

Digital Profiling of Circulating Extracellular Vesicles at Single-Upconversion Nanoparticle Sensitivity and Resolution

by Guan HUANG

Thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

under the supervision of Prof. Dayong Jin,
Dr. Gungun Lin, Dr. Ying Zhu

University of Technology Sydney
Faculty of Science

01/07/2022

CERTIFICATE OF ORIGINAL AUTHORSHIP

I, Guan Huang declare that this thesis is submitted to fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Mathematical and Physical Sciences, Faculty of Science, at the University of Technology Sydney.

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This research is supported by an Australian Government Research Training Program, Guangzhou Elite Program Scholarship and Translation Cancer Research Network PhD Scholarship Top-up Award.

Signature of Student:

Production Note:

Signature removed prior to publication.

Date: 01 July 2022

Acknowledgements

First and foremost, I would like to thank my principle supervisor Distinguished Professor Dayong Jin for his supervision throughout the PhD project. You helped me look at research in a way that optimized my time at the bench and by understanding what was required to tackle a problem effectively and efficiently. At the same time, I am very grateful to your insightful comments, expertise and guidance in driving my research in the right direction.

I am thankful for having had the opportunity to work with such passionate and hardworking fellow researchers, Dr. Yinghui Chen, Dr. Gungun Lin and Dr. Ying Zhu. Thank you for your always supportive and willing to meet if I have questions or concerns. I would like to acknowledge my colleagues and collaborators who contributed to my research. Thanks to our IBMD colleagues, Dr. Yongtao Liu for optical knowledge sharing and optical system setup, Dr. Shihui Wen for UCNPs knowledge sharing and providing me with UCNPs at a high constant quality, Dr. Qian Peter Su for TIRF setup, Dejiang Wang for cell transfection, Mahnaz Maddahfar for providing polymers, Dr. Yuan Liu for microfluidic fabrications, and everyone in IBMD group. Thank you, Haoqi Mei for spending a lot of time in Western blotting and Dr. Yan Liao for providing materials to plasmid amplification. And this acknowledgement could never be complete without the collaborators from Garvan Institute of Medical Research. My thanks to A/Professor David Gallego-Ortega for his input in the project and for his support on preclinical models. I am grateful to Laura Rodriguez de la Fuente for enumerable amount of time and effort to helping me extract blood from mouse models. Thanks all, everything from experimental design to scientific writing has been invaluable over the years I've been here.

“In a PhD student's life, research is a 24x7 thing, if we aren't physically doing experiments, we most likely are mentally preparing for one.” I could not have done this without the love and support from my parents. I also want to thank my loving and warm boyfriend Peng Nie who listened with enthusiasm and curiosity to the minutiae of the project itself, and with great sympathy and understanding whenever I was feeling overwhelmed, anxious and hopeless. And I want to thank all my friends for helping me have a life outside my research. I am blessed to have you all in my life.

Finally, I would like to acknowledge the support provided by Australian Government Research Training Program, Guangzhou Elite Program Scholarship and Translation Cancer Research Network PhD Scholarship Top-up Award through which most of the work in this dissertation was funded. I sincerely apologize if I have forgotten to acknowledge anyone.

List of publications

1. **G. Huang**, Y. Zhu, S. Wen, H. Mei, Y. Liu, D. Wang, M. Maddahfar, Q. P. Su, G. Lin, Y. Chen and D. Jin. Single Small Extracellular Vesicle (sEV) Quantification by Upconversion Nanoparticles. *Nano Letters*. 22, 3761–3769 (2022).
2. **Guan Huang**, Yongtao Liu, Dejiang Wang, Ying Zhu, Shihui Wen, Dayong Jin*. Upconversion Nanoparticles for Super-resolution Imaging of Single Small Extracellular Vesicles (in preparation)
3. **Guan Huang**, Laura Laura Rodriguez de la Fuente, David Gallego-Ortega, Ying Zhu, Yongtao Liu, Dayong Jin*. Preclinical detection of circulating EVs (in preparation)
4. **Huang, G.**; Lin, G.; Zhu, Y.; Duan, W.; Jin, D. Emerging Technologies for Profiling Extracellular Vesicle Heterogeneity. *Lab on a Chip* **20**, 2423–2437 (2020) (highlighted on front cover)
5. Chen, Y, Shimoni, O, **Huang, G**, Wen, S, Liao, J, Duong, HTT, et al. Upconversion nanoparticle-assisted single-molecule assay for detecting circulating antigens of aggressive prostate cancer. *Cytometry Part A*. 2021; 1– 11.
6. Liu, Y., Lin, G., Bao, G., Guan, M., Yang, L., Liu, Y., Wang, D., Zhang, X., Liao, J., Fang, G., Di, X., **Huang, G.**, Zhou, J., Cheng, Y., and Jin, D. Stratified Disk Microrobots with Dynamic Maneuverability and Proton-Activatable Luminescence for in Vivo Imaging. *ACS Nano* 2021 15 (12), 19924-19937
7. Lin, G., Liu, Y., **Huang, G.**, Chen, Y., Makarov, D., Lin, J., Quan, Z. and Jin, D. 3D Rotation-Trackable and Differentiable Micromachines with Dimer-Type Structures for Dynamic Bioanalysis. 2021. *Adv. Intell. Syst.*, 3: 2000205.
([1,2,3,4,5] are closely related to my PhD program)

