectra of 5%Ni doped ZnO colloids in methanol with Z60, Z61 and TiO₂ capping agents. Samples were tested after stored in ambient condition for 7 weeks.

Figure 4.38 Optical absorption spectra of 5%Co doped ZnO colloids in methanol with Z60, Z61 and TiO₂ capping agents. Samples were tested after stored in ambient condition for 7 weeks.

4.2.3 Quantum yield measurements

As mentioned in Chapter 3.3.6, quantum yield is defined as the number of emitted photons divided by the number of absorbed photons. The quantum yield (QY) is a measure of the optical quality of the nanocrystal. This is more important for bio-imaging as every single crystal is used as a marker independently. The loss of absorbed energy is mostly due to the surface defect which causes non-radiative emission. In addition, doping by a different cation may cause lattice defects resulting in reduced QY. Therefore, we studied the QYs of the pure ZnO, TiO₂ capped ZnO and Co doped ZnO to indicate the optical quality of our synthesised nanocrystals.

Fluorescein and Anthracene standard solutions were first cross-calibrated. The standard QY values reported in the original literature were 0.79 and 0.27 respectively. The calculated values for these two standard materials were 0.7854 and 0.2715 respectively, which have a deviation of less than 1% from the standard values. Then the QYs of the test samples were calculated using Fluorescein as the standard. The QYs of uncapped and TiO₂ capped ZnO in methanol were 0.9417 and 0.7963 respectively. The data used in these calculations are listed in Table 4.10, and the plot of integrated PL against absorption is shown in Figure 4.39.

Figure 4.39 Plot of integrated PL intensity against UV-Vis absorbance of the standard and test samples. The gradient of each sample is obtained from the trend line equation. All trend lines have good fittings of higher than 97%. Legend “Fluor” refers to Fluorescein, “Anth” refers to Anthracene, “ZnTi” means the TiO₂ capped ZnO.

Table 4.10 QY results of standard and pure ZnO, TiO₂ capped ZnO test samples and the data used for the calculations.

Compound / Solvent	Gradient	Refractive Index	Measured QY	Literature QY
Fluorescein / 0.01M NaOH	3.7758	1.340	0.7854	0.79
Anthracene / Ethanol	1.2576	1.361	0.2715	0.27
ZnO / Methanol	4.5826	1.328	0.9417	-
ZnO-TiO ₂ / Methanol	3.8752	1.328	0.7963	-

We noticed higher PL emission under UV excitation from TiO₂ capped ZnO than the pure ZnO (shown in Fig. 4.24). However, due to the higher absorbance of this QD, the total quantum yield is lower than the pure ZnO. This may be related to the capping mechanism, where the capping agent creates surface defects, which lower the quantum efficiency, but modify the bandgap to give emission at a longer wavelength. Both uncapped and TiO₂ capped ZnO QDs possess higher quantum yields than the Fluorescein standard material, and are therefore, useful in bio-imaging applications.

To further confirm the QYs of our ZnO samples, we performed the QY tests again using the Fluorescein and Anthracene as the standard samples and measured the QYs of 5%Co and 10%Co doped ZnO. The same four standard solutions were prepared. Four test solutions were made: 5% Co doped ZnO in methanol in concentrations of 1.5×10^{-1} mol/L and 7.5×10^{-2} mol/L; and 10%Co-ZnO in methanol in concentrations of 1.5×10^{-1} mol/L and 7.5×10^{-2} mol/L. Photoluminescence emission and absorbance spectra were measured for all the standard solutions and test solutions with excitation source at 325 nm. Figure 4.40 shows the PL emission spectra for 5%Co and 10%Co doped ZnO in different concentrations. It is seen that in higher concentration, 5%Co-ZnO and 10%Co-ZnO have quite the same PL intensity, but in lower concentration, 5%Co-ZnO shows higher PL intensity. Meanwhile, we noticed that higher concentration results in higher PL intensity. This implies that for higher PL emission, we could use higher concentration ZnO nanocrystals in colloidal solution. As the PL intensity is directly affected by the monomer concentration, we need to measure the absorption in order to judge the QY of the nanocrystal sample.

Figure 4.40 PL emission spectra of 5%Co and 10%Co doped ZnO in different concentrations (0.075 mol/L and 0.15 mol/L respectively).

The integrated PL intensity was calculated by the area under the PL curve in the wavelength range from 370 to 600 nm. A graph was plotted using integrated PL intensity against the absorbance as shown in Figure 4.41. A trend line was added to each curve with the intercept at zero, in order to obtain the gradient of the line. Using Equation (24), we calculated the quantum yield of the 5%Co and 10%Co doped ZnO. The values are shown in Table 4.11. For the two standard samples, we obtained 0.789 and 0.254 respectively, with a deviation of $\pm 5\%$ from the standard values. Then the QYs of the test samples were calculated using fluorescein as a standard, thus obtaining 0.779 and 0.931 for 5%Co and 10%Co doped ZnO respectively. The 10%Co doped ZnO possesses higher quantum yield than the 5%Co-ZnO. We noticed that the PL intensities of them (5%Co and 10%Co doped ZnO) were quite the same, but the later absorbed less photon, as a result, the ratio of PL over Abs% of the 10%Co-ZnO is higher. Both 5%Co and 10%Co doped ZnO nanocrystals possess high QYs (higher than most of the reported QY values as mentioned

in Section 2.1.4), and therefore, should be suitable for bio-imaging applications.

Figure 4.41 Plot of integrated PL intensity against UV-Vis absorbance of the standard and test samples. The gradient of each sample is obtained from the trend line equation. All trend lines have good fittings of higher than 99%. Legend “Fluor” refers to Fluorescein, “Anth” refers to Anthracene, “5%Co” means the 5%Co-ZnO, “10%Co” means the 10%Co-ZnO.

Table 4.11 QY results of standard and 5%Co, 10%Co-ZnO test samples and the data used for the calculations.

Compound / Solvent	Refractive Index	Gradient	Measured QY	Literature QY
Fluorescein / 0.01M NaOH	1.340	3.8212	0.789	0.79
Anthracene / ethanol	1.361	1.1926	0.254	0.27
5%Co with ZnO / methanol	1.328	3.8418	0.779	-
10%Co with ZnO / methanol	1.328	4.5928	0.931	-

Comparing the QY values of Fluorescein and Anthracene in Table 4.10 and 4.11, it is seen that the measurement error between batches is within 0.02.

4.3 Modelling of photoluminescence spectra of ZnO nanocrystals

Optical properties of bulk ZnO materials have been studied intensively. Very different defect origins, such as oxygen vacancy (V_o) [70], zinc vacancy (V_{Zn}) [168], interstitial zinc (Zn_i), and substitutional Cu^{2+} on zinc site etc. have been suggested to be responsible for the green emission band of ZnO (in Figure 4.21). As mentioned in Chapter 2.1.5, several models have been used to fit the PL emission LO band, but we adopted the multimode Brownian Oscillator (MBO) model taking into account both the electron-LO phonon coupling and the dissipative effect of the phonon-bath modes. For our nanocrystals of size 2-5 nm, we did not see the LO emission band due to surface defects. Only when we used dry powder applied on a Si-wafer, a second peak on PL spectrum was detected on Ni-doped ZnO. For the pure ZnO, we shall model the absorption and emission spectra together to simulate the full optical properties of the ZnO nanocrystals.

The modelling software used in this research is based on the MBO model derived by Zhao *et al.* [169]. The full derivations of equations are attached in Appendix II. For our single crystal nanoparticles, we adopted a simple form of MBO model in which only a single primary oscillator (i.e. LO phonon) was considered and its coupling strength with the bath modes was assumed to be a constant (i.e. $\gamma_j(\omega) = \text{constant}$) to compute PL spectra.

The PL line shape can be obtained from the following equation [86]:

$$I_{PL}(\omega) = \frac{1}{\pi} \text{Re} \int_0^\infty \exp[i(\omega - \omega_{eg}^0 + \lambda)t - g^*(t)] dt \quad (26)$$

where $g(t)$ is the spectral response function, can be expressed in terms of the frequency-domain correlation function, $C''(\omega)$,

$$g(t) = \frac{1}{2\pi} \int_{-\infty}^\infty d\omega \frac{C''(\omega)}{\omega^2} [1 + \coth(\beta\hbar\omega/2)] [e^{-i\omega t} + i\omega t - 1] \quad (27)$$

$$C''(\omega) = \frac{2\lambda\omega_i^2\omega\gamma_i}{\omega^2\gamma_i^2 + (\omega_i^2 - \omega^2)^2} \quad (28)$$

Therefore, the parameters in the model that need to be set or obtained are:

γ_j (cm^{-1}): being the spectral distribution function describing the coupling between the primary oscillator and the secondary bath oscillators,

ω_j (cm^{-1}): being the angular frequency of the j th nuclear mode of the primary (bath) oscillator,

S: being the well known dimensionless Huang-Rhys factor characterizing the strength of electron-LO phonon interactions [170,171],

T (K): being the temperature of the PL measurement,

ω_{eg} : being the 0-0 transition electronic energy gap eV, corresponding to zero phonon line (ZPL).

Figure 4.42 shows the Raman scattering spectrum of 5%Ni doped ZnO at room temperature. The sample was prepared by depositing a thick layer of 5%Ni-ZnO powder on a Si-wafer. Therefore, the PL spectrum is different from those measured on colloids (shown in Section 4.2.1). From the spectrum, we notice a peak at 429 cm^{-1} , which corresponds to the zero-phonon line of ZnO (437 cm^{-1} reported), but has shifted a bit due to quantum confinement effect. The two additional peaks at about 813 and 891 cm^{-1} should be due to the Ni doping. This was also detected by Cong *et al.* [123], where they found two additional modes at 813 and 891 cm^{-1} in the Raman spectra of the $\text{Zn}_{0.97}\text{Ni}_{0.03}\text{O}$ nanoparticles, besides the zero-phonon line at 437 cm^{-1} . These two modes were associated with Ni^{2+} occupation at the Zn sites, because the ionic radius of Ni^{2+} (0.55) is less than that of Zn^{2+} (0.60), lattice defects were introduced or intrinsic host-lattice defects were activated when Ni^{2+} ions occupied the Zn sites.

Figure 4.42 Raman spectrum of 5%Ni-ZnO powder at room temperature.

Figure 4.43 shows the measured and modelled PL excitation and emission spectra of pure ZnO capped by Z60. Perfect fitting was obtained using the following parameters:

$$\gamma_j = 438 \text{ cm}^{-1}, \omega_j = 891 \text{ cm}^{-1}, S = 6.02, T = 298 \text{ K}, \omega_{eg} = 3.60 \text{ eV}.$$

In the modelling process, the stoke shift $2\lambda_j = 2S\hbar\omega_j$ was kept constant as it is the material's intrinsic property, while the values of ω_j and S were varied, until perfect fitting between model curves and experimental curves was obtained. In the best fit parameters, the transition energy of pure electronic transition (ZPL) is 3.60 eV (ω_{eg}), higher than the fundamental band gap (~ 3.37 eV) of ZnO, implying the quantum confinement effect due to the 1-2 nm crystal size. The Huang-Rhys factor $S = 6.02$ is close to the previous studies ($S = 6.5$ obtained by Shi and Kuhnert on ZnO solid) [86,89]. The ω_j value of 891 cm^{-1} corresponds to the mode in the Raman spectrum. The relatively small γ_j indicates little coupling between the primary oscillator and the secondary bath oscillators. This is obvious because only one peak is observed on the emission curve. The perfect fitting of the model curves with the experimental curves indicates that the MBO model is suitable

for our synthesized ZnO nanocrystals.

(Parameters: 438, 891, 6.02, 298, 3.60)

Figure 4.43 Measured and modelled PL excitation and emission spectra of pure ZnO capped by Z60.

Figure 4.44 shows the measured and modelled PL spectra of 5%Ni doped ZnO. The fitting parameters obtained for the model are:

$$\gamma_j = 2400 \text{ cm}^{-1}, \omega_j = 4000 \text{ cm}^{-1}, S = 1.10, T = 298 \text{ K}, \omega_{eg} = 3.74 \text{ eV}.$$

It is noticed that there are two emission peaks in the spectrum indicating some coupling between the primary oscillator and the secondary bath oscillators, therefore, γ_j value is higher. Although the Huang-Rhys factor (S) obtained is smaller than that obtained in Fig. 4.43, it is still higher than 1, indicating intermediate electron-LO phonon coupling [170],

which is responsible for the observed PL side band in the lower eV side. The transition energy of purely electronic transition (ZPL) ($\omega_{eg} = 3.74$ eV) is higher than the fundamental band gap (~ 3.37 eV) of ZnO, implying quantum confinement effect caused by the 2 nm crystal size. This 2-peak PL line shape is a typical intermediate damped mode, in which the emission spectrum is slightly asymmetrical, and because of that, the Brownian oscillator is no longer a continuous coherent oscillator. This could be caused by the Ni^{2+} substitution of Zn sites, and the ionic radius of Ni^{2+} is smaller than that of Zn^{2+} , introducing lattice defects or intrinsic host-lattice defects. The high ω_j value is the result of this substitution. In conclusion, the MBO theoretical model fits to the experimental curve, indicating the good suitability of the MBO model for pure ZnO and doped ZnO nanocrystals in the quantum size range. For the modelling of doped ZnO and more complex materials, more variables may be required to build into the software, so that more complex PL shapes can be modelled.

