2019

DEVELOPMENT AND CHARACTERIZATION OF NANOMATERIALS FOR PULMONARY DRUG DELIVERY APPLICATIONS

Elisa A. Torrico Guzman
University of Rhode Island, torrico.elisa@gmail.com

Follow this and additional works at: https://digitalcommons.uri.edu/oa_diss

---

Recommended Citation

Torrico Guzman, Elisa A., "DEVELOPMENT AND CHARACTERIZATION OF NANOMATERIALS FOR PULMONARY DRUG DELIVERY APPLICATIONS" (2019). *Open Access Dissertations*. Paper 864.
https://digitalcommons.uri.edu/oa_diss/864

This Dissertation is brought to you for free and open access by DigitalCommons@URI. It has been accepted for inclusion in Open Access Dissertations by an authorized administrator of DigitalCommons@URI. For more information, please contact digitalcommons-group@uri.edu.

DEVELOPMENT AND CHARACTERIZATION OF NANOMATERIALS FOR
PULMONARY DRUG DELIVERY APPLICATIONS

BY

ELISA A. TORRICO GUZMAN

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
CHEMICAL ENGINEERING

UNIVERSITY OF RHODE ISLAND

2019

DOCTOR OF PHILOSOPHY DISSERTATION
OF
ELISA A. TORRICO GUZMAN

APPROVED:

Dissertation Committee:

Major Professor   Samantha A. Meenach

Geoffrey Bothun

Sara Rosenbaum

Nasser H. Zawia
DEAN OF THE GRADUATE SCHOOL

UNIVERSITY OF RHODE ISLAND

2019

ABSTRACT

Chronic pulmonary diseases such as lung cancer and cystic fibrosis represent significant global health concerns because of their resulting high mortality and low life expectancy. Pulmonary drug delivery has demonstrated advantages over other delivery routes due to the ability to use lower concentrations of drug, reduction in systemic toxicity, and offering better targeting to the area of disease. Effective application of dry powder therapeutics requires the optimization of particle deposition in the lungs and avoidance of physiologic defense mechanisms such as mucosal entrapment, mucociliary clearance, and macrophage engulfment. Evaluation of aerosol pulmonary therapeutic parameters demands accurate *in vitro* models that can appropriately simulate the pulmonary environment, such that the results can accurately translate to *in vivo* testing. In addition, there is a need in the development of nanoparticle-based therapeutics capable of delivering chemopreventive agents owing to their poor water solubility and large concentration requirements for efficacy.

This dissertation was aimed at: (i) developing and evaluating resveratrol-loaded nanoparticles capable of preventing *in vitro* tumors spheroid growth, (ii) developing and characterizing dry powder paclitaxel-loaded nanocomposite microparticles, and evaluating their efficacy in the growth inhibition of lung cancer tumor spheroids using an *in vitro* three-dimensional air interface culture model, and (iii) developing and characterizing PEGylated nanocomposite microparticles, and evaluating their ability to penetrate mucus via an artificial mucus transport model.

Biodegradable nanoparticles were formulated with appropriate size for cellular uptake (~200 nm). The nanoparticles demonstrated tunable, pH-dependent sustained release for up to 4 days using 3-hour acetalated dextran. The nanoparticles showed increased colloidal stability with hydrophobic therapeutic agents and improved mucus-penetrating capabilities when coated with vitamin E poly(ethylene glycol) (VP5k). Nanoparticles loaded with resveratrol, a natural chemopreventive agent, showed superior inhibition of tumor growth when dosed in comparison to free resveratrol. Finally, nanocomposite nanoparticles loaded with paclitaxel (PTX), a toxic chemotherapeutic agent, showed comparable efficacy in comparison to free PTX.