Conferences

1. Background-free single luminescent nanoparticle assay for detecting tumor-derived extracellular vesicles, Oral Poster, Thomas Ashworth CTC & Liquid Biopsy Symposium 2021, Sydney, Australia
2. Profiling Single Extracellular Vesicles from Single Cells, Oral Presentation, Australasian Extracellular Vesicles Conference 2021, Auckland, New Zealand

Award

1. Vice-Chancellor's Postgraduate Research Conference Fund 2021, UTS, Australia.

2. TCRN PhD Scholarship Top-up Awards 2020, Translational Cancer Research Network, Australia
3. Australasian Extracellular Vesicles Conference Outstanding student presentation award 2020, ANZSEV, Australia and New Zealand

Structure of Thesis

This thesis has five chapters. Chapter 1 is on the basic knowledge and literature review. Chapter 2-4 are the three core result chapters reporting the experiments, the research results and discussions of developing nanoparticles and imaging tools to detect and qualify the surface biomarkers of single extracellular vesicles (EVs). In chapter 2, the types and numbers of EV surface markers have been quantified and profiled at single nanoparticle sensitivity. In chapter 3, super-resolution imaging techniques have been used to digitize the number of nanoparticles on single EVs. In chapter 4, the developed technology platform has been applied to prognose the cancer metastasis on two mouse models, both relevant breast cancer cohort. Chapter 5 is the conclusion of this thesis and discussions for future works. I organize the five chapters following the flowchart below:

