Miniature fluidic devices for nanoparticle synthesis with biomedical applications

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**MINIATURE FLUIDIC DEVICES FOR
NANOPARTICLE SYNTHESIS
WITH BIOMEDICAL APPLICATIONS**

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SCHOOL OF ELECTRICAL AND ELECTRONIC ENGINEERING

2016

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Abstract

Nanoparticles exhibit very distinct characteristics from their bulk counterparts, therefore there is great interest in studying the properties of these nanomaterials for various applications such as biophotonics, photovoltaics, and nanomedicine. Miniature devices not only provide the conveniences of small physical footprints and system portability, but also serve as excellent platforms to give us insight into the molecular interactions at reduced length and volume scales, requiring less amount of sample and hastening the sample processing time. Therefore, the union of these two concepts is the motivation behind this thesis as miniaturization of reaction vessels can provide better control over the reaction conditions, enabling higher reproducibility and tighter size distributions. As a result, nanocrystals with superior qualities like high monodispersity and narrow emission linewidths can be easily reproduced with less chemical wastage.

In this thesis, synthesis of several nanoparticles using millimetre dimensioned channels in miniaturized devices are presented. Firstly, plasmonic nanoparticles were synthesized for photodynamic therapy. By varying the flow rate and molar ratios of the precursors, nanoparticles with different shapes, sizes, and crystal structures were fabricated. Secondly, monodispersed semiconductor nanoparticles with interesting optical characteristics were also produced. Modifying the relative ratio of the reactants and their injection rate yielded particles with different morphologies. These nanoparticles were then used for cell imaging. Lastly, preliminary studies on a dye encapsulated micelle constructed using a chip based millifluidic device were carried out. The goal is to extend this approach to increase the drug loading efficiency of multimodal fluorescent particles. The polymer was used for drug delivery can encapsulate chemotherapy drugs to enable controlled drug release in the cellular environment. Adjusting the relative flow rate of the injected streams, different mixing regimes could be

achieved, affecting considerations such as the sizes of the particles. These factors are critical and have a profound impact on the drug loading efficiency. Therefore, the miniaturization of nanoparticle synthesis methods provides windows of opportunity to integrate such devices for real-time spectroscopy and analysis of these nanoparticles and subsequent incorporation with other fluidic technologies such as biosensing and flow cytometry.

Table of Contents

Abstract	iii
Table of Contents	v
List of Figures	viii
Nomenclature and Abbreviations	xii
Acknowledgements	xv
Chapter 1 Introduction	1
1.1 Motivation and Objectives	1
1.2 Major Contributions	2
1.3 Thesis Organization	4
Chapter 2 Literature Review	6
2.1 Miniature Fluidic Devices	6
2.1.1 Principles of Fluidic Flow	7
2.1.2 Nanofluidic Devices	10
2.1.3 Microfluidic Devices	11
2.1.4 Millifluidic Devices	13
2.2 Nanoparticle Synthesis	15
2.2.1 Synthesis Methods	15
2.2.2 Colloidal Synthesis - Hot injection	16
2.2.3 Nanoparticle Synthesis using Fluidic Devices	17
2.2.4 Millifluidic Devices VS State-Of-The-Art Synthesis Methods	29
2.3 Conclusion	32
Chapter 3 Synthesis of Copper Sulphide Nanocrystals for Photothermal Therapy	34
3.1 Introduction	34
3.2 Materials and Methods	35
3.2.1 Materials	35
3.2.2 Millifluidic Chip Fabrication	36
3.2.3 Synthesis of Cu _{2-x} S NCs	36
3.2.4 Cu _{2-x} S NCs Characterization	37
3.2.5 Ligand Exchange of Cu _{2-x} S NCs from Organic to Aqueous Phase	38
3.2.6 Cell Culture of Macrophage Cells	38
3.2.7 Cytotoxicity of Cu _{2-x} S NCs	38
3.2.8 Cellular Uptake of GSH capped Cu _{2-x} S NCs and NIR Laser Illumination	39
3.2.9 <i>In vitro</i> Cell Imaging	39

3.3	Results and Discussion.....	40
3.3.1	Millifluidic Chip Synthesis of Cu _{2-x} S NCs	40
3.3.2	Characterization of Cu _{2-x} S NCs	44
3.4	Photothermal Therapy Application of Cu _{2-x} S NCs	58
3.4.1	Cellular Uptake of Glutathione capped Cu _{2-x} S Nanocrystals and Near-Infrared Laser Illumination.....	58
3.5	Conclusion and Further Recommendations	66
Chapter 4	Cadmium Sulphide Nanoparticle Synthesis and Applications in Bioimaging ..	69
4.1	Introduction	69
4.2	Materials and Methods	73
4.2.1	Chemicals.....	73
4.2.2	Fabrication of Millifluidic Chip.....	73
4.2.3	Synthesis of CdS Nanocrystals in Millifluidic Chip.....	74
4.2.4	Characterization of CdS Nanocrystals	74
4.2.5	Ligand Exchange of CdS Nanocrystals with Mercaptosuccinic Acid	75
4.2.6	Cell Culture and Bioimaging of Macrophage Cells using Millifluidic CdS Nanocrystals.....	75
4.3	Results and Discussion.....	76
4.3.1	Effect of Injection Flow Rate/Residence Time.....	78
4.3.2	Effect of Different Cd:S Molar Ratios.....	83
4.4	Bioimaging with Macrophage Cells.....	87
4.5	Conclusion.....	90
Chapter 5	Preliminary Work and Future Studies - Hydrodynamic Flow Focusing and Nanoprecipitation Using Millifluidic Chips	92
5.1	Self-Assembly of Polymer Nanoparticles	92
5.1.1	Bulk Mixing Techniques for Polymer Synthesis	92
5.2	Hydrodynamic Flow Focusing	93
5.3	Problem of Drug Loading Efficiency in AIE Luminogens	94
5.4	Preliminary Results - Encapsulation of Organic Dye (DCM) with mPEG-DSPE Phospholipid	96
5.4.1	Simulation of Hydrodynamic Flow Focusing in Millifluidic Chip	96
5.4.2	Experimental Methods and Materials	100
5.5	Results and Discussion.....	102
5.6	Future Studies.....	106
Chapter 6	Conclusion and Further Recommendations	107
6.1	Conclusion.....	107

6.2 Further Recommendations	108
Author's Publication	109
References	110

List of Figures

<i>Figure 1-1 Graphical abstract outlining the nanoparticles investigated in this thesis and their corresponding biomedical applications.</i>	5
Figure 2-1 Selected biophotonic applications using different miniaturised devices working at milli-, micro-, and nanoscale level, along with the corresponding detectable biological systems at the corresponding length scale.	7
Figure 2-2 (a) Schematic of the electric double layer, showing the accumulation of counter-ions on the charged surface, along with the diffuse layer and Debye length, LD . The bold line indicates the electrical potential profile, ψ , which becomes more significant with shrinking dimensions. Reproduced from [10] with permission of The Royal Society of Chemistry. Comparison of the surface charge effects on (b) a microchannel versus (c) a nanochannel. (d) In a microchannel, the electrical potential decays to the bulk value, while (e) the nanochannel exhibits an electrical potential at the center of the channel. Furthermore, (g) the nanochannel shows a higher counter-ion concentration (orange coloured positive charges) compared to co-ions (blue coloured negative charges), unlike (f) the microchannel, where equal concentration of both charges exist in the middle of the channel. Reprinted with permission from [6]. Copyright (2005) American Chemical Society.....	10
Figure 2-3 Schematic drawing of DNA unravelling in a nanofluidic device by means of nanopillars. b) Stretching of DNA molecules by applying an electric field across the channel to drive the DNA from the pillar-free region into the pillared region, and c) the subsequent recoiling of DNA after the electric field is turned off and the DNA retracts back to the pillar-free region. Reprinted figure with permission from [17]. Copyright (2002) by the American Physical Society.	11
Figure 2-4 Various microfluidic chip designs mimicking different vital organs in our body serving as a platform for in vitro drug testing and cell culture. Reproduced from [25] with permission of The Royal Society of Chemistry.	13
Figure 2-5 (A) Schematic drawing of millifluidic chip with miniaturized traps (T) positioned in the row array (R), (B) 3D CAD drawing of the chip, and (C) actual fabricated device using SLA technique. Reproduced from [30] with permission of AIP Publishing.	14
Figure 2-6 LaMer plot showing nanoparticle formation showing the nucleation (Phase II) and growth (Phase III) of the nanoparticles. Reproduced from [35] with permission of The Royal Society of Chemistry.....	16
Figure 2-7 (a) Continuous flow, (b) liquid-liquid segmented flow and (c) liquid-gas fluid slug segmented flow, and (d) hydrodynamic flow focusing. Reprinted with permission from [39]. Copyright 2006 John Wiley & Sons, Inc (b and c).	18
Figure 2-8 Millifluidic flow reactor setup for Au NP synthesis, with b) absorbance and transmission electron microscope (TEM) images of the as-synthesized Au nanorods (NRs). The TEM images and the absorbance curves correspond to Au NRs with aspect ratios of (i) 1.5, (ii) 2.0, (iii) 2.5, (iv) 3, and (v) 3.5, with respective scale bars of 50 nm. Reprinted with permission from [41]. Copyright (2013) American Chemical Society.....	21
Figure 2-9 a) Schematic drawing and b) actual microfluidic channel with droplet jet injector seen under a microscope. Transverse cross sections of the chip c) before and d) after the step. e) Lateral cross section before and after the step showing the increase in channel height from 45 μm to 90 μm . Adapted with permission from [43]. Copyright (2005) American Chemical Society.....	22

Figure 2-10 (a) Simulated and actual microfluidic chips with different channel dimensions, (b) the corresponding experimental results of the microfluidic chip synthesized BSA-CdTe quantum dots (QDs), (c) photoluminescence, and (d) photostability characterizations of the microfluidic (MF) and benchtop (BT) BSA-QDs. Reproduced from [50] with permission of The Royal Society of Chemistry.....	24
Figure 2-11 Bioimaging of RAW264.7 mice macrophage cells and Panc-1 human pancreatic cells labelled with the microfluidic (MF) (a) BSA-QDs, (b) MPA-QDs, and (c) FA-QDs. Reproduced from [50] with permission of The Royal Society of Chemistry.....	26
Figure 2-12 T-shaped microfluidic chip for assembly of high molecular weight chitosan supplement (HMCS) nanoparticles via hydrodynamic flow focusing. Reprinted with permission from [51]. Copyright 2014 John Wiley & Sons, Inc.	27
Figure 2-13 Hydrodynamic size of unloaded chitosan nanoparticles synthesized using the hydrodynamic focusing microfluidic chip at different flow ratios. DS I, II and III refer to HMCS with different degrees of substitutions. Reprinted with permission from [51]. Copyright 2014 John Wiley & Sons, Inc.	28
Figure 2-14 Paclitaxel (PTX) drug release profile of the chip synthesized chitosan nanoparticles as a function of the environmental pH to mimic circulation around tumour cells. Reprinted with permission from [51]. Copyright 2014 John Wiley & Sons, Inc.	29
Figure 2-15 Schematic representation of generic (a) bench top and (b) miniature fluidic synthesis setups. Adapted from (a) [55] and (b) [56] under the CC BY 4.0 license.	29
Figure 3-1 (a) Annotated schematic design of the millifluidic chip, b) actual chip with sulphur and copper precursors during the synthesis, c) top view, and d) cross-section view of pristine PDMS chip. Figure reproduced from Cheung et al. [93].....	42
Figure 3-2 TEM images showing the size and morphology of the Cu _{2-x} S NCs synthesized at different flow rates of (a) 750 μ L/min, (b) 1000 μ L/min, (c) 2000 μ L/min, (i) 3000 μ L/min, (j) 4000 μ L/min, and (k) 5000 μ L/min, along with histograms depicting the corresponding size distributions (d, e, f, l, m, and n) and aspect ratios (g, h, o, p, and q) of the particles. The molar ratio of Cu:S was maintained at 1:2. Figure reproduced from Cheung et al. [93].....	46
Figure 3-3 a) Powdered XRD patterns and b) absorbance spectra of Cu _{2-x} S NCs synthesized under different flow rates with the Cu:S molar ratio fixed at 1:2. Figure adapted from Cheung et al. [93].	47
<i>Figure 3-4 TEM images showing the size and morphology of the Cu_{2-x}S NCs synthesized at different cadmium to sulphur (Cd:S) molar ratios of (a) 1:10, (b) 1:6, (c) 1:2, (j) 2:1, (k) 6:1, and (l) 10:1, along with histograms depicting the corresponding size distributions (d, e, f, m, n, and o) and aspect ratios (g, h, i, p, q, and r) of the particles. The injection flow rate of the precursors were maintained at 3000 μL/min. Figure reproduced from Cheung et al. [93]....</i>	50
Figure 3-5 (a) XRD patterns, (b) Vis/NIR absorbance spectrum, and (c) EDX measurements of Cu _{2-x} S NCs synthesized with different Cu:S molar ratios with flow rate fixed at 3000 μ L/min.	52
Figure 3-6 HRTEM images of single nanoparticles corresponding to the different crystal compositions of covellite (a and c), djurleite (e and g), roxbyite (i), and spionkopite (k) with annotated lattice spacing. The associated FFT spot diffraction patterns accompanying the HRTEM images for covellite (b and d), djurleite (f and h), roxbyite (j), and spionkopite (l). Figure reproduced from Cheung et al. [93].	53
Figure 3-7 TEM images of ligand-exchanged GSH-Cu _{2-x} S NCs seen under (a) high magnification and (c) low magnification. (b) Size distribution of GSH-Cu _{2-x} S NCs and its Vis-	

NIR absorption spectrum with full width half maximum spanning from 898 nm to 1428 nm.	59
Figure 3-8 Cytotoxicity effects of different concentrations of GSH-Cu _{2-x} S NCs on RAW264.7 macrophage cells. The red dotted line indicates 50% cell viability, defining 13.5 μ M and 27 μ M as suitable concentration levels based on the IC ₅₀ benchmark. Reproduced from [93] with permission of The Royal Society of Chemistry.	60
Figure 3-9 a) Fiber-coupled laser used as the near infra-red (NIR) source for illumination of the cells. b) The NIR laser light illumination was performed inside the incubator with the laser light being delivered via the optical fiber into the incubator.	60
Figure 3-10 (a) The 6-well cell culture plate together with the optical mount holding the fiber in place. (b) NIR laser illumination of the cells in the cell culture well as seen using a NIR viewer.	61
Figure 3-11 Microscope images of RAW264.7 mice macrophage cells with different Cu _{2-x} S NC concentrations after being subjected to NIR irradiation at 915 nm with power densities of 36.7 W/cm ² and 52.1 W/cm ² for 15 minutes. Red fluorescence observed after staining with propium iodide (PI) to indicate regions of cell damage or death. The inset at the top left hand corner shows NIR laser irradiation of the cells in the cell culture plate through a NIR viewer. Reproduced from [93] with permission of The Royal Society of Chemistry.	63
Figure 3-12 Annotated microscope images showing regions of cell death induced by NIR irradiation of the Cu _{2-x} S NCs and their corresponding affected regions and power densities. Reproduced from [93] with permission of The Royal Society of Chemistry.	63
Figure 3-13 Absorption curves with their corresponding laser irradiation wavelengths and power density requirement for photothermal effect - A) 808 nm at 24 W/cm ² , 40 W/cm ² , 64 W/cm ² [118], B) 980 nm, 1.41 W/cm ² [122], C) 980 nm, 0.51 W/cm ² [119], D) 915 nm (this work) at 36.7 W/cm ² , 52 W/cm ² .	65
Figure 4-1 a) Annotated schematic and b) actual millifluidic chip for the synthesis of cadmium sulfide nanocrystals (CdS NCs). Simulations reflecting CdS NC (c) concentration and (d) fluid velocity flow inside the chip.	77
Figure 4-2 TEM images of CdS NCs produced under different flow rates and Cd:S precursor molar ratios.	77
Figure 4-3 Absorbance curves of selected batches of CdS NCs showing the high reproducibility of nanoparticle fabrication using these millifluidic chip reactors.	80
Figure 4-4 TEM images and size distributions of CdS nanocrystals synthesized in millifluidic chips at different flow rates of 5000 μ L/min (a and b), 3000 μ L/min (c and d), 1500 μ L/min (e and f), 750 μ L/min (g and h), and 375 μ L/min (i and j) respectively. The Cd:S molar ratio (MR) was kept constant at 1:6.	81
Figure 4-5 a) Absorbance curves and b) photoluminescence spectra of CdS NCs formed at different flow rates with Cd:S molar ratio fixed at 1:6.	82
Figure 4-6 Millifluidic (mF) CdS NCs under ambient light (a) without and (b) with ultraviolet (UV) irradiation. (c) Reddish glow of mF CdS defect state emission in the dark, illuminated with a UV torchlight.	83
Figure 4-7 Millifluidic chip synthesized CdS NCs seen under (a) ambient light and (b) upon illumination with ultraviolet (UV) light and emitting greenish-blue light due to band edge emissions.	83
Figure 4-8 TEM images of CdS nanocrystals fabricated using millifluidic chips at three different molar ratios of (a) Cd:S = 1:3, (b) 0.10M of Cd and 0.6M of S, and (c) 0.05M of Cd	

and 0.3M of S. Their corresponding size distributions (b, e, h) and aspect ratios (c, f, i) are also presented. The flow rate was kept constant at 1500 $\mu\text{L}/\text{min}$	85
<i>Figure 4-9 (a) Absorbance and (b) photoluminescence spectra of CdS NCs synthesized using different molar ratios. The flow rate was kept constant at 1500 $\mu\text{L}/\text{min}$.</i>	85
<i>Figure 4-10 Powered X-ray diffraction (XRD) patterns of millifluidic chip synthesized CdS at (a) different flow rates and (b) molar ratios.</i>	86
Figure 4-11 In vitro bioimaging of RAW264.7 macrophage cells with and without CdS-MSA NCs. The red fluorescence observed is due to the emission of the CdS-MSA NCs upon illumination with ultraviolet (UV) light.	88
Figure 4-12 Photoluminescence spectrum of ligand exchanged CdS-MSA nanocrystals.	88
Figure 5-1 Pictorial illustration of (A) the nanoprecipitation and (B) miniemulsion methods of synthesizing polymers and nanoparticles. Reprinted from [175], under CC BY.	92
Figure 5-2 Schematic drawing describing the interaction between the solvent and non-solvent molecules during hydrodynamic flow focusing. Reproduced from [162] with permission of The Royal Society of Chemistry.	93
Figure 5-3 Non-planar hexaphenylsilole (HPS) exhibiting restricted intramolecular rotation (RIR) during aggregation, resulting in light emission. This phenomenon is thus coined as aggregation-induced emission (AIE). Reproduced from [180] with permission of The Royal Society of Chemistry.	95
Figure 5-4 a) Annotated diagram showing simulation model with the dark blue region inside the millifluidic chip corresponding to deionized water in the carrier inlets and the dark red region indicating the DCM-mPEG-DSPE sample flow. Selected velocity and concentration profiles showing the focusing of the middle sample stream and their cross sectional concentration distribution for the relative flow ratios of $R=0.1$ (b, c and d) and 1 (e, f and g).	99
<i>Figure 5-5 Graph showing the change in the width of the focused stream with different relative flow ratios (R) along with miniature diagrams reflecting the focused stream width. The inset shows the actual millifluidic chip used for hydrodynamic flow focusing.</i>	100
Figure 5-6 a) Schematic and b) actual hydrodynamic flow focusing millifluidic chip with two side carrier streams (containing DI H_2O) and an inlet for the DCM-mPEG-DSPE mixture.	102
Figure 5-7 a) Unfiltered output mixture from hydrodynamic flow focusing millifluidic chip. b) Pour the mixture through the filter paper to remove large dye precipitates. c) Dye residue collected on filter paper and d) clear filtrate containing micelles.	103
Figure 5-8 DCM dye encapsulated micelle solution a) under normal ambient light, b) with UV illumination in ambient conditions, and c) under UV illumination in the dark.	104
Figure 5-9 Photoluminescence spectrum of DCM dye encapsulated micelle solution.	104
Figure 5-10 a) Hydrodynamic diameter of DCM-mPEG-DSPE micelles and b) their corresponding relative variances as a function of the relative flow ratio, R. BT represents the micelles assembled using macroscale benchtop method of vigorous stirring.	105
Figure 5-11 FESEM image of dye encapsulated micelles synthesized using the millifluidic chip with $R=0.05$	106

Nomenclature and Abbreviations

3D	Three dimensional
ABS	Acrylonitrile butadiene styrene
ACQ	Aggregation-caused quenching
AIE	Aggregation-induced emission
AR	Aspect ratio
Au	Gold
Ag	Silver
BT	Bench top
CAD	Computer-aided design
CdSe	Cadmium selenide
Cu-OAm	Copper-oleylamine complex
Cu _{2-x} S	Copper sulfide
CO ₂	Carbon dioxide
CTAB	Cetyl trimethyl ammonium bromide
CVD	Chemical vapour deposition
DCM	4-(Dicyanomethylene)-2-methyl-6-(4-dimethylaminostyryl)-4H-pyran
DI water	Deionized water
DMEM	Dulbecco's modified eagle's medium
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DOX	Doxorubicin
DRIE	Deep reactive ion etching
EDX	Energy-dispersive X-ray spectroscopy
FBS	Fetal bovine serum
FDM	Fused deposition modelling
FFT	Fast fourier transform
FITC	Fluorescein isothiocyanate
FWHM	Full width half maximum
FITC	Fluorescein isothiocyanate
GSH	L-guthathione

HAuCl ₄ ·3H ₂ O	Gold tetrachloroaurate trihydrate
HF	Hydrofluoric acid
HMCS	High molecular weight chitosan supplement
HPS	Hexaphenylsilole
HRTEM	High resolution transmission electron microscope
LED	Light emitting diode
LoC	Lab-on-chip
LSPR	Localized surface plasmon resonance
MBE	Molecular beam epitaxy
MHA	Mercaptohexanoic acid
mPEG-DSPE	Methoxy-poly (ethylene glycol)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N
MSA	Mercaptosuccinic acid
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide
NaBH ₄	Sodium borohydride
NaOH	Sodium hydroxide
NC	Nanocrystal
NIL	Nanoimprint lithography
NIR	Near infra-red
NR	Nanorod
OA	Oleic acid
OAm	Oleylamine
ODE	Octadecene
PBS	Phosphate buffered saline
PDF	Powder diffraction file
PDMS	Polydimethylsiloxane
PI	Propidium iodide
PLA	Polylactic acid
PMMA	Poly(methyl methacrylate)
PTFE	Polytetrafluoroethylene
PTT	Photothermal therapy
PTX	Paclitaxel

QD	Quantum dot
<i>Re</i>	Reynold's number
RIE	Reactive ion etching
S-OA	Sulphur-oleic acid complex
S-OAm	Sulphur-oleyamine complex
SA/V	Surface area is to volume
Se	Selenium
SEM	Scanning electron microscope
SLA	Stereolithography
Te	Tellurium
TEM	Transmission electron microscope
THF	Tetrahydrofuran
TOP	Tri-n-octylphosphine
UV-Vis-NIR	Ultraviolet/Visible/Near Infra-red
XRD	X-ray diffraction

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Chapter 1 Introduction

1.1 Motivation and Objectives

Nanoparticles refer to materials in the range of 1 – 100 nm, in this size domain the material behaves different from their macroscopic bulk forms in terms of physical and chemical properties. This therefore gives rise to the study of these materials and how they can be exploited for various applications such as in electronics for lighting displays, sensors for monitoring heavy metal ions in water, as well nanomedicine where nanoparticles can serve as drug delivery agents to transport the drug to targeted regions in the body.