Spray-dried nanocomposite microparticles demonstrated excellent aerosol performance when evaluated with an *in vitro* Next Generation Impactor for dry powder inhaler formulations, with a tunable targeted deposition depending on the formulation and spray drying conditions. These results highlight multiple beneficial characteristics of inhaled drug delivery with nanocomposite microparticles for lung cancer treatment, including sustained release over a long period of time, tunable deposition to the desired area within the bronchopulmonary tract, and improved mucus penetration. Additionally, the results observed in our 3D cell culture models differed from those observed in a 2D monolayer of cells, demonstrating the importance of accurate *in vitro* models to allow for appropriate translational expectations.

ACKNOWLEDGMENT

First, I would like to express my deepest gratitude to my advisor, Dr. Samantha Meenach, for her continuous support and guidance throughout my Ph. D. studies and certainly during the preparation of my dissertation. Her passion and vision led towards an exciting research area, which has kept me motivated over these past years. I thank her for all of the opportunities she has provided for my professional development, encouraging me to participate in outreach activities, promoting my independence in the progress of our projects, and supporting my involvement in various conferences, during which I improved valuable skills for my future. Finally, I thank Dr. Meenach for her patience, understanding, and great empathy when I required it most.

In addition to my advisor, I would like to thank my committee members: Dr. Geoffrey Bothun, Dr. Sara Rosenbaum, and Dr. Deyu Li for their insightful comments and questions, prompting me to expand my research perspective. Also, thank you to Dr. Jyothi Menon for kindly agreeing to be the chair for my dissertation defense.

I would like to thank Dr. Michael Greenfield for his warm welcome to URI and his guidance in the many steps involved in my Ph.D. curricula. I would like to thank Dr. Everett Crisman for his very much appreciated training and supervision in SEM and XRD since I first began using the equipment, and also to Dr. Irene Andreu for her readiness to help with problems during the use of SEM and XRD in the past months. Similarly, I would like to thank Dr. Al Bach from INBRE for his patience and meticulous training with various equipment, and recently for his enormous help with NMR.

I want to thank all my lab mates, especially former students, Qihua Sun, who helped me with initial training in the lab, along with Zimeng Wang, who has been a great person to discuss my research with when I needed some extra insight, even after his graduation. Also, thank you to current student, Golam Jakaria, for providing thoughtful suggestions during experiments and helping with confocal imaging. Finally, I want to thank all undergraduate and high school students for their help and interest in my projects, especially Mitch Gravely, now a Ph.D. student, who has helped in one of the projects included in this dissertation.

I would like to extend a special thanks to Kevin Greene, my best friend and boyfriend, who has supported me in happy, hard, and stressful times during the past 8 years, and also because he had the patience to go with me to the lab during weekends whenever I needed to.

Finally, I want to thank all my family for being my invaluable emotional support, especially my parents, René and Rosario '*Charo*', for believing in me and always encouraging me to do great things. Thank you, Dad and Mom, for your love and for being my greatest role models. I love you both infinitely. To my grandparents, who in their own way showed me that with love and perseverance everything can be possible. Also, my little sister, Ericka, whose energetic and happy stories have always fueled my spirits every time we talk. And finally, my great aunt, Dora, who in her 98 years has been a shining example of a truly independent woman and who has encouraged me to expand my mind in many different ways.

Thank you all, and thanks to God for the blessings and all above.

¡Gracias!