Since Cu^{2+} and Co^{2+} are also smaller in ionic radii than Zn^{2+} , we observed similar PL line shapes as the Ni-doped ZnO. However, all these PL studies were conducted on the dry powders. The original synthesized ZnO colloids have the perfect PL line shape with only red shifted peak for some of the doped and capped ZnO as demonstrated in section 4.2.1. Since we are using colloids for bio-imaging applications, we did not repeat the modelling for Cu and Co doped ZnO powders. The modelling of the pure ZnO and Ni doped ZnO have sufficiently demonstrated the suitability of the MBO model for ZnO nanocrystals.

(Parameters: 2400, 4000, 1.10, 298, 3.74)

Figure 4.44 Measured and modelled PL spectra of 5%Ni doped ZnO.

Chapter Five: Bio-Imaging Tests and Cytotoxicity Study

Bio-imaging is one of the potential applications of our doped ZnO nanocrystals. The major advantages of ZnO over Cd-based quantum dots are the better intrinsic optical properties, the less toxic synthesis process and the bio-compatible core material. Therefore, bio-imaging and cytotoxicity tests are performed as the ultimate goal of this research. Bio-imaging tests were performed on both human cells and plant cells. The results are presented in the following sections.

5.1 Bio-imaging on human cells

As introduced in the section 3.3.7, a human osteosarcoma (tumour) cell line (MG-63 from ATCC) derived from human bone was used for both bio-imaging and cytotoxicity study. Human histiocytic lymphoma cell line (U-937 from ATCC) derived from human blood was also tested on bio-imaging. Another cell line L929 that originated from mouse subcutaneous connective tissue was used to check the cytotoxicity of ZnO nanoparticles. Figure 5.1 shows the images of the cells without adding nanoparticles. These pictures were taken by a microscope using different colour filters.

(a) MG-63 cells, Scale bar = 100μm

(b) L929 cells, Scale bar = 50μm

(c) U937 cells, Scale bar = 20μm

Figure 5.1. Images of the original cells without nanocrystals added.
(a) MG-63, (b) L929 and (c) U937 cells without nanoparticles added, taken by Leica DMIL microscope (different colour filters were used).

Figure 5.2 (a) and (b) shows the bio-imaging pictures of MG-63 cells labelled by ZnO-Z61 and ZnO-TiO₂ nanocrystals. It is seen that a dual colour image is obtained using ZnO-Z61 with a blue colour in the nuclei and a turquoise colour in the cytoplasm. While

in the images labelled by ZnO-TiO₂, only blue colour is seen on the cytoplasm part of the cells. This is either due to the larger particle size of ZnO-TiO₂ nanoparticles that they could not penetrate into the nuclei, or due to the too low emission that cannot be detected. These colours correspond to the PL emission wavelengths, where the ZnO-TiO₂ emits in the deep blue-violet side, and ZnO-Z61 emits in blue-turquoise range. Figure 5.2 (c) and (d) shows the bio-images of U937 cell labelled by 5%Co-ZnO-Z60 and MG-63 cells labelled by the uncapped 5%Co-ZnO. As mentioned in Section 4.2.1 (Figure 4.31), that uncapped 5%Co-ZnO had stable colloids, and can be used for bio-imaging, but the PL emission is at 390 nm (blue-violate colour), while Z60 capped 5%Co emits at 420 nm (blue turquoise colour). The colours of the images are very well matched with the PL emission peaks. In the meantime, the Z60 capped 5%Co-ZnO showed dual colour image with blue colour in the nucleus and turquoise colour in the cytoplasm. This dual colour imaging should be due to the same principle as the ZnO-Z61 labelled cells. This is a very interesting and useful feature as it shows that our quantum dots are able to label both the nuclei and the cytoplasm simultaneously. Two colour or multi-colour imaging is currently done in multiple steps using different QDs. We shall discuss this aspect in more detail in the next Chapter.

Figure 5.2 (e) and (f) shows the bio-images of Mg-63 cells labelled by 5%Cu-ZnO-Z60 and 10%Ni-ZnO-Z61 respectively. Only one colour in the cytoplasm part can be observed on both images. This is because either the larger size of the particles that could not penetrate through the wall of the nuclei, or, the poor compatibility between the nanoparticles and the nuclei that does not allow absorption of the nanocrystals into the nuclei.

(a) ZnO-Z61

(b) ZnO-TiO₂

(c) 5%Co-ZnO-Z60

(d) 5%Co-ZnO

(e) 5%Cu-ZnO-Z60

(f) 10%Ni-ZnO-Z61

Figure 5.2 Bio-imaging pictures of all the tested cells.
 (a) MG-63 cells labelled by ZnO-Z61 nanocrystals, (b) MG-63 cells labelled by ZnO-TiO₂ nanocrystals, (c) U937 cells labelled by 5%Co-ZnO-Z60, (d) MG-63 cells labelled by uncapped 5%Co-ZnO, (e) MG-63 cells labelled by 5%Cu-ZnO-Z60 nanocrystals, (f) MG-63 cells labelled by 10%Ni-ZnO-Z61.

Comparison to the bio-imaging by other nanoparticles:

The best bio-images reported so far were labelled by CdSe-ZnS core-shell QDs coated with anti-body, streptavidin, biotin, or peptides (refer to Chapter 2). In the double colour images in Fig. 2.11 and Fig. 2.14(a), the cytoplasm parts are labelled in green with QDs, and the nuclei are labelled by organic dyes (blue and red respectively). It is well known that the organic dyes suffer photobleaching, therefore, are not suitable for longer term bio-imaging and tracking purposes. Our dual colour image is from the same high quality QD, therefore, suitable for long term imaging and tracking purposes. If it is necessary, the organic dyes can also be added to label the nuclei, which will be the same as the Cd-based QDs. Another difference is that the Cd-based QDs were coated with specific anti-bodies, therefore, only suitable for the specific cell. While our QDs are more generic, can be used to label several types of cells as demonstrated above (suitability for other cells need to be tested out).

Compared to Fig. 2.13 and Fig. 2.14(b), which were labelled by glutaraldehyde or polymer coated QDs (more generic QDs), our images (Fig. 5.2 (a, c, and d)) are much clearer than these images. The detailed structures in the cell, both in nucleus and cytoplasm, are clearly shown in our images. Therefore, our QDs possess at least three advantages over the other markers: (1) unique dual colour labelling on both nucleus and cytoplasm simultaneously, (2) generic marker suitable for several types of cells, (3) bright and long lasting imaging compared to polymer and protein coated QDs.

5.2 Cytotoxicity test by MTT method

All the QDs synthesized in this study were tested on their cytotoxicities on MG-63 cells using the MTT method. The absorbance values at wavelength 490nm, which is defined by the One-Solution Assay, were measured by a 96-well plate reader, and the relative percentage to the reference cell (without QD addition) was calculated. All the data points are from the average of 5-6 measurements, and each QD was tested at least in 4 wells. The percentage values in relation to the concentrations of QDs added (up to 1000 $\mu\text{g/mL}$) are plotted in Figure 5.3. The standard deviations of the values are about 10%. It is seen from the figure that TiO_2 and Aps capped nanoparticles are more toxic to cells than other capping agents. Cu- and Ni- doped ZnO are slightly more toxic than Co-doped and pure ZnO. The safe nanocrystals in all concentrations are: Z60 and Z61 capped pure ZnO, Co-doped ZnO and Cu-doped ZnO as they are not toxic at all up to 1000 $\mu\text{g/mL}$. Concentrations beyond 1000 $\mu\text{g/mL}$ were not tested because this concentration is already much higher than the required concentration for both *in vivo* and *in vitro* bio-imaging applications. Reported injection concentrations for mouse *in vivo* tests are in the range of 10 to 250 $\mu\text{g/mL}$ [13,22,172], and maximum concentration for *in vitro* tests on live cells is 100 $\mu\text{g/mL}$ [9,173]. For pure cytotoxicity tests for QDs, a concentration between 62.5 to 1000 $\mu\text{g/mL}$ was reported [13]. Cytotoxicity of QDs was already found in 62.5 $\mu\text{g/mL}$ concentration. The toxic threshold for cells is consistently reported to be in the tens of p.p.m. range (tens of $\mu\text{g/mL}$) [174]. Therefore, our concentration of 1000 $\mu\text{g/mL}$ is the highest concentration reported so far.

Figure 5.3 Cell viability results tested by MTT method. (a) ZnO and 5%Co doped ZnO, (b) 5%Cu and 10%Ni doped ZnO. Legends: concentration of nanocrystals added in unit of $\mu\text{g/mL}$. Standard deviations of the values are about 10%.

Comparison of the synthesized ZnO QD with commercial CdSe based QD on cytotoxicity

A commercial CdSe/ZnS core-shell quantum dots product was purchased from Evident

Technologies (USA). The QDs are in size of about 3.5 nm, capped with tri-n-octylphosphine oxide (TOPO) and suspended in chloroform in concentration of 10mg/ml. To make the QDs water soluble, ligand exchange and re-suspension in water were performed according to the suggested procedures [175]. In detail, 15 g mercaptoacetic acid (MAA) was added into 1 ml of the original QD chloroform solution and reacted for at least 2 hrs. When precipitation was seen, the suspension was centrifuged at 5000 rpm for 10 min. and the QDs were washed by chloroform for 5 times to ensure complete removal of the TOPO and excess ligand (because TOPO is soluble in chloroform only). Then the chloroform was evaporated at room temperature for overnight, and the modified QDs were re-suspended in 1 ml purified water by ultrasonication to obtain clear solution. This QD suspension was then further diluted by purified water to obtain the required concentrations for cytotoxicity study. Two types of cells, MG-63 and L929, were used for this study. Figure 5.4 shows the comparison among 5%Co-ZnO-Z60, ZnO-Z60 nanocrystals and commercial CdSe/ZnS QD on Mg-63 (a) and L929 (b) cells respectively.

It is seen from Figure 5.4(a), the cell viability dropped to 50% at 300 $\mu\text{g/ml}$ concentration of commercial CdSe QD, while the cells with 5%Co-ZnO-Z60 and ZnO-Z60 added were in good condition up to 500 $\mu\text{g/ml}$. In the test on L929 cells, the QD concentration was lowered because it was found that this cell line derived from small animals were more sensitive to the QDs than the MG-63 cell derived from human bone. It is seen from Figure 5.4(b) that cell viability dropped to 50% when 80 $\mu\text{g/ml}$ of commercial QDs were added, or 200 $\mu\text{g/ml}$ of (doped) ZnO QDs were added. This trend is the same as that on MG-63 cell. Therefore, it can be concluded that the cytotoxicity of commercial CdSe based QD is relatively higher than the (doped) ZnO QDs synthesized in this research.

Figure 5.4 Cell viability results comparing synthesized ZnO QDs to commercial QD product. (a) On MG-63 cells, (b) on L929 cells.

Comparability of the two types of particles

Although there are commercial QD water solutions, they are all in very low concentrations (4-10 $\mu\text{g/ml}$) for direct *in vivo* injection use. For cytotoxicity study,

concentrations ranging from 100 to 1000 $\mu\text{g/ml}$ are required. Available commercial high concentration QDs are in either toluene or chloroform because these solvents are polar, which help to maintain the particle size. Toluene is very toxic with high boiling point (higher than water), not suitable for ligand exchange and redispersion in water by the commonly adopted vaporization method. After detailed discussion with the technical staff in Evident Technologies, QDs capped by TOPO dissolved in chloroform was purchased. It is noticed in the above-described ligand exchange procedures, a precipitation step was inevitable because of the solvent used in the commercial QDs. This would cause particle agglomeration resulting in larger particles. However, there is no precipitation step in our ZnO nanoparticle preparation process. This is the difference between the two types of particles, which can not be avoided. Nevertheless, after the ligand exchange for the commercial QDs, the MAA capped QDs became water soluble and readily absorbed by cells. In the same condition, ZnO nanoparticles were also water soluble and readily absorbed by cells. Therefore, the two types of particles can be reasonably compared.

To confirm if the chloroform was completely evaporated, a TGA scan was performed on the QD powder after ligand exchange. Figure 5.5 shows the TGA graph. It is seen that about 4% weight loss was detected after heating up to 100°C in 16 min. This could be the chloroform residual or absorbed moisture since the MAA capped QD surface was water soluble and the QDs were exposed to air for overnight. In future experiments, cytotoxicity by chloroform in amount equivalent to 4%QD may be tested to confirm any effect from the chloroform residual, or a drying step for QD powder by heating up to 60°C before redispersion in water should be performed to ensure complete evaporation of chloroform.

Figure 5.5 TGA graph of commercial CdSe-ZnS QD powder after ligand exchange.