Since the size nanoparticles as its name suggests is in the nanometer scale, miniaturization of fabrication systems for nanoparticles can provide much better control over the reaction conditions. With better control of the reaction parameters, nanocrystals with more superior qualities such as high monodispersity and narrow emission linewidths can be easily reproduced with less chemical wastage. The development of this exciting field would greatly benefit from increased attention and contributions from various branches of study such as biology, chemistry, physics, engineering, material science and medicine.

Current well-established colloidal nanoparticle synthesis methods requires skilled expertise and some element of luck due to variations in reaction conditions such as temperature gradients, mixing inhomogeneities inherently present in macroscopic batch synthesis techniques. Therefore there is a need to reduce these variables and provide a stable and reproducible platform to aid in the synthesis of these nanomaterials. This will spur development not just in the area of nanoparticle synthesis but also other fields such as nanomedicine and

optoelectronics where nanomaterials can play a huge role serving as probes, delivery vectors, and materials with tunable electrical and optical characteristics.

Therefore, the objective of this thesis is to realise the potential of a new regime of fluidic technology in which nanoparticles can be produced in increased quantities with highly reproducible characteristics, thereby rendering them useful for subsequent applications and hopefully transitioning the technology into commercialization. Thus in this thesis, simple, customizable millifluidic systems were used to synthesize nanoparticles with reduced temperature fluctuations and reaction uncertainties in the synthesis conditions so as to fabricate nanocrystals with desirable qualities such as high monodispersity, crystallinity, and unique optical characteristics.

1.2 Major Contributions

The major contributions of this thesis to the research community is in the utilization of millimeter-scale 3D printing technology to develop an easy to use platform to synthesize various types of nanoparticles. Compared to the traditional flask synthesis methods, temperature and mixing conditions can be precisely controlled due to the reduced working dimensions. The ability to directly print the designed mould for millifluidic chips reduces the laborious fabrication procedures required in microfluidics where a master mould is required to make subsequent chips. Through the work of each chapter, we hope to showcase the potential of millifluidic synthesized nanoparticles for different biomedical applications, thereby encouraging more developments in the field especially transitioning from not only producing the nanoparticles, but also how to make use of these nanomaterials to improve theranostics and device efficiencies. The following states a short excerpt of each contribution:

1. A novel plasmonic material – copper sulphide Cu_{2-x}S , was manufactured using passive mixing in a millifluidic chip and used for cell killing. The shape of the Cu_{2-x}S nanocrystals could be varied between the spherical and elongated rod conformations by adjusting the injection flow rates and molar ratios of the reactants. In addition, different crystallographic structures ranging from copper-rich djurleite to copper-deficient covellite were obtained. As a result of the flexibility in material design, localized surface plasmonic peaks could be tuned from 1115 nm to 1246 nm. Therefore, these nanocrystals were used for photothermal therapy to annihilate cells. The temperature of these treated cells (containing Cu_{2-x}S) increased when exposed to near infra-red laser light causing cell death, while untreated cells remained viable.
2. Semiconductor cadmium sulphide (CdS) nanoparticles were fabricated in organic solvents using the millifluidic chip and applied in bioimaging because of their photoluminescence. Highly reproducible sizes and monodispersed CdS nanoparticles were obtained by modifying the on-chip residence time of the reactants. This method provided access to quantum-confined nanoparticles or quantum dots about 1-2 nm in diameter, which are challenging to obtain via bulk synthesis methods. Photoluminescence of the nanoparticles was also absent in bulk synthesis using similar protocols. Imaging of cells which have absorbed the CdS nanoparticles were carried out by illuminating the cells at 350 nm and observing the fluorescence imaging. Cells containing CdS will show up in red due to the emission wavelength of the CdS nanoparticles, therefore they can be used to track cell signalling pathways or as optical probes for drug monitoring.

3. Self-assembly of polymer nanoparticles in the form of dye encapsulated micelles using hydrodynamic flow focusing in a millifluidic chip were studied in an attempt to find a solution to increase the drug loading efficiency of a nanoaggregate system.

1.3 Thesis Organization

This thesis is arranged in 6 chapters, beginning with the introduction in Chapter 1, which lays out the motivation and objectives of this thesis as well as the major contributions of this work. Chapter 2 gives a brief overview of miniature fluidic devices, touching briefly on nanofluidics, microfluidics and millifluidics, before focusing on nanoparticle formation in the bulk and micro regimes, followed by examining the applications of these nanoparticles.

The third chapter discusses about the fabrication of plasmonic copper sulphide (Cu_{2-x}S) nanocrystals in a home-made millifluidic chip. The synthesis procedure and characterisation results are presented, showing excellent size and shape control. The Cu_{2-x}S nanocrystals are induced to be taken up by cells and exposed to near infra-red irradiation to achieve selected cell-killing using thermal effects enhanced by the nanocrystals.

Chapter 4 deals with the production of small semiconductor cadmium sulphide (CdS) quantum dots and nanocrystals with photoluminescence. We demonstrate that millifluidic chip synthesized CdS nanocrystals have outstanding and highly reproducible size control. Changes in morphology was also observed by changing reaction parameters such as residence time and precursor molar concentrations. The synthesized CdS nanocrystals were then converted to dissolve in aqueous solvents and employed as an optical probe for fluorescence imaging of cells.

The fifth chapter presents some preliminary data using hydrodynamic flow focusing in millifluidic chips for controlled and homogenous mixing. This initial testing was carried out using a model of an organic dye encapsulated with phospholipids, which will form micelles to protect the hydrophobic dye in the core. This serves as preliminary studies for subsequent applications for drug delivery. Figure 1-1 summarizes the different nanoparticles and their respective applications discussed in this work. Lastly, this thesis concludes with chapter 6, summarising the contributions of this work to the research fields and potential areas to explore in future.

Figure 1-1 Graphical abstract outlining the nanoparticles investigated in this thesis and their corresponding biomedical applications.

Chapter 2 Literature Review

2.1 Miniature Fluidic Devices

Miniaturized fluidic systems aim to scale down macroscale processes to milli-, micro- and nanoscale dimensions and provide solutions to the shortcomings of the conventional macroscale benchtop processes. Miniaturized fluidic devices are generally used to speed up the processing and analysis time as multiplexing and automation of operations can be achieved in these systems. Owing to their relatively small dimensions, smaller amounts of reagents and analytes are needed, this is especially useful in situations where samples are very limited or in low-resource settings. In addition, being a low-cost alternative to conventional methods, an inexpensive miniaturized device also offers accessibility to the masses and disposability after use.

The origin of miniaturized fluidic devices was a result of consolidated progress in the fields of molecular analysis, biodefence, molecular biology, and microelectronics [1] into what would become known as the field of microfluidics. Currently it is defined as the study of precision manipulation of minute fluid volumes for various reaction processes. The diversity of its beginnings is a reflection of the interdisciplinary nature of the microfluidics field: combining physics, chemistry, biochemistry, engineering, and nanotechnology. Furthermore, the introduction of soft lithography in the 1990s by Whitesides' group [2] accelerated the fabrication of various miniaturized systems through a cheaper microfabrication alternative compared to standard photolithography. Thus, a burst of research developments in the field of microfluidic devices was initiated.

Figure 2-1 Selected biophotonic applications using different miniaturised devices working at milli-, micro-, and nanoscale level, along with the corresponding detectable biological systems at the corresponding length scale.

2.1.1 Principles of Fluidic Flow

Generally, fluidic flow can exist in two forms – laminar flow and turbulent flow. Laminar flow refers to parallel layers of liquid moving in a well-controlled and orderly manner, while turbulent flow is chaotic and uncontrollable. An indication of the operating fluid regime can be deduced from the Reynolds number (Re). Re , is a dimensionless parameter given by the ratio of inertial forces to the viscous forces due to the fluid velocity and viscosity respectively. It is expressed as:

$$Re = \frac{\rho u D_h}{\eta} \quad \text{Equation 2-1}$$

where ρ is the fluid density, η is the fluid viscosity, D_h is the hydraulic diameter or characteristic length for the system, and u is the fluid velocity. The effects of scaling is reflected in the fluidic flow through the Re . Inertial forces decrease proportionally with the

reduction of D_h , thereby resulting in a smaller Re . Consequently, this reduction of D_h means that viscosity becomes more dominant. A low Re signifies a Stokes' flow, translating to laminar flow. For channel flows where Re values approach 2300 transition flow sets in [3], and Re exceeding 4000 denote turbulent flow.

For reliable and predictable device performances, a well-defined and reproducible flow is desirable. Therefore, miniaturized fluidic devices with small channel dimensions, where the critical dimension is usually the channel diameter, exhibit Re values much less than 100 operate in the laminar flow regime. In addition, these small-scale systems offer a marked increase in the surface area to volume (SA/V) ratio, where surface forces dominate over gravitational forces [4, 5].

Fluid flow in channels is most commonly actuated by pressure or electrokinetics, with pressure-driven flow being widely adopted because of its simplicity and easy implementation using external devices, such as syringe pumps. However, fluidic resistance may dampen these flows as large hydrodynamic pressures may be required to actuate the fluids. This is evident from the governing equations of an incompressible, Newtonian fluid where the fluidic resistance in a cylindrical tube is expressed as:

$$R = \frac{8\eta L}{\pi r^4} \quad \text{Equation 2-2}$$

where η is the fluid viscosity, L refers to the channel length, and r being the channel radius. Based on *Equation 2-2*, the fluidic resistance is inversely proportional to the fourth power of the channel radius, hence as the channel diameter decreases, resistance experienced by the fluid in the channel increases rapidly.

Alternatively, electrokinetics which involves the application of an external electric field to transport the fluid through the channel by utilizing mechanisms such as electroosmosis and electrophoresis, can be used to drive fluids. An important concept here is the occurrence of the electric double layer (EDL), which brings about surface-charge-governed ion transport [5] present particularly in nanochannels. The changing electrical potential near the surface results in spontaneous formation of an EDL, comprising of a Stern layer and a diffuse layer as illustrated in Figure 2-2a.

Although a bulk solution may be of neutral charge, surface charges can exist at the solid-liquid interface. In this case, surfaces interacting with an aqueous solution can gain a net surface charge. Due to electrostatic attraction, ions with opposing charges to the surface ions (i.e. counter-ions) accumulate and form a shielding layer along the charged surface, forming a layer known as the Stern layer. Co-ions (ions with the same polarity as the ions in the Stern layer but of smaller mass) are repelled away from the layer, and contribute as free ions in the fluid forming the diffuse layer. The zeta potential ζ is at the slip plane between the two layers (Figure 2-2a). The thickness of such a layer is determined by the Debye length, which is the distance where the surface charge decays to $\frac{1}{e}$ of its original charge towards the bulk value. In aqueous solutions, the Debye length is found to be between 1 – 100 nm [6]. As observed from Figure 2-2d, in microchannels, the EDL is negligible because the electrical potential decays rapidly to the bulk value, and the surface charges are not significant enough to electrostatically manipulate ions. However, the effects of the EDL become more pronounced as the channel dimensions approach the Debye length, making this phenomenon unique to nanochannels [7, 8]. In the case of the nanochannel, an electrical potential is present in the center of the channel (Figure 2-2e). Mainly, the EDL introduces non-uniform motion and electric fields

perpendicular in direction to the fluid flow. Therefore, the resultant axial and transversal fluxes of electrostatic fields can be exploited to separate and disperse the analyte ions [9].

Figure 2-2 (a) Schematic of the electric double layer, showing the accumulation of counter-ions on the charged surface, along with the diffuse layer and Debye length, L_D . The bold line indicates the electrical potential profile, ψ , which becomes more significant with shrinking dimensions. Reproduced from [10] with permission of The Royal Society of Chemistry. Comparison of the surface charge effects on (b) a microchannel versus (c) a nanochannel. (d) In a microchannel, the electrical potential decays to the bulk value, while (e) the nanochannel exhibits an electrical potential at the center of the channel. Furthermore, (g) the nanochannel shows a higher counter-ion concentration (orange coloured positive charges) compared to co-ions (blue coloured negative charges), unlike (f) the microchannel, where equal concentration of both charges exist in the middle of the channel. Reprinted with permission from [6]. Copyright (2005) American Chemical Society.

2.1.2 Nanofluidic Devices

Nanofluidics refer to the manipulation of fluid within or around nanometer structures and its applications. Perhaps the most important development in nanofabrication has been the introduction of nanoimprint lithography (NIL) [11], which works by mechanically deforming the resist material to achieve feature sizes as small as 10 nm [12]. Chou *et al.* devised nanochannels fabricated by NIL [13], where a mould with the designed pattern is produced by electron beam lithography (EBL) followed by reactive ion etching (RIE). The mould is then pressed against the substrate coated with a resist layer, such as poly(methyl methacrylate) (PMMA). During the imprinting step, the resist is heated to a temperature above its glass transition temperature. The mould is then removed and the pattern on the resist layer is fully developed using RIE with oxygen. Other nanolithography techniques reported include focused

ion beam, interferometric lithography, and sphere lithography [14]. Fluidic devices with nanochannels have been used to stretch single DNA molecules for length measurements and optical mapping [15-17].

Figure 2-3 Schematic drawing of DNA unravelling in a nanofluidic device by means of nanopillars. b) Stretching of DNA molecules by applying an electric field across the channel to drive the DNA from the pillar-free region into the pillared region, and c) the subsequent recoiling of DNA after the electric field is turned off and the DNA retracts back to the pillar-free region. Reprinted figure with permission from [17]. Copyright (2002) by the American Physical Society.

Nanofabrication may not be achievable using soft lithography as the resolution limitations in conventional photolithography may not be sufficient to achieve the critical dimensions required for nanofluidics [14]. Although promising in the possibility to manipulate DNA molecules, in terms of fabrication techniques, nanofluidics has yet to see a revolution [18] similar to soft lithography for microfluidics, thereby limiting the scale of complexity that can be achieved with nanometric channels.

2.1.3 Microfluidic Devices

Microfluidics is the most mature and well-studied fluidic domains, benefiting from the fabrication technologies made available from microelectronics. Its channels are in the range of micrometers. A microfluidic chip typically consist of a channel layer and a substrate layer, with common substrates such as glass, silicon, and polymers [19]. Glass and silicon microfluidic

chips can be patterned using surface micromachining, buried-channel techniques, including deep reactive ion etching (DRIE) and chemical vapour deposition (CVD), or bulk micromachining [20]. These materials are particularly useful for high-temperature processing, high aspect ratio devices (up to 20:1), or electrode integration. However, the fabrication of glass and silicon-based microfluidic chips requires specialized equipment, hazardous chemicals such as hydrofluoric acid (HF) and cleanroom conditions for fabrication, making it expensive and rendering it less accessible for many researchers working on miniaturized devices.

More recently, polymers have replaced the silicon substrate to be the dominant material for microchannel fabrication due to its low cost and ease for rapid prototyping, with the most common methods being casting or hot embossing [21] for replication. In particular, soft lithography reduces the need to repeatedly perform photolithography to fabricate microfluidic devices. The master is typically created using standard lithographic techniques such as photolithography, electron beam lithography, and micromachining. Once the master mould is produced, an elastomeric polymer is poured over it, after which the polymer can be peeled off once it is cured [22]. This process can then be repeated to make replicas of the microfluidic chip just by using the master mould. Elastomeric polymers are flexible cross-linked polymer chains which can stretch or compress with force. PDMS is a notable elastomeric polymer widely used in soft lithography because of its low cost, biocompatibility, optical transparency, and gas permeability [23]. Furthermore, due to its permeability to oxygen, nitrogen, and carbon dioxide, viable cell cultures can be maintained, thus enabling PDMS chips to be used for long-term cell imaging studies. Microfluidic chips have wide applications in the biomedical field ranging from implants and tools for drug delivery [24], nanomedicine, organ-on-chip [25], diagnostic tools such as flow cytometry [26] and lab-on-chip platforms [27].

Figure 2-4 Various microfluidic chip designs mimicking different vital organs in our body serving as a platform for in vitro drug testing and cell culture. Reproduced from [25] with permission of The Royal Society of Chemistry.

2.1.4 Millifluidic Devices

As its name suggests, millifluidic devices refer to channel dimensions in the millimeter scale. Such channel sizes can be seen directly with the naked eye and are less stringent in terms of their fabrication process. Millifluidic channels moulds can be fabricated using three-dimensional (3D) printers, thereby serving as a low-cost alternative. This is an attractive option compared to lithography, which requires cleanroom facilities and is thus more expensive and time-consuming.

Unlike the other fabrication techniques mentioned earlier, 3D printing not only enables the creation of a mould to construct the chip, it has the added advantage of directly printing the actual chip designed using computer-aided design (CAD) drawings. Some commonly used 3D printing technologies include fused deposition modelling (FDM) and stereolithography (SLA). FDM works by melting thermoplastics like acrylonitrile butadiene styrene (ABS) and polylactic acid (PLA) by extruding the plastic filament through a heated nozzle and depositing it layer by layer to build the finished product. SLA, on the other hand, utilizes photocurable

liquid resins, which are deposited as a fresh layer and cured only if the ultraviolet laser scans across it. Similarly, this process is then repeated layer by layer until the model is complete. In fact, depending on the resolution of the 3D printer and the technology used, 3D printing can achieve high enough resolution to fabricate micrometer structures as well [28].

Compared to microfluidic devices, fabricating millimeter dimensioned structures using the laborious procedures of conventional lithography appears to be an overkill and a seemingly waste of resources. Therefore, the advent of 3D printing fits comfortably to fill this gap and enable rapid prototyping of millifluidic structures. Hence, there has been much interest in millifluidic-based bioanalytical devices for cell culture, manipulation, drug screening and nanomaterial synthesis [29-31].

Figure 2-5 (A) Schematic drawing of millifluidic chip with miniaturized traps (*T*) positioned in the row array (*R*), (B) 3D CAD drawing of the chip, and (C) actual fabricated device using SLA technique. Reproduced from [30] with permission of AIP Publishing.

Regime, Critical Operating Length [m]	Fluidic Channel Characteristics	Advantages	Limitations
Nanofluidic, 10^{-9}	<ul style="list-style-type: none"> Ultra-high SA/V ratio Greater interfacial effects such as strong electrostatic interaction between charged surface and ions in channel (EDL formation) Low fluid volumes Liquid considered as ensemble of molecules 	<ul style="list-style-type: none"> Single macromolecule confinement Valence-based macromolecule separation Suitable for DNA sequencing 	<ul style="list-style-type: none"> Incomplete theoretical framework Non-standard components Non-standard characterization Lacks a disruptive fabrication technology Difficult repeatability
Microfluidic, 10^{-6}	<ul style="list-style-type: none"> Higher surface tension High SA/V ratio Low fluid volumes 	<ul style="list-style-type: none"> Developed theoretical framework Single cell scale 	<ul style="list-style-type: none"> Prone to clogging and air bubbles Non-standard components

	<ul style="list-style-type: none"> • Liquid considered as continuum 	<ul style="list-style-type: none"> • Rapid prototyping using soft lithography • Wide range of working flow rates • Suitable for flow cytometry, sensing, and bioanalysis. 	<ul style="list-style-type: none"> • Non-standard characterization
Millifluidic, 10⁻³	<ul style="list-style-type: none"> • Lower surface tension • Lower SA/V ratio • Higher fluid volumes • Liquid considered as continuum 	<ul style="list-style-type: none"> • Cell population scale • Non-lithographic rapid prototyping using 3D printing • Able to process viscous fluids • Suitable for nanoparticle synthesis and large-scale <i>in vitro</i> bioanalysis 	<ul style="list-style-type: none"> • Low resolution features • Non-standard components • Non-standard characterization • Limited range of working flow rates

Table 2-1 Overview of characteristics of miniaturized fluidic devices, its advantages, and limitations.

2.2 Nanoparticle Synthesis

2.2.1 Synthesis Methods

Nanoparticles are materials in the order of 1-100 nm in size and have been shown to have various biomedical and optoelectronic applications. For example, nanoparticles have been used as drug delivery vehicles for the treatment of various chronic diseases such as cancer and also as imaging probes for the visualization of tumours and marking of various cells. In addition, they can also be employed as light emitting diodes (LEDs) due to their electroluminescence properties and energy harvesting solar cells as their bandgaps can be tuned by utilizing quantum dots (QDs) of different sizes [32, 33]. There are many methods to synthesize nanomaterials or nanoparticles and they can be broadly categorised into solid-phase, vapour-phase, liquid-phase, and two-phase approaches [34]. Examples of solid-phase and vapour-phase deposition techniques include chemical vapour deposition (CVD), molecular beam epitaxy (MBE), sputtering, ball milling, and laser ablation. However, in the context of this work, the topic of interest is focused on liquid-phase colloidal synthesis of nanoparticles.

2.2.2 Colloidal Synthesis - Hot injection

Figure 2-6 LaMer plot showing nanoparticle formation showing the nucleation (Phase II) and growth (Phase III) of the nanoparticles. Reproduced from [35] with permission of The Royal Society of Chemistry.