DEDICATION

To my Dad, René,

My hero, my inspiration and my angel in Heaven

and

To my Mom, Charo,

My heroine, my strength and my angel on Earth

PREFACE

This dissertation is presented in manuscript format in accordance with The University of Rhode Island Graduate School Guidelines and is comprised of six chapters. The first chapter is an introduction and overview of the dissertation, which includes the objectives and significance of the work. The second chapter, entitled “Pulmonary Delivery of Nanocarriers” involves in part a review of literature in contribution to the book chapter entitled “Routes of Administration for Nanocarriers” submitted as chapter 6 in the book *Nanoparticles for Biomedical Applications* (Elsevier S&T Books). The third chapter, entitled “Evaluation of the Cancer-Preventive Effect of Resveratrol-Loaded Nanoparticles on the Formation of Lung Tumor Spheroids” is a manuscript in preparation for the submission to the journal *Nanomedicine: Nanotechnology, Biology, and Medicine*. The fourth chapter, entitled “Development and Evaluation of Paclitaxel-Loaded Aerosol Nanocomposite Microparticles and Their Efficacy Against Lung Cancer Tumor Spheroids” is a manuscript in preparation to submit to *Journal of Biomedical Nanotechnology*. The fifth chapter, entitled “Development and Characterization of Aerosol Nanocomposite Microparticles Capable of Mucus Penetration” is being prepared for submission to the journal *Colloids and Surfaces B: Biointerfaces*. The sixth chapter is a summary of the main conclusions of this dissertation and future directions related to this area of research.

TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGMENT	iv
DEDICATION	vi
PREFACE	vii
TABLE OF CONTENTS	viii
LIST OF FIGURES	xvii
LIST OF TABLES	xxv
CHAPTER 1	1
INTRODUCTION	1
1.1. Overview and Motivation	1
1.2. Objectives and significance	5
REFERENCES	10
CHAPTER 2	13
Pulmonary Delivery of Nanocarriers	13
2.1. Introduction	13
2.2. Lung Physiology	13
2.3. Delivery Devices	14
2.3.1. Metered dose inhalers (MDIs)	14

2.3.2. Nebulizers	15
2.3.3. Dry Powder Inhaler (DPI).....	16
2.4. Particle Fate	17
2.5. Applications.....	21
2.6. Challenges, Advantages, and Limitations	22
2.7. Potential applications	24
REFERENCES.....	26
CHAPTER 3.....	33
Evaluation of the cancer-preventive effect of resveratrol-loaded nanoparticles on the formation of lung tumor spheroids	33
ABSTRACT	34
KEYWORDS	34
3.1. Introduction	35
3.2. Methods	38
3.2.1. Synthesis of Acetalated Dextran	38
3.2.2. Nuclear Magnetic Resonance Analysis of Acetalated Dextran	39
3.2.3. Preparation of Resveratrol-Loaded Ac-Dex Nanoparticles	39
3.2.4. Particle Morphology and Shape Analysis via Scanning Electron Microscopy	40

3.2.5. Particle Size, Size Distribution, and Zeta Potential Analysis of Nanoparticles	40
3.2.6. Analysis of Nanoparticle Drug Loading.....	40
3.3. <i>In Vitro</i> Cell Studies.....	41
3.3.1. Two-Dimensional Cell Culture and Cell Viability	41
3.3.2. RSV Nanoparticle Cellular Uptake	42
3.3.3. Formation and Evaluation of 3D Multicellular Spheroids	42
3.3.4. Growth Inhibition of Multicellular Spheroids Exposed to Free RSV and RSV NP	43
3.3.5. Clonogenic Assay.....	43
3.4. Statistical Analysis.....	44
3.5. Results	44
3.5.1. Characterization of Acetalated Dextran	44
3.5.2. Characterization of Nanoparticles.....	44
3.5.3. Cell Studies	46
3.6. Discussion.....	56
3.7. Conclusions	60
3.8. Acknowledgments.....	60
REFERENCES.....	62
CHAPTER 4.....	69

Development and Evaluation of Paclitaxel-Loaded Aerosol Nanocomposite Microparticles and Their Efficacy Against Lung Cancer Tumor Spheroids	69
ABSTRACT	70
KEYWORDS	70
4.1. Introduction	71
4.2. Materials and Methods	74
4.2.1. Materials	74
4.2.2. Synthesis of acetalated dextran (Ac-Dex)	75
4.2.3. Nuclear magnetic resonance (NMR) analysis of acetalated dextran (Ac-Dex)	76
4.2.4. Preparation of PTX-loaded Ac-Dex nanoparticles (PTX NP)	76
4.2.5. Formulation of PTX nanocomposite microparticles (PTX nCmP) via spray drying	77
4.2.6. Particle morphology and shape analysis via scanning electron microscopy (SEM)	78
4.2.7. Particle size, size distribution, and zeta potential analysis of NP and nCmP	78
4.2.8. Analysis of PTX loading in PTX NP and PTX nCmP	79
4.2.9. <i>In vitro</i> PTX release from NP and nCmP	80
4.2.10. Differential scanning calorimetry (DSC)	80
4.2.11. Powder X-ray diffraction (PXRD)	80