5.3 Cytotoxicity observation on small animal cells

Since no cell proliferation occurred in the Mg-63 cells, we used L929 cells derived from the mouse subcutaneous connective tissue to further observe the cytotoxicity behaviour. Accelerated tests were conducted by adding ZnO nanoparticles in 100 $\mu\text{g/mL}$ concentration to the cells directly after the cell subcultivation on 12-well culture flask. Normally the cells are allowed to grow 1 day before nanocrystals are added; therefore, this test is considered an abnormal or accelerated test. The cell growing status was observed by a microscope on the 2nd and 3rd days without renewing the medium. Microscopic images were taken and compared to the control cells (see Figure 5.1(b)). Figure 5.6 shows the cell images on the 3rd day using pure ZnO without capping, 5%Co-ZnO-Z60, ZnO-TiO₂, and commercial CdSe/ZnS core shell QDs respectively. It is seen from the figures that about 40% cell proliferation can be seen on the cells labelled by

pure ZnO without capping (images (a)), while no cell proliferation is seen on the cells labelled by the 5%Co-ZnO-Z60 (images (b)). Slow growth is observed on the image (c) labelled by ZnO-TiO₂, which could be related to the slight acidic condition of this NC (an acidic catalyst was used for the hydrolysis of the TIP precursor). More than 50% dead cells with obvious cell blebbing are observed on the cells labelled by CdSe/ZnS QDs (image (d)). This could be due to the cytotoxicity of this QD. From these comparative studies, we found that the L929 cell line from small animals is more sensitive to the QDs than the human cells. Relatively, the 5%Co-ZnO-Z60 QDs are not harmful for the cells, but pure ZnO is slightly harmful, and ZnO-TiO₂ NCs are more harmful to the cells. The commercial CdSe/ZnS QDs caused severe cell proliferation and blebbing. These observed results are in line with the cytotoxicity results tested by MTT method described in section 5.2, where 50% cell viability was measured with 80 µg/mL of commercial QD or with 200 µg/mL of ZnO-Z60 and 5%Co-ZnO-Z60. These observations again proved that small animal cells are more sensitive to the addition of nanocrystals, and a low concentration should be used for bio-imaging and *in vivo* tests. A separate *in vivo* bio-imaging test in rat model has been conducted. The amount of injected ZnO colloid was only 50µM/L (about 4 µg/mL), which did not cause any negative effect on the rat, and clear optical emission was detected in the kidney, liver and lung of the rat. This means that the tested dosages are more than required for bio-imaging of animal and human cells. Therefore, we can conclude that the bio-markers are non-toxic when used for bio-imaging.

Figure 5.6 L929 cells growth status after abnormal and accelerated tests. 100 $\mu\text{g/mL}$ of QDs were added immediately after the cell subculture. Images were taken on the 3rd day. (a) pure ZnO nanocrystals without capping were added, (b) 5%Co-ZnO-Z60 nanocrystals were added, (c) ZnO-TiO₂ nanocrystals were added, (d) commercial CdSe/ZnS QDs were added. All are under the same magnification, scale bar = 50 μm . Image of control cells is shown in Fig.5.1 (b).

5.4 Bio-imaging and cytotoxicity tests on plant cells

5.4.1 Bio-imaging on plant cells

Following the procedures described in section 3.3.8, the plant cross-section fluorescence images were taken by the Leica True Confocal Scanner (TCS) SP2 confocal laser scanning microscope. The wavelength ranges of the green, yellow and red channels are summarized below (same as Table 3.3):

Channel 1	500 – 550	Green
Channel 2	550 – 600	Yellow
Channel 3	600 - 750	Red

Figure 5.7 shows the confocal images of the reference sample without colloidal nanocrystal added. There is no colour emission detected in the first two channels, while some red emission can be seen in the 3rd channel, and this is due to the autofluorescence of plant’s own internal sugars or structures. The cross-section structure of the plant is clearly seen.

Figure 5.7 Confocal images for reference sample without nanocrystal added using 100 x optical magnifications

Figure 5.8 shows the confocal images of plants using ZnO-Z60 nanocrystal. Luminescence was detected in all three channels, with the highest intensity of luminescence in the vascular regions of the plant, which is the region of transportation of water and minerals. This emission is especially prominent in both the phloem and xylem tissue areas. Some fluorescence can also be found surrounding individual cells (see the higher magnification images in (b) and (c)), around the cell wall area of the ground tissue, which might indicate that the colloidal nanocrystals have diffused through the vascular tissue.

Figure 5.8 Confocal images of plant cross-sections labelled by ZnO-Z60 colloidal nanocrystals.

(a) using 100x optical magnifications, (b) zoom into the vascular region using 5x digital zoom, (c) zoom at single cell using 10x digital zoom.

The yellow and green emissions are due to the absorbed nanocrystals, and the red region is attributed to autofluorescence as seen in the control. From the single cell image within the ground tissue region (in image (c)), we can observe that fluorescence is present prominently around the cell wall. In accordance with the fluorescence images above, the yellow region has the brightest fluorescence, followed by green, then red.

Figure 5.9 shows the confocal images of plants using ZnO-Z61 nanocrystal. Bright luminescence was detected in all three channels, which is very similar to the

characteristics of ZnO-Z60 (Figure 4.27). However, from the single cell image, we can see relatively blurred images in the vascular region, indicating more non-specific absorption of this nanocrystal by the plant.

Figure 5.9 Confocal images of plant cross-sections labelled by ZnO-Z61 colloidal nanocrystals.
(a) using 100x optical magnifications, (b) zoom into the vascular region using 5x digital zoom.

No visible fluorescence was detected in the cross-section of the plant labelled by ZnO-TiO₂, indicating no uptake of ZnO-TiO₂, and this may be due to the larger particle size of this nanoparticle that could not transport inside the plant.

Figure 5.10 shows the confocal images of plants using 5%Cu doped ZnO-Z60 nanocrystals. Luminescence was detected in all three channels, with higher luminescence at the vascular region. Compared to the images in Figure 5.8, the cell walls are not labelled very well, but nanocrystal penetration seems deeper through the vascular tissue, indicating a higher absorption by the plant. There are traces of bright red fluorescence staining at the junctions of the cell walls. This is in accordance with the study of native microbial aggregates on mung bean sprout cotyledons as determined by confocal

scanning laser microscopy [176]. In nature, bacteria are often organized in aggregates or biofilms rather than as solitary cells on plant surfaces to protect them from adverse environmental conditions. Under the confocal laser scanning microscopy, cells with compromised membranes (attacked by dead bacteria) will emit red fluorescence, and these red fluorescent appear to be less compact and sparsely distributed. The Cu doped ZnO can be considered an adverse environment for the mung bean plant because of the high concentration nanocrystals added, thus causing the plant's cells to be attacked by the bacterial exopolymer materials (such as exopolysaccharides) and the cell membranes to be compromised. The overall intensity of luminescence is lower than that in Figure 5.6, this agrees with the photoluminescence results that 5%Cu-ZnO-Z60 in water has lower intensity than the corresponding pure ZnO quantum dots (see Figure 4.28 and 4.29).

Figure 5.10 Confocal images of plant cross-sections labelled by 5%Cu-ZnO-Z60 colloidal nanocrystals.

(a) using 100x optical magnifications, (b) zoom into the vascular region using 5x digital zoom, (c) zoom at single cell using 10x digital zoom.

Figure 5.11 shows the confocal images of plants using 5%Cu doped ZnO-Z61 nanocrystals. Luminescence was detected in all three channels, with higher luminescence in green and yellow, however, there was no significant clarity in imaging the internal structures of the cross-sections, thus producing only relatively blurred images that indicate the general regions of uptake, such as the vascular regions. From the magnified images, the overall fluorescence was observed, but cell walls were not clearly defined by the illumination.

Figure 5.11 Confocal images of plant cross-sections labelled by 5%Cu-ZnO-Z61 colloidal nanocrystals.
(a) using 100x optical magnifications, (b) zoom into the vascular region using 5x digital zoom.

Figure 5.12 shows the confocal images of plants using 5%Cu-ZnO-TiO₂ nanocrystals. Luminescence was detected in all the three channels, more prominently in the central vascular region, with higher luminescence in yellow and green ranges.

Figure 5.12 Confocal images of plant cross-sections labelled by 5%Cu-ZnO-TiO₂ colloidal nanocrystals.
(a) using 100x optical magnifications, (b) zoom into the vascular region using 5x digital zoom.

In the magnified image of the ground tissue region (b), clear fluorescence was present prominently around the cell wall with yellow colour being the brightest. This could be due to the larger particle size of this nanocrystal that could only transport in the centre region tissues.

Figure 5.13 shows the confocal images of plants using 10%Ni-ZnO-Z60 nanocrystals. Luminescence was detected in all three channels, with higher intensity in the yellow range. Some fluorescence can be seen surrounding individual cells, around the cell wall area of the ground tissue, thus proving that the nanocrystals have diffused through the vascular tissue. In comparing ZnO-Z60 and 5%Cu-ZnO-Z60, fluorescence intensity is lower indicating Ni doped ZnO has decreased the photoluminescence ability. This is not in line with the PL results (Figure 4.28), where the Ni doped ZnO produced higher intensity than Cu doped ZnO nano-particles. This reverse phenomenon observed during the bio-imaging is related to the uptake of the Ni doped ZnO nanoparticles by the membrane surface and the transportation of the Ni-ZnO nanoparticles in the plant. Although all dopants have been doped into the ZnO lattices, the activities of the differently doped ZnO at the membrane surface could be different as the crystal structures and other physical and chemical properties are different. Zhang *et al.* [177] studied the effect of membrane surface charge on nickel uptake by purified mung bean root protoplasts and found the activity of Ni at the membrane surface is the major determinant of the rate of Ni influx into mung bean protoplasts. The lower emission intensity on plant labelled by Ni-ZnO could be due to less amount of nanoparticles on the cells, which could be caused by the less activity of Ni doped ZnO at the membrane surface, thus fewer nanoparticles were transported to the cells.

Figure 5.13 Confocal images of plant cross-sections labelled by 10%Ni-ZnO-Z60 colloidal nanocrystals.
(a) using 100x optical magnifications, (b) zoom into the vascular region using 5x digital zoom.

Figure 5.14 shows the confocal images of plants using 10%Ni-ZnO-Z61 nanocrystals. Luminescence was detected in all the three channels without significant clarity in the internal structures of the cross-sections, as a result, only relatively blurred images that indicate the general regions of uptake, including the vascular regions. Autofluorescence in the red region in the magnified image (b) appears brighter as the vascular channel in the stem part has a better absorption of water and others nutrients.

Figure 5.14 Confocal images of plant cross-sections labelled by 10%Ni-ZnO-Z61 colloidal nanocrystals.
(a) using 100x optical magnifications, (b) zoom into the vascular region using 5x digital zoom.

Figure 5.15 shows the confocal images of plants using 10%Ni-ZnO- TiO₂ nanocrystals. Luminescence was detected in all three channels, but with a much lower intensity than 5%Cu-ZnO-TiO₂, however, this is in line with the PL results that all TiO₂ capped nanocrystals have lower PL intensity than Z60 and Z61 capped nanocrystals as shown in Table 4.9 (excitation at 488 nm). Fluorescence appears around the cell walls of the plant. The lower luminescence of TiO₂ capped nanocrystals may be due to the relative larger particle size. The larger size in comparison to the space in the transportation vein inside the plant hampers the flow of the nanocrystals, and results in less absorption by the plant cells.

Figure 5.15 Confocal images of plant cross-sections labelled by 10%Ni-ZnO-TiO₂ colloidal nanocrystals.
(a) using 100x optical magnifications, (b) zoom into the vascular region using 5x digital zoom.

Figure 5.16 shows the confocal images of plants using 5%Co-ZnO-Z60 nanocrystals. Luminescence was detected in all three channels, with significant clarity and selectivity on the vascular cylinder region only. The green and yellow channels show the whole cells on the surface and cell walls of those lower cells. It is interesting that the autofluorescence (red channel) shows the inversed imaging area of the plant cross-section

with the green and yellow channels. In this autofluorescence, no clear cell shape and cell walls can be observed. Conversely, the areas labelled by QDs are clearly shown. This proved the significance of bio-imaging by QDs. Compared to the Cu and Ni doped ZnO nanocrystals, 5%Co-ZnO-Z60 nanocrystals possess more specific labelling characteristics, which can mark the cells and cell walls in the vascular cylinder region with clear and detailed structures.

Figure 5.16 Confocal images of plant cross-sections labelled by 5%Co-ZnO-Z60 colloidal nanocrystals.
(a) using 100x optical magnifications, (b) zoom into the vascular region using 5x digital zoom.

5.4.2 Quantitative analyses of confocal images

In the last section, we described the fluorescence intensities qualitatively by visualizing the confocal images. A quantitative measure of the intensity can be performed by the Leica TCS SP2 confocal microscope for selected 10 locations on one image. The mean intensity ($\bar{A}$) and standard deviation (d) of the random samples for each channel was calculated. The results for the three channels are summarized in the following tables:

Table 5.1 Statistical fluorescence intensity data of the plant images.
Mean and standard deviation of samples (a, plant without nanocrystal added), green channel (b), yellow channel (c) and red channel (d) using sample size of 4.