The process of nanoparticle formation is best explained using the LaMer plot [36] as illustrated in Figure 2-6, with three phases occurring in this process. In the first phase (Phase I), the monomer concentration of the precursors increases and exceeds the supersaturation level (S). In order to form highly monodispersed nanoparticles, it is essential that no seeds are present, otherwise heterogeneous nucleation will occur, resulting in a series of particles with different sizes. At this point, due to the high activation energy of the homogeneous nucleation process, no particle formation occurs yet and the concentration of the monomers continues to build up. Then in Phase II (nucleation stage), as the monomer concentration reaches the critical supersaturation level (S_c), the energy of the system is now sufficient to overcome the energy barrier and causes rapid formation of nuclei (i.e. burst nucleation). As a result of the burst nucleation, the monomer concentration starts to drop rapidly until it falls below the critical S_c value and proceeds to Phase III, known as the growth stage. In this phase, further nucleation cannot be sustained, hence the particles start to increase in size, constituting the growth process.

Therefore, burst nucleation is highly desirable to produce monodispersed nanoparticles as all the nuclei are formed simultaneously and the growth conditions are identical.

Hot injection is a synthesis method first developed by Murray et al. [37] to induce spontaneous nucleation in order to produce nanoparticles with uniform sizes (i.e. high monodispersity). It involves quickly injecting a high concentration of precursor into a hot solution containing surfactants thereby giving rise to supersaturation. This corresponds to Phase II of the LaMer plot as described earlier, hence resulting in burst nucleation and the spontaneous generation of numerous nuclei which reduces the free energy of the hot supersaturated solution. In addition, further nucleation is inhibited due to the decrease in temperature as a result of the addition of precursors at room temperature. This then causes the number of particles to stagnate or even decrease (i.e. Ostward ripening), indicating the initiation of the subsequent growth stage (Phase III of the LaMer plot) [38]. The segregation of the nucleation and growth stages is crucial to fabricate nanoparticles with high monodispersity.

2.2.3 Nanoparticle Synthesis using Fluidic Devices

Increasing miniaturization of laboratory systems and devices has led to the integration of functional components in miniaturized devices known as lab-on-chip (LoC) devices. In particular, for the field of nanosynthesis of materials for biomedical applications, these LoC systems possess many unique characteristics favourable for nanoparticle synthesis. For instance, LoC devices require only small amounts of samples, have quick reaction times and moreover, provide unique, controlled micro environments which are crucial for nanosynthesis. These microenvironments are paramount to control crucial nanoparticle characteristics, such as morphology, size, and surface chemistry.

Traditionally, in conventional benchtop (BT) nanoparticle synthesis as described in Section 2.2.2, reactants are added into a three neck flask. For certain synthesis protocols where an inert environment is necessary to prevent oxidation of the reactant species, nitrogen (N_2) or argon (Ar) gas supply is fed into the three neck flask. A magnetic stirrer bar is also added to allow mixing of the reactants by physical agitation. The reaction mixture can be heated directly on a hot plate, heating mantle or by immersion in a water or oil bath with a temperature probe to regulate the temperature of the reaction mixture.

Figure 2-7 (a) Continuous flow, (b) liquid-liquid segmented flow and (c) liquid-gas fluid slug segmented flow, and (d) hydrodynamic flow focusing. Reprinted with permission from [39]. Copyright 2006 John Wiley & Sons, Inc (b and c).

On the other hand, for nanoparticle synthesis in miniature channels, the reaction mixtures are fed into the inlets of the chip using tubes connected to syringe pumps. The channels serve as the reaction vessel for microfluidic synthesis as the fluid traverses the entire chip. There are two main categories of fluid flow in the microchannels, namely continuous flow (single phase) or segmented flow (two phase). The continuous fluid flow scheme involves a single uninterrupted fluid stream composed of the reactant solutions (Figure 2-7a). Mixing in the continuous fluid flow scheme occurs via passive diffusion between the laminar flow reactions mixture. Another flow regime is the segmented flow scheme, which can be further subdivided

into gas-liquid segmented flow and liquid-liquid segmented flow. In contrast to a single fluid stream, the gas-liquid segmented flow has the reaction mixtures trapped in liquid slugs separated by gas bubbles (Figure 2-7c), while the reaction mixture forms a droplet carried by an immiscible carrier fluid for the case of the liquid-liquid segmented flow scheme (Figure 2-7b). The segmented flow scheme promotes rapid mixing within the discrete droplets in the otherwise laminar fluid flow in the microchannels and also prevents channel fouling as the contact between reactant droplets and the channel walls are minimized compared to the continuous flow regime. Lastly, there is another continuous flow regime known as hydrodynamic flow focusing which consists of two miscible fluid streams as shown in Figure 2-7d. By controlling the flow rates of the centre stream and the side streams, a narrow jet width can be obtained enabling rapid mixing between the two liquids.

2.2.3.1 Metal Nanoparticles

Metal nanoparticles such as gold (Au) and silver (Ag) are widely studied because when electromagnetic radiation with wavelengths larger than the size of the particles impinges on the nanoparticles, it couples with the particles and generates electron charge oscillations at the interface between the medium and the metal particles known as surface plasmons. In nanoparticles, these surface plasmons are known as local surface plasmons resonance (LSPR) as the surface plasmon is confined within the nanoparticle and is highly sensitive to changes in its surrounding medium, thereby enabling applications such as biosensing, cancer treatment and bioimaging [40].

Lohse et al. [41] developed a millifluidic synthesis scheme made up of commercially available tubes and materials (Figure 2-8) to achieve high throughput, gram-scale production of Au nanoparticles with improvements in the yield for seeded Au nanorod (NR) growth in terms of

efficiency of conversion from the Au precursor (gold tetrachloroaurate trihydrate, $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$) into the eventual Au nanoparticles. In their work, they investigated the synthesis of spherical Au nanoparticles as well as nanorods via two methods, namely direct synthesis by reduction of the gold precursors in the growth solution and seed-mediated growth using Au nanoparticle seeds with the growth solution enriched with silver nitrate. The growth solution consists of the gold salt $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, ligands such as sodium citrate, mercaptohexanoic acid (MHA) or cetyl trimethyl ammonium bromide (CTAB), as well as supporting reagents such as aqueous sodium borohydride (NaBH_4) or deionized water depending on the ligand used. As illustrated in Figure 2-8, these solutions were driven into millifluidic tubes using the peristaltic pump and mixed via diffusion as they traversed the Y-mixer before they were characterized in the observation cell to determine its size and shape. Since the flow rate in this study was optimized, they reported different particle sizes and morphologies by varying the reaction precursors such as the compositions of the growth solution and the ligands used and the method of synthesis. Figure 2-8b shows the absorbance curves and transmission electron microscope (TEM) images showing Au NRs with different aspect ratios (AR) ranging from 1.5 to 3.5. Different sizes, shapes and ARs of the Au nanoparticles give rise to different LSPR peaks, thus such a fabrication platform allows the tuning of the LSPR peak to the desired wavelength region or even combining different configurations to extend the plasmonic band. Although the footprint of this system is relatively large compared to chip-based systems, it demonstrates the potential of continuous flow millifluidic systems to scale up the production of Au NPs by enabling high throughput rates of up to as much as 2.5 mg/min and yet still retaining control over the shape and size of the particles.

Figure 2-8 Millifluidic flow reactor setup for Au NP synthesis, with b) absorbance and transmission electron microscope (TEM) images of the as-synthesized Au nanorods (NRs). The TEM images and the absorbance curves correspond to Au NRs with aspect ratios of (i) 1.5, (ii) 2.0, (iii) 2.5, (iv) 3, and (v) 3.5, with respective scale bars of 50 nm. Reprinted with permission from [41]. Copyright (2013) American Chemical Society.

2.2.3.2 Semiconductor Nanoparticles

Group II-IV semiconductor nanoparticles are one of the most well-studied semiconductor nanoparticles, with cadmium chalcogenides (i.e. CdE, where E= sulphur (S), selenium (Se) and tellurium (Te)) being popular candidates of interest. Since in section 2.2.3.1, continuous flow synthesis has been discussed, we will discuss segmented flow or droplet flow synthesis in this section. In fact, this was the strategy adopted by Chan et al. when they investigated the growth of cadmium selenide (CdSe) nanocrystals in glass-based microfluidic chip reactors as they started with the continuous flow scheme [42] before moving on to the droplet flow [43].

Segmented flow as illustrated and described in section 2.2.3 can be implemented as either as a plug (Figure 2-7b), where the reactant droplets are separated by an immiscible carrier fluid, or a slug (Figure 2-7c) in which pockets of inert gas are introduced to separate the reactant droplets. The plug configuration is preferred as contact between the reactants and the channel wall can be minimised, if not avoided, eliminating particle size variations as a result of velocity dispersion in the channel. The slug configuration, although offers a slight improvement as compared to the continuous flow regime, still makes contact with the channel walls where the fluid velocity is confined by the boundary condition to be zero. Therefore, some dispersion is still experienced and may translate to larger particle size distributions and lower monodispersity.

Figure 2-9 a) Schematic drawing and b) actual microfluidic channel with droplet jet injector seen under a microscope. Transverse cross sections of the chip c) before and d) after the step. e) Lateral cross section before and after the step showing the increase in channel height from

45 μm to 90 μm . Adapted with permission from [43]. Copyright (2005) American Chemical Society.

Figure 2-9 presents the droplet jet injector was reported by Chan et al. [43] for the microfluidic synthesis of CdSe using cadmium oxide and oleic acid to form the cadmium oleate precursor and tri-n-octylphosphine (TOP), selenium (Se) powder and octadecene (ODE) to form the selenium precursor (TOPSe) and reaction at high temperatures of around 300 °C. Perfluorinated polyether (PFPE) was used as the inert carrier fluid to isolate the reactants. By varying the flow rates of both the reactant stream and the carrier streams, different droplet sizes and stream stabilities were observed. By optimizing parameters such as the reaction temperature and flow rates (consequently, residence times), spherical and highly monodispersed CdSe nanocrystals of around 3.8 nm diameter were fabricated as seen in the TEM image in Figure 2-9g.

In addition to studying the material parameters, it is also essential to study the feasibility of using these particles synthesized from miniature reaction vessels for function applications. Semiconductor nanoparticles have the unique property of size dependent properties such as tunable emission wavelengths due to quantum confinement effects. Therefore, semiconductor nanoparticles smaller than the exciton Bohr radius of the bulk material are known as quantum dots (QDs). The emission wavelength of these QDs can be tuned by controlling their physical sizes. The photoluminescence of the QD is the reason why these nanoparticles are widely used in bioimaging applications. Compared to traditional fluorescent organic dyes with narrow excitation peaks and broad emission spectrums [44], QDs have broad excitation spectrums and narrow emission peaks. This unique property enables multiple fluorescent QDs markers (with different emission wavelengths) to label different cellular regions of interest to be observed while using a single excitation wavelength [45, 46]. Besides that, QDs are more resistant to

photobleaching as compared to organic dyes [47], retaining their ability to fluoresce under continuous exposure at the excitation wavelength, thereby allowing studies which require uninterrupted monitoring of fluorescence emission such as the elucidation of signalling pathways [48] and cancer metastasis [49].

Figure 2-10 (a) Simulated and actual microfluidic chips with different channel dimensions, (b) the corresponding experimental results of the microfluidic chip synthesized BSA-CdTe quantum dots (QDs), (c) photoluminescence, and (d) photostability characterizations of the microfluidic (MF) and benchtop (BT) BSA-QDs. Reproduced from [50] with permission of The Royal Society of Chemistry.

Hu et al. demonstrated a detailed study of seed-mediated microfluidic chip synthesis of cadmium tellurite (CdTe) QDs conjugated with bovine serum albumin (BSA) and folic acid (FA) biomolecules for imaging macrophage and pancreatic cancer cells [50]. In this work, the effect of parameters such as the reaction temperature and channel dimensions (800 mm in length, 200 μm in height and widths of 200 μm, 400 μm and 600 μm) on the quality of the QDs produced (Figure 2-10a and b) were investigated. In addition, the photoluminescence and photostability of the microfluidic (MF) chip synthesized CdTe-BSA QDs and the conventional benchtop (BT) method were compared. As presented in Figure 2-10c and d, it was discovered

that the microfluidic chip could produce QDs of comparable quality and stability in a drastically shorter duration.

RAW264.7 mice macrophages and Panc-1 human pancreatic cancer cells were seeded onto cover glasses with Dulbecco's modified eagle's medium (DMEM) in a 6-well plate prior to treatment with the QDs. The cells were then treated with the BSA-QD, 3-mercaptopropionic acid QD (MPA-QD) and FA-QD formulations and incubated for 4 hours. The treated cells were then rinsed thrice with phosphate-buffered saline after the incubation and observed under a microscope. The microscope images in Figure 2-11a and c show the RAW264.7 and Panc-1 cells labelled using the as-synthesized BSA-QDs and FA-QDs. The bright green fluorescence signals are a clear indication that the microfluidic BSA-QDs and FA-QDs provide excellent contrast and hence can be used as optical contrast agents for cell imaging. In addition, folic acid (FA) functions as a targeting ligand as folate receptors are overexpressed in many types of cancer cells. Lastly, the reason for inclusion of the middle row MPA-QDs (Figure 2-11b), despite MPA not being a biomolecule, is to indicate that uptake of the QDs into the cell occurred via a specific bio-mediated pathway and was not due to passive diffusion.

Figure 2-11 Bioimaging of RAW264.7 mice macrophage cells and Panc-1 human pancreatic cells labelled with the microfluidic (MF) (a) BSA-QDs, (b) MPA-QDs, and (c) FA-QDs. Reproduced from [50] with permission of The Royal Society of Chemistry.

2.2.3.3 Polymer Nanoparticles

Most anticancer drugs such as camptothecin and paclitaxel are hydrophobic in nature, making it challenging to deliver these drugs directly to the infected sites. Polymer nanoparticles thus are ideal drug delivery agents to encapsulate these hydrophobic drugs and transport them to the tumour cells due to advantages such as biodegradability, reduced toxicity, and abundance.

Majedi et al. [51] made use of hydrodynamic flow focusing in a T-shaped microfluidic chip where the dimensions of the mixing channel was 150 μm (width) by 60 μm (height) by 1 cm (length) to assemble chitosan nanoparticles loaded with a cancer chemotherapy drug - paclitaxel. Chitosan is a polysaccharide which is popularly used as a drug delivery carrier because it is biodegradable, easily chemically functionalized and sensitive to pH changes [52]. By injecting the hydrophobically-modified chitosan supplement solution (HMCS) along with the paclitaxel chemotherapy drug (i.e. the polymeric stream - dark green region in Figure 2-12)

and having the other two inlets (i.e. carrier stream, light green regions) infused with water (an immiscible solution), a tightly focused fluid stream was formed. Varying the relative flow rates of the polymeric stream and the carrier stream, different mixing regimes were attained, resulting in fabrication of nanoparticles with different sizes, compactness and surface charges.

Figure 2-12 T-shaped microfluidic chip for assembly of high molecular weight chitosan supplement (HMCS) nanoparticles via hydrodynamic flow focusing. Reprinted with permission from [51]. Copyright 2014 John Wiley & Sons, Inc.

The chitosan nanoparticles were labelled with fluorescein isothiocyanate (FITC) dye so that the cellular uptake of these nanoparticles could be quantified by measuring the mean fluorescence intensity at different nanoparticle concentrations with using flow cytometry. The human breast adenocarcinoma (MCF-7) cells were employed as the cell model in this case study. Figure 2-13 showed that the microfluidic chip synthesized nanoparticles were smaller in size as compared to the bulk synthesized nanoparticles. The microfluidic HMCS nanoparticles exhibited enhanced cellular internalization as compared to the bulk synthesized nanoparticles, which were attributed to the reduced particle sizes, increased compactness and higher surface charge of the microfluidic synthesized nanoparticles [53, 54].

Figure 2-13 Hydrodynamic size of unloaded chitosan nanoparticles synthesized using the hydrodynamic focusing microfluidic chip at different flow ratios. DS I, II and III refer to HMCS with different degrees of substitutions. Reprinted with permission from [51]. Copyright 2014 John Wiley & Sons, Inc.

The microfluidic chip synthesized chitosan nanoparticles enabled controlled release of the paclitaxel (PTX) drug over time where the drug release rates vary according to the environmental pH. Figure 2-14 depicts the *in vitro* simulation of the cellular environment as the nanoparticles circulate in the body (pH=7.4) and maintaining their drug load, encounter tumour cells (pH=6.5) and subsequently causing an increase in the drug release rate, and being engulfed by the lysosome after they enter the tumour cell (pH=5.5) where the nanoparticles rapidly offload the drug. Therefore, the microfluidic chip synthesized chitosan nanoparticle are capable of carrying and delivering the anticancer drug to the targeted tumour cells due to their pH sensitivity and the process can be monitored by measuring the fluorescence emission as the nanoparticles are labelled with FITC dye.

Figure 2-14 Paclitaxel (PTX) drug release profile of the chip synthesized chitosan nanoparticles as a function of the environmental pH to mimic circulation around tumour cells. Reprinted with permission from [51]. Copyright 2014 John Wiley & Sons, Inc.

2.2.4 Millifluidic Devices VS State-Of-The-Art Synthesis Methods

Figure 2-15 Schematic representation of generic (a) bench top and (b) miniature fluidic synthesis setups. Adapted from (a) [55] and (b) [56] under the CC BY 4.0 license.

Current state-of-the-art synthesis techniques such as hot-injection [37, 57], non-injection [58], solvothermal synthesis [59], and microwave-assisted synthesis [60, 61] largely involve the use of macroscopic reaction flasks and pressurized chambers where heating and mixing are carried out. These methods seek to find solutions and optimised parameters at which nanoparticle nucleation and growth processes can be separated so as to produce highly monodispersed nanoparticles. For ease of reference, these techniques will be collectively termed as bench top

(BT) methods in subsequent discussions as they are usually performed on a wet chemical bench in a laboratory setting.

Adv/Disadv	Description	Bench-Top (BT)	Millifluidic (mF)
Adv ✓	Length of reaction	Long ~ hours to days	Short ~ mins
Adv ✓	Ease of reproducibility	Dependent on sensitivity of reaction to expt conditions	Highly reproducible
Adv ✓	Scaling up NP production	Challenging. Cannot scale up reaction volume directly. Even if parallel reaction flasks, will take very long	Easy. Can use multiple chips in parallel and increase volume of stock solution
Disadv ✗	Reactant leakage	Less risk unless flask stoppers are dislodged	High risk if chip is not well sealed
Adv ✓	Real-time characterization and optimization	On-going reaction, cannot change parameters in the midst	Due to time:spatial resolution, can change parameters at the inlet and collect its output

Table 2-2 Advantages and drawbacks of millifluidics compared to conventional bench-top synthesis methods.

Firstly, the bench top methods generally take longer durations compared to millifluidic synthesis methods. The BT techniques usually involve long reaction durations in the range of several hours and even days, for example skutterudite nanocrystals (CoSb_3) requires 24-72 hours for particle formation [62]. However, continuous flow millifluidic chip synthesis takes only at most several minutes (Chapter 3 to Chapter 5) as the duration over which the precursors can interact and mix on the chip depends on the injection rate as well as the total fluid capacity of the chip. This means that we can potentially reduce the synthesis time required for nanoparticle fabrication and speed up particle production rate to yield more nanoparticles over a fixed duration.

Secondly, reproducible batches of nanoparticles are challenging for BT techniques as the nucleation kinetics of nanoparticle synthesis are sensitive to experimental conditions such as inhomogeneous mixing and localized heating of the reaction mixture. The mixing time in bulk macroscopic reaction flasks is relatively long compared to the nucleation time, hence resulting in variations in temperatures, pH and reactant concentrations at different regions in the reaction vessel, thereby leading to polydispersity and poor batch reproducibility [56]. On the other hand, miniature reaction vessels such as millifluidic and microfluidic systems provides excellent heat mass transfer due to the large surface area to volume ratio [63] provided by the miniature channels and reproducible mixing conditions as laminar flow is maintained within the fluidic channels [64]. Therefore, the miniature millifluidic chip environment provides a well-controlled reaction environment conferring superior batch to batch reproducibility which is demonstrated subsequent work in Section 4.3.1.

Thirdly, scaling up the reaction to improve nanoparticle production is tricky for BT methods as the reaction volume in the flask cannot be scaled up directly as it would exacerbate the problems of inhomogeneous mixing and localised heating causing deterioration of the nanoparticle quality [65]. Millifluidic devices can alleviate this problem as several chips can be operated in parallel to increase the output rate of the nanoparticles produced and the amount of stock solution can be increased while maintaining the quality of the nanoparticles produced.

Nevertheless, reactant leakage is a conundrum that plagues all fluidic devices, thus millifluidic devices are not spared. Although millifluidic technology is capable of processing larger volumes and is less susceptible to channel fouling compared to microfluidic devices [66], should there be any leakages in the chip due to sealing issues, catastrophic damage can result, rendering the chip void. On the other hand, reactant leakage is almost impossible for BT

techniques unless the flask stoppers are dislodged or the glassware is damaged and the reaction mixtures are spilled.

Lastly, real-time characterization and optimization is possible for the fluidic setup as the temporal progress of chemical reactions can be spatially resolved on the chip as specific positions can be probed [67] to carry out in situ nanoparticle characterizations. Based on the results, the input parameters can then be modified and thus optimization can be achieved. The same cannot be done for BT methods as the reaction is an on-going process occurring in the whole vessel, no doubt aliquots can be extracted and characterized, but optimization would require numerous trials requiring fresh preparation of the precursors.

Therefore, we believe that miniature millifluidic reaction vessels can help to improve the quality, reproducibility, throughput, and ease of production of nanoparticles. The subsequent chapters provide a glimpse of some nanoparticles synthesized using millifluidic chips and the use of these nanoparticles for biomedical applications, offering an insight into the immense potential of this technology.

2.3 Conclusion

There are many other categories of nanoparticles like magnetic nanoparticles [68], graphene nanoparticles [69], mesoporous silica [70, 71] just to mention a few. It would take an entire book to cover all the different types of nanoparticles available, thus the purpose of this chapter is just to give a flavour of the possibilities in nanoparticle synthesis brought about by miniature fluidic devices. The interested reader may find the associated references listed above useful.