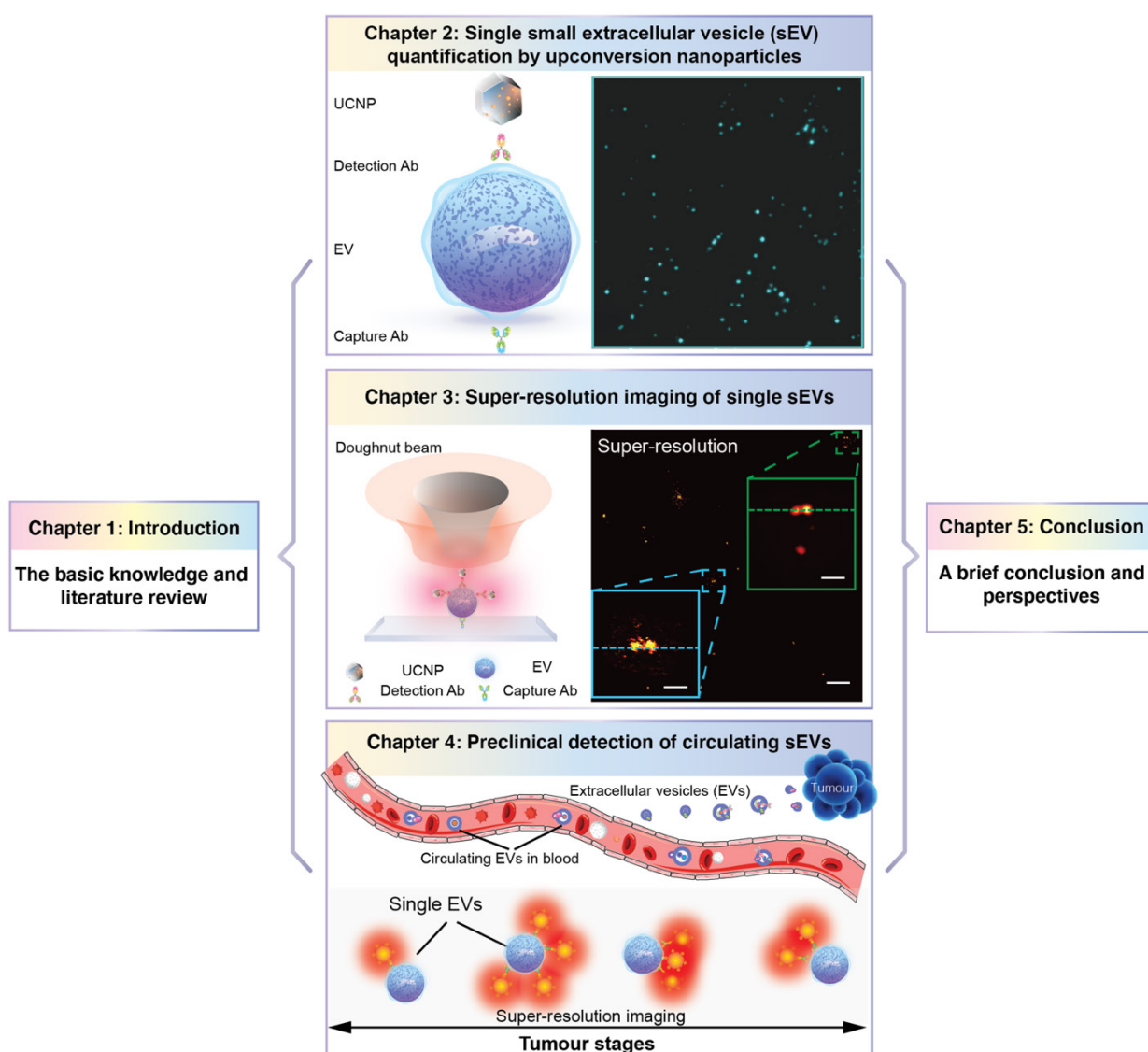


Table of Contents

Acknowledgements	i
List of publications	ii
Structure of Thesis	iv
Table of Contents	v
Abstract	1
Statement of Contribution of Authors for chapter 1	2
Chapter 1 Introduction	3
1.1 Liquid biopsy in cancer diagnostics and management	3
1.2 Liquid biopsy biomarkers.....	4
1.2.1 Circulating tumour cells (CTC)	4
1.2.2 Circulating tumour DNA (ctDNA)	5
1.2.3 Circulating RNA	5
1.2.4 Circulating Proteins	5
1.2.5 Circulating extracellular vesicles	6
1.3 Role of EVs in liquid biopsy	7
1.4 EV separation and concentration techniques.....	8
1.5 EV characterisation techniques	9
1.6 Strengths of circulating EVs in liquid biopsy.....	10
1.7 Limitations of circulating EVs in liquid biopsy	11
1.7.1 Heterogeneity - A hidden world beneath population averages	12
1.7.2 Current Approaches for EV Heterogeneity Analysis	13
1.8 Nanomaterials for EV detection	22
1.8.1 Magnetic nanoparticles (MNPs)	23
1.8.2 Graphene oxide (GO).....	23
1.8.3 Gold nanoparticles (AuNPs)	23
1.8.4 Quantum dots (QDs)	24
1.8.5 Semiconducting polymer dots (Pdots).....	24
1.8.6 Carbon nanotubes (CNT)	26
1.8.7 Upconversion nanoparticles (UCNPs).....	27
1.9 UCNPs in bio-applications	27
1.9.1 Mechanism of UCNPs.....	27
1.9.2 Synthesis of UCNPs	29
1.9.3 Surface modification of hydrophobic UCNPs	29
1.9.4 UCNPs in biosensing	32

1.10 EVs and super-resolution microscopy.....	36
1.10.1 Single EV imaging technique	37
1.10.2 UCNPs in super-resolution techniques	39
1.10.3 Diffraction limit in microscopy.....	39
1.10.4 UCNP-based STED-like super-resolution nanoscopy	40
1.10.5 Photon transition system in UCNPs.....	40
1.10.6 Saturation effect with doughnut-shaped beam.....	41
1.11 Aim.....	43
Statement of Contribution of Authors for Chapter 2.....	45
Chapter 2 Single small extracellular vesicle (sEV) quantification by upconversion nanoparticles	46
Abstract.....	46
2.1 Introduction	46
2.2 Methods	48
2.2.1 Cell culture.....	48
2.2.2 EVs isolation from Cell culture medium.....	48
2.2.3 Cryogenic Electron Microscopy.....	49
2.2.4 Nanoparticle tracking analysis.....	49
2.2.5 Western blotting.....	49
2.2.6 Flow cytometry	50
2.2.7 Synthesis of NaYF ₄ :20%Yb ³⁺ ,2%Er ³⁺	51
2.2.8 Transmission electron microscopy.....	51
2.2.9 Synthesis of POEGMEA ₁₃ -b-PMAEP ₇	51
2.2.10 Surface modification of UCNPs.....	52
2.2.11 Characterization of functionalised UCNPs.....	52
2.2.12 TIRF setup and imaging.....	52
2.2.13 LENS assay.....	53
2.2.14 Image and data processing	53
2.3 Results	54
2.3.1 Production and isolation of cancer cell-derived EVs.....	54
2.3.2 Characterization of Cancer cell-derived EVs.....	55
2.3.3 Preparation and characterisation of LENS.....	57
2.3.4 Quantification of EpCAM ⁺ EVs from cell line model.....	63
2.4 Conclusion and discussion	67
Statement of Contribution of Authors for Chapter 3.....	69
Chapter 3 Super-resolution imaging of single sEVs.....	70
Abstract.....	70
3.1 Introduction	70
3.2 Methods	72
3.2.1 Cell transformation.....	72
3.2.2 EV isolation.....	73