(a) Control plant sample

	Green	Yellow	Red	Trans	
Control	13.38	33.22	17.30	123.30	(mean)
	2.00	3.94	3.09	14.18	(std. dev.)

(b) Green fluorescence (range: 500-550nm)

	ZnO	5%Cu-ZnO	10%Ni-ZnO	5%Co-ZnO	
Z60	62.53	52.54	29.93	54.1	(mean)
	8.13	9.48	4.21	4.7	(std. dev.)
Z61	77.98	54.44	30.20	63.5	(mean)
	10.05	12.45	2.41	12.1	(std. dev.)
TiO ₂	2.28	41.24	37.03	71.7	(mean)
	0.17	11.98	5.78	10.2	(std. dev.)

(c) Yellow fluorescence (range: 550-600nm)

	ZnO	5%Cu-ZnO	10%Ni-ZnO	5%Co-ZnO	
Z60	89.66	65.00	59.06	67.3	(mean)
	12.81	10.72	5.25	7.1	(std. dev.)
Z61	121.43	74.58	49.37	83.9	(mean)
	13.49	16.34	2.49	18.6	(std. dev.)
TiO ₂	7.95	58.19	54.02	85.5	(mean)
	0.63	15.16	7.12	19.3	(std. dev.)

(d) Red fluorescence (range: 600-750nm)

	ZnO	5%Cu-ZnO	10%Ni-ZnO	5%Co-ZnO	
Z60	41.95	26.26	20.89	15.6	(mean)
	10.96	9.52	3.28	2.1	(std. dev.)
Z61	50.99	36.23	24.16	17.8	(mean)
	7.25	15.49	9.79	5.6	(std. dev.)
TiO ₂	3.58	27.63	25.42	18.9	(mean)
	0.29	11.49	7.39	6.8	(std. dev.)

From the statistical data of all three channels, we can clearly see that all the biomarkers except TiO₂ capped ZnO nano-particles exhibit higher intensity relative to the control sample, meaning that they can be used for plant cell labelling. TiO₂ capped ZnO nano-particles were not able to label the plant section due to the larger particle size that could not be transported inside the narrow veins of the plant. Particle size below 4 nm is appropriate for *in vivo* bio-imaging. In analyzing the green, yellow and red fluorescence individually, the yellow channel intensities of almost all the nanocrystals are higher than green channel. This is because most of the emission peaks which are around 570nm, are in the yellow channel range. Z61 capped ZnO nano-particles have higher emission intensity than Z60 capped ZnO. But Z60 capped ZnO nanoparticles have smaller size, which allows the nanocrystals penetrate into the cells and cell walls, thus providing more detailed cell structures. The fluorescence intensities are high enough for detection and analysis of cells. Therefore, we can conclude that Z60 capped ZnO biomarkers provide superior labelling characteristics in green and yellow channels.

In comparing across each row of green and yellow channels, we can see that doped ZnO nanocrystals have lower emission intensities than the pure ZnO. This is in line with the conclusions on PL spectra results. As the absorption spectra showed in Section 4.2.2, all the doped ZnO nanocrystals had a second absorption peak in visible range corresponding

to lower bandgap energy, while the undoped ZnO had only the UV absorption. This lower bandgap energy leads to lower emission energy resulting in lower emission intensity in imaging. The bandgap energies of the undoped and doped ZnO nanocrystals are summarized in Table 5.2. As analyzed in Section 4.2.2, the lower energy levels are related to lattice defects induced by the substitution of Zn^{2+} sites by the dopants Cu^{2+} , Co^{2+} or Ni^{2+} cations because these cations have smaller ionic radius than that of Zn^{2+} cation. Therefore, the higher the dopant level, the more defects are introduced into the ZnO nanocrystal, and the lower bandgap energy is. Thus dopants such as Cu, Co and Ni can be used for bandgap modification, but the doping concentration should be carefully controlled. Nevertheless, the emission intensities of 5% dopants were high enough for bio-imaging as shown above, and they showed sharper images with better absorption at the cell walls of the plant cells.

Table 5.2 Comparisons of bandgap energies of undoped and doped ZnO nanocrystals

Nanocrystal	Z60 capped	Z61 capped
Undoped ZnO	3.542 eV	3.542 eV
5%Cu-doped ZnO	2.033 eV	1.959 eV
5%Ni-doped ZnO	2.331 eV	2.138 eV
5%Co-doped ZnO	2.384 eV	2.339 eV

5.4.3 Cytotoxicity observation on plant

The growing status of the mung bean sprouts after adding nanocrystals were observed and compared to the control sample (without nanocrystal added). It was found that the nanocrystal concentration had significant effects on the plant growth. When using the highly concentrated colloids (0.15 mol/L or 32.7 g/L), the bean sprout changed yellowish on the 2nd day, and the growing length was obviously shorter than the control sample; thus the growth was highly effected. When using lower concentration of 5 g/L, the bean

sprout was growing with green leaves without obvious yellowing; thus the cytotoxicity was acceptable. When reducing the concentration to the level required for *in vivo* bio-imaging (14 to 100 mg/L), no cytotoxicity was observed. Among the Cu, Co, and Ni doped ZnO nanocrystals, Co doped ZnO was found to be least or non-toxic up to the concentration of 5 g/L. Cu doped ZnO was comparable to Co doped ZnO, and Ni doped ZnO was most toxic when concentration was higher than 500 mg/L. Therefore, both Co and Cu doped ZnO are safe for bio-imaging of plant systems.

Chapter Six: Discussion

6.1 Influencing factors for small and stable particle size

Based on the results presented in Sections 4.1 and 4.2, we found that the most significant influencing factor for particle size is the concentration of Zinc Acetate precursor. When 0.3 mol/L was used, the smallest particle size obtained (capped by APTES) was 20 nm, but when concentration was reduced to 0.15 mol/L, particle size below 10 nm was obtained (capped by APTES, Z60 and Z61). This is because, for a higher concentration, there are more materials available for nucleation and growth in a fixed volume of solution. When high enough temperature and reaction time are provided, all the nuclei will grow according to Eq. (9), and there is a higher chance for particles to meet and agglomerate together in higher concentration. In order to control the particle growth, we need to lower the concentration and use a surface capping agent to limit the growth. However, too low a concentration will cause wider size distribution as explained in Figure 2.13. Therefore, an optimum precursor concentration should be used. In this study, we have found that the optimum precursor concentration is 0.15 mol/L. The reaction temperature is determined by the boiling point of the solvent. Considering the bio-compatibility and the increasing emphasis on “green” chemistry and processes [93, 105], the temperature should be as low as possible. Therefore, we selected methanol as the synthesis solvent as its boiling temperature is only 65°C, which is lower than most of the common solvents. The refluxing time is an effective parameter for the control of nucleation and particle growth. When the optimum refluxing time (e.g. 6.5 hours) was used, the particle growth was under control, thus allowing the conjugation of capping agent onto the particle surface. Too long refluxing time results in immediate precipitation and larger particle size; too short refluxing time results in incomplete chemical reaction and nucleation. Therefore, an

optimum refluxing time is required for a controlled particle size. In summary, the optimum synthesis parameters for small and stable particle size of pure ZnO nanocrystals are: precursor concentration 0.15 mol/L, reaction temperature $67 \pm 1^\circ\text{C}$, refluxing time 6 hours and 30-45 min., fast cooling by ice water to 4°C , and add capping agent immediately in proper amount.

6.2 Mechanism of ZnO formation

The mechanism of formation of ZnO in this synthesis process consists of three steps. (1) Starting from the dissolution of zinc acetate in methanol, an intermediate phase consisting layered zinc hydroxyacetate (LZHA) is formed. The chemical formula may be $\text{Zn}(\text{OH})_{1.5}(\text{CH}_3\text{CO}_2)_{0.5} \cdot 0.5\text{H}_2\text{O}$ [221], or $\text{Zn}_5(\text{OH})_8(\text{CH}_3\text{CO}_2)_2 \cdot 2\text{H}_2\text{O}$ [108], or other forms of methoxyacetate complexes, which are temporary phases. (2) Raising the temperature leads to progressive and complete dissolution of the intermediate phases in methanol. (3) The low hydrolysis ratio (defined as H , the molar ratio of H_2O to Zn ions, $H < 25$ is low, $H > 50$ is high) and the high reaction temperature lead to the formation of oxides [178]. In our synthesis process, no additional water was added; the hydrolysis was by the hydrate in the $\text{Zn}(\text{Ac})_2 \cdot 2\text{H}_2\text{O}$ precursor itself. Therefore, H is very low. The temperature was at $67\text{--}68^\circ\text{C}$, which is higher than the boiling point of methanol (65°C), meaning the reaction was in the superheated condition. These parameters ensure the complete conversion of the intermediate complexes into oxides. The overall chemical reaction to form LZHA is:

The conversion of LZHA into ZnO is:

Since water is supplied only from the precursor $\text{Zn}(\text{Ac})_2 \cdot 2\text{H}_2\text{O}$, the reaction is slow and the nucleation and particle growth are also slow. This process condition prevents too fast nucleation and particle growth, and ensures the separation of nucleation stage and particle growth stage, in order to allow the introduction of capping agents and controlled growth. Some authors reported the layered hydroxide zinc acetate in their final products, where a reaction temperature of 60°C and a hydrolysis ratio higher than 2 were used [108,179]. These further confirmed that a higher temperature than the boiling point of the solvent and low hydrolysis ratio are very important for obtaining pure oxide material.

The study by Poul *et al.* [103] showed that acetate plays two roles in this synthesis process. Firstly, this anion deprotonates the solvent leading to a methoxy group, and secondly, it also acts as a complexing agent towards the cation leading to the formation of intermediate complex. It was found that chloride or sulphate did not have the same function; no oxide could be formed. In the presence of acetic acid as shown in Eq. (24) and (25), the intermediate complexes are hydrolyzed, oxides are formed through polymerization and condensation, which are the same principle as the sol-gel route [180] as described in Equations (14) to (17). Therefore, the intermediate product of acetic acid acts as the catalyst for the formation of oxides.

Similar chemical reactions were reported for Cobalt acetate, Nickel acetate and Cu acetate precursors in polyol medium [179,181]. Therefore, as long as the above parameters are carefully controlled, stoichiometric doping of these materials into Zinc acetate can be accurately controlled, and Co, Ni or Cu doped ZnO can be achieved. Due to the slightly different electropositive level of these dopants, slightly different solubilities and intermediate complexes are expected. A metastable solid solution in the ZnO-CoO

system was reported [167], thus indicating that Co has better solubility in ZnO and could form a metastable state. This was also observed in our study that Co doped ZnO was stable without the capping agent. This colloid was able to label the MG-63 cells with a blue- violet colour as shown in Fig. 5.2(d).

6.3 Mechanisms of surface modifications and bioconjugations

Besides all the synthesis parameters, the most important technique for size control is the use of surface capping agents. There are many candidates for surface modification, but for bio-applications, the choices of chemical groups are limited to amino-, thiol-, carboxyl- and silanol- only due to their bio-compatibilities with most of the cells. Of course, many polymers can be used to coat the particles, but the particle size is increased due to the large molecular size of the polymer. In this research, we only selected one polymer (PVP) as the capping agent for comparison with other capping agents. It has been proven (Fig. 4.6) that polymer is not suitable for surface capping of single nanocrystal because the molecular size is too large compared to the size of the single nanocrystal. In addition, the binding affinity of polymer to ZnO surface is low compared to other capping agents. Table 6.1 lists the coordinating groups that strongly bind to the ZnO surfaces, that make the particles separate, and the protruding groups point outwards into the solution, thus facilitating water solubility and stability of particles of all the capping agents used in this research.

Generally, ZnO can be stabilized by coating with titania, silica, silane, carboxylic and polymeric coatings [2]. The coating process is a self-assembly process and occurs due to several types of forces. They are namely intermolecular forces and chemical

Table 6.1 Structural formulas and coordinating groups of the capping agents

Capping agent / Chemical formula	Structural formula	Coordinating groups	
		Binding	Protruding
APTES / $\text{H}_2\text{NC}_3\text{H}_6\text{Si}(\text{OC}_2\text{H}_5)_3$		-Si-OC ₂ H ₅	-NH ₂
TEOS / $\text{Si}(\text{OC}_2\text{H}_5)_4$		-Si-OH	-OC ₂ H ₅
TiO ₂ / $\text{Ti}(\text{OC}_3\text{H}_7)_4$		-Ti-OH	-Ti-OH
MS / $\text{HO}_2\text{C}_2\text{H}(\text{SH})\text{CH}_2\text{CO}_2\text{H}$		-SH (thiol)	-COOH
PVP / $(\text{C}_6\text{H}_9\text{OH})_n$ Z60 / $\text{H}_2\text{NC}_2\text{H}_4\text{NHC}_3\text{H}_6\text{-Si}(\text{OCH}_3)_3$ Z61 / $\text{H}_2\text{NC}_2\text{H}_4\text{NHC}_3\text{H}_6\text{-Si}(\text{OH})_3$		C=O (carbonyl) -Si-OCH ₃ -Si-OH	-CH-CH ₂ - -NH ₂ -NH ₂

bondings. Intermolecular forces such as van der Waals are classified as physisorption, while chemical bondings such as ionic bonding are classified as chemisorption. No surface reactions are involved for physisorption, and the forces are weak with long range bonding. Conversely, chemisorption involves surface reaction like dissociation and

reconstruction. They are mainly strong and short range.