From the review presented, a brief overview of the technologies related to miniature fluidic devices was put forth with special emphasis on nanoparticle synthesis in these miniature

reaction vessels. Microfluidics has been the forerunner in fluidic technology, but recent advances in fabrication techniques such as rapid prototyping have enabled other fluidic regimes like millifluidics to emerge as a formidable force. The ability of millifluidic channels to process larger reactant volumes and retain control over localized parameters such as temperature and mixing efficiencies makes it an attractive alternative reaction vessel. In addition, millifluidic channels being larger in dimension, provides advantages such as easy visualization of fluidic regimes and are less prone to clogging, which would otherwise be detrimental to the chip. Lastly, there is a pressing need to look beyond pure scientific exploration of nanoparticle fabrication in miniature reaction chambers focusing on properties such as high crystallinity and uniform physical characteristics like shape and size, but also to investigate if these as-synthesized materials can be made into functional devices or probes for healthcare and consumer electronics.

Chapter 3 Synthesis of Copper Sulphide Nanocrystals for Photothermal Therapy

3.1 Introduction

In order to explore the capabilities of continuous-flow millifluidic in organic phase synthesis, we demonstrate the shape and size control of Cu_{2-x}S nanocrystals (NCs) using a continuous-flow millifluidic chip in this chapter. The Cu_{2-x}S NCs were chosen in this study because Cu_{2-x}S are novel plasmonic NCs which have a large range of carrier (hole) densities extending from 10^{17} to 10^{23} cm^{-3} and relatively high thermal conductivities, making them attractive for photovoltaics, biophotonics, single molecular detection and disease therapy [72-75].

Aside from the traditional macroscale bench top synthesis methods, NP fabrication using flow synthesis methods are also attractive. One of major categories is continuous-flow microfluidic synthesis. Monodispersed nanoparticles can be fabricated by precise sequential addition of the precursors downstream on the chip with uniform temperature distribution [76]. In addition, since the input kinetic energy can be easily tuned by varying the flow rates, size-tunable nanoparticles such as Au [77, 78], CdS [79], Fe_3O_4 [80], and CdSe [42, 81], can be achieved. Other continuous-flow studies have also shown that properties such as the crystal/phase of the nanoparticles can be altered [82], magnetic intensity can be enhanced [83] and the reduced full-width-half-maximum of the fluorescent emission [84]. Even the complex core-shell CdSe/ZnS can be produced using the simple continuous-flow microfluidic synthesis [85]. However, there are many instances which exposes the disadvantages for microfluidic chip synthesis such as the sophisticated operational control, time consuming fabrication, relatively low throughput rate and high fabrication cost [31, 86, 87].

Therefore, another alternative to flow synthesis is the millifluidic chip fabrication. Millifluidic synthesis is gathering interest among researchers for the fabrication of NPs such as Cu nanoclusters, Cu and Fe₂O₃ [87-89]. Millifluidics boasts comparative advantages like ease-of-fabrication, high throughput rate and suitability for other applications such as radiation-based X-ray absorption spectroscopy [31, 41] aside from similar advantages as microfluidics such as uniform heating and interaction time between precursors. At the same time, empirical research have shown that the continuous-flow millifluidic synthesis is capable of controlling the size of NP such as Ag and Au as well [85, 90, 91].

In this study, we found that by varying the flow rates and copper to sulphur molar ratio, different sizes and shapes of Cu_{2-x}S NCs could be produced. The photothermal effect obtained from these NCs could then be used to annihilate RAW264.7 cells upon near infra-red (NIR) laser irradiation.

3.2 Materials and Methods

3.2.1 Materials

Copper (I) chloride (99.995%, Sigma Aldrich), sulphur (analytical grade, Sigma Aldrich), oleylamine (>50.0%, Tokyo Chemical Industry Co., Ltd.), oleic acid (>85.0%, Tokyo Chemical Industry Co., Ltd.), toluene (>99.5%, Tokyo Chemical Industry), chloroform (ACS standard, Merck Pte Ltd), and absolute ethanol ($\geq$ 99.5 %, Merck Pte Ltd) were used in the synthesis of the Cu_{2-x}S nanoparticles. The 184 Sylgard silicone elastomer kit from Dow Corning was used to fabricate the polydimethylsiloxane (PDMS) continuous-flow millifluidic chip. RAW264.7 mice macrophage cell line (ATCC, Manassas, VA, USA), Dulbecco's Modified Eagle's Medium ([DMEM], Hyclone), fetal bovine serum ([FBS], Hyclone), penicillin-streptomycin (Gibco, Life Technologies, SG, Singapore) were used in cell culturing,

while cell viability was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium-bromide) assay kit (Sigma Aldrich), dimethylsulfoxide ([DMSO], Sigma Aldrich) and Apoptosis Detection Kit I (BD Pharmingen, San Diego, USA). All chemicals were used as received without further purification. Deionized (DI) water mentioned in the experiments was purified by a Milli-Q water purification system.

3.2.2 Millifluidic Chip Fabrication

The millifluidic chip was made from a 3D printed mould designed using the software SketchUp and printed in acrylonitrile butadiene styrene (ABS) plastic. The polydimethylsiloxane (PDMS) resin mixture was made by mixing the base resin and the curing agent in the volume ratio of 1:10 using the 184 Sylgard silicone elastomer kit (Dow Corning) and degassed before pouring it into the ABS mould. The filled mould was then baked for at least 4-5 hours at 65 °C on a hotplate. When the PDMS was fully cured, it was carefully removed from the mould and sealed with a glass slide. Holes were made at the inlets and outlets of the chip with a puncher and polytetrafluoroethylene (PTFE) tubes were inserted. The interface between the tubes and the inlets and outlets were reinforced and sealed with PDMS to prevent leakage of the precursors as they were injected into the chip. The fabrication of such a millifluidic chip with reasonably accurate dimensions and reproducibility is inexpensive, convenient and relatively easy, making it highly suitable for mass production.

3.2.3 Synthesis of Cu_{2-x}S NCs

The synthesis protocol used was similar to Liu *et al.* [92]. Briefly, 10 mL of oleic acid (OA) was added to dissolve sulphur (0.5 mmol, 1 mmol, 6 mmol or 10 mmol). The mixture was then stirred and heated at 120 °C. Similarly, 10 mL of oleylamine (OAm) was added to copper (I) chloride (0.5 mmol, 1 mmol, 6 mmol or 10 mmol) and also heated at 120 °C with stirring for

half an hour. After 30 minutes, the temperature of the copper-oleylamine (Cu-OAm) mixture was increased to 200 °C before being cooled to 120 °C at which the mixture was then withdrawn in a syringe. The sulphur-oleic acid (S-OA) mixture was also likewise withdrawn in another syringe. Meanwhile, the PDMS chip was pre-heated at 120 °C on a hotplate before a syringe pump (KD Scientific) was used to inject the two precursor mixtures simultaneously into the chip. The millifluidic chip was operated in the continuous-flow regime. The precursors were mixed in the initial winding section to initiate the nucleation process, while the subsequent straight section allowed particle growth. Lastly, further growth of the Cu_{2-x}S NCs was quenched by adding absolute (99%) ethanol near the end of the chip. Finally, the nanocrystals were purified by centrifuging the mixture at 8000 rpm for 2 minutes, decanting the supernatant and redispersing the pellet in chloroform.

The injection flow rate of the precursors were varied (150 $\mu\text{L}/\text{min}$, 1000 $\mu\text{L}/\text{min}$, 2000 $\mu\text{L}/\text{min}$, 3000 $\mu\text{L}/\text{min}$, 4000 $\mu\text{L}/\text{min}$ and 5000 $\mu\text{L}/\text{min}$), while the ratio of copper to sulphur was kept constant at 1:2 and the characteristics of the synthesized Cu_{2-x}S particles were studied. Besides that, Cu_{2-x}S particles synthesized at different copper to sulphur molar ratios (1:10, 1:6, 1:2, 2:1, 6:1 and 10:1) with a fixed flow rate of 3000 $\mu\text{L}/\text{min}$ were also investigated.

3.2.4 Cu_{2-x}S NCs Characterization

Transmission electron microscope (TEM) images were captured using a JEOL JEM-2011 TEM to examine the morphology and size of the NCs. The UV-Vis-NIR absorbance spectra were measured using a PerkinElmer Lambda 950 UV-Vis-NIR Spectrophotometer System. Powdered X-ray diffraction (XRD) measurements were obtained using the Rigaku SmartLab powder XRD diffractometer to determine the crystal phase. Energy-dispersive X-ray spectroscopy (EDX) measurements were performed in conjunction with the INCAx-ACT

detector from Oxford Instruments on the JEOL JSM-5600LV scanning electron microscope (SEM) to corroborate the changes in crystal phase.

3.2.5 Ligand Exchange of Cu_{2-x}S NCs from Organic to Aqueous Phase

The Cu_{2-x}S NCs were originally synthesized and dispersed in organic solvents and thus have to be converted into the aqueous phase to enable biocompatibility for subsequent uptake into the cells. Therefore, ligand exchange with L-gutathione (GSH) was employed to convert the nanocrystal into the aqueous phase. 1 mmol of reduced GSH was dissolved in 1 mL of deionized (DI) water with the pH of the solution adjusted to pH 10-11 by adding an appropriate amount of sodium hydroxide (NaOH) solution. Following that, 1 mL of the as-synthesized Cu_{2-x}S NCs dispersed in chloroform was added to the mixture. The reaction mixture was then stirred vigorously for 30 minutes with simultaneous addition of absolute ethanol. Lastly, the mixture was centrifuged at 8000 rpm for 2 minutes and redispersed in deionized (DI) water from a Milli-Q water purified system.

3.2.6 Cell Culture of Macrophage Cells

The cell culture medium for the RAW264.7 mice macrophage cells (American Type Culture Collection) consisted of Dulbecco's modified eagle's medium (DMEM, Hyclone), complemented with 10% fetal bovine serum (FBS), penicillin (100 µg/mL, Gibco) and streptomycin (100 µg/mL, Gibco). The cells were then incubated in a humidified environment with 37°C and 5% carbon dioxide (CO₂).

3.2.7 Cytotoxicity of Cu_{2-x}S NCs

The 96-well plate was seeded with a density of 1×10^4 RAW264.7 cells/well and allowed to adhere for 24 hours. Varying concentrations of the GSH capped Cu_{2-x}S NCs were added to the macrophage cells and incubated for a further 24 hours. The MTT (3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide) assay (Sigma) was performed to determine cell viability. 20 μ L of 5 mg/mL MTT mixed with phosphate buffered saline (PBS) was added to each well and incubated for another 4 hours. 150 μ L of 100% dimethylsulfoxide (DMSO, Sigma) was added and the cell plate was gently shook to dissolve the precipitate. Then, a microplate reader (Bio-Rad) was used to measure the absorbance readings with an excitation wavelength of 490 nm. The absorbance of the sample wells were subsequently normalized with the control well and expressed as percentages, with the viability of untreated cells taken as 100%.

3.2.8 Cellular Uptake of GSH capped Cu_{2-x}S NCs and NIR Laser Illumination

A 6-well plate was seeded with the macrophage cells at a density of 1×10^5 cells/well in DMEM culture medium. 13.5 μ M and 27 μ M concentrations of Cu_{2-x}S were added to the macrophage cells based on results from the MTT cytotoxicity studies. The treated cells were rinsed three times with PBS buffer (pH = 7.2) after 4 hours of incubation. The near infra-red (NIR) illumination source used was a multimode fiber coupled diode laser (K915FA3RN- 30.00W, BWT Beijing Ltd.) with centre wavelength of 915 ± 10 nm and numerical aperture of 0.22. The fiber end was straight cleaved and directed onto the cells. Different power densities were obtained by adjusting the output optical power of the diode laser. The NIR laser light was shone on each spot at a fixed power density level for 15 minutes.

3.2.9 *In vitro* Cell Imaging

The cells were incubated for 30 minutes after the NIR illumination and the apoptosis assay was performed using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, San Diego, USA) according to the manufacturer's protocol which was mentioned in [70]. In summary, cold PBS was used to wash the cells twice before

suspending in the binding buffer. Following which, 5 μL of fluorescein isothiocyanate (FITC) Annexin V and 5 μL of propidium iodide (PI) dyes were added and gently swirled before incubating for another 15 minutes in the dark at room temperature. *In vitro* cell imaging was accomplished using a fluorescence microscope (Eclipse-Ti, Nikon), where damaged or dead cells were stained with PI and appeared red upon illumination with light at 525 nm excitation wavelength.

3.3 Results and Discussion

3.3.1 Millifluidic Chip Synthesis of Cu_{2-x}S NCs

The homemade millifluidic chip illustrated in Figure 3-1 consisted of channels with dimensions of 1.5 mm (width) by 1.5 mm (height) and spanned a total length of 545 mm. Therefore, the total volume of fluid housed within the chip was 1227 mm^3 (or 1227 μL). Using this chip, Cu_{2-x}S NCs of various shapes and sizes were synthesized. Varying the injection flow rates of the precursors yielded various shapes such as triangular prisms (having sides with 13.5 nm by 12.9 nm by 11.7 nm) at 150 $\mu\text{L}/\text{min}$, rods (aspect ratios of 1.3–3.4) at 2000–3000 $\mu\text{L}/\text{min}$ and spheres (3.6 nm and 8.8 nm in diameter) at 4000–5000 $\mu\text{L}/\text{min}$.

Using copper to sulphur precursor molar ratios (Cu:S) of 1:10, 2:1, 6:1, and 10:1, spherical Cu_{2-x}S NCs (6.4–12.6 nm in diameter) were produced, while 1:6 and 1:2 molar ratios resulted in rod shaped NCs. In addition to morphological alterations, changes in the crystallographic structure of the NCs and their corresponding localized surface plasmon resonance (LSPR) peaks were also observed. The Cu_{2-x}S NCs ranged from covellite ($x = 1$, CuS) for molar ratios of 1:10, 1:6 and 1:2, before transiting to roxbyite ($x = 0.25$, $\text{Cu}_{1.75}\text{S}$) at 2:1 and becoming djurleite ($x=0.03$, $\text{Cu}_{1.97}\text{S}$) for 6:1 and 10:1. The

LSPR absorption peaks also red-shifted from 1115 nm to 1644 nm as the molar ratio was varied from sulphur rich ratios (1:10) to copper rich ratios (10:1). These results are summarized in

Table 3-1 supplemented with additional information such as the aspect ratios, throughput time and yield of the NCs.

Figure 3-1 (a) Annotated schematic design of the millifluidic chip, b) actual chip with sulphur and copper precursors during the synthesis, c) top view, and d) cross-section view of pristine PDMS chip. Figure reproduced from Cheung et al. [93].

The synthesis of the Cu_{2-x}S NCs were accomplished using the millifluidic chip, which consists of 2 inlets for the sulphur and copper precursor solutions, a zig-zag section to facilitate passive mixing of the precursors, followed by straight sections to allow formation of the nuclei and subsequent growth of the NCs (Figure 3-1a). An additional inlet towards the end of the channel injects ethanol into the mixture to quench the reaction. Lastly, the products of the reaction are collected at the outlet and purified before storage for further characterization and applications. The nanoparticle fabrication process is best exemplified in Figure 3-1b, where mixing of the precursors occurred in the winding zig-zag channel, followed by formation of a light green section which signified nucleation and the gradual darkening of the solution indicated particle growth.

Bonding the PDMS layer to the glass slide as shown in Figure 3-1c helps to provide uniform heating to the different sections on the chip so as to reduce temperature fluctuations experienced by the particles. With the increased surface area to volume ratio provided by the

millifluidic channels (Figure 3-1c and d), the contact between the channels and the heating surface was enhanced, thereby providing a homogenous environment for particle growth.

This millifluidic chip based reactor offers a simple, compact and robust platform whereby the enclosed channels protect the precursors from oxidation without the need of an inert gas (argon or nitrogen) atmosphere as with the case of macroscale synthesis. This is especially vital in the synthesis of Cu_{2-x}S NCs as the highly reactive copper precursor can be easily oxidized to the copper (II) (Cu^{2+}) state. In addition, nanoparticle synthesis in this millifluidic chip promises high reproducibility stemming from the well-controlled incubation environment in the chip. The low Reynolds number conferred by the small channel sizes, ensures laminar flow where mixing conditions are predictable and can be engineered by channel design, flow rate, and flow regimes. Using such a miniature synthesis platform, the amount of chemical waste generated can be minimised, moving a step closer towards green and sustainable chemistry [94].

Table 3-1 Summary of characteristics of Cu_{2-x}S nanocrystals synthesized using the millifluidic chip. Table reproduced from Cheung *et al.* [93].

Molar Ratio (Cu:S)	Flow Rate ($\mu\text{L}/\text{min}$)	Shape	Dimension AR: Aspect Ratio D: Diameter (nm) L: Length (nm) W: Width (nm) P: Perimeter (nm)	Aspect Ratio	Throughput Time (s)	Yield (%)	Composition	LSPR Peak (nm)
1:10	3000	Spherical	D: 6.4	1.2 ± 0.3	25	86	Covellite	1115
1:6	3000	Rod	L: 12.0, W: 5.3	2.3 ± 0.5	25	87	Covellite	1150
1:2	3000	Rod	L: 13.9, W: 4.3	3.4 ± 1.0	25	90	Covellite	1272
2:1	3000	Spherical	D: 8.8	1.1 ± 0.1	25	93	Roxbyite	1315
6:1	3000	Spherical	D: 11.9	1.1 ± 0.2	25	94	Djurleite	1471
10:1	3000	Spherical	D: 12.6	1.1 ± 0.1	25	97	Djurleite	1644
1:2	150	Prism	P: $13.5 \times 12.9 \times 11.7$	N.A.	490	40	Low Yield*	1281
1:2	1000	Spherical	L: 7.8, W: 6.2	1.3 ± 0.4	72	91	Covellite	1246
1:2	2000	Rod	L: 12.0, W: 5.3	2.3 ± 0.4	37	87	Covellite	1266
1:2	4000	Spherical	D: 8.8	1.1 ± 0.1	18	78	Spionkopite	1293
1:2	5000	Spherical	D: 3.6	1.2 ± 0.2	15	67	Spionkopite	1277

* – No specific conclusion can be drawn due to large variation of crystallinity.

N.A – not applicable

3.3.2 Characterization of Cu_{2-x}S NCs

3.3.2.1 Effect of Flow Rate on Cu_{2-x}S NCs

At the slowest flow rate of $150 \mu\text{L}/\text{min}$ corresponding to the residence time of 490 s, slightly longer than 8 minutes, prism-shaped Cu_{2-x}S NCs with average edge lengths of 13.5 nm by 12.9 nm by 11.7 nm (Figure 3-2a) were obtained. Upon increasing the injection flow rate to $1000 \mu\text{L}/\text{min}$, a high yield of spherical particles with an aspect ratio of 1.3 ± 0.4 were produced (Figure 3-2b). Further increase in the injection speed to $2000 \mu\text{L}/\text{min}$ and $3000 \mu\text{L}/\text{min}$ gave rise to nanorods with aspect ratios of 2.3 ± 0.4 and 3.4 ± 1.0 respectively Figure 3-2c and i). We postulate that the nanorod formation could be due to prolonged nucleation. At such high flow rates, down at the molecular scale there was insufficient time for the precursor molecules to form enough nuclei, thereby causing the excess free precursor molecules to attach onto reactive facet of the nuclei, resulting in rod-shaped NCs. Choi *et al.* also reported a similar

mechanism for rod-shaped nanoparticle formation, suggesting that reactive facet binding in seed mediated nanoparticle growth could result in morphological changes [95].

Figure 3-2 TEM images showing the size and morphology of the Cu_{2-x}S NCs synthesized at different flow rates of (a) 750 $\mu\text{L}/\text{min}$, (b) 1000 $\mu\text{L}/\text{min}$, (c) 2000 $\mu\text{L}/\text{min}$, (i) 3000 $\mu\text{L}/\text{min}$, (j) 4000 $\mu\text{L}/\text{min}$, and (k) 5000 $\mu\text{L}/\text{min}$, along with histograms depicting the corresponding size distributions (d, e, f, l, m, and n) and aspect ratios (g, h, o, p, and q) of the particles. The molar ratio of Cu:S was maintained at 1:2. Figure reproduced from Cheung et al. [93].

However, at extremely high flow rates of 4000 $\mu\text{L}/\text{min}$ and 5000 $\mu\text{L}/\text{min}$, the shapes of the fabricated NCs reverted back to spherical NCs with diameters of 8.8 nm and 3.6 nm respectively (Figure 3-2j and k). The observation of very small particles with sizes of 2 – 4 nm in diameter led us to believe that when the injection flow rate exceeded 3000 $\mu\text{L}/\text{min}$, there was inadequate time for particle growth, inhibiting adsorption of the precursors to the reactive surface of the nuclei, therefore elongation of the rod-shaped NCs could not be sustained.

Figure 3-3a shows the powdered XRD patterns of the NCs fabricated at different flow rates. Major peaks were observed at 2-theta values of 29.25°, 31.76°, 32.82°, and 47.89° for NCs synthesized at flow rates from 1000 – 3000 $\mu\text{L}/\text{min}$ corresponding to the (1 0 2), (1 0 3), (0 0 6), and (1 1 0) planes of covellite (CuS , Powder Diffraction File (PDF) card number 01-078-2391). The high resolution transmission electron microscope (HRTEM) images Figure 3-6a and c) indicate lattice distances of 2.815 Å and 1.898 Å and the fast fourier transform (FFT) analysis (Figure 3-6b and d) are consistent with the conclusion from the XRD pattern, further validating the hexagonal covellite structure.

At faster flow rates of 4000 – 5000 $\mu\text{L}/\text{min}$, the XRD patterns (Figure 3-3a) resembled spionkopite ($\text{Cu}_{1.39}\text{S}$, PDF 00-036-0380) with major peaks at 29.00°, 32.21°, 47.57°, and 50.08° matching the (6 0 5), (6 0 8), (6 6 0), and (6 0 19) planes. Likewise, Figure 3-6k indicated a lattice distance of 1.910 Å corresponding to the (6 6 0) hexagonal spionkopite crystal plane.

The spionkopite structure was also consistent with the FFT diffraction spot pattern depicted in Figure 3-6l.

Due to localized surface plasmon resonance (LSPR) effects, Cu_{2-x}S NCs exhibited absorbance peaks in the near infra-red (NIR) region. By varying the injection flow rates, the LSPR peaks can be adjusted from 1246 nm to 1293 nm as presented in Figure 3-3b. This narrow LSPR shift of 47 nm was because the Cu:S ratio was fixed at 1:2, thus both the variation in the degree of copper deficiency and crystallographic composition were not significant, resulting in only copper deficient covellite ($x=1$) and spionkopite ($x=0.6$) structures. Therefore, we attribute the observed LSPR peak shift, when the flow rate was altered, to the quantum confinement effects of Cu_{2-x}S NCs, which was also reported by Luther *et al.* [96] and Saldanha *et al.* [97].