4.2.12. Karl Fischer coulometric titration	81
4.2.13. <i>In vitro</i> aerosol dispersion performance with a Next Generation Impactor (NGI)	81
4.2.14. Tapped density and theoretical aerodynamic size analysis of nCmP	83
4.2.15. <i>In vitro</i> cell studies	84
4.2.16. Statistical analysis	87
4.3. Results	88
4.3.1. Characterization of acetalated dextran (Ac-Dex)	88
4.3.2. Nanoparticle characterization	88
4.3.3. Nanocomposite microparticle characterization	91
4.3.4. Karl Fischer titration	92
4.3.5. Solid-state analysis of particle formulations	92
4.3.6. Density and theoretical MMAD of nCmP	94
4.3.7. <i>In vitro</i> aerosol performance of nCmP	94
4.3.8. <i>In vitro</i> cell analysis of PTX NP and PTX nCmP	96
4.4. Discussion	102
4.5. Conclusions	108
4.6. Acknowledgements	109
REFERENCES	110
CHAPTER 5	117

Development and characterization of aerosol nanocomposite microparticles capable of mucus penetration.....	117
ABSTRACT	118
KEYWORDS	118
5.1. Introduction	119
5.2. Materials and Methods	122
5.2.1. Materials	122
5.2.2. Synthesis of Acetalated Dextran	123
5.2.3. Synthesis of Vitamin E Poly(ethylene glycol).....	124
5.2.4. Nuclear Magnetic Resonance Analysis of Acetalated Dextran and Vitamin E Poly(ethylene glycol).....	124
5.2.5. Preparation of Curcumin-Loaded Ac-Dex Nanoparticles coated with PVA and VP5k.....	125
5.2.6. Formulation of Curcumin Nanocomposite Microparticles (CUR nCmP) via Spray Drying.....	126
5.2.7. Particle Morphology and Shape Analysis via Scanning Electron Microscopy	126
5.2.8. Particle Size, Size Distribution, and Zeta Potential Analysis of Nanoparticles	127
5.2.9. Analysis of CUR Loading in NP and nCmP.....	127
5.2.10. <i>In Vitro</i> Drug Release from NP and nCmP.....	128

5.2.11. Solid-State Analysis of NP and nCmP	128
5.2.12. Karl Fischer Coulometric Titration	129
5.2.13. nCmP <i>In Vitro</i> Aerosol Dispersion Performance with a Next Generation Impactor.....	129
5.2.14. Tapped Density and Theoretical Aerodynamic Analysis of nCmP	131
5.2.15. Preparation of Artificial Mucus	132
5.2.16. Analysis of Freeze-Dried Artificial Mucus Morphology	132
5.2.17. Evaluation of Nanoparticle Penetration through Artificial Mucus	132
5.2.18. Statistical Analysis	133
5.3. Results	133
5.3.1. Characterization of Ac-Dex and VP5k.....	133
5.3.2. Nanoparticle Characterization.....	134
5.3.3. Microparticle Characterization	139
5.3.4. Water Content via Karl Fischer Titration	140
5.3.5. Solid-State Analysis of NP and nCmP	140
5.3.6. Density and Theoretical MMAD of nCmP.....	143
5.3.7. <i>In Vitro</i> Aerosol Performance of nCmP	143
5.3.8. Artificial Mucus and Penetration Studies of Nanoparticles	145
5.4. Discussion.....	147
5.5. Conclusions	153