APTES has 2 types of reactive groups namely the silanol and amino groups. The silanol groups are highly reactive, and can form covalent bonds with -OH groups on ZnO surface. In addition, the trisilanol group has a high enthalpy of adsorption [182] and as such they are adsorbed readily onto ZnO surfaces forming encapsulation based on siloxane units. The strong siloxane encapsulation prevents further growth of ZnO particles and improves its optical properties. The high polarity of trisilanol group gave APTES colloidal good stability. This helps to explain the good results obtained for various analyses thus proving the APTES effective capping ability. Figure 6.1 (a) illustrates the surface capping mechanism of APTES on ZnO particles.

As observed on the TEOS coated particles, hydrolyzed TEOS also acted as a good stabilizer and prevented particle coalescence. The silica show strong stability at neutral pH since its isoelectric point is at pH 2 [183]. The hydroxyl side chain groups make TEOS capped ZnO stable in water solutions and are suitable for *in-vivo* bio-applications. They could be further functionalized with targeting ligands (such as peptides, antibodies or small-molecule inhibitors) that allow the conjugation of ZnO nanoparticles to biological systems. The slightly poorer capping ability of TEOS compared to APTES was because the Si-OH had an equivalent tendency to join together or being adsorbed onto ZnO surface. Hence the particles could aggregate and form larger particles after aging and long term storage as shown in Figure 6.1(b).

Hydrolyzed TiO₂ also has hydroxyl side groups, which can condense onto ZnO surface, similar to SiO₂. However, the Ti-OH is much more reactive than the Si-OH, and therefore, the tendency of joining within Titania itself is higher. In the meantime, the isoelectric

point of TiO_2 is at around pH 6 [184], which is about the same as the ZnO-methanol solution, thus it is not stable, compared to the APTES colloids. TiO_2 may have effectively capped onto the individual ZnO particles, but agglomeration among the TiO_2 capped particles would still occur due to the pH of the solution. The surface reaction for particle aggregation is similar to TEOS as illustrated in Figure 6.1(b).

Mercaptosuccinic acid, like the APTES, has two functional groups, which are the thiol and carboxyl groups. The thiol group formed a thiolate bond with ZnO. These covalent bondings helped to cap and immobilize the ZnO particles. However, the carboxyl group of MS acid was highly polar and hydrophilic. They tend to form hydrogen bonding among themselves (as shown in Fig. 6.1 (c)) as well as with the methanol medium. As a result they enhanced the precipitation of ZnO particles, thus leading to agglomeration in the colloidal [184]. Meanwhile, the binding affinity of thiols to ZnO surface is only moderate, as reported by other authors [185]. These explain the observations of the white particulates observed upon addition of MS acid as shown in Fig.4.6(f).

Polyvinylpyrrolidone (PVP) is a homopolymer with a carbonyl group, $\text{C}=\text{O}$. Due to the difference of electronegativities of carbon and oxygen, $\text{C}=\text{O}$ bond became moderately polar. A slight positive charge was accumulated on the carbon and this positive polarity allowed it to bind onto the surface of ZnO particles. However, because of the moderate polarity of PVP, the effectiveness of the functional group adsorbed on ZnO surface would depend on the acidic or basic strength of the solvent [186, 187]. It was noted that PVP had a lower basic strength than methanol, and thus the carbonyl group was unable to adsorb strongly onto ZnO. This resulted in poor dispersion stability and the ZnO particles were more easily coagulated together.

Z60 and Z61 have similar molecular structure with APTES, but they are with longer side chains containing double amino groups. This makes the Z60 and Z61 capped particles more stable (see Figure 6.1(e)) and with a higher bio-conjugation ability. This is the key novelty of this research as no other publication has ever reported the use of these materials as capping agent or for bio-applications. In comparing Z60 and Z61, Z61 is fully hydrolyzed, therefore, has three $-OH$ groups, while Z60 is only partially hydrolyzed by the water content in the colloid under room temperature. The fewer $-OH$ groups in Z60 reduced the chance of particle agglomeration, and is therefore more stable than Z61.

Figure 6.1 Capping mechanisms of different capping agents. Capping by APTES (a), TEOS (b), MS (c) and TiO_2 (d) respectively, showing APTES has better stability, TEOS, TiO_2 and MS have the possibility of joining two particles together, and Z61 (e), binding to ZnO surface and making ZnO particle stabilized.

In summary, APTES was found to be effective to cap ZnO particles thereby providing small particle size and stable colloidal solution. The amino side groups are readily suitable for bio-imaging applications. But the Z60 and Z61 are better than APTES in terms of their smaller size and more stable colloids, and their better optical properties and stronger bio-imaging ability. TEOS and TiO_2 also showed some promising capping capabilities, but their long term stability of colloids was not satisfactory (TEOS) nor were their optical properties good enough (TiO_2). The other capping agents (MS, PVP) did not show a good capping capability, and were therefore not selected for further optical and bio-imaging studies.

Once the ZnO surface has been modified by functional groups such as amino, thiol, and carboxyl, biomolecules such as proteins, enzymes, antibodies, oligonucleotides, etc. can be linked to the nanoparticles following standard covalent bioconjugation protocols as discussed in Section 2.3.1. Figure 6.2 further illustrates the chemical reactions of surface groups with the biomolecules. Carboxyl-modified nanoparticles have pendent carboxylic acids, making them suitable for covalent coupling of proteins and other amine-containing biomolecules via water-soluble carbodiimide reagents [131]. Disulfide-modified oligonucleotides can be immobilized onto thiol-functionalized nanoparticles by disulfide-coupling chemistry [188]. Amine-modified nanoparticles (APTES, Z60 and Z61 modified ZnO nanocrystals) can be coupled to a wide variety of haptens and drugs via succinimidyl esters, iso(thio)cyanates and carboxylic acid. An advantage for this carboxylate-amine condensation is that most of the proteins contain primary amine and carboxylic acid groups; therefore, they do not need any further chemical modifications before bioconjugation to cells [7].

Figure 6.2 Bioconjugation protocols for the attachment of biomolecules onto the surfaces of nanoparticles. Surface modifications by carboxyl group (a), thiol group (b) and amino group (c) respectively [189].

6.4 Effects of dopants on ZnO particle size and optical properties

The doping of ZnO nanocrystals in this study has played the major role for the successful bio-applications of ZnO NCs. Since ZnO intrinsically emit in UV range, doping and surface modification made the photoluminescence emission shift to visible range, making the bio-images colourful and clearly visible within the cells. This is the major function of dopants. However, since the substitution of Zn^{2+} sites by the doping cations with smaller ionic radii generates inevitable lattice defects, there may be some side effects on the properties of ZnO. First, we looked at the effects of dopants types and concentrations on the particle size. We calculated the average particle size of each type of doped ZnO nanoparticles within 30 days after synthesis. Figure 6.3 shows the plots of the average particle sizes of all the Cu, Ni and Co doped ZnO capped by Z60, Z61, TiO_2 and APTES (Aps) respectively. There is no clear correlation between particle size and dopant concentration for all the dopants. Cu doped ZnO NCs showed relatively smaller size than

Co and Ni doped ZnO NCs. We attempted to link this behaviour to the ionic radii of the dopants, but did not find a direct correlation as the ionic radius of Co^{2+} ($r=0.58$) is smaller than that of Zn^{2+} (0.60), although they are slightly larger than Cu^{2+} ($r=0.57$). The effects of surface capping agents on particle size were more obvious. Generally, all the Z60, Z61 and APTES capped ZnO particles were in smaller size (measured 1 to 2 nm), and all the TiO_2 capped ZnO NCs were relatively larger (measured 4 to 5 nm). This further confirmed the importance of surface capping in size control. Meanwhile, this was good and convenient in our materials design because we could control the size by synthesizing parameters, and surface capping and modifying the optical properties by adjusting the dopant type and doping concentration.

Figure 6.3 Effects of dopants and their concentrations on particle size. (average size within 30 days after synthesis). The standard deviations of these size data are between 10 to 30%.

The influences of dopants and their concentrations on PL properties have been demonstrated clearly in Section 4.2.1 and summarized in Table 4.9. In general, all the

three dopants (Cu, Ni, Co) could shift the PL emission peaks to visible wavelength range, making coloured and clearly visible bio-images on both human cells and plant systems. In terms of the PL emission red shift values (excitation at 325 nm), Co caused about 40 to 45 nm red shift, Ni caused about 30 nm, and Cu caused about 20nm red shift. For excitation at 488 nm, all the doped ZnO NCs emit in green range (520 to 580 nm), which is not very critical. The PL intensity was affected by all the dopants, with Ni doped ZnO having higher intensity than Co and Cu (excitation at 325 nm). The later two dopants were comparable. When excited at 488 nm, Co and Ni doped ZnO nanocrystals had similar high intensities, while Cu doped ZnO had the lowest intensity. Similar bandgap changes for Cu, Co and Ni doped ZnO were also detected by other authors, and lower PL intensities have also been reported [122, 190]. As indicated by the PL spectra of uncapped 5%Co-ZnO and capped 5%Co-ZnO (Figure 4.31), the capping agents also helped in the red-shift of PL emission to different extents. The recently reported blue emission from ZnO QDs was from the formation of surface ZnO/oleic acid complexes [33]. Therefore, the PL properties of NCs are the results of combined effects by both doping and surface capping. Due to the well controlled synthesis process and surface capping techniques, all our nanoparticles possessed well defined PL emissions, without unwanted peaks from surface defects or doping defects such as a triplet PL structure from uncontrolled Cu impurity [71], green emission from O vacancies (V_o) or Zn interstitials (Zn_i) that have been reported by many other researchers [69, 72].

The bio-imaging colours in cells matched very well with the PL emission wavelengths as demonstrated in Figure 5.2. It must be highlighted that the bio-imaging intensity and labelling quality (detailed cell structures) are determined by more factors, such as the cell uptake of NCs, conjugation strength, and cytotoxicity. We will discuss the bio-imaging mechanism in the next section.

6.5 Mechanism of dual colour bio-imaging

The major mechanism of bio-imaging is the uptake of nanoparticles by cells through endocytosis [13, 49, 191]. Nanoparticles are then transported to vesicular compartments around the nucleus of the cell where they remain [134, 192]. Some authors have claimed receptor-mediated uptake [13], whereas others have reported non-specific uptake. As observed by many authors that living cells selectively ingest colloidal nanocrystals, the detailed transfer mechanisms are still not well understood. Certainly, the ingestion of nanoparticles depends very much on the particular protocol. In our study, we found that the surface chemistry and the particle size of the nanocrystals are the most important factors. As shown in Fig. 2.17 and 6.2, the surface chemical groups of QDs must be able to conjugate to the biomolecules. In addition, hydroxylated QDs were also used for *in situ* hybridization with oligonucleotides [170], which are usually in the nuclei of the cells. After the required surface conditions are met, for the nanoparticles to penetrate through the wall of nucleus, the size of the nanoparticles should be as small as possible. The dual colour images in Fig. 5.2 can be explained as follows: the blue colour emission in the nuclei was caused by the PL emission from the smaller nanocrystals. These particles could penetrate into the nucleus and emit in a blue-shifted wavelength compared to the relatively larger particles, which remain in the cytoplasm region and emit in a longer wavelength, therefore, in turquoise colour. The multi-colour imaging is usually obtained by pre-mixing different QDs or adding different QDs in a sequence [193], in which, cross-effects are inevitable, but these complex procedures are not preferred. Since our nanoparticles were in the quantum confinement range because the exciton Bohr radius of ZnO is 2.34 nm [194], we can conclude that for high quality bio-imaging, the ZnO QDs must be in the quantum confinement range, and preferably below 5 nm for the labelling of nuclei. Therefore, for the dual colour high quality bio-imaging of cells by ZnO

nanocrystals, the surface capping groups of the nanocrystals must be able to conjugate to the cells, and the size of the nanoparticles must be in the quantum confinement range, ideally between 5 nm.

Chapter Seven: Conclusions and Future Work

7.1 Conclusions

In the present research, a soft chemical synthesis method was adopted to synthesize pure ZnO nanocrystal colloids and Co, Ni, and Cu doped ZnO nanocrystals. The process parameters were optimized towards controlled particle size within quantum confinement range (below 10 nm), stable colloidal solutions for more than 30 days, and excellent photoluminescence properties, thus exhibiting good bio-imaging capabilities. Several types of surface capping agents were utilized to control the growth of the nanoparticles and to functionalize the surfaces for further bio-imaging applications. The materials properties were characterized in terms of chemical purity, nanocrystal shape, size, lattice structures and size growing trends. The effects of surface capping agents, dopant type, and concentration on both the materials properties and optical properties were investigated. The photoluminescence excitation and emission properties were measured and successfully modelled by the multimode Brownian oscillator model. Bio-imaging was conducted using the synthesized nanocrystals on both human cells and plant cells. Finally, the cytotoxicities of all the nanocrystals on human cells and plant cells were evaluated.