Figure 3-3 a) Powdered XRD patterns and b) absorbance spectra of Cu_{2-x}S NCs synthesized under different flow rates with the Cu:S molar ratio fixed at 1:2. Figure adapted from Cheung *et al.* [93].

3.3.2.2 Effect of Molar Ratio on Cu_{2-x}S NPs

The effect of the molar ratio of copper and sulphur at a constant flow rate of 3000 $\mu\text{L}/\text{min}$ was also investigated for both cases where copper is in excess and also when sulphur is in excess. For the case of Cu:S molar ratio where the number of mole of S-oleic acid (S-OA) precursor is greater than the copper-oleylamine (Cu-OAm) precursor, morphological changes were observed. At the Cu:S molar ratio of 1:10, spherical Cu_{2-x}S NCs with a diameter of 6.4 nm as presented in Figure 3-4a. Decreasing the number of moles of sulphur to 6 mmol and 1 mmol (i.e. Cu:S=1:6 and 1:2) yielded nanorods with aspect ratios of 2.3 ± 0.5 and 3.4 ± 1.0 (Figure 3-4b and c).

On the other hand, when copper is in excess (i.e. number of moles of Cu-OAm is greater than S-OA) negligible morphological changes were observed, however the as-synthesized Cu_{2-x}S NCs exhibited varied crystallographic compositions. The basic case of Cu:S=1:2 corresponded to the copper-deficient covellite (CuS), while a slight increase in molar ratio to 2:1 gave rise to roxbyite (Cu_{1.75}S) and lastly, the copper-rich djurleite (Cu_{1.97}S) was formed at 6:1 and 10:1. The NCs synthesized at these three molar ratios are spherical with varying diameters of 8.8 nm (2:1), 11.9 nm (6:1), and 12.6 nm (10:1). Minor changes in the shapes of the NCs produced under different Cu:S molar ratios could be due to the alterations of the crystal compositions, which might have caused steric hindrance and impeded the binding of free precursor molecules to the reactive facet of the nuclei.

Under standard atmospheric conditions, covellite (CuS), anilite (Cu_{1.75}S), digenite (Cu_{1.8}S), djurleite (Cu_{1.95}S), and chalcocite (Cu₂S) are different forms of copper sulphide that can exist by modifying the stoichiometric factor in Cu_{2-x}S [98]. Our millifluidic chip enabled the synthesis of additional configurations like spionkopite (Cu_{1.39}S) and roxbyite (Cu_{1.75}S). We

believe that the high pressure conditions within the millifluidic chip due to the enclosed environment and small channel dimensions could have contributed to the range of crystallographic compositions of copper sulfide NCs formed. X-ray diffraction (XRD) analysis was carried out to elucidate the compositions of the NCs formed under the different fabrication conditions.

The crystal composition of NCs with excess sulphur (e.g. Cu:S=1:2, 1:6, and 1:10) matched covellite, similar to the NCs obtained by varying only the flow rate. But upon increasing the Cu:S molar ratio, a crystallographic structure resembling roxbyite ($\text{Cu}_{1.75}\text{S}$) was unveiled. The (16 0 0), (18 2 1), (20 0 1), (0 16 0), and (8 8 6) planes of roxbyite coincided with the major peaks at 26.59° , 31.20° , 34.06° , 46.84° , and 48.90° (Figure 3-5a). Figure 3-6i shows the 1.938 Å lattice distance corresponding to the monoclinic roxbyite (0 16 0) crystal plane. Similarly, the FFT analysis in Figure 3-6j also corroborates the same crystal structure and relevant planes of roxbyite. At the other end of the spectrum where the Cu:S molar ratios were 6:1 and 10:1, NCs with copper-rich djurleite compositions were produced instead. From Figure 3-5a, dominant peaks at 26.27° , 37.62° , 46.13° , 46.31° , and 48.65° matching the djurleite (0 0 4), (8 0 4), (0 4 6), (0 8 0), and (12 0 4) crystal planes. The HRTEM images of single particles depicted in Figure 3-6e and g reveal lattice distances of 1.870 Å and 2.389 Å respectively, consistent with the monoclinic djurleite (12 0 4) and (8 0 4) planes (PDF 00-023-0959). The FFT analysis (Figure 3-6f and h) lend further support to the djurleite structure.

Figure 3-4 TEM images showing the size and morphology of the Cu_{2-x}S NCs synthesized at different cadmium to sulphur (Cd:S) molar ratios of (a) 1:10, (b) 1:6, (c) 1:2, (j) 2:1, (k) 6:1, and (l) 10:1, along with histograms depicting the corresponding size distributions (d, e, f, m, n, and o) and aspect ratios (g, h, i, p, q, and r) of the particles. The injection flow rate of the precursors were maintained at 3000 $\mu\text{L}/\text{min}$. Figure reproduced from Cheung et al. [93].

In addition to changes in crystal composition, the LSPR peak was also varied from 1115 nm to 1644 nm by modifying the Cu:S molar ratios (Figure 3-5b). The near infra-red (NIR) LSPR peaks show that the Cu_{2-x}S NCs are highly self-doped (p-type) semiconductor quantum dots with copper-deficient stoichiometric properties as suggested by Alivisatos' group [96]. The LSPR spectrum is closely related to the stoichiometry of copper (i.e. x -value of Cu_{2-x}S NCs). A larger value of x in the Cu_{2-x}S NC introduces more holes in the copper deficient crystal structure, thereby creating more free hole carriers in the nanocrystal. Denser free carriers provide an environment with higher mobility for free charges on the surface to resonate. Therefore an increase in the x -value, results in shifting of the LSPR peak to a higher resonant frequency (blue-shift). Kriegel and Xie suggested that the LSPR spectrum shift in copper chalcogenide nanocrystals can be manipulated by the oxidation (inducing more holes) and reduction (holes trapping) of Cu_{2-x}S NCs [99, 100]. Moreover, Liu et al. revealed that free carrier holes can be trapped by the deprotonated solvents [92]. They showed that the deprotonated oleic acid can trap free holes on Cu_{2-x}S NCs, resulting in red-shifting of the LSPR absorbance peak. Herein, our study has shown that it is possible to obtain a much wider LSPR spectra shift of 529 nm (1115 nm to 1644 nm) by controlling the Cu:S molar ratios as compared to changing the injection flow rate.

In this synthesis protocol, oleylamine serves as surfactant [101] to passivate the hole carriers on the surface of Cu_{2-x}S NCs, thus decreasing the average carrier (hole) density of the nanocrystals and resulting in a red-shift of the LSPR peak [102]. Previously, in Figure 3-5a, it was observed that the XRD patterns exhibited the covellite ($x = 1$) composition for 1:2 Cu:S ratio, roxbyite ($x = 0.25$) for 2:1 Cu:S ratio, and djurleite ($x = 0.06$) for 6:1 and 10:1 Cu:S ratios. The corresponding LSPR spectra for these composites red-shifted as the value of x decreases (Figure 3-5b). This decrease of x indicates the formation of copper-rich nanocrystals. The red-

shift in the LSPR peaks can be attributed to increased passivation of the nanocrystals, which gives rise to lower carrier densities in the crystals as discussed above.

Figure 3-5 (a) XRD patterns, (b) Vis/NIR absorbance spectrum, and (c) EDX measurements of Cu_{2-x}S NCs synthesized with different Cu:S molar ratios with flow rate fixed at 3000 μL/min.

Mixed phases may be present in some samples as the experimental XRD patterns were not perfectly fitted with single phase. Thus, to verify the properties of the nanocrystals, EDX measurements were carried out by drop-casting the Cu_{2-x}S nanocrystal samples on a silicon wafer and measuring the elemental composition of copper and sulphur at different positions on the covered wafer (Table 3-2). There might be multiple phases present in the Cu_{2-x}S nanocrystals especially for samples with higher copper composition. Figure 3-5c corroborates this as can be observed from the significantly larger standard deviations of the copper in both cases of the 6:1 and 10:1 (Cu:S) molar ratios.

Figure 3-6 HRTEM images of single nanoparticles corresponding to the different crystal compositions of covellite (a and c), djurleite (e and g), roxbyite (i), and spionkopite (k) with annotated lattice spacing. The associated FFT spot diffraction patterns accompanying the HRTEM images for covellite (b and d), djurleite (f and h), roxbyite (j), and spionkopite (l). Figure reproduced from Cheung et al. [93].

Table 3-2 EDX composition ratios compared against the XRD fittings

Cu:S Molar Ratio	XRD Crystal Phase	EDX Composition Ratio	
		Cu	S
10:1	Djurleite and Chalcocite Cu_2S	2.26 ± 0.53	1
6:1	Chalcocite Cu_2S , Digenite $\text{Cu}_{1.8}\text{S}$	1.27 ± 0.22	1
2:1	Roxbyite 00-023-0958	1.02 ± 0.03	1
1:2	Spionkopite 00-036-0380	0.88 ± 0.04	1
1:6	Covellite 01-078-2391	0.86 ± 0.03	1
1:10	Covellite 01-078-2391	0.70 ± 0.05	1

Table 2 Brief summary of the synthesis condition, shape and plasmonic absorption of the current Cu_{2-x}S semiconductor nanocrystals.

Shape	Synthesis Method	Copper Source	Sulphur Source	Molar Ratio (Cu:S)	Dimension	LSPR Peak (nm)	Year	Ref.
					AR: Aspect Ratio D: Diameter (nm) L: Lateral (nm) T: Thickness (nm) W: Width (nm) P: Perimeter (nm)			
Disk	Thermolysis	Copper(II) chloride	Postassium (I) ethylxanthate	1:4	L: 15.2 T: 3.8	1130-1260*	2013	[103]
	Hot Injection	Copper(I) chloride	Sulphur Powder	1:4	L: 13-21 T: 5**	1112-1276**	2013	[104]
	Solventless Synthesis	Copper nitrate	1-dodecanethiol	1:6	L:24.5 T:4.4	In-plane: 3100 Out-Plane:1800	2011	[105]
	Thermolysis	Copper nitrate	1-dodecanethiol	1:6	AR: 4.65-7.10*	In-plane: 3100 Out-Plane:1700	2012	[106]
Hexagonal	Hot Injection	Copper(II) acetylacetonate	ammonium diethyldithiocarbamate	1:1.25	D:5	1250	2012	[99]
Platelet-like	Heat up Procedure	Copper(I) Chloride	Sulphur Powder	1:2	D: 13-13.7* T: 5-5.3*	1090-1250*	2013	[100]
Rod	Colloidal Approach	copper acetate	tert-dodecanethiol	1:16	Length: 10-100	NA	2012	[107]
	Cation Exchange	Cu ⁺ ions	Sulphur Powder	NA	NA	1128	2013	[108]
Spherical	Facile Approach	Copper(II) acetylacetonate	Sulphur Powder	2:1	D: 2.8-9.7***	1270-2400*	2014	[97]
	Hot Injection	Copper(II) acetylacetonate	ammonium diethyldithiocarbamate	1:1.25	D:2-6***	1770	2011	[96]
	Hot Injection	Copper(I) chloride	Sulphur Powder	1:1	D:6.6-20***	990-1180*	2013	[109]
	Solventless Synthesis	Copper nitrate	1-dodecanethiol	1:6	D: 4.0	1600	2011	[105]
	Sono-electro-chemical / Hydro-thermal	Cupric sulfate	Sodium thiosulfate	1:2	D: 5-20	NA	2009	[98]
	Hot Injection	Copper(I) chloride	Sulphur Powder	1:2	D: 2.8-13.5	1070-1230**	2013	[110]
	Non-Injection Approach	Copper(I) chloride	Sulphur Powder	2:1	D: 6.5	1800	2015	[111]
	Aqueous Synthesis	Copper(I) chloride	Thioacetamide	2:1	D: 2.7-7.2	NA	2015	[112]
Spherical	Continuous-Flow Millifluidic Synthesis	Copper(I) chloride	Sulphur Powder	1:10 / 2:1 / 6:1 / 10:1	D: 6.4-12.6 ⁺ D: 3.6-8.8 [^]	1115-1644 ⁺ 1277-1293 [^]	Our Work	
Rods				1:2 / 1:6	AR: 2.3-3.4 ⁺ AR: 1.3-3.4 [^]	1150-1272 ⁺ 1246-1272 [^]		
Prism				1:2	P: 14x13x12	1281		

*The change is attributed to post-synthesis reactions such as reduction, oxidation or aging.

**The change is attributed to different surfactant or composition molar ratios.

***The change is attributed to different synthesis conditions such as reaction time or temperature.

+The change is attributed to different precursor molar ratios.

[^]The change is attributed to different flow rates.

NA: Not Applicable / Not Available.

Table 2 consolidates some of the recent work on Cu_{2-x}S NCs, highlighting areas of interest such as the morphology, copper and sulphur precursors, NC dimensions, LSPR peaks as well as parameters studied. To the best of our knowledge, this is first demonstration of continuous-flow organic synthesis of Cu_{2-x}S NCs. Also, our maximum LSPR peak shift of 529 nm is also one of the widest peak shifts based on the existing literature. In addition, various morphologies were also obtained in this study, which will hopefully provide greater insight for those working in the field. The synthesis protocol adapted in this work was relatively simple and “green” as compared with the conventional approach in which the thiolic and phosphonic ligands such as trioctylphosphine oxide were involved. Stringent oxygen free environment was required to protect these toxic and easily oxidized during the conventional synthesis.

The localized surface plasmonic resonance (LSPR) is caused by the interaction of the electromagnetic field of the incident light on free carriers in a metal (or heavily-doped semiconductor nanoparticles, e.g. Cu_{2-x}S). Factors that affect the LSPR absorption peak include the refractive index of the surrounding medium, size, shape, material composition, and charge carrier densities of the nanoparticles. These factors boil down to the complex dielectric function, which results in shifting of the LSPR peaks.

This is evident from the extinction cross section ($\sigma_{ext}(\lambda)$) of a spherical nanoparticle based on Mie theory given by [113]:

$$\sigma_{ext}(\lambda) = \frac{24\pi^2 N a^3 \epsilon_m^{3/2}}{\lambda \ln(10)} \frac{\epsilon_i(\lambda)}{(\epsilon_r(\lambda) + 2\epsilon_m)^2 + \epsilon_i(\lambda)^2} \quad \text{Equation 3-1}$$

where N is the finite number of polarizable elements which can interact with the electromagnetic field, a is the radius of the spherical nanoparticle, ϵ_m refers to the dielectric constant of the surrounding medium, λ is the wavelength of the incident light, ϵ_r and ϵ_i are

the real and imaginary components of the dielectric constant of the nanoparticles, and $\chi=2$ is the shape factor for a spherical nanoparticle.

The resonance condition for LSPR is fulfilled when σ_{ext} is very large, indicating extremely strong absorption and scattering phenomena at a particular wavelength or frequency. This necessitates that ϵ_r is approximately $-2\epsilon_m$, hence narrowing the selection of materials to metals such as Au, Ag, Cu etc. The imaginary ϵ_i values should be negligible. The Drude model for the dielectric function of a material ($\epsilon(\omega)$) with free charge carriers was adopted [96, 114], where

$$\epsilon(\omega) = 1 - \frac{\omega_p^2}{\omega(\omega + i\gamma)} \quad \text{Equation 3-2}$$

consists of ω_p as the bulk plasmon oscillation frequency associated with the free carriers and γ represents the damping frequency, which reflects the plasmon bandwidth. The Clausius-Mossotti relation (Equation 3-3) describes the polarizability of sub-wavelength nanoparticles (α) [96, 114, 115],

$$\alpha = 3\epsilon_0 V \frac{\epsilon(\omega) - \epsilon_m}{\epsilon(\omega) + 2\epsilon_m} \quad \text{Equation 3-3}$$

with the volume of the nanoparticle as V , permittivity of free space as ϵ_0 , and the dielectric constant of the surrounding medium as ϵ_m . Therefore, when the denominator of Equation 3-3 tends to zero (i.e. real part of dielectric constant $\epsilon_r = -2\epsilon_m$, assuming that the imaginary part of the dielectric function has a weak frequency dependence and is thus negligible), the polarizability is resonantly enhanced.

$$\epsilon_r(\omega) = 1 - \frac{\omega_p^2}{\omega^2 + \gamma^2} \quad \text{Equation 3-4}$$

The dielectric function in Equation 3-2 can be simplified and represented by Equation 3-4. Thus taking into consideration the resonance condition and substituting $\varepsilon_r = -2\varepsilon_m$ into Equation 3-4, where $\omega = \omega_{sp}$ gives Equation 3-5

$$\begin{aligned} -2\varepsilon_m &= 1 - \frac{\omega_p^2}{\omega_{sp}^2 + \gamma^2} \\ \Rightarrow \therefore \omega_{sp} &= \sqrt{\frac{\omega_p^2}{1 + 2\varepsilon_m} - \gamma^2} \end{aligned} \quad \text{Equation 3-5}$$

which denotes the LSPR frequency (ω_{sp}). In addition, incorporating the definition of ω_p ,

$$\omega_p^2 = \frac{n e^2}{\varepsilon_0 m_{eff}} \quad \text{Equation 3-6}$$

where N represents the density of the free charge carriers, e refers to the charge of an electron and m_{eff} is the effective mas of the charge carrier (electrons or holes depending on the plasmonic material). Substituting Equation 3-6 into Equation 3-5, we get

$$\begin{aligned} \omega_{sp} &= \sqrt{\frac{\omega_p^2}{1 + 2\varepsilon_m} - \gamma^2} \\ \therefore \omega_{sp} &= \sqrt{\frac{N_h e^2}{\varepsilon_0 m_h (1 + 2\varepsilon_m)} - \gamma^2} \end{aligned} \quad \text{Equation 3-7}$$

the LSPR frequency (ω_{sp}) in this case for copper sulphide (Cu_{2-x}S) nanoparticles where the free charge carriers are holes, thus h is the subscript associated with the free charge carriers as expressed in Equation 3-7. Therefore, finally with this expression, we can see that the carrier density is directly proportional to the LSPR frequency, which corroborates our experimental data in Chapter 3, where lower LSPR frequencies (or longer LSPR wavelengths) were observed with increasingly copper rich Cu_{2-x}S nanoparticles (i.e. smaller x values with less copper cation vacancies and hence lower carrier densities).

3.4 Photothermal Therapy Application of Cu_{2-x}S NCs

Photothermal therapy is one of the well-known promising applications of copper sulfide nanoparticles for cancer therapy [116]. It involves the use of near infra-red (NIR) laser light to induce sufficient temperature elevations in tumor cells containing the nanoparticles to induce cell death. The idea behind photothermal therapy is to selectively annihilate the cancerous cells upon the NIR irradiation, leaving the neighboring healthy tissues unscathed, thereby achieving targeted cancer therapy. Currently, gold (Au) nanostructures of various morphologies as well as carbon-based nanomaterials such as carbon nanotubes (CNTs) and graphene have been widely studied as photothermal agents [103-106]. Cu_{2-x}S NCs have only recently emerged as potentially contenders for photothermal therapy due to advantages like cost-effectiveness in terms of synthesis and low cytotoxicity [117].

3.4.1 Cellular Uptake of Glutathione capped Cu_{2-x}S Nanocrystals and Near-Infrared Laser Illumination

Figure 3-7 TEM images of ligand-exchanged GSH- Cu_{2-x}S NCs seen under (a) high magnification and (c) low magnification. (b) Size distribution of GSH- Cu_{2-x}S NCs and its Vis-NIR absorption spectrum with full width half maximum spanning from 898 nm to 1428 nm.

The diameter of the GSH capped Cu_{2-x}S NCs (6.4 ± 1.5 nm) (Figure 3-7) was much smaller than the Au nanostructures and CNTs, favouring endocytosis and efficient uptake of the NCs *in vitro* and clearance *in vivo*. In addition, continuous-flow synthesized Cu_{2-x}S NCs have the added edge of reproducibility as the synthesis parameters are better controlled. In this section, we demonstrate a proof-of-concept experiment showing the successful application of GSH capped aqueous phase Cu_{2-x}S NCs in RAW264.7 mice macrophage cell line for photothermal therapy.

Figure 3-8 Cytotoxicity effects of different concentrations of GSH- Cu_{2-x}S NCs on RAW264.7 macrophage cells. The red dotted line indicates 50% cell viability, defining 13.5 μM and 27 μM as suitable concentration levels based on the IC_{50} benchmark. Reproduced from [93] with permission of The Royal Society of Chemistry.

Based on the IC_{50} value from the MTT (3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltertrazolium bromide) cytotoxicity assay presented in Figure 3-8, GSH capped Cu_{2-x}S NC concentrations at 13.5 μM and 27 μM were chosen to investigate the subsequent effect of Cu_{2-x}S NCs as agents for photothermal ablation. A fiber coupled laser diode with center wavelength of 915 ± 10 nm was used as the NIR laser source (Figure 3-9). The macrophage cells were irradiated with power densities of 36.7 W/cm^2 and 52.1 W/cm^2 by varying the laser diode driving current and keeping the beam diameter fixed at around 1.5 mm.

Figure 3-9 a) Fiber-coupled laser used as the near infra-red (NIR) source for illumination of the cells. b) The NIR laser light illumination was performed inside the incubator with the laser light being delivered via the optical fiber into the incubator.

Figure 3-10 (a) The 6-well cell culture plate together with the optical mount holding the fiber in place. (b) NIR laser illumination of the cells in the cell culture well as seen using a NIR viewer.

After laser irradiation, the cells were stained with propidium iodide (PI). PI is a fluorescent dye which is impermeable to the cell membrane of a viable cell and only stains the nuclei of dead or damaged cells which have lost their membrane integrity. Therefore, regions which showed up in red indicate regions of cell death. Despite NIR irradiation at a lower power density of 21.7 W/cm^2 , no visible cell death was observed, hence we postulate that 21.7 W/cm^2 was too low for efficient photothermal conversion. Therefore, when the power density was increased to 36.7 W/cm^2 and 52.1 W/cm^2 , distinct regions of cell death were observed after staining with PI as depicted in Figure 3-11 and Figure 3-12. Figure 3-11 shows no significant cell death when cells without incubating with Cu_{2-x}S NCs were irradiated with NIR laser light. Similarly, no cell deaths were observed in the samples inoculated with $27 \mu\text{M}$ Cu_{2-x}S NCs without laser light irradiation. These results agree with the findings of Li et al. [118], which also showed no apparent cell death for cells without neither copper sulfide nanoparticles nor laser illumination.