3.2.3 Synthesis of NaYF ₄ :40%Yb ³⁺ ,4%Tm ³⁺ nanocrystals.	73
3.2.4 Fabrication of LENS.	73
3.2.5 GFP-LENS colocalization procedure.....	73
3.2.6 Dual-LENS assay procedures.	73
3.2.7 Deconvolution methods for wide-field imaging	74
3.2.8 Super-resolution setup.	74
3.3 Results	75
3.3.1 Optical performance evaluation of LENS doped with different kinds of emitters	75
3.3.2 Co-localization and comparison of EpCAM-mGFP EV with LENS.....	79
3.3.3 Single EV imaging by super-resolution microscope.....	82
3.3.4 Future direction of the technique - Size related steric hindrance.....	85
3.4 Conclusion and discussion	88
Statement of Contribution of Authors for Chapter 4.....	89
Chapter 4 Preclinical detection of circulating EVs.....	90
Abstract.....	90
4.1 Introduction	90
4.1.1 Limitations in breast cancer clinical management.....	90
4.1.2 Role of EVs in tumour metastases	91
4.1.3 Role of EVs in indexing the stage of metastatic tumour progression	92
4.1.4 Murine preclinical cancer modelling	93
4.2 Methods	99
4.2.1 Experimental Animal Models	99
4.2.2 Intraductal mammary carcinoma implantation.	99
4.2.3 Blood and lungs collection.....	100
4.2.4 Plasma isolation from fresh blood.....	100
4.2.5 EVs isolation from Plasma.....	100
4.3 Results	101
4.3.1 Preclinical prognosis of Syngeneic Models.....	102
4.3.2 Preclinical prognosis of GEMMs.....	107
4.4 Conclusion and discussion	113
Chapter 5 Conclusion and Perspectives.....	115
5.1 Conclusion	115
5.2 Technical limitations	116
5.3 Clinical translation limitations	117
5.4 Perspectives	118
Reference	122

Abstract

Circulating extracellular vesicles (EVs) carry significant information about the progression stages of tumour sites. Quantification of low-abundant EVs and statistical profiling of the heterogeneity of single EVs, particularly from liquid biopsy sampling, will guide clinical decisions on the stages of tumour progression. However, the nanoscopic sizes (typically 40-200 nm) and the extremely small quantity of cargo materials demand the high detection sensitivity, stability, resolution and throughput to be simultaneously achieved.

Nanotechnology has been broadly used in the field of liquid biopsy. This thesis explores a new strategy for ultra-sensitive, photo-stable, and super-resolution immunoassay of single EVs, which is based on the development, bio-conjugation and application of upconversion nanoparticles (UCNPs). In chapter 2, we apply UCNPs for direct enumeration of single CD9 and EpCAM positive EVs ($CD9^{+}EpCAM^{+}EVs$). The achieved single-molecule sensitivity results in a femtomolar detection limit (1.8×10^6 EVs mL⁻¹), which was nearly 3 orders of magnitude lower than the standard enzyme-linked immunosorbent assay (ELISA). Compared with previous luminescence resonance energy transfer (LRET) method using UCNPs for detection of EVs, our technique achieves single tumour-derived sEV quantification. In chapter 3, we report super-resolution imaging technique for single $CD9^{+}EpCAM^{+}EV$ analysis. The upconversion luminescence of single UCNPs can nonlinearly response to a donut-shaped scanning beam, so that a resolution better than 40 nm can be achieved beyond the diffraction limit. In chapter 4, with the ultra-sensitivity and photo stability achieved by UCNPs as well as super resolution offered by a donut-shaped scanning, the preclinical translation capability of the integrated technology platform has been examined by two types of breast cancer mouse models. Our results suggest that the population of cancer-derived circulating EVs, detected and classified by the number of UCNPs, can be used to monitor the metastatic tumour progression, including non-metastasis/high-metastasis and low-metastasis/high-metastasis mouse models. Furthermore, we find that the number of UCNPs on single EVs can be used to index the stage of metastatic tumour progression. In chapter 5, we discuss the challenges and opportunities of this thesis towards clinical translation, which suggests a new scope of research by integrating nanotechnology, microscopy imaging and lab-on-a-chip devices for EV research and applications. This thesis presents a viable approach of using the EVs-based liquid biopsy for tumour diagnosis and prognosis.

Key words: extracellular vesicles, upconversion nanoparticles, super-resolution, cancer metastases, liquid biopsy