The following conclusions can be drawn based on the experimental and modelling results:

- (i) Through investigations on the chemical synthesis processes, it has been proven that the selection of suitable solvent and precursors, low hydrolysis ratio, superheated condition (temperature higher than the boiling point of the solvent), suitable refluxing time, and stoichiometric doping are the key factors to ensure the achievement of pure ZnO and accurately doped ZnO nanocrystals, which are

stabilized in colloidal solutions. The optimum synthesis parameters for small and stable particle size of pure ZnO and doped ZnO nanocrystals are: precursor concentration 0.15 mol/L, reaction temperature of $66.5 \pm 0.5^\circ\text{C}$, refluxing time of 6 hours and 30-45 minutes, fast cooling by ice water to $4-5^\circ\text{C}$, and add capping agent immediately in proper amount.

(ii) It has been proven that surface capping is very effective in controlling the particle size in the chemical synthesis processes. The capping agents selected in this study have covered almost all the potential bio-compatible surface chemical groups, including amino-, thiol-, carboxyl-, hydroxyl-, and polymers. The most stable and smallest particle size was obtained by using the double amino- containing capping agents, Z60 and Z61. This is a new discovery in this research as no earlier work has been reported. Smaller particle size below 10 nm has been achieved using Z60 and Z61 as capping agents for both pure ZnO and doped ZnO. The colloids of these nanoparticles were stable for 30-45 days. Particles capped by the single amino-group was also small, but the colloids were less stable (about 30 days). TiO_2 capped particles have slightly larger size and slightly less stability (about 30 days). The nanocrystals have spherical shapes even in their grown up state due to restriction by the strong capping agent. The lattice structures of all the synthesized ZnO nanocrystals are the Wurtzite P63mc hexagonal structure. With doping by Co, Ni, or Cu cations, the Zn^{2+} sites are substituted by the doping cations, and the lattice structures showed layered crystal structures. This layered structure was confirmed by HRTEM images on the larger particles, where layered structures were presented within particles and on the surfaces of particles.

(iii) The optical properties of all the pure ZnO and doped ZnO capped by different capping agents have been examined. This is the first comprehensive study on the photoluminescence and bandgap modifications of ZnO by the dopants and surface

capping. The PL emission wavelengths and intensities of the synthesized nanocrystals are the combined effects of the dopant and the surface capping. In general, TiO_2 capping does not shift the emission peaks of pure ZnO and doped ZnO. The TiO_2 capped pure ZnO nanocrystals had a higher intensity than the pure ZnO (uncapped), but all the doped ZnO capped by TiO_2 had lower intensities than the other Z60 and Z61 capped nanocrystals. Therefore, Z60 and Z61 are the preferred capping agents due to the higher PL emissions and red shifted bandgaps. The Z60 and Z61 capped pure ZnO had PL emission red shift of 20-30 nm, 5%Cu doped ZnO, 10-20 nm, 10%Ni doped ZnO, 20-40 nm, and 5%Co doped ZnO, 20-50 nm. Through these bandgap modifications, the intrinsically UV emission ZnO nanocrystals have been rendered excellent in capability for bio-imaging applications.

- (iv) The quantum yields of the synthesized nanoparticles are in the range of 78% to 94%, which is higher than most of the quantum dots reported. The PL emission line shapes are modelled by the multimode Brownian oscillator model. The perfect fitting of theoretical curves to experimental curves was obtained for pure ZnO capped by Z61. The good fitting was obtained on 5%Ni doped ZnO, which has a side band in the PL curve. These proved that the MBO model is also suitable for ZnO nanocrystals in the quantum size range. For the modelling of doped ZnO and more complex materials, more variables may be built into the software so that better fitting and understanding of all the influencing factors for complex PL shapes can be obtained.
- (v) Bio-images on human tumour cells demonstrated the excellent bio-conjugation properties of the surface modified ZnO nanocrystals as well as their high emission intensities after their uptake by the cells. These indicate the high quality of the synthesized nanocrystals. The unique dual colour images are the results of quantum

confinement effects by small size range and the high bio-compatibility of the nanocrystal. Bio-images on plant cells confirmed the suitability of the nanocrystals for imaging plant systems. The nanocrystals were absorbed and well transported inside the plant; therefore, stronger PL emissions were detected, especially in the vascular region and cell walls. Cytotoxicity tests demonstrated that our nanocrystals are non-toxic up to the concentration of 1000 $\mu\text{g/mL}$, which is higher than all the required dosages for *in vitro* and *in vivo* bio-imaging applications.

In summary, the pure ZnO and Co-doped ZnO nanocrystals synthesized in this research have met the required properties simultaneously for high quality bio-imaging of human cells and plant systems. The unique dual colour high quality bio-imaging capability has been realized by the combination of nano-size, suitable surface functional groups, high intrinsic optical properties, the non-toxic nature and the high colloidal stability of the synthesized nanocrystals. Therefore, these bio-markers are suitable for both *in vitro* and *in vivo* bio-imaging applications. These bio-markers can be further attached with specific antibodies for specific cancer detection.

7.2 Future work

Because of the promising results in this research, more efforts should be made in the following aspects to expand the knowledge in this field, and to explore more applications using high quality nanocrystals.

- 1) Further studies on the doping mechanisms and the lattice changes due to the substitution of Zn^{2+} by Co^{2+} , Cu^{2+} and Ni^{2+} dopants as well as other dopants should be conducted. This may be especially useful for growing nanowires as a preferential

growth plane.

- 2) Bio-imaging tests on other human cells and further imaging and cytotoxicity tests on small animals should be performed. *In vivo* tests on rat have been started already.
- 3) Improvement of the MBO modelling software to include more variable to suit more complex PL line shapes should be carried out.
- 4) Studies on using ZnO nanocrystals as drug delivery carriers by attaching medicines to the surfaces, or through antibody-antigen, biotin, peptide and other protocols as listed in Fig.2.16 and Table 2.3 should be conducted. This is especially useful for cancer early detection and medicine.

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Publications

Journal papers:

1. Y.L. Wu, A.I.Y. Tok, F.Y.C. Boey, X.T. Zeng and X.H. Zhang, "Chemical Synthesis of ZnO Nanocrystals", IEEE Transactions on Nanotechnology, Vol. 6, No. 5, p497-503, 2007.
2. Y.L. Wu, A.I.Y. Tok, F.Y.C. Boey, X.T. Zeng and X.H. Zhang, "Surface Modification of ZnO Nanocrystals", Applied Surface Science, Vol. 253, p5473-5479, 2007.
3. Y.L. Wu, C.S. Lim, S. Fu, A.I.Y. Tok, H.M. Lau, F.Y.C. Boey, X.T. Zeng, "Surface modifications of ZnO quantum dots for bio-imaging", NANOTECHNOLOGY, Vol. 18, p215604 (9pp), 2007.
4. Y L Wu, S Fu, A I Y Tok, X T Zeng, C S Lim, L C Kwek, and F C Y Boey, "A dual colored bio-marker made of doped ZnO nanocrystals" NANOTECHNOLOGY, Vol. 19, p345605 (9pp), 2008.

Conference paper:

5. Y.L. Wu, S. Fu, A.I.Y. Tok, X.T. Zeng, C.S. Lim, and F.C.Y. Boey, "Band gap modification of zinc oxide nanocrystals by doping and surface modification for bio-imaging", Conference proceedings, 5th International Symposium on Nanomanufacturing (ISNM-5), Singapore-MIT alliance, 23 – 25 January 2008.
6. Linda Y.L. Wu, G.J. Loh, S. Fu, A.I.Y. Tok, X.T. Zeng, L.C. Kwek, and F.C.Y. Boey, "Doped ZnO Colloids for Cancer Detection - Bio-Imaging and Cytotoxicity Study", MRS 2009 Spring Meeting, San Francisco, CA, USA, 13-17 April 2009.

Appendix I to VIII

Appendix I Description of MBO theory and parameters

The Brownian oscillator model introduces dissipation mechanisms into the harmonic quantum oscillators by coupling the primary oscillators to a continuous distribution of secondary oscillators. In this model, the system is taken to be a two electronic-level system with some primary nuclear coordinates coupled linearly to the electronic systems:

$$H = |g\rangle H_g \langle g| + |e\rangle H_e \langle e| + H' \quad (\text{A1.1})$$

where

$$H_g = \sum_j \left[\frac{p_j^2}{2m_j} + \frac{1}{2} m_j \omega_j^2 q_j^2 \right] \quad (\text{A1.2})$$

$$H_e = \hbar \omega_{eg}^0 + \sum_j \left[\frac{p_j^2}{2m_j} + \frac{1}{2} m_j \omega_j^2 (q_j + d_j)^2 \right] \quad (\text{A1.3})$$

$$H' = \sum_n \left[\frac{p_n^2}{2m_n} + \frac{1}{2} m_n \omega_n^2 \left(Q_n - \sum_j \frac{c_{nj} q_j}{m_n \omega_n^2} \right)^2 \right] \quad (\text{A1.4})$$

Here p_j (P_n), q_j (Q_n), m_j (m_n), and ω_j (ω_n) represent the momentum, the coordinate, the mass, and the angular frequency of the j th (n th) nuclear mode of the primary (bath) oscillators, respectively. d_j is the displacement for the j th nuclear mode in the excited electronic state. H' describes the bath oscillators and their coupling to the primary oscillators with a coupling strength C_{nj} . The linear absorption and the relaxed fluorescence line shapes can be obtained from a spectral broadening function $g(t)$. Using a second-order cumulant expansion, the spectral broadening function $g(t)$ can be expressed in terms of the frequency-domain correlation function $C''(\omega)$:

$$g(t) = \frac{1}{2\pi} \int_{-\infty}^{\infty} d\omega \frac{C''(\omega)}{\omega^2} [1 + \coth(\beta \hbar \omega / 2)] [e^{-i\omega t} + i\omega t - 1] \quad (\text{A1.5})$$

$$C''(\omega) = -i \int_{-\infty}^{\infty} dt e^{-i\omega t} \tilde{C}(t) \quad (\text{A1.6})$$

$$\tilde{C}(t) = -\frac{1}{2\hbar^2}[\langle U(t)U(0)\rho_{\frac{1}{2}} \rangle - \langle U(0)U(t)\rho_{\frac{1}{2}} \rangle] \quad (\text{A1.7})$$

Where $U \equiv H_e - H_g$ is the collective bath coordinate representing the coupling of the chromophore to its environment responsible for spectral shifts and broadening.

We note that $C''(\omega)$ is in fact the imaginary part of the frequency-domain correlation function $C(\omega)$ with its real part $C'(\omega)$ related to $C''(\omega)$ by:

$$C'(\omega) = \coth \frac{\beta \hbar \omega}{2} C''(\omega) \quad (\text{A1.8})$$

The function $C''(\omega)$ is a summation of individual contributions from each primary oscillator:

$$C''(\omega) = \sum_j C_j''(\omega) \quad (\text{A1.9})$$

where

$$C_j''(\omega) = \frac{2\lambda_j \omega_j^2 \omega \gamma_j(\omega)}{\omega^2 \gamma_j^2(\omega) + [\omega_j^2 + \omega \Sigma_j(\omega) - \omega^2]^2} \quad (\text{A1.10})$$

Here $2\lambda_j$ is the Stokes shift for the j th mode,

$$2\lambda_j = \frac{m_j \omega_j^2 d_j^2}{\hbar} \quad (\text{A1.11})$$

$\gamma_j(\omega)$ represents the spectral distribution of the coupling,

$$\gamma_j(\omega) = \frac{\pi}{m_j} \sum_n \frac{c_{nj}^2}{2m_n \omega_n^2} [\delta(\omega - \omega_n) + \delta(\omega + \omega_n)] \quad (\text{A1.12})$$

One can alternatively write $\lambda_j = S_j \hbar \omega_j$ where S_j is the dimensionless Huang-Rhys factor characterizing the strength of electro-LO phonon interactions. The value of S_j can vary from much less than unity (e.g. $S=0.2$ for weak coupling) to equal to greater than unity (e.g. $S=10$ for strong coupling).

To facilitate numerical computations, the real and imaginary parts of the line shape

function $g(t) = g'(t) + ig''(t)$ can be written in terms of $C''(\omega)$ as follows:

$$g'(t) = \frac{1}{\pi} \int_0^\infty d\omega \frac{1 - \cos(\omega t)}{\omega^2} \coth(\beta \hbar \omega / 2) C''(\omega) \quad (\text{A1.13})$$

$$g''(t) = \frac{i}{\pi} \int_0^\infty d\omega \frac{\sin(\omega t) - \omega t}{\omega^2} C''(\omega) \quad (\text{A1.14})$$

The PL line shape can be then obtained from the spectral response function, $g(t)$:

$$I_{\text{PL}}(\omega) = \frac{1}{\pi} \text{Re} \int_0^\infty \exp[i(\omega - \omega_{\text{eg}}^0 + \lambda)t - g^*(t)] dt \quad (\text{A1.15})$$

where $g^*(t)$ is the complex conjugate of $g(t)$. Using Equation (A1.15) and the computer software, after input the PL emission spectrum, we can calculate the following parameters:

- γ_j (cm^{-1}): being the spectral distribution function describing the coupling between the primary oscillator and the secondary bath oscillators,
- ω_j (cm^{-1}): being the angular frequency of the j th nuclear mode of the primary (bath) oscillator,
- S: being the dimensionless Huang-Rhys factor characterizing the strength of electron-LO phonon interactions
- T (K): being the temperature of the PL measurement,
- ω_{eg} : being the 0-0 transition electronic energy gap eV, corresponding to zero phonon line (ZPL).