In addition, it can be seen that both the concentration of the Cu_{2-x}S NCs as well as the NIR power density played crucial roles in photothermal ablation as relatively well-defined circular

regions were observed after the laser treatment (Figure 3-12). With the same power density of 52.1 W/cm^2 , the diameter of the region of cell death was $571 \text{ }\mu\text{m}$ for the lower Cu_{2-x}S NC concentration of $13.5 \text{ }\mu\text{M}$ as compared to $1472 \text{ }\mu\text{m}$ for the higher $27 \text{ }\mu\text{M}$ concentration. This indicates that more extensive cell death could be induced if a higher concentration of NCs was used. However, it should be noted that a balance between the photothermal effect and cytotoxicity of Cu_{2-x}S (MTT assay in Figure 3-8) should be considered for further biological applications. At a fixed concentration of $27 \text{ }\mu\text{M}$, the higher power density of 52.1 W/cm^2 resulted in a zone of cell death almost twice in diameter compared to that of the lower power density of 36.7 W/cm^2 , spanning $1472 \text{ }\mu\text{m}$ and $784 \text{ }\mu\text{m}$ respectively as annotated in Figure 3-12. This shows that the region of cell death could be enhanced either by increasing the output power of the laser or reducing the diameter of the laser beam. Nonetheless, this may not be desirable for subsequent transition to clinical trials as the power densities are way above the maximum permissible exposure (MPE) limits (0.54 W/cm^2 for 915 nm) [111]. Therefore, superstructures have been engineered to enhance the NIR absorption and photothermal conversion efficiency so that the same photothermal effect can be achieved albeit at much lower power densities near the MPE limit [119]. Alternatively, some groups have proposed the use longer NIR wavelengths in the range of $1000 - 1350 \text{ nm}$ because of the advantages of deeper penetration depth and higher MPE ($\sim 1 \text{ W/cm}^2$) at these wavelengths [120, 121].

Figure 3-11 Microscope images of RAW264.7 mice macrophage cells with different Cu_{2-x}S NC concentrations after being subjected to NIR irradiation at 915 nm with power densities of 36.7 W/cm^2 and 52.1 W/cm^2 for 15 minutes. Red fluorescence observed after staining with propium iodide (PI) to indicate regions of cell damage or death. The inset at the top left hand corner shows NIR laser irradiation of the cells in the cell culture plate through a NIR viewer. Reproduced from [93] with permission of The Royal Society of Chemistry.

Figure 3-12 Annotated microscope images showing regions of cell death induced by NIR irradiation of the Cu_{2-x}S NCs and their corresponding affected regions and power densities. Reproduced from [93] with permission of The Royal Society of Chemistry.

The purpose of the photothermal therapy (PTT) application was to demonstrate a proof-of-concept application for the millifluidic chip Cu_{2-x}S nanocrystals. This proof-of-concept

application provides a holistic scheme of work from nanoparticle synthesis to its functional application for cell annihilation. We believe that our work serves as a good reference to facilitate the transition of fundamental scientific research from the laboratory to real world applications. Table 3-3 lists some recent literature for comparison to provide some perspective and help address concerns regarding the laser power density and nanoparticle concentration. The relatively high laser power density employed in this work could be mainly attributed to the non-alignment between the plasmonic peak of Cu_{2-x}S and the near infra-red laser emission wavelength used. From Table 3-3 and Figure 3-13, it is evident that different laser wavelengths were employed. Comparing [118] and [122] which both utilised spherical copper sulfide nanoparticles, a drastic difference in laser power density of 40 W/cm^2 and 1.41 W/cm^2 was observed. The corresponding absorption characteristics of the nanoparticles (Figure 3-13C), 980 nm appears to be very close to the absorption peak of the nanoparticles in [122] and thereby resulting a relative low laser power. Therefore, a longer wavelength laser source or blue shifting the absorbance of the Cu_{2-x}S nanoparticles are two possible solutions to reduce the laser power density for photothermal therapy.

Figure 3-13 Absorption curves with their corresponding laser irradiation wavelengths and power density requirement for photothermal effect - A) 808 nm at 24 W/cm², 40 W/cm², 64 W/cm² [118], B) 980 nm, 1.41 W/cm² [122], C) 980 nm, 0.51 W/cm² [119], D) 915 nm (this work) at 36.7 W/cm², 52 W/cm².

Table 3-3 Comparison of Cu_{2-x}S nanoparticles for photothermal therapy applications.

NP Mineral	Morphology	Size	Laser Power Density	NP Concentration	Cell Line	Ref
Hexagonal covellite CuS	Spherical	3nm	24 W/cm ² and 40 W/cm ² , @808 nm for 5 mins and 64 W/cm ² for 3 mins	384 μM (@24 W/cm ²), 192 μM and 384 μM (@40 W/cm ²)	HeLa	[118]
μ-Cu _{2-x} S	Spherical (nanodots)	12nm	1.41 W/cm ² @980 nm (CW) for 5 mins	6.25, 12.5, 25, 50, 100 μg/mL	HeLa	[122]
CuS super-structures (Hexagonal phase covellite)	Star-shaped intersecting hexagonal plates (starfruit-like)	Hexagonal nanoplates edge length ~500–800 nm, average thickness 50 nm	0.51 W/cm ² @980 nm for 5 mins	250 μg/mL	HeLa	[119]

The working concentrations for this study (i.e. 13.5 μM and 27 μM) were determined using the IC_{50} benchmark, was moderate as other similar work employed higher concentrations (192 μM and 384 μM) [118]. The concentration of nanocrystals used for photothermal therapy also depends on the light-to-heat conversion efficiency. We believe that a matched absorption would possibly enable lower concentrations of Cu_{2-x}S to be used and subsequently lower laser power densities to activate the photothermal effects.

Lastly, although limited photothermal conversion efficiency was provided by the as-synthesized spherical Cu_{2-x}S , there is certainly potential for further development and study of the Cu_{2-x}S nanocrystals for photothermal and other biomedical applications. Since high conversion efficiencies have been demonstrated by Tian *et al.* [119] using specially engineered Cu_{2-x}S superstructures, exhibiting about 50% improvement in photothermal conversion efficiency, morphological control and engineering should be further examined to improve the functionality of Cu_{2-x}S nanomaterials.

3.5 Conclusion and Further Recommendations

Table 3-4 Summary of characteristics of millifluidic chip synthesized Cu_{2-x}S NCs. Reproduced from [93] with permission of The Royal Society of Chemistry.

Molar Ratio (Cu:S)	Flow Rate ($\mu\text{l}/\text{min}$)	Shape	Dimension	Aspect Ratio	Composition	LSPR Peak
			AR: Aspect Ratio D: Diameter L: Length W: Width P: Perimeter			
1:10	3000	Spherical	D: 6.4nm	1.2 ± 0.3	Covellite	1115nm
1:6	3000	Rod	L: 12.0nm W: 5.3nm	2.3 ± 0.5	Covellite	1150nm
1:2	3000	Rod	L: 13.9nm W: 4.3nm	3.4 ± 1.0	Covellite	1272nm
2:1	3000	Spherical	D: 8.8nm	1.1 ± 0.1	Roxbyite	1315nm
6:1	3000	Spherical	D: 11.9nm	1.1 ± 0.2	Djurleite	1471nm
10:1	3000	Spherical	D: 12.6nm	1.1 ± 0.1	Djurleite	1644nm

1:2	150	Prism	P: 13.5 x 12.9 x 11.7nm	Not Applicable	*Low Yield	1281nm
1:2	1000	Rod	L: 7.8nm W: 6.2nm	1.3 ± 0.4	Covellite	1246nm
1:2	2000	Rod	L: 12.0nm W: 5.3nm	2.3 ± 0.4	Covellite	1266nm
1:2	4000	Spherical	D: 8.8nm	1.1 ± 0.1	Spionkopite	1293nm
1:2	5000	Spherical	D: 3.6nm	1.2 ± 0.2	Spionkopite	1277nm

In summary, the input flow rates and copper to sulfide ratios in this continuous-flow millifluidic chip provided substantial control over the shapes and sizes of the Cu_{2-x}S NCs. This surfactant-free approach enabled the fabrication of triangular prism, spherical and rod-shaped NCs with varying aspect ratios by modifying physical parameters and chemical ratios. Subsequently, characterizations conducted using TEM, FFT analysis and powdered XRD revealed that the NCs compositions varied from copper-deficient covellite (CuS), spionkopite ($\text{Cu}_{1.39}\text{S}$), roxbyite ($\text{Cu}_{1.75}\text{S}$) to copper-rich djurleite ($\text{Cu}_{1.94}\text{S}$) formation under different synthesis conditions.

Since the LSPR spectra obtained from Cu_{2-x}S NCs were due to the highly self-doped (p-type) semiconductor QDs with copper-deficient stoichiometries, therefore by varying the Cu:S molar ratios, the LSPR peak could be tuned in the NIR region from 1115 nm to 1644 nm. In addition to the synthesis of Cu_{2-x}S NCs, we have also demonstrated that the photothermal application of these nanoparticles could effectively induce cell death in RAW264.7 mice macrophage cells upon NIR laser irradiation.

All in all, the continuous-flow millifluidic synthesis chip is a valuable platform for organic synthesis of nanoparticles to study the nucleation kinetics of the reactions as it offers excellent

control over various parameters. We believe that its potential for synthesizing different nanostructures and compositions has not been fully explored. Further investigations on the mechanisms for different shape formation and designs of more advanced functional structures will be carried out in subsequent work.

Chapter 4 Cadmium Sulphide Nanoparticle Synthesis and Applications in Bioimaging

4.1 Introduction

Quantum dots (QDs) are semiconductor nanoparticles with all three dimensions in the order of the exciton Bohr radius of the bulk material. For the case cadmium sulphide (CdS), the excitonic Bohr radius is 2.8 nm [123], which is much smaller than other semiconductors cadmium chalcogenides like cadmium selenide (CdSe – 5.6 nm [124]) and cadmium telluride (CdTe – 7.3 nm [125]). Therefore, synthesis of such small nanoparticles is challenging using bulk synthesis methods. Studies on colloidal CdS began as early as 1981 by Gratzel et al. [126] when they investigated the electrolysis of water driven by the irradiation of light on CdS nanoparticles with ruthenium (IV) oxide catalyst. Following which many preparation methods such as hot-injection [57, 127], solvothermal [128, 129], non-injection [110, 130] and microwave-assisted synthesis [131, 132] emerged over time. However, amongst these techniques, the size, morphology and crystalline quality of the CdS nanoparticles varies with some involving the use of elevated temperatures, pressures, highly explosive organometallic compounds, and long reaction durations. Therefore, we believe that miniature reaction vessels can help to improve the quality, reproducibility and ease of production of luminescent CdS nanocrystals (NCs).

Cadmium sulfide (CdS) semiconductor nanoparticles are of interest in various applications such as optoelectronic applications particularly in photovoltaics and photoactivation [133-136], bioimaging [137] and biosensing [138]. At the nanoscale where quantum confinement effects come into play, these nanoparticles can exhibit very different optical and chemical properties from the bulk material. CdS nanocrystals exhibit rich morphologies, ranging from spherical, nanorods, nanocubes, tripods, tetrapods, candy corn, arrow-shaped [127, 139] just to mention

a few. Therefore, understanding and control of the shapes, sizes and surface characteristics of CdS nanoparticles are crucial to developing devices with novel characteristics such as quantum dot-sensitized solar cells with higher stability compared to thin-film solar cells made from the same material [140].

Miniature reaction chambers provide the advantages of a well-controlled reaction conditions such as reproducible mixing conditions enabled by manipulation of laminar fluid flow in engineered channel designs or active micromixers [141, 142], excellent heat and mass transfer due to the increased surface area to volume ratio [143] provided by the miniature channel dimensions. In addition, on chip synthesis provides a spatial to temporal mapping of the reaction dynamics [144]. Traditional macroscale QDs fabrication methods such as organometallic synthesis involves the use of highly toxic precursors and generation of considerable chemical waste like solvents, surfactants, unreacted precursors and unwanted reaction byproducts. On the other hand, microfluidic and millifluidic synthesis are greener approaches as the quantity of required chemicals are much reduced and the homogenous reaction conditions result in less waste products due to more efficient reaction kinetics [94].

Due to the potential advantages of miniature systems, many groups have also explored CdS nanoparticle synthesis using miniature reaction vessels such as capillary tubes, microreactors, microfluidic chips, and even microorganisms like bacteria (Table 4-1). Although there is great promise in these miniscule technologies, each is still under development and has different limitations which need to be tackled. For example, microscale systems involving capillary tubes offer simplicity in terms of implementation and adaptation to macroscale injection methods, but is constrained by the limited geometric designs and relatively larger footprints [41]. Biosynthesis methods, on the other hand, despite enabling the fabrication of

biocompatible nanoparticles using green chemistry at room temperatures, suffers from low throughput, long synthesis durations [145], and requires further investigation into the purification processes of these nanoparticles as biomolecules might still be attached to their surfaces. Miniature fluidic channels or lab-on-chip (LoC) devices in addition to the advantages offered by capillary tubes, provide additional flexibility for various designs to generate reproducible mixing within the channel. However, most of the existing on-chip synthesis of CdS nanoparticles are based on aqueous solvents with reaction temperatures around 25-30 °C, yielding poor crystalline nanoparticles.

In this work, synthesis of CdS nanocrystals (NCs) ranging from 6.7 nm long and 2.6 nm wide multipod arms to spherical NCs with quantum confined sizes of 2.4 nm with observations of morphologies such as nanorods and multipods, aside from spherical nanoparticles was demonstrated. The as-synthesized NCs synthesized in less than 3.5 mins exhibited good crystallinity as substantiated by the high resolution transmission electron microscope (HRTEM) images as well as the powder x-ray diffraction (XRD) patterns. Size variations along with changes in the optical properties of these as-synthesized CdS NCs were also achieved by varying the residence time, and molar ratios of the precursors. In this work, we employed oleylamine (OAm) as both the surfactant and coordinating solvent for the reaction. Oleylamine has been successfully proven to control shape and size of nanocrystals in traditional benchtop methods as demonstrated by Yong et al. [127] and Joo et al.[139], where elemental sulphur was added to metal-oleylamine complexes to form semiconducting metal sulfide nanoparticles. Therefore, we feel that there is great potential in extending their application in millifluidic synthesis methods. We first discuss the effects of residence time on the chip on the NCs formed, followed by examining the effect of varying the the molar ratios of the Cd and S precursors. Lastly, the millifluidic chip synthesized CdS NCs were employed in a proof of concept

demonstration for *in vitro* bioimaging and toxicity study using RAW264.7 mice macrophage cells.

Table 4-1 Synthesis methods of cadmium sulphide nanocrystals / quantum dots (CdS NCs/QDs) in miniature reaction vessels

Synthesis Method	Reaction Condition/ Vessel	τ_R	Absorption Excitonic Peak	Photoluminescence (Y/N)	Size and Shape ⁺ D: Diameter L: Length W: Width	Crystal Phase	Ref
Microfluidic -continuous Flow Capillary Microreactor (Organic)	Capillary microreactor in oil bath at 180 – 260 °C	68 s	389 – 453nm HWHM ~12 nm MSC peak at 320 nm.	Y. 391 – 463 nm. Weak PL due to high conc of surface defects	Spherical. D: 3.0 – 5.4 nm [*]	Zinc blende.	2010 [146]
Millifluidic reactor (Organic)	Reactants mixed prior to entering the stainless steel coil maintained at 270 °C.	3 min	425 nm	Y. 443 nm	D: 4.25 nm [*]	N. A.	2015 [147]
Biosynthesis - extracellular microbial synthesis (Aqueous)	White rot fungus at 37 °C.	12h	~450 nm	458 nm, FWHM <30 nm	Spherical. D: 1.5 - 2.0 nm (TEM), 2.56nm average (from XRD scherrer's eqn)	Cubic zinc blende.	2014 [148]
	Bacteria from living abalone at 30 °C.	7 days	375 nm (broad band-edge absorption)	Y. 590 nm and 610 nm	Spherical. D: 50-100 nm(~90nm average)	Cubic zinc blende.	2016 [145]
Microfluidics -chip based droplet segmented flow (Aqueous)	Microfluidic channels, reaction at room temperature.	75 ms	290 – 370nm	360nm	N. A. † D: 1.32 nm, 1.64 nm, 2.51 nm *	N. A. † Particles synthesized at room temp were not very crystalline.	2004 [149]
	Adjust relative inlet flow rates to control droplet ratio.	N.A.	427 – 477 nm	Y. 618 nm	Spherical D: 4.2 – 8.2 nm	N.A.	2005 [150]
Microfluidic - capillary microreaction system (Organic)	Capillary microreactor with heater at 260- 285 °C	20 s	411-459 nm	Y. ~420-460 nm Noticeable trap state emission.	Spherical. D: 3.74 – 5.66 nm [*]	Zinc blende [‡]	2009 [151]
Microfluidics - continuous flow millifluidic chip (Organic)	Millimeter dimensioned channels, reaction at 170 °C	25 – 98 s	393-426nm. MSC peak at 361 nm.	Y. Band-edge ~430 nm, defect states: ~530–390 nm	Spherical. D: 2.4 – 5.8 nm Bipods and Multipods. L: 3.5 – 6.7 nm W: 2.6 – 5.6 nm	Mixed Phases of wurtzite and zinc blende.	Our Work

τ_R – Residence / reaction time

Photoluminescence (Y/N) - Presence of photoluminescence (Yes/No)

MSC – Magic-size cluster

⁺ – Sizes listed are the diameters of the nanoparticles unless otherwise stated.

^{*} – Size calculated from excitonic peak of absorption spectrum according to [24].

[‡] – Not stated explicitly, inferred from X-ray diffraction (XRD) pattern.

[†] Stated that particles synthesized at room temp were not very crystalline, hence was not characterized.

N. A. – Not applicable (due to lack of data / description).

4.2 Materials and Methods

4.2.1 Chemicals

Cadmium chloride and sulphur from Sigma Aldrich, oleyamine (>50.0%, Tokyo Chemical Industry Co., Ltd.). Toluene (>99.5%, Chemicals Testing and Laboratory, Singapore), ethanol (95 %, Aik Moh Pte Ltd, Singpaore) were used in the synthesis of the cadmium sulphide nanoparticles. The 184 Sylgard silicone elastomer kit from Dow Corning was used to fabricate the polydimethylsiloxane (PDMS) millifluidic chip. RAW264.7 mice macrophage cell line (ATCC, Manassas, VA, USA), Dulbecco's Modified Eagle's Medium ([DMEM], Hyclone), fetal bovine serum ([FBS], Hyclone), penicillin-streptomycin (Gibco, Life Technologies, SG, Singapore) were used in cell culturing. All the above chemicals were used as purchased without further purification. Deionized (DI) water mentioned in the experiments was purified by a Milli-Q water purification system.

4.2.2 Fabrication of Millifluidic Chip

The millifluidic channels were designed using the free software SketchUp and the mould was printed in acrylonitrile butadiene styrene (ABS) material using the UP Plus 2 3D printer (PP3DP). The Sylgard 184 Elastomer Kit was used for the polydimethylsiloxane (PDMS) mixture based on a 10:1 ratio of the base resin to the curing agent. The base and curing agent were then mixed homogeneously before degassing the mixture in a dessicator to remove the bubbles. The PDMS mixture was then poured into the mould, degassed and heated cured on a hotplate at 70 °C for at least 4 hours. The PDMS was then carefully removed from the mould and bonded to a glass slide. Holes were then made at the inlets and outlet of the chip and polytetrafluoroethylene (PTFE) tubes used to deliver the precursors and nanocrystal product were then inserted and sealed to the chip.

4.2.3 Synthesis of CdS Nanocrystals in Millifluidic Chip

The synthesis protocol used is similar to [127] and [139]. Briefly, 6 mmol of sulphur powder was dissolved in 10 mL of oleylamine and stirred at room temperature in a flask until all the powder has completely dissolved. In another three neck flask, 1 mmol of cadmium chloride (CdCl_2) was dissolved in 10 mL of oleylamine, heated and stirred at 170 °C under nitrogen (N_2) flow for 20 minutes, before the temperature was raised further to 200 °C for another 10 minutes. Both the sulphur-oleylamine (S-OAm) and the cadmium-oleylamine (Cd-OAm) mixtures were then withdrawn using separate syringes and injected into the PDMS millifluidic chip.

The precursors were delivered into the millifluidic chip via the syringe pump (KD Scientific), where the injection flow rates of the precursors were set at 3000 $\mu\text{L}/\text{min}$, 1500 $\mu\text{L}/\text{min}$, and 750 $\mu\text{L}/\text{min}$ and the products of the reaction was collected at the outlet of the chip. In addition, different molar ratios of the precursors were also used. The molar ratios of cadmium to sulphur (Cd:S) were varied from 1:3 (1 mmol of CdCl_2 and 3 mmol of S), 1:6, 1:12 and 3:1 (3 mmol of CdCl_2 and 1 mmol of S). Toluene was added near the end of the chip to quench the reaction and stop further growth of the nanocrystals. To purify the CdS NCs, 2 parts of ethanol were added to 1 part of the output solution were mixed before centrifuging at 8000 rpm for 3 minutes. The supernatant was decanted and the pellet was redispersed in toluene for subsequent storage of the NCs.

4.2.4 Characterization of CdS Nanocrystals

The ultraviolet-visible (UV-Vis) absorbance spectrum of the CdS nanocrystals was measured using a Shimadzu UV-2450 spectrophotometer, while the photoluminescence emission spectrum was collected using a HORIBA Jobin Yvon Fluorolog-3 Fluorometer. Transmission electron microscope (TEM) images were obtained using a JEOL JEM-2011 TEM. The Rigaku

SmartLab powder X-ray diffractometer was used to carry out the powdered X-ray diffraction (XRD) measurements to determine the crystallographic structures present.

4.2.5 Ligand Exchange of CdS Nanocrystals with Mercaptosuccinic Acid

For 5 mg of CdS with oleylamine ligands, 150 mg of mercaptosuccinic acid (MSA) should be used. The MSA was dissolved in 3mL of chloroform with stirring, then the as-synthesized CdS with oleylamine ligands from the previous section was added. Subsequently, ammonium hydroxide ($\text{NH}_3 \cdot \text{H}_2\text{O}$) and deionized water ($\text{DI H}_2\text{O}$) were added to the mixture in the volumetric ratio of 3:7 and stirred vigorously overnight. If the ligand exchanged process is completed, the mixture should exhibit two distinct layers with the yellow CdS NCs in the top layer and a clear bottom layer. Extract the top layer carefully and flocculate the NCs using ethanol, decant the supernatant and dissolve the pellet using deionized water for further use.

4.2.6 Cell Culture and Bioimaging of Macrophage Cells using Millifluidic CdS Nanocrystals

RAW264.7 mice macrophage cells (American Type Culture Collection) were cultured with Dulbecco's modified eagle's medium (DMEM, Hyclone), supplemented with 10% FBS, penicillin (100 $\mu\text{g}/\text{mL}$, Gibco) and streptomycin (100 $\mu\text{g}/\text{mL}$, Gibco) in an incubator at 37°C and 5% CO_2 . The cells were seeded at a density of 1×10^5 cells/well with DMEM culture medium. 3.8 mg/mL of CdS-MSA were added to the macrophage cells and incubated for 4 hours. Then, the treated cells were rinsed thrice with PBS buffer ($\text{pH} = 7.2$) and fixed with 4 % formaldehyde. *In vitro* microscope images were obtained using a fluorescence microscope (Eclipse Ti-U Inverted Microscope System, Nikon) and a filter cube with 350 nm excitation wavelength and a red emission filter with a centre wavelength of 630 nm.

4.3 Results and Discussion

The dimension of the channel on the miniature chip is 1.5 mm (width) by 1.5 mm (height), spanning a total length of approximately 545 mm and capable of carrying a total fluid capacity of 1227 mm³ or 1227 μ L. Due to the millimeter dimensions of the channel, we term the chip as a millifluidic chip. There are five sections in this chip, three inlets (two precursor inlets and another inlet for toluene) and one outlet as depicted in Figure 4-1b. The sulphur and cadmium precursors are injected into the chip via separate inlets before the two streams meet at the Y-shaped region. Next, the streams encounter a winding section, which folds the fluids and promotes mixing [152] as illustrated by the flow velocity profile in Figure 4-1d. After mixing, the solution then flows into the straight regions where nucleation and growth takes place to form the CdS NCs indicated by the increase in CdS concentration simulated in Figure 4-1c. Finally, the reaction is quenched by injecting toluene near the end of the chip and flushing the CdS NCs towards the outlet.

The base of the millifluidic chip is bonded to a glass slide to allow uniform heating of the entire chip and reduce temperature fluctuations of particles at different regions of the chip. Our continuous flow fluidic chip boasts a simple passive mixing environment providing stable conditions for incubation of the nanoparticles. In addition, we have shown previously that such a chip can enable the synthesis of nanoparticles with precursors that are prone to oxidation without the needing to purge the channels with inert gases [93].

Figure 4-1 a) Annotated schematic and b) actual millifluidic chip for the synthesis of cadmium sulfide nanocrystals (CdS NCs). Simulations reflecting CdS NC (c) concentration and (d) fluid velocity flow inside the chip.

Examples of CdS NCs with different morphologies and sizes synthesized using our millifluidic chip are shown in Figure 4-2. By varying parameters such as the injection flow rate, dominantly spherical particles could be obtained with sizes ranging from 2.4 – 5.8 nm. In addition, morphologies such as tripods and bipods were also observed.

Figure 4-2 TEM images of CdS NCs produced under different flow rates and Cd:S precursor molar ratios.

Table 4-2 Summary of size, morphology, absorption excitonic peaks and photoluminescence peaks of CdS NCs synthesized under different flow rates and molar ratios.

Flow Rate ($\mu\text{L}/\text{min}$)	Molar Ratio (Cd:S)	Precursor Conc. (Cd, S) (mol/L)	Shape	Average Dimensions* (nm)	Excitonic Peak (nm)	PL Peak and FWHM (nm)	τ_R (s)	Crystal Structure (TBC)
5000	1:6	0.10, 0.6	Spherical	2.4 ± 0.4	361 (MSC), 385	425 ± 27	15	Just beginning to crystallize
3000	1:6	0.10, 0.6	Spherical	2.8 ± 0.4	361 (MSC), 401	427 ± 21	25	Greenockite (W)
1500	1:6	0.10, 0.6	Spherical, Some bipods and tripods	3.3 ± 0.5	410	426 ± 23	49	Hawleyite (zb)
750	1:6	0.10, 0.6	Spherical	3.5 ± 0.5	419	434 ± 19 , and 594 ± 75 (Bimodal)	98	Greenockite (W)
375	1:6	0.10, 0.6	Spherical	3.07 ± 0.7	426	449 ± 25	196	Hawleyite (zb)
1500	1:12	0.10, 1.2	Spherical and bipods	5.8 ± 0.9 , 5.6 ± 0.1	409	425 ± 27	49	Greenockite (W)
1500	1:3	0.10, 0.3	Tripods	6.7 ± 2 , 2.6 ± 0.6	361 (MSC), 393	427 ± 21	49	Mixed Phase

Conc. – concentration

* - determined using TEM images

FWHM – full wave half maximum

PL – photoluminescence

τ_R – residence time

4.3.1 Effect of Injection Flow Rate/Residence Time

Different injection flow rates were studied to examine their effect on the CdS NCs formed. The injection flow rate determines the residence time (τ_R) of the fluid in the chip. Unfortunately, the reaction time in bulk macroscale synthesis methods cannot be directly translated into the residence time for miniature reactors as the latter involves reactions at the molecular level [153]. Compared to the conventional bench top synthesis using the same reactants [127, 139], our homemade millifluidic chip set with a flow rate of 5000 $\mu\text{L}/\text{min}$ and 3000 $\mu\text{L}/\text{min}$ corresponding to a residence time of merely 15s and 25s produced quantum-confined nanocrystals or quantum dots with an average size of 2.4 ± 0.4 nm and 2.8 ± 0.4 nm (Figure 4-4a to d), bordering the exciton Bohr radius of CdS. Upon decreasing the flow rate to 1500 $\mu\text{L}/\text{min}$ and 750 $\mu\text{L}/\text{min}$, larger nanocrystals were obtained with average diameters of 3.3 ± 0.5 nm (Figure 4-4e and f) and 3.5 ± 0.3 nm (Figure 4-4g and h) respectively. Based on the TEM

images, the CdS NCs were mostly spherical in shape, with some bipods and tripods observed at 1500 $\mu\text{L}/\text{min}$.

The ultraviolet-visible (UV-Vis) absorbance curves in Figure 4-5a show the first excitonic peaks for all the CdS NCs synthesized at different flow rates. The position of the excitonic peaks can be used to estimate the size of the NCs based on empirical work done by Yu et al. [154]. The absorbance spectra in Figure 4-5a further corroborates the particle sizes determined from the TEM images with peak values at 385 nm, 401 nm, 410 nm, 419 nm, and 426 nm corresponding to flow rates of 5000 $\mu\text{L}/\text{min}$, 3000 $\mu\text{L}/\text{min}$, 1500 $\mu\text{L}/\text{min}$, 750 $\mu\text{L}/\text{min}$, and 375 $\mu\text{L}/\text{min}$ respectively. The increase in the residence time due to slower injection flow rates enabled the particles to increase in size due to the longer growth period.

Besides that, a consistent additional peak was observed at 361 nm for the absorbance spectra of CdS NCs synthesized at high flow rates of 5000 $\mu\text{L}/\text{min}$ and 3000 $\mu\text{L}/\text{min}$, exhibiting linewidths as narrow as 14 nm. The presence of this discrete peak indicates the formation of a magic-size cluster (MSC), which degrades in intensity over time before spontaneously red-shifting to another distinct wavelength instead of gradually red-shifting over time [155]. This is in stark contrast to the red-shifted peak before it, which progressively shifts from 385 nm to 426 nm as the residence time increase from 15 s to 196 s, while the MSC peak remains constant at 361 nm as indicated in Figure 4-5a. In addition, we demonstrated the ability of our device to produce different batches of CdS NCs using the same parameter of 5000 $\mu\text{L}/\text{min}$ and showed that the same MSC peak at 361 nm (Figure 4-3a) could be replicated. Figure 4-3 also shows other synthesis parameters which exhibited almost identical absorbance spectra, providing compelling evidence for the reliability and stability of the millifluidic chip as a miniature reaction vessel.

Figure 4-3 Absorbance curves of selected batches of CdS NCs showing the high reproducibility of nanoparticle fabrication using these millifluidic chip reactors.

These as-synthesized CdS NCs emitted visible light when irradiated with ultraviolet (UV) light with an excitation wavelength of 350 nm. From Figure 4-5b, NCs produced using the slower flow rate of 750 $\mu\text{L}/\text{min}$ exhibited a bimodal emission with the longer wavelength peak at around 594 nm being higher in intensity and broader compared to the band edge emission at 434 nm. On the other hand, particles fabricated using the high flow rates of 1500 $\mu\text{L}/\text{min}$ and above had a dominant peak at the band edge (~ 427 nm) with a trailing edge spreading over to the longer wavelength region.

Figure 4-4 TEM images and size distributions of CdS nanocrystals synthesized in millifluidic chips at different flow rates of 5000 $\mu\text{L}/\text{min}$ (a and b), 3000 $\mu\text{L}/\text{min}$ (c and d), 1500 $\mu\text{L}/\text{min}$ (e and f), 750 $\mu\text{L}/\text{min}$ (g and h), and 375 $\mu\text{L}/\text{min}$ (i and j) respectively. The Cd:S molar ratio (MR) was kept constant at 1:6.

The red-shifted broadband photoluminescence emission was attributed to the sulphur defect states [156]. For this set of CdS NCs, the molar ratio of the Cd and S precursors were fixed at 1:6, with S being in excess. Wei et al. [157] showed that the band edge photoluminescence of a CdS QD terminated with sulphur was quenched, while a cadmium terminated CdS QD was brighter. Therefore, due to the excess sulphur precursors employed, it was likely that the band edge emission was quenched, resulting in the dominance of the long wavelength trap states in the emission spectrum. Figure 4-6 and Figure 4-7 show the colour changes in the CdS NC solutions due to the defect trap states and band edge emissions. The photoluminescence of the CdS NCs could be further enhanced by coating ZnS, a wide band gap material, in order to confine the exciton to the core of the NC. Techniques such as successive ionic layer adsorption and reaction (SILAR) and thermal cycling with a single precursor (TC-SP) have been demonstrated to improve the quantum yield of the core-shell CdS/ZnS NC from around 6% to 30% (SILAR) [158, 159] and 50% (TC-SP) [158].

Figure 4-5 a) Absorbance curves and b) photoluminescence spectra of CdS NCs formed at different flow rates with Cd:S molar ratio fixed at 1:6.

Figure 4-6 Millifluidic (mF) CdS NCs under ambient light (a) without and (b) with ultraviolet (UV) irradiation. (c) Reddish glow of mF CdS defect state emission in the dark, illuminated with a UV torchlight.

Figure 4-7 Millifluidic chip synthesized CdS NCs seen under (a) ambient light and (b) upon illumination with ultraviolet (UV) light and emitting greenish-blue light due to band edge emissions.

4.3.2 Effect of Different Cd:S Molar Ratios

In addition to the initial Cd:S molar ratio of 1:6, in order to study the influence of the relative ratios of cadmium and sulphur on the size and morphology of the NCs formed, two additional Cd:S precursor molar ratios were employed – namely 1:3, 1:6 and 1:12. In all three cases, the ratio of 1 corresponds to 0.1M of the cadmium precursor. Figure 4-8 depicts the TEM images of the NCs formed at a fixed flow rate of 1500 $\mu\text{L}/\text{min}$ but with different molar ratios of 1:3 (Figure 4-8a, b, c), 1:6 ((Figure 4-8d, e, f), 1:12 ((Figure 4-8g, h, i) together with their size

distributions. Attempts were made to increase the molar ratio of the cadmium precursor instead of just increasing the sulphur precursor, however they were unsuccessful as the cadmium precursor is more reactive, thus when present in excess, it coagulates very quickly, hence causing blockages in the tubes and channels. Therefore the change in molar ratio was confined to only increasing the amount of sulphur used in the reaction.

However, the CdS NCs produced using the 3:1 molar ratio (i.e. cadmium is in excess of sulphur) was of poor quality and did not yield any meaningful TEM images. Based on the TEM images in Figure 4-8, it is observed that the molar ratio of 1:3 (Cd:S) gave rise to substantial amounts of tripod structures having average arm lengths of 6.7 ± 2 nm and diameters of 2.6 ± 0.6 nm and aspect ratios of 2.58 ± 0.6 . The molar ratio of 1:6 yields a mixture of spherical and multipod structures, however majority are spherical. For the extreme case of Cu:S = 1:12, large particles with lengths of 5.8 ± 0.9 and widths of 5.6 ± 0.1 , seem to resemble bipods, however based on the measured dimensions, it seems that they might be spherical as the distribution of the aspect ratio is 1.0 ± 0.1 . The absorbance and photoluminescence spectra of the CdS NCs synthesized using the Cu:S molar ratio of 1:3 show that these NCs exhibits different optical characteristics from the 1:6 and 1:12 cases, with smaller particle sizes and also gives rise to a bimodal fluorescence peak consisting of the narrow band edge emission at 434 nm and a broadband defect emission at the longer wavelength of 594 nm.

Figure 4-8 TEM images of CdS nanocrystals fabricated using millifluidic chips at three different molar ratios of (a) Cd:S = 1:3, (b) 0.10M of Cd and 0.6M of S, and (c) 0.05M of Cd and 0.3M of S. Their corresponding size distributions (b, e, h) and aspect ratios (c, f, i) are also presented. The flow rate was kept constant at 1500 $\mu\text{L}/\text{min}$.

Figure 4-9 (a) Absorbance and (b) photoluminescence spectra of CdS NCs synthesized using different molar ratios. The flow rate was kept constant at 1500 $\mu\text{L}/\text{min}$.

Figure 4-10 Powered X-ray diffraction (XRD) patterns of millifluidic chip synthesized CdS at (a) different flow rates and (b) molar ratios.

Lastly, although the synthesis of CdS in miniature reaction vessels have been studied by many groups as shown earlier in Table 4-1, some have reported that the particles exhibited poor crystal quality, while others mostly reported cubic zinc blende structures. Based on the XRD measurements depicted in Figure 4-10, in terms of the flow rate, we can observe that initially at 5000 $\mu\text{L}/\text{min}$, the residence time is only 15s, and therefore a relatively weak signal is captured and this could mean that the crystal structure is just beginning to form. Subsequently as the flow increases, there are two main peaks at 44° and 52° , both of which could be contributed by either the hexagonal wurtzite Greenockite (43.7° and 51.9° corresponding to $\langle 1\ 1\ 0 \rangle$ and $\langle 1\ 1\ 2 \rangle$ planes) or cubic zinc blende Hawleyite (44.0° and 52.2° corresponding to $(2\ 2\ 0)$ and $(3\ 1\ 1)$ planes) structures. The subsequent emergence of a tiny peak at 36° matching the $\langle 1\ 0\ 2 \rangle$ plane of wurtzite. Therefore, we conclude that most our nanocrystals exists in mixed phases

of wurtzite and zinc blende, where the presence of the 36° peak indicates the slight dominance of wurtzite structure.

4.4 Bioimaging with Macrophage Cells

QDs have been used for biological imaging as magnetic resonance imaging (MRI) contrast agents [160, 161], optical imaging probes [162], and near infrared imaging probes [163, 164] due to properties such as their wide absorption spectrum, narrow emission spectrum, and improved photostability compared to organic dyes [165]. However, concerns involving the use of QDs include photoblinking and heavy metal toxicity [166]. Here, we demonstrated fluorescence imaging of the RAW264.7 mice macrophage cell line with CdS NCs which have been ligand exchanged with mercaptosuccinic acid (MSA). These CdS-MSA NCs can be used as optical probes to monitor the toxicity of the nanoparticles and investigate the breakdown of CdS in the cells. We believe that the small dimensions of the millifluidic chip synthesized CdS NCs would serve as an excellent model to study the cytotoxicity of such nanoparticles and their excretion pathway.

3.27 μ M CdS-MSA NCs were added to RAW264.7 mice macrophage cells and incubated for 24 hours before imaging under ultraviolet (UV) light illumination. The NCs were readily taken up by the macrophages as indicated by the numerous red regions observed in the dark field as well as the overlaid images in Figure 4-11, where all the cells in the full field of view observed using a 20X objective lens glowed in red upon UV illumination. The red fluorescence is indicative of the presence of CdS-MSA NCs based on both the photoluminescence spectrum in Figure 4-12 showing a peak at around 620 nm as well as the control experiment shown in Figure 4-11 where no fluorescence was observed for cells which were not treated with the NCs.

Figure 4-11 *In vitro* bioimaging of RAW264.7 macrophage cells with and without CdS-MSA NCs. The red fluorescence observed is due to the emission of the CdS-MSA NCs upon illumination with ultraviolet (UV) light.

Figure 4-12 Photoluminescence spectrum of ligand exchanged CdS-MSA nanocrystals.

Toxicology concerns have always been a point of contention for cadmium (Cd) based nanoparticles due to issues such as heavy metal ion leakage upon interaction with biological fluids as well as oxidative stress as a result of over expression of reactive oxygen species [167]. However, studies regarding the toxicity of nanoparticles with different morphologies are limited [168, 169] and sometimes inconclusive due to the different cell models used and the complex nature of the subject [170].

Nanoparticles have large surface area to volume ratios, thus quantum confinement effects such as size, shape, surface charge, and surface passivation contribute significantly to the toxicity of the nanoparticles. Therefore, we believe that our millifluidic chip serves as an excellent platform to synthesize highly reproducible nanoparticles with good monodispersity, which would then serve as models for detailed studies on the effects of NC size, morphologies and surface modifications on nanoparticle toxicity.

A few strategies can be employed to reduce the toxicity of the NCs, one of which being coating the bare NCs with a zinc sulfide (ZnS) layer [158, 159] or coating with silica reverse micelles [171]. This reduces the probability of leakage of heavy metal ions such as Cd^{2+} into the cells, thereby reducing the cytotoxicity effects.

Recent studies have shown that the nanoparticle shape can affect pathways of the nanoparticle and its excretion efficiency. Chu et al. [172] discovered that nanoparticles with sharp edges such as nanostars and nanodiamonds are able to penetrate the endosome membrane remain in the cytosol after entering the cell, while particles with rounded edges such as spherical nanoparticles remained within the endosome, before being expelled from the cell via exocytosis.

The nanoparticles with sharp edges on the other hand remained stationary inside the cell and could not be excreted.

Likewise, the size of the nanoparticles also affect its cellular interaction. There appears to be a preference for optimal nanoparticles with sizes around 30-50 nm for maximal endocytotic cellular uptake [173]. Also, small nanoparticles are more toxic than larger nanoparticles as they have a larger surface area to mass ratio compared to that of bigger nanoparticles, hence this increases the probability of the small particles interacting with the surrounding biomolecules and triggering an adverse response [174].

Therefore as cells are very sensitive to interactions with nanoparticles, it is not surprising that nanoparticle shape and size engineering are of great interest to the biomedical research community. For imaging purposes, this means that if differently nanoparticle morphologies are used, they could be used to locate and image different cellular components. Likewise, quantum dots and nanocrystals with different sizes will exhibit photoluminescence at different wavelengths and thus can be used to as fluorescent tags by functionalizing the surface of these nanoparticles. However, it should be noted that there might be a trade-off between intensity of the photoluminescence and the size of the CdS nanocrystals because of the diminished quantum confinement effect with increasing particle sizes. Also, the concentration of the CdS nanocrystals used for bioimaging are limited by its toxicity, thus a balance must be struck between these conflicting parameters.

4.5 Conclusion

In this work, we have demonstrated that CdS nanoparticles can be fabricated with easy and high reproducibility using a simple millifluidic chip. Changing the residence time (or injection flow rate) of the reactants in the millifluidic chip enabled precise control over nucleation and

growth conditions and facilitating the synthesis of spherical CdS nanocrystals from as small as 2.4 ± 0.4 nm to 5.8 ± 0.9 nm to multipod structures with arms spanning 6.7 ± 2 nm long and 2.6 ± 0.6 nm wide. The novelty in this work is the synthesis of highly reproducible nanocrystals even the presence of magic sized clusters can be replicated with ease. In addition, multipod structures were also observed in short reaction durations of less than 2 minutes. Lastly, we also show that these luminescent nanocrystals can be employed as fluorescent probes for cell imaging and toxicity studies. Therefore, we feel that there is great potential of millifluidic chip devices in nanoparticle synthesis as there is flexibility in the design to promote mixing and this miniature fluidic environment provides a stable and constant environment suitable for nucleation and growth of nanoparticles.

Chapter 5 Preliminary Work and Future Studies - Hydrodynamic Flow Focusing and Nanoprecipitation Using Millifluidic Chips

5.1 Self-Assembly of Polymer Nanoparticles

5.1.1 Bulk Mixing Techniques for Polymer Synthesis

Figure 5-1 Pictorial illustration of (A) the nanoprecipitation and (B) miniemulsion methods of synthesizing polymers and nanoparticles. Reprinted from [175], under CC BY.

Nanoprecipitation and bulk mixing techniques are common preparation methods for self-assembly of polymers. Nanoprecipitation (Figure 5-1A) involves dissolving the polymer in a solvent miscible with water, for instance tetrahydrofuran (THF) or dimethylformamide (DMF). This solvent mixture is then added dropwise to deionized water under vigorous sonication. Lastly, the organic solvent (e.g. THF) is then evaporated by heating at 90 °C (i.e. boiling point of THF). The reduced solubility of the polymers in water compounded by the cross-linking due to the hydrophobic interactions among the polymer chains, induce the aggregation of the polymers to form suspended particles in the remaining aqueous solution.

Similar to nanoprecipitation procedure, the miniemulsion method shown in Figure 5-1B, also requires the polymer to be dissolved in aprotic organic solvents (without O-H or N-H) bonds such as dichloromethane or chloroform. However, instead of removing the organic solvent, an

aqueous surfactant solution (stabilizing ligand) is added and stirred vigorously, by means of vortexing or ultrasonication to form oil-in-water emulsion droplets. Finally, the organic solvent is removed by vaporization to yield the encapsulated hydrophobic polymer. As reflected in Figure 5-1B, the surfactant is amphiphilic (having both hydrophilic and hydrophobic segments), therefore the hydrophobic tails of the surfactants face inwards making contact with the hydrophobic polymer, while the hydrophilic heads of the surfactants face outwards protecting the encapsulated polymer from the aqueous environment.