The S factor is a key parameter characterizing the electron-LO-phonon coupling strength, which has been proven to govern the relative intensity of the ZPL and its successive sidebands.

Appendix II Calculation of capping amount

Calculation of capping agents to be added to ZnO colloids

The principle is that 2 molecules of the capping agent should be used for every 1 nm² of the surface area of the ZnO quantum dot.

Assuming 0.03 moles of ZnO in 160g of solution,

In 20 g of solution, $0.03 \text{ mol} \div 8 = 0.00375 \text{ mol}$ of ZnO

$$\begin{aligned}\text{Mass of ZnO} &= 0.00375 \text{ mol} \times M_r \text{ of ZnO} = 0.00375 \text{ mol} \times 81.4084 \text{ g/mol} \\ &= 0.3053 \text{ g}\end{aligned}$$

$$\begin{aligned}\text{Total volume of ZnO} &= 0.3053 \text{ g} \div \text{density of ZnO} = 0.3053 \text{ g} \div 5.606 \text{ g/cm}^3 \\ &= 0.0545 \text{ cm}^3 \\ &= 5.446 \times 10^{19} \text{ nm}^3\end{aligned}$$

Assuming that the particle size is 1 nm in diameter, we can calculate the volume of each particle.

$$\text{Volume of a particle} = \frac{4}{3} \pi r^3 = \frac{4}{3} \pi (0.5 \text{ nm})^3 = 0.5236 \text{ nm}^3$$

$$\begin{aligned}\text{Total number of ZnO particles} &= (5.446 \times 10^{19} \text{ nm}^3) \div 0.5236 \text{ nm}^3 \\ &= 1.040 \times 10^{20} \text{ particles}\end{aligned}$$

$$\begin{aligned}\text{Total surface area of the ZnO particles} &= 4\pi r^2 \times (1.040 \times 10^{20} \text{ particles}) \\ &= 4\pi (0.5 \text{ nm})^2 \times 1.040 \times 10^{20} \text{ particles} \\ &= 3.267 \times 10^{20} \text{ nm}^2\end{aligned}$$

Following the principle as stated above,

$$\begin{aligned}\text{Total number of molecules of capping agent needed} &= (3.267 \times 10^{20} \text{ nm}^2) \times 2 \\ &= 6.535 \times 10^{20} \text{ molecules}\end{aligned}$$

Since 1 mole = 6.02×10^{23} molecules,

$$\begin{aligned}\text{Number of moles of capping agent needed} &= (6.535 \times 10^{20} \text{ molecules}) \div (6.02 \times 10^{23} \text{ molecules}) \\ &= 0.001086 \text{ moles}\end{aligned}$$

For each separate capping agent, we can calculate the weight needed.

Z60: Mr of 222

$$\begin{aligned}\rightarrow \text{Mass needed} &= 0.001086 \text{ moles} \times 222 \\ &= \underline{\underline{0.241 \text{ g}}}\end{aligned}$$

Z61: Mr of 180.0855

$$\begin{aligned}\rightarrow \text{Mass needed} &= 0.001086 \text{ moles} \times 180.0855 \\ &= \underline{\underline{0.195 \text{ g}}}\end{aligned}$$

3-Aminopropyl Triethoxysilane: Mr of 221.37

→ Mass needed = 0.001086 moles x 221.37

$$= \underline{0.240 \text{ g}}$$

TiO₂ sol:

Total weight of the sol-gel solution

= (2 x 87.83 g) of ethanol + 1.77 g of DI H₂O + 0.87 g of HCl + 5 g of Ti(OPr)₄

= 183.3 g

However, we expect a 2% loss in the total volume of solution after stirring.

Resultant weight of sol-gel solution = 183.3 g x 98%

$$= 179.634 \text{ g}$$

As the number of moles Ti will not change, we can calculate the amount present.

Number of moles of Ti(OPr)₄ in original solution = 5 g / 284.26

$$= 0.017589 \text{ moles}$$

Mass of TiO₂ needed = (0.001086 mol ÷ 0.017589 moles) x 179.634 g

$$= 11.0908 \text{ g}$$

SiO₂ sol:

Total weight of the sol-gel solution

= (161 g) of ethanol + 12.28 g of DI H₂O + 0.33 g of HCl + 47.30 g of TEOS

= 220.91 g

Resultant weight of solution after 2% weight loss = 216.49 g

Original Number of moles of TEOS = 47.30/208.33 = 0.227044 moles

Mass of SiO₂ sol needed = (0.001086 mol ÷ 0.227044 moles) x 216.49 g

$$= 1.0355 \text{ g}$$

Spreadsheet for Capping Amount Calculation

Calculation of amount of capping agent (based on 2 molecules on 1 nm ² of surface)																
Part. Size (nm)	Part. Area (nm ²)	Mol ZnO in 20g	Mass ZnO (in 20g)	Std Vol ZnO (nm ³)	Vol/particle (nm ³)	No of particle	Total part area (nm ²)	Molecules Cap 2 Mole 1nm ²	Moles Cap	Mass cap APTES	Mass sol-gel SiO2	Mass Ms Mr=150.15	Mass Mp Mr=196.34	Mass PVP Mr=1000	Mass Z60 Mr=222	Mass Z61 Mr=180.085
1	3.14159	0.003797	0.309	5.508E+19	0.5235983	1.05196E+20	3.30481E+20	6.60963E+20	0.001097398	0.242930904	1.046385739	0.164774248	0.215463	0.5486988	0.243622	0.19762484
2	12.56636	0.003797	0.309	5.508E+19	4.1887867	1.31494E+19	1.65241E+20	3.30481E+20	0.000548699	0.121465452	0.523192869	0.082387124	0.107732	0.2743494	0.121811	0.09881242
5	78.53975	0.003797	0.309	5.508E+19	65.449792	8.41564E+17	6.60963E+19	1.32193E+20	0.00021948	0.048586181	0.209277148	0.03295485	0.043093	0.1097398	0.048724	0.03952497
6	113.09724	0.003797	0.309	5.508E+19	113.09724	4.87016E+17	5.50802E+19	1.1016E+20	0.0001829	0.040488484	0.174397623	0.027462375	0.035911	0.0914498	0.040604	0.03293747
10	314.159	0.003797	0.309	5.508E+19	523.59833	1.05196E+17	3.30481E+19	6.60963E+19	0.00010974	0.02429309	0.104638574	0.016477425	0.021546	0.0548699	0.024362	0.01976248
20	1256.636	0.003797	0.309	5.508E+19	4188.7867	1.31494E+16	1.65241E+19	3.30481E+19	5.48699E-05	0.012146545	0.052319287	0.008238712	0.010773	0.0274349	0.012181	0.00988124
25	1963.4938	0.003797	0.309	5.508E+19	8181.224	6.73252E+15	1.32193E+19	2.64385E+19	4.38959E-05	0.009717236	0.04185543	0.00659097	0.008619	0.021948	0.009745	0.00790499
30	2827.431	0.003797	0.309	5.508E+19	14137.155	3.89613E+15	1.1016E+19	2.20321E+19	3.65799E-05	0.008097697	0.034879525	0.005492475	0.007182	0.01829	0.008121	0.00658749
35	3848.4478	0.003797	0.309	5.508E+19	22449.279	2.45354E+15	9.44232E+18	1.88846E+19	3.13542E-05	0.006940883	0.029896735	0.004707836	0.006156	0.0156771	0.006961	0.00564642
40	5026.544	0.003797	0.309	5.508E+19	33510.293	1.64368E+15	8.26203E+18	1.65241E+19	2.74349E-05	0.006073273	0.026159643	0.004119356	0.005387	0.0137175	0.006091	0.00494062
45	6361.7198	0.003797	0.309	5.508E+19	47712.898	1.15441E+15	7.34403E+18	1.46881E+19	2.43866E-05	0.005398465	0.023253016	0.00366165	0.004788	0.0121933	0.005414	0.00439166
50	7853.975	0.003797	0.309	5.508E+19	65449.792	8.41564E+14	6.60963E+18	1.32193E+19	2.1948E-05	0.004858618	0.020927715	0.003295485	0.004309	0.010974	0.004872	0.0039525
60	11309.724	0.003797	0.309	5.508E+19	113097.24	4.87016E+14	5.50802E+18	1.1016E+19	1.829E-05	0.004048848	0.017439762	0.002746237	0.003591	0.009145	0.00406	0.00329375

Appendix III Product description of MG-63 cell line

Product Description

Before submitting an order you will be asked to read and accept the terms and conditions of ATCC's Material Transfer Agreement or, in certain cases, an MTA specified by the depositing institution.

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Cell Biology

ATCC® Number:	CRL-1427™	Order this Item	Price:	
Designations:	MG-63		Depositors:	A Billiau
Biosafety Level:	1		Shipped:	frozen
Medium	& See Propagation		Growth Properties:	adherent
Serum:				
Organism:	Homo sapiens (human)		Morphology:	fibroblast
Source:	Organ: bone Disease: osteosarcoma			
Cellular Products:	interferon			
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.			

Related Cell Culture Products

Applications:	transfection host(technology from amaxa Roche FuGENE® Transfection Reagents)
Receptors:	transforming growth factor beta (TGF-beta) RI, expressed transforming growth factor beta (TGF-beta) RII, expressed
DNA Profile (STR):	Amelogenin: X,Y CSF1PO: 10,12 D13S317: 11 D16S539: 11 D5S818: 11,12 D7S820: 10 TH01: 9.3 TPOX: 8,11 vWA: 16,19
Cytogenetic Analysis:	This is a hypotriploid human cell line. The modal chromosome number was 66 occurring in 44% of cells. The rate of cells with higher ploidies was 2.0%. Eighteen to 19 marker chromosomes were common to all cells.
Age:	14 years
Gender:	male
Ethnicity:	Caucasian
Comments:	High levels of interferon production can be induced using polyinosinic - polycytidylic acid, cycloheximide and actinomycin D.
Propagation:	ATCC complete growth medium: The base medium for this cell line is .ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003. To

	make the complete growth medium, add the following components to the base medium: heat-inactivated fetal bovine serum to a final concentration of 10%. Atmosphere: air, 95%; carbon dioxide (CO ₂), 5% Temperature: 37.0°C
--	--

Subculturing: **Protocol:**

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor.
3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).
Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.
4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.
5. Add appropriate aliquots of the cell suspension to new culture vessels.
6. Incubate cultures at 37°C.

Subcultivation Ratio: A subcultivation ratio of 1:4 to 1:8 is recommended
Medium Renewal: 2 to 3 times per week

Preservation:	Freeze medium: Complete growth medium supplemented with 5% (v/v) DMSO Storage temperature: liquid nitrogen vapor phase
Related Products:	Recommended medium (without the additional supplements or serum described under ATCC Medium):ATCC 30-2003
References:	1058: Billiau A, et al. Human interferon: mass production in a newly established cell line, MG-63. Antimicrob. Agents Chemother. 12: 11-15, 1977. PubMed: 883813 23014: Takeuchi Y, et al. Relationship between actions of transforming growth factor (TGF)-beta and cell surface expression of its receptors in clonal osteoblastic cells. J. Cell. Physiol. 162: 315-321, 1995. PubMed: 7860639 32445: Yee A, et al. Biochemical characterization of the human cyclin-dependent protein kinase activating kinase. J. Biol. Chem. 271: 471-477, 1996. PubMed: 8550604

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Appendix IV Product description of U937 cell line

Product Description

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Cell Biology

ATCC®
Number:
Designations:
Biosafety
Level:
Medium
Serum:
Organism:

CRL-1593.2™
U-937
1
& See Propagation
Homo sapiens (human)

Order this Item

Price:

Depositors:
Shipped:

Growth Properties:

Morphology:

H Koren
frozen
suspension
monocyte

Source:
Cellular
Products:

Disease: histiocytic lymphoma
lysozyme; beta-2-microglobulin (beta 2 microglobulin); tumor necrosis factor (TNF), also known as tumor necrosis factor alpha (TNF-alpha, TNF alpha), after stimulation with phorbol myristic acid (PMA)

Permits/Forms:

In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

Related Cell Culture Products

Restrictions:

The original U-937 cell line was established by Dr. K. Nilsson's laboratory in 1974 and he has requested the following: (1) In all papers reporting any use of this cell line or any derivatives thereof a direct reference should be made to Sundstrom and Nilsson (Int. J. Cancer 17: 565-577, 1976). (2) Any proposed commercial use of the cells should be negotiated with Professor Kenneth Nilsson, Rudbeck Laboratory, SE-751 85 Uppsala, Sweden. (3) No distribution of any of the cells or sublines derived therefrom should be made to third parties; (4) The cells should be used for non-clinical, non-commercial research only.

Isolation:

Isolation date: 1974

Applications:

transfection host(technology from amaxa
Roche FuGENE® Transfection Reagents)

Receptors:

complement (C3)

DNA Profile
(STR):

Amelogenin: X
CSF1PO: 12
D13S317: 10,12
D16S539: 12
D5S818: 12
D7S820: 9,11
TH01: 9.3
TPOX: 8,11
vWA: 15

Age:

37 years

Gender:	male
Ethnicity:	Caucasian
Comments:	<p>The U-937 cell line was derived by Sundstrom and Nilsson in 1974 from malignant cells obtained from the pleural effusion of a patient with histiocytic lymphoma.</p> <p>Studies since 1979 have shown that U-937 cells can be induced to terminal monocytic differentiation by supernatants from human mixed lymphocyte cultures, phorbol esters, vitamin D3, gamma interferon, tumor necrosis factor (TNF) and, retinoic acid.</p> <p>The cells are negative for immunoglobulin production and Epstein-Barr virus expression.</p> <p>The cells express the Fas antigen, and are sensitive to TNF and anti-Fas antibodies.</p> <p>In 1994, PCR and cytogenetic analyses showed that a number of stocks of U-937 were contaminated with the human myeloid leukemia cell line, K-562.</p> <p>In the earliest stocks available, the level of contamination was 0.6%. [40484]</p> <p>Distribution was discontinued in March 1994, except if required for patent purposes.</p> <p>Anyone who wishes to receive a sample of this original material should contact the Head of the ATCC Patent Depository.</p> <p>A stock of CRL-1593 found to be free of K-562 was propagated continuously for 8 weeks and tested weekly by PCR.</p> <p>Distribution and seed stocks give DNA profiles characteristic of U-937 only.</p> <p>Such preparations are now offered as authentic U-937 (ATCC CRL-1593.2) and are believed to be free of second subpopulations.</p>
Propagation:	<p>ATCC complete growth medium: The base medium for this cell line is ATCC-formulated RPMI-1640 Medium, Catalog No. 30-2001. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%.</p> <p>Atmosphere: air, 95%; carbon dioxide (CO2), 5%</p> <p>Temperature: 37.0°C</p>
Subculturing:	<p>Protocol: Cultures can be maintained by the addition of fresh medium or replacement of medium. Alternatively, cultures can be established by centrifugation with subsequent resuspension at 1 to 2 X 10⁵ viable cells/ml.</p> <p>Interval: Maintain cell density between 1 X 10⁵ and 2 X 10⁶ viable cells/ml.</p> <p>Medium Renewal: Add fresh medium every 3 to 4 days (depending on cell density)</p>
Preservation:	<p>Freeze medium: Complete growth medium supplemented with 5% (v/v) DMSO</p> <p>Storage temperature: liquid nitrogen vapor phase</p>
Related Products:	<p>Recommended medium (without the additional supplements or serum described under ATCC Medium): ATCC 30-2001</p> <p>recommended serum: ATCC 30-2020</p>
References:	<p>1080: Ralph P, et al. Lysozyme synthesis by established human and murine histiocytic lymphoma cell lines. J. Exp. Med. 143: 1528-1533, 1976. PubMed: 1083890</p> <p>21866: . Gene expression during normal and malignant differentiation. London: Academic Press; 1985.</p> <p>21876: . International symposium on new trends in human immunology and cancer immunotherapy. Paris: Doin Editeurs; 1980.</p> <p>22906: Koren HS, et al. In vitro activation of a human macrophage-like cell line. Nature 279: 328-331, 1979. PubMed: 450085</p> <p>22912: Gidlund M, et al. Natural killer cells kill tumour cells at a given stage of differentiation. Nature 292: 848-850, 1981. PubMed: 7266653</p> <p>23049: Olsson I, et al. Induction of differentiation of the human histiocytic lymphoma cell line U-937 by 1 alpha,25-dihydroxycholecalciferol. Cancer Res. 43: 5862-5867, 1983. PubMed: 6315218</p> <p>23103: Morimoto H, et al. Overcoming tumor necrosis factor and drug resistance of human tumor cell lines by combination treatment with anti-Fas antibody and drugs or toxins. Cancer Res. 53: 2591-2596, 1993. PubMed: 7684321</p> <p>29094: Giovannangeli C, et al. Accessibility of nuclear DNA to triplex-forming oligonucleotides: The integrated HIV-1 provirus as a target. Proc. Natl. Acad. Sci.</p>

USA 94: 79-84, 1997. PubMed: 8990164
29139: Brigino E, et al. Interleukin 10 is induced by recombinant HIV-1 Nef protein involving the calcium/calmodulin-dependent phosphodiesterase signal transduction pathway. Proc. Natl. Acad. Sci. USA 94: 3178-3182, 1997. PubMed: 9096366
40484: Reid YA, et al. Cell Line Cross-contamination of U-937. J. Leukocyte Biol. 57: 804, 1995. PubMed: 7759961
58042: Sundstrom C, Nilsson K. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int. J. Cancer 17: 565-577, 1976. PubMed: 178611

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Appendix V Product description of L929 cell line

Product Description

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Cell Biology

ATCC® Number:	CCL-1™	Order this Item	Price:	
Designations:	NCTC clone 929 [L cell, L-929 , derivative of Strain L]		Depositors:	WR Earle
Biosafety Level:	1		Shipped:	frozen
Medium	& See Propagation		Growth Properties:	adherent
Serum:				
Organism:	Mus musculus (mouse)		Morphology:	fibroblast

Source: **Tissue:** subcutaneous connective tissue; areolar and adipose
Strain: C3H/An

Permits/Forms: In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

Related Cell Culture Products

Isolation:	Isolation date: March, 1948
Applications:	testing [92346] [92380] [92382] [92389] [92404] toxicity testing [21469] [21470] [21606] transfection host(technology from amaxa Roche FuGENE® Transfection Reagents)
Tumorigenic:	Y
Reverse Transcript:	positive
Antigen Expression:	H-2k
Cytogenetic Analysis:	modal chromosome number = 66; range = 65 to 68. There were approximately 20 to 30 marker chromosomes present in each metaphase spread. A high percentage of those markers were common to most analyzed cells. A long metacentric chromosome with secondary constriction was noted in 77/100 cells.
Age:	100 days
Gender:	male
Comments:	NCTC clone 929 (Connective tissue, mouse) Clone of strain L was derived in March, 1948. Strain L was one of the first cell strains to be established in continuous culture, and clone 929 was the first cloned strain developed. The parent L strain was derived from normal subcutaneous areolar and adipose tissue of a 100-day-old male C3H/An mouse. [25770] Clone 929 was established (by the capillary technique for single cell isolation)

	from the 95th subculture generation of the parent strain. [21404] Tested and found negative for ectromelia virus (mousepox).
Propagation:	ATCC complete growth medium: The base medium for this cell line is ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003. To make the complete growth medium, add the following components to the base medium: horse serum to a final concentration of 10%. Atmosphere: air, 95%; carbon dioxide (CO ₂), 5% Temperature: 37.0°C
Subculturing:	Protocol: <ol style="list-style-type: none"> 1. Remove and discard culture medium. 2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor. 3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes). Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal. 4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting. 5. Add appropriate aliquots of the cell suspension to new culture vessels. 6. Incubate cultures at 37°C. <p>Subcultivation Ratio: A subcultivation ratio of 1:2 to 1:8 is recommended Medium Renewal: 2 to 3 times per week</p>
Preservation:	Freeze medium: Complete growth medium supplemented with 5% (v/v) DMSO Storage temperature: liquid nitrogen vapor phase
Related Products:	Recommended medium (without the additional supplements or serum described under ATCC Medium): ATCC 30-2003 derivative: ATCC CCL-1.1 derivative: ATCC CCL-1.2 derivative: ATCC CCL-1.3 derivative: ATCC CCL-1.4
Bioreactive Factors:	Growth Factors: T cell growth factor (TCGF)
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92404: Plastic containers for intravenous injection. Geneva (Switzerland):International Organization for Standardization/ANSI;ISO ISO 15747:2003.

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Appendix VI Particle size distributions of different size ranges

Size d.nm	Mean Intensity %	Std Dev Intensity %	Size d.nm	Mean Intensity %	Std Dev Intensity %	Size d.nm	Mean Intensity %	Std Dev Intensity %	Size d.nm	Mean Intensity %	Std Dev Intensity %
0.4000	0.0		5.615	0.0		78.82	0.0		1108	0.0	
0.4632	0.0		6.503	0.0		91.28	0.0		1281	0.0	
0.5365	0.0		7.531	0.0		105.7	0.0		1484	0.0	
0.6213	0.0		8.721	0.0		122.4	0.0		1718	0.0	
0.7195	0.0		10.10	0.0		141.8	0.0		1990	0.0	
0.8332	0.0		11.70	0.0		164.2	0.0		2305	0.0	
0.9648	0.0		13.54	0.0		190.1	0.0		2689	0.0	
1.117	0.0		15.69	0.0		220.2	0.0		3091	0.0	
1.294	2.8		18.17	0.0		255.0	0.0		3580	0.0	
1.489	34.9		21.04	0.0		295.3	0.0		4145	0.0	
1.736	45.1		24.36	0.0		342.0	0.0		4801	0.0	
2.010	17.1		28.21	0.0		396.1	0.0		5580	0.0	
2.328	0.0		32.67	0.0		458.7	0.0		6439	0.0	
2.696	0.0		37.84	0.0		531.2	0.0		7456	0.0	
3.122	0.0		43.82	0.0		615.1	0.0		8635	0.0	
3.615	0.0		50.75	0.0		712.4	0.0		1.000e4	0.0	
4.187	0.0		58.77	0.0		825.0	0.0				
4.848	0.0		68.06	0.0		955.4	0.0

(a) Average size 1.6 nm

Size d.nm	Mean Volume %	Std Dev Volume %	Size d.nm	Mean Volume %	Std Dev Volume %	Size d.nm	Mean Volume %	Std Dev Volume %	Size d.nm	Mean Volume %	Std Dev Volume %
0.400	0.0		5.61	0.0		78.8	7.0		1110	0.0	
0.483	0.0		6.50	0.0		91.3	2.5		1280	0.0	
0.536	0.0		7.53	0.0		106	0.5		1480	0.0	
0.621	0.0		8.72	0.0		122	0.0		1720	0.0	
0.719	0.0		10.1	0.0		142	0.0		1990	0.0	
0.833	0.0		11.7	0.0		164	0.0		2300	0.0	
0.965	0.0		13.5	0.0		190	0.0		2670	0.0	
1.12	0.0		15.7	0.0		220	0.0		3090	0.0	
1.29	0.0		18.2	0.0		255	0.0		3580	0.0	
1.50	0.0		21.0	0.0		295	0.0		4150	0.0	
1.74	0.0		24.4	0.0		342	0.0		4800	0.0	
2.01	0.0		28.2	0.0		396	0.0		5560	0.0	
2.33	0.0		32.7	0.0		459	0.0		6440	0.0	
2.70	0.0		37.8	8.4		531	0.0		7460	0.0	
3.12	0.0		43.8	20.8		615	0.0		8630	0.0	
3.62	0.0		50.7	25.4		712	0.0		1.00e4	0.0	
4.19	0.0		58.8	21.4		825	0.0				
4.85	0.0		68.1	13.9		955	0.0

(b) Average size 52 nm

Size	Mean	Std Dev	Size	Mean	Std Dev	Size	Mean	Std Dev	Size	Mean	Std Dev
% Volume	% Volume	% Volume	% Volume	% Volume	% Volume	% Volume	% Volume	% Volume	% Volume	% Volume	% Volume
0.4	0		5.615	0		78.82	20.1		1106	0	
0.4632	0		6.503	0		91.28	22.32		1281	0	
0.5365	0		7.531	0		105.7	18.86		1484	0	
0.6213	0		8.721	0		122.4	12.91		1718	0	
0.7195	0		10.1	0		141.8	7.184		1990	0	
0.8332	0		11.7	0		164.2	3.034		2305	0	
0.9649	0		13.54	0		190.1	0.8073		2669	0	
1.117	0		15.89	0		220.2	0.08733		3091	0	
1.294	0		18.17	0		255	0		3580	0	
1.499	0		21.04	0		295.3	0		4145	0	
1.736	0		24.36	0		342	0		4801	0	
2.01	0		28.21	0		396.1	0		5560	0	
2.328	0		32.87	0		458.7	0		6439	0	
2.696	0		37.84	0		531.2	0		7456	0	
3.122	0		43.82	0		615.1	0		8635	0	
3.615	0		50.75	0		712.4	0		1e+004	0	
4.187	0		58.77	3.057		825	0				
4.849	0		68.06	11.65		955.4	0

(c) Average size 104 nm

Appendix VII JCPDS No.36-1451 data sheet

Appendix VIII JCPDS No.21-1486 data sheet