5.2 Hydrodynamic Flow Focusing

Figure 5-2 Schematic drawing describing the interaction between the solvent and non-solvent molecules during hydrodynamic flow focusing. Reproduced from [162] with permission of The Royal Society of Chemistry.

Mixing via passive diffusion produces homogeneous mixtures with excellent reproducibility, however such processes are passive and requires long diffusion lengths and consequently increased time durations [176]. On the other hand, bulk macroscale mixing as mentioned in section 5.1.1, despite being more time efficient, generates turbulent flow which is difficult to control, thus consistency and reproducibility are common issues plaguing such mixing techniques. Therefore, another continuous laminar fluidic flow regime known as hydrodynamic flow focusing is of interest to the fluidic community as it can provide fast mixing with repeatable results [177]. As depicted in Figure 5-2, the central stream containing the solvent with the reactants is focused into a narrow stream by the side streams carrying the non-solvent.

The focused width (w_f) can be tuned by controlling the relative injection flow ratios of the central sample stream and the side carrier streams. Because of this reduced stream width, solvent molecules and non-solvent molecules can interact by weaving in and out of between the two liquids as the diffusion length was significantly reduced [162]. Therefore, by adjusting the relative flow rates of the two input streams, different regimes of slow mixing and rapid mixing can be achieved to provide well-controlled reaction conditions required for particle nucleation and growth.

5.3 Problem of Drug Loading Efficiency in AIE Luminogens

Unlike conventional quantum dots (QDs) and other fluorophores which suffer detrimental effects of aggregation-caused quenching (ACQ) when they are present in large concentrations, there is a novel class of fluorophores known as aggregation-induced emission (AIE) luminogens which exploits the natural phenomenon of aggregation to emit light. These materials were first discovered in 2001 by Tang Ben Zhong's group [178, 179]. They found that a group of organic compounds containing carbon-silicon (C-Si) bonds (i.e. siloles) were non-emissive when dissolved in solvents, but luminescent when aggregated in poor solvents or in the solid-state. Later, the mechanism behind this phenomenon was attributed to restriction of the intramolecular rotation (RIR). As shown in Figure 5-3, the AIE luminogen such as hexaphenylsilole (HPS) can be visualized as rotating propeller blades. Therefore, when the particles are aggregated, it results in steric hindrance and inhibits non-radiative radiation via rotation, thus activating the radiative emission pathway and emitting visible light [180].

Figure 5-3 Non-planar hexaphenylsilole (HPS) exhibiting restricted intramolecular rotation (RIR) during aggregation, resulting in light emission. This phenomenon is thus coined as aggregation-induced emission (AIE). Reproduced from [180] with permission of The Royal Society of Chemistry.

In a recent work by Xue et al. [181], an AIE luminogen – carboxylated tetraphenylethylene (TPE-COOH) was investigated as a fluorescence probe and delivery vector for the chemotherapy drug doxorubicin (DOX). They utilised bulk mixing methods similar to those described in 5.1.1, adding the TPE-COOH dissolved in dimethylsulfoxide (DMSO) to deionized water and vortexing the mixture. DOX was loaded by similarly adding the DOX solution into the previous mixture. However, the drug loading efficiency of DOX was limited to only 10%, beyond which the polydispersity of the nanoparticles increased significantly.

We propose implementing the nanoprecipitation method and adapting the TPE-COOH nanoaggregates protocol for our millifluidic device to attain monodispersed drug-loaded nanoaggregates with increased loading efficiencies. As discussed earlier in section 5.2, the miniature fluidic environment provides a well-controlled environment to facilitate reproducible mixing conditions. In addition, we are using devices with millimeter dimensions instead of microfluidics which require stringent cleanroom conditions to fabricate the master mould. The advantages of this millifluidic system include the ease of fabrication as well as the ability to process larger volumes within the same period of time, while retaining the advantages of

microfluidics. Besides that, these millifluidic devices can enable automation of the mixing process, thus reducing the possibility of variations due to human error and inconsistencies.

In the following section, a preliminary study based on 4-(Dicyanomethylene)-2-methyl-6-(4-dimethylaminostyryl)-4H-pyran (DCM) dye with poly(ethylene glycol)–distearoylphosphatidyl ethanolamine (mPEG-DSPE) was employed as an analogous system to study the feasibility of employing the millifluidic chip-based hydrodynamic flow focusing to achieve monodispersed nanoaggregates of the AIE luminogen encapsulated within micelles. DCM is an organic dye, which is hydrophobic and insoluble in water but soluble in an organic solvent like the dimethyl sulfoxide (DMSO). Although the mechanism behind the fluorescence of AIE luminogens differ from that of the organic dyes, they are similar in the sense that both are soluble in DMSO and will aggregate in aqueous solvents such as deionized water (DI H₂O). Therefore, sections 5.4.1 and 5.4.2 will discuss the simulation results and some preliminary data based on this analogous model.

5.4 Preliminary Results - Encapsulation of Organic Dye (DCM) with mPEG-DSPE Phospholipid

5.4.1 Simulation of Hydrodynamic Flow Focusing in Millifluidic Chip

The hydrodynamic flow focusing was modelled using the finite element method software Comsol Multiphysics. The laminar flow and transport of diluted species models were implemented in the simulation to couple the Navier-Stokes equation (Equation 5-1) with the continuity equation (Equation 5-2) and the mass balance equation (Equation 5-3) to solve for the flow velocity (u) and concentration (c) of the sample species,

$$\rho(u \cdot \nabla)u = -\nabla p + \nabla \cdot \eta(\nabla u + (\nabla u)^T) = F \quad \text{Equation 5-1}$$

$$\nabla \cdot u = 0 \quad \text{Equation 5-2}$$

$$\nabla \cdot (-D\nabla c + cu) = 0 \quad \text{Equation 5-3}$$

where ρ refers to the density of the fluid, u is the fluid velocity, p is the fluid pressure, η is the dynamic viscosity of the fluid, F is the sum of the external forces applied to the liquid, D is the diffusion coefficient, and c is the concentration of the reaction species. The simulation was done based on a two-dimensioned geometry. The fluid parameters were set according to that of water and the flow rates at the carrier inlets were fixed at 2 mL/min, while the sample injection flow rate was varied from 0.1 mL/min to 3 mL/min corresponding to relative flow ratios $\left(R = \frac{FR_{sample}}{FR_{carrier}} \right)$ of 0.05 to 1.5. The boundary conditions were set such that the velocity at the channel walls were zero, the pressure at the outlet was zero and the input sample concentration was given an arbitrary value of 1 mol/m³.

The concentration scale bar was set according to the concentration of DCM-mPEG-DSPE in the millifluidic chip, thus a concentration of zero shown as dark blue regions correspond to the regions with DI water as there was no DCM-mPEG-DSPE present. Conversely, dark red regions and intermediate colours indicate the presence of the dye and phospholipid mixture. Figure 5-4 presents the simulation results showing the velocity and concentration profiles of selected R values, namely $R=0.1$ and $R=1$. An overview of the millifluidic chip together with the constituents of its inlets is shown in Figure 5-4a, where the region of interest is circled in green. Figure 5-4b and e illustrate the fluid velocities within the chip at $R=0.1$ and $R=1$ respectively, comparing both figures it is obvious that the flow in all 3 inlets are approximately equal for $R=1$ (Figure 5-4e), while the flow is faster in the two side carrier channels compared to the middle sample channel for the case of $R=0.1$. This is consistent with our definition of R .

The concentration profile on the other hand, is more intriguing, as it provides insights into the mixing of the fluids inside the chip. Based on Figure 5-4c and f, firstly, it is observed that the

width of the middle sample inlet appears to decrease in width and forming a focused stream. Secondly, the width of the focused stream changes for different R values. Figure 5-4d and g are the graphical plots of the concentration distribution across the millifluidic channel as illustrated by the dotted line in Figure 5-4c. Since the concentration profile is reflective of the carrier and sample fluid streams, the full width half maximum (FWHM) of the concentration distribution (Figure 5-4d and g) can be inferred to be the width of the focused stream.

Figure 5-5 summarises the changes in the width of the focused stream as a function of R values. At small R values of 0.05 and 0.1, the focused stream appears to be very narrow and the concentration of the sample within this stream drops significantly as indicated by the change in colour of the stream from red to light blue and yellow. This means that there is mixing between the water and the sample streams and it is precisely this interaction between the streams that causes rapid mixing.

On the other hand, as R increases (i.e. sample flow increases in magnitude relative to the carrier flows), what appears to be a relatively undisturbed focused central stream begins to develop ($R=0.50$ and above in Figure 5-5). The difference in this focused stream as evidenced from the concentration profiles of the simulated millifluidic channels in Figure 5-5, shows a central stream with a reduced width but yet retaining the initial concentration of the loaded dye and phospholipid mixture (centre channel is deep red). Therefore, this isolated centre stream is preferred for applications like flow cytometry where the aim is to focus the cells in a single file manner for detection and segregation, while the smaller R values are preferred for applications such as ultrafast mixing. In this chapter, we investigate the effect of the value of R on the production of dye encapsulated phospholipid micelles, showing some preliminary results and its potential to be extended for future studies.

Figure 5-4 a) Annotated diagram showing simulation model with the dark blue region inside the millifluidic chip corresponding to deionized water in the carrier inlets and the dark red region indicating the DCM-mPEG-DSPE sample flow. Selected velocity and concentration profiles showing the focusing of the middle sample stream and their cross sectional concentration distribution for the relative flow ratios of $R=0.1$ (b, c and d) and 1 (e, f and g).

Figure 5-5 Graph showing the change in the width of the focused stream with different relative flow ratios (R) along with miniature diagrams reflecting the focused stream width. The inset shows the actual millifluidic chip used for hydrodynamic flow focusing.

5.4.2 Experimental Methods and Materials

5.4.2.1 Materials

4-(Dicyanomethylene)-2-methyl-6-(4-dimethylaminostyryl)-4H-pyran (DCM) laser dye was (Exciton, Ohio, USA), 5 kDa (molecular weight) poly(ethylene glycol)-distearoylphosphatidyl ethanolamine (5k MW mPEG-DSPE) (Laysan Bio, Inc., Arab, AL, USA), chloroform (ACS standard, Merck Pte Ltd, Singapore), and dimethylsulfoxide ([DMSO], Sigma Aldrich, Singapore) were used in the synthesis of the polymer nanoparticles. The 184 Sylgard silicone elastomer kit from Dow Corning was used to fabricate the polydimethylsiloxane (PDMS) hydrodynamic flow focusing millifluidic chip. Whatman Grade 1 filter paper with a pore size of 11 μm was acquired from Sigma Aldrich, Singapore. All chemicals were used as received without further purification. Deionized (DI) water mentioned in the experiments was purified by a Milli-Q water purification system.

5.4.2.2 Bulk Method

The protocol was adapted from Hu et al [182]. In essence, 2 mL of DCM dye (1 mg/mL concentration) dissolved in chloroform was added to 9 mg of mPEG-DSPE according to the mass ratio of 1:4.5. The chloroform in the mixture was removed by placing the mixture in a vacuum dessicator to remove the volatile chloroform solvent via vaporisation. Then, 10 mL of deionized water was added to the dry residue and the resultant solution was stirred overnight. Excess DCM dye present in the solution was removed via filtration using an 11 μm pore size filter paper (Whatman). Lastly, the filtrate was centrifuged at 5000 rpm for 10 minutes to remove any residual DCM dye. The supernatant was extracted as it contained the organic DCM dye encapsulated by the mPEG-DSPE micelle.

5.4.2.3 Millifluidic Chip Hydrodynamic Flow Focusing Method

Similar to the bulk method, 2 mL of DCM dye was added to 9 mg of mPEG-DSPE but the solvent used was changed to dimethyl sulfoxide (DMSO) instead of chloroform. Unlike the bulk method, the organosulphur solvent was not evaporated, instead it was injected into the millifluidic chip along with two carrier streams of deionized water. The output mixture was collected at the outlet of the chip. This mixture was then filtered using the 11 μm pore size filter paper (Whatman) to remove the residual precipitated DCM dye. Again, excess DCM dye was removed by centrifugation at a low speed of 5000 rpm for 10 minutes. The supernatant containing the micelle-DCM dye complex was extracted and retained for subsequent characterization.

5.4.2.4 Characterization of Micelles

The average particle size was determined using dynamic light scattering measurements. These measurements were performed using the NanoBrook 90Plus Particle Size Analyzer (Brookhaven Instruments, Holtsville, USA). Each data point was obtained by measuring three

samples with the same parameters. Scanning electron microscope imaging was done by placing droplets of the as-synthesized micelle solution on a silicon wafer and dried in a vacuum dessicator, following which a thin layer of gold was sputtered onto the samples and imaged using a field emission scanning electron microscope (JEOL FESEM 6340F). The photoluminescence emission spectrum was collected using a HORIBA Jobin Yvon Fluorolog-3 Fluorometer.

5.5 Results and Discussion

Figure 5-6 a) Schematic and b) actual hydrodynamic flow focusing millifluidic chip with two side carrier streams (containing DI H₂O) and an inlet for the DCM-mPEG-DSPE mixture.

Based on the simulation design in section 5.4.1, a 3D printed mould with uniform cross-section channel dimensions of 1 mm by 1 mm was fabricated. The length of the channel from the point where all 3 streams are in contact until the outlet spans 32 mm in length. As shown in Figure 5-6a and b, the bright orange DCM-mPEG-DSPE mixture dissolved in DMSO was delivered into the middle stream, which was flanked by two carrier streams containing deionized water (DI H₂O). Similar to the simulation, the injection flow rate of the side carrier streams were fixed at 2 mL/min, while the flow rate of the centre sample stream is varied from 0.1 mL/min to 3 mL/min to generate relative flow ratios of 0.05 to 1.5 (i.e. $R = \frac{FR_{sample}}{FR_{carrier}}$). As seen in Figure

5-6, the width of the centre sample stream was reduced indicating that hydrodynamic flow focusing has been achieved as predicted by the modelling in section 5.4.1 and Figure 5-5.

Figure 5-7 a) Unfiltered output mixture from hydrodynamic flow focusing millifluidic chip. b) Pour the mixture through the filter paper to remove large dye precipitates. c) Dye residue collected on filter paper and d) clear filtrate containing micelles.

The output solution obtained at the outlet of the millifluidic chip is shown in Figure 5-7a. This solution was then filtered using an 11 μm pore size filter paper and a clear filtrate was obtained (Figure 5-7d). The residue collected on the filter paper (Figure 5-7c) appeared to be excess DCM dye which was not encapsulated by the PEGylated phospholipid (mPEG-DSPE) as DCM is hydrophobic and thus insoluble in water. Since the filtrate is clear, the only visual inspection method to verify if there is any dye present in the solution is to use ultraviolet (UV) light illumination since the absorbance wavelength of the dye falls in the UV region. Figure 5-8 shows the red glow from the DCM dye in a seemingly transparent solution containing the micelle solution. This glow is further validated by Figure 5-9, which shows the photoluminescence spectrum of the same micelle solution measured using the fluorometer with excitation wavelength set at 400 nm.

Figure 5-8 DCM dye encapsulated micelle solution a) under normal ambient light, b) with UV illumination in ambient conditions, and c) under UV illumination in the dark.

Figure 5-9 Photoluminescence spectrum of DCM dye encapsulated micelle solution.

The diameter of the micelles were determined using the dynamic light scattering (DLS) technique. This gives the hydrodynamic size of the micelles presented in Figure 5-10a and b. Generally the size of the micelles synthesized using the millifluidic chips ($R=0.05$ to 1.5) are smaller than those synthesized using the bulk mixing method (BT). Referring back to the simulation results in Figure 5-4 and Figure 5-5, we would expect the micelles formed at $R=0.25$ and exhibit desirable qualities such as monodispersed sizes brought about by fast mixing aided by hydrodynamic flow focusing.

However, Figure 5-10a shows relatively modest results of around 114 to 129 nm diameters (with the exclusion of $R=0.1$) for R values of 0.5 and less. The relative variance on the other

hand appear to be promising with values less than 0.5 (except $R=0.1$). For the case of $R=0.1$, the size of the particles seem unusually large at around 260 nm with a considerable relative variance values of 1.22. The cause of this anomaly is unknown and further experimentation is required to gather more information. On the other hand, for larger R values (i.e. $R=1$ and 1.5), the hydrodynamic diameters of the micelles appear to be very small at 14 nm and 62 nm respectively, but the relative variance values are much higher at 0.77 and 0.64. Since at large R values, the centre stream only experiences minimal interaction with the carrier stream as shown in the simulation results of Figure 5-4 and Figure 5-5, where only the side surfaces in contact with the carrier show some change in concentration, while the middle stream remains undisturbed. Therefore, the mixing was not adequate and hence could produce some micelles but with high polydispersity.

Figure 5-10 a) Hydrodynamic diameter of DCM-mPEG-DSPE micelles and b) their corresponding relative variances as a function of the relative flow ratio, R . BT represents the micelles assembled using macroscale benchtop method of vigorous stirring.

Figure 5-11 FESEM image of dye encapsulated micelles synthesized using the millifluidic chip with $R=0.05$.

5.6 Future Studies

The results presented above are purely preliminary data from the initial testing stages of micelle formation by implementing hydrodynamic flow focusing in millifluidic chips. Although we acknowledge that additional results are necessary in order to draw more accurate deductions, the work so far gives us a glimmer of hope that efficient mixing can be achieved in the millifluidic environment. Both the simulation results and the preliminary experimental data seem corroborate the existence of a distinct cutoff regime for efficient mixing. We believe that with more thorough testing and optimization such as tuning the relative flow rates with narrower intervals and varying the dye and PEGylated phospholipid ratios as well as their concentrations, more encouraging results can be obtained.

Besides that, based on the SEM image of the millifluidic chip synthesized micelles (Figure 5-11), the shape of the micelles might be rod shaped or elongated, thus measurements using the DLS technique might not be the best method to characterize these particles. Either further experimentation to seek to control the shape of the micelles or other characterization methods such as particle counting using SEM or TEM images could be considered subsequently.

Chapter 6 Conclusion and Further Recommendations

6.1 Conclusion

From the work presented in this thesis, it is evident that the millifluidic chip provides a flexible and easy to use system capable of regulating nanoparticle formation and growth with excellent reproducibility. Millifluidic synthesis of nanoparticles allows fine control over reaction parameters such as temperature, mixing time, relative concentration of precursors. The large surface area to volume ratio enhances heat and transfer enabling homogenous heating of the miniature reaction chamber. It also allows spatial resolution of the temporal reaction progress, which would be very helpful for multistep reactions.

Three classes of nanoparticles were studied, namely plasmonic, semiconductor, and polymer nanoparticles. For the plasmonic and semiconductor nanoparticles, we showed that uniform sized nanoparticles could be obtained by flowing the reactants through the chip and adjusting parameters like the flow rate, molar ratio and reactant concentrations. Notable results include the wide tunability range of the plasmonic Cu_{2-x}S LSPR peak from 1115 nm to 1644 nm and the narrow size control of CdS nanoparticles with excellent reproducibility as well as the batch replication of magic-sized CdS clusters. Exploratory work on self-assembly of dye encapsulated micelles in the millifluidic chip reactor was also presented in hope of extending the control over the reaction conditions to another mixing regime.

Applications of the synthesized nanoparticles for applications such as photothermal therapy and bioimaging also show that these nanoparticles besides being studied in terms of crystal growth and particle formation, are practical materials that can be used for applications that can

improve aspects of healthcare such as therapy and diagnostics or even be used to improve device efficiencies for optoelectronics.

6.2 Further Recommendations

The applications proposed in this thesis are focused in the biomedical field, such as cell imaging, cancer therapy, and drug loading applications. Nanoparticles are nanomaterials with flexible properties, for example the band gap of semiconductor nanoparticles can be varied by changing the size of the particles due to quantum confinement effects. Therefore, combining both millifluidic chip synthesized Cu_{2-x}S nanocrystals and CdS quantum dots could be incorporated into solar cells to harvest solar energy into electricity.

Besides looking into other avenues where these chip-synthesized nanoparticles can play a role, there is plenty of room to investigate the synthesis of complex nanoparticles such as ternary and quaternary nanoparticles. If the synthesis of these nanoparticles can be simplified using such miniature platforms, widespread adoption of nanoparticle manufacturing will be possible. This would then benefit the research community at large as novel materials can be generated and subsequent studies in various fields such as fundamental science, engineering, and medicine. For instance, personalized medicine in terms of treatment plans and allergy reactions can be simulated *in vitro* and accounted for when designing specific drugs suitable for the patient.

Lastly, the larger millimetre dimensioned structures may facilitate the incorporation of optical characterization tools such as UV/Vis absorbance and small angle X-ray scattering (SAXS) measurements which would enable real-time monitoring of the nanoparticles produced. This would then enable better quality control over the output products and quick rectification of reaction parameters to observe changes in the nanoparticles.

Author's Publication

Journal Papers

1. T.-L. Cheung, **L. Hong**, N. Rao, C. Yang, L. Wang, W. J. Lai, *et al.*, "The non-aqueous synthesis of shape controllable Cu₂-xS plasmonic nanostructures in a continuous-flow millifluidic chip for the generation of photo-induced heating," *Nanoscale*, vol. 8, pp. 6609-6622, 2016. **(Co-author/Equal contribution)**
2. **L. Hong**, T.-L. Cheung, N. Rao, Q. Ouyang, Y. Wang, S. Zeng, C. Yang, C. Dang, P. H. J. Chong, L. Liu, W.-C. Law, and K.-T. Yong, "Millifluidic chip synthesis of luminescent CdS Nanocrystals for Bioimaging". **(Submitted)**
3. Q. Ouyang, S. Zeng, L. Jiang, **L. Hong**, G. Xu, X.-Q. Dinh, *et al.*, "Sensitivity Enhancement of Transition Metal Dichalcogenides/Silicon Nanostructure-based Surface Plasmon Resonance Biosensor," *Scientific Reports*, vol. 6, p. 28190, 2016.

Book Chapter

1. A. M. Soehartono, **L. Hong**, G. Yang, P. Song, H. K. S. Yap, K. K. Chan, P. H. J. Chong, K.-T. Yong, "Miniaturized fluidic devices and their biophotonic applications", in *Handbook of Photonics for Biomedical Engineering*, H. P. A. Ho, D. Kim, and G. M. Somekh, Eds., ed Dordrecht: Springer Netherlands.

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