5.6. Acknowledgments.....	153
REFERENCES.....	154
CHAPTER 6.....	162
CONCLUSIONS AND FUTURE WORK	162
6.1. Conclusions	162
6.2. Future Work.....	164
APPENDIX A	165
Supplementary Material for Chapter 3	165
A.1. Experimental details.....	166
A.1.1. Materials	166
A.1.2. Synthesis of Acetalated Dextran (Ac-Dex)	167
A.1.3. Nuclear Magnetic Resonance (NMR) Analysis of Acetalated Dextran (Ac-Dex).....	167
A.1.4. Preparation of RSV-Loaded Ac-Dex Nanoparticles (RSV NP)	168
A.1.5. Particle Morphology and Shape Analysis via Scanning Electron Microscopy (SEM).....	169
A.1.6. Particle Size, Size Distribution, and Zeta Potential Analysis	169
A.1.7. Analysis of Nanoparticle Drug Loading.....	169
A.1.8. <i>In Vitro</i> Cell Studies	170
A.1.8.1. RSV NP Cellular Uptake	171

A.1.8.2. Two-Dimensional (2D) Cell Culture and Cell Viability	171
A.1.8.3. Formation of 3D Multicellular Spheroids (MCS)	172
A.1.8.4. Growth Inhibition of MCS	173
A.1.8.5. Clonogenic assay	174
A.1.9. Statistical Analysis	174
REFERENCES	178
APPENDIX B	179
Supplementary Material for Chapter 5	179

LIST OF FIGURES

Figure 1.1 Schematic of Paclitaxel nCmP delivery via inhalation.....	4
Figure 1.2 Schematic of (Left) the formation of multicellular tumor spheroids (MCS) in ultra-low attachment 96-well plates via centrifugation and (Right) morphology of solid tumors.	6
Figure 1.3 Schematic of (Top) the fabrication of the alginate hydrogel scaffold and (Bottom) formation of 3D multicellular spheroid (MCS) in an air interface cell culture (AIC) for the evaluation of Paclitaxel nCmP.....	8
Figure 1.4 Schematic of the mucus penetrating properties of vitamin E poly(ethylene glycol) coated nanoparticles (VP5k NP) compared to poly(vinyl alcohol) nanoparticles (PVA NP).....	9
Figure 2.1 Particle deposition based on the aerodynamic diameter of particles. Adapted from [19].	17
Figure 2.2 Cellular and non-cellular pulmonary barriers for nanocarriers including those present in the central lung/upper airways (bronchial) and peripheral/lower airways (alveoli). Adapted from [28].	20
Figure 3.1 Representative scanning electron micrographs of (Left) blank nanoparticles and (Right) resveratrol-loaded nanoparticles. Scale bar = 500 nm.	46
Figure 3.2 Viability analysis of blank nanoparticles without resveratrol (Blank NP) and uptake of curcumin (CUR)-loaded NP (CUR NP). (A) Relative viability of A549 cells exposed to Blank NP (*p < 0.05, **p < 0.01, ***p < 0.001, and n ≥ 3, which represent comparison to the control with no exposure to NP). (B) Confocal images of CUR NP uptake in A549 cells, showing CUR NP (green)	

and cell membrane (red), scale bar = 20 μm . (C) Fluorescence quantification of CUR NP uptake in A549 cells.....47

Figure 3.3 Optimization of A549 multicellular spheroid (MCS) formation. (A) Growth analysis of A549 MCS grown at different initial cell seeding concentrations; (B) Fluorescence images showing MCS morphology and viability on days 1 and 5, where green cells represent live cells and red cells represent dead cells (Scale bar = 300 μm); and (C) Bright-field images of A549 cells grown at 5,000 cells/MCS for 22 days (Scale bar = 500 μm). $**p < 0.01$ which represents comparison of 8,000 cells/MCS to 5,000 and 3,000 cells/MCS on day 22. ...49

Figure 3.4 Evaluation of multicellular spheroid (MCS) growth prevention using different resveratrol (RSV) formulations, including (A) free RSV (RSV) and (B) RSV-loaded nanoparticles (RSV NP). Control-D refers to media with 0.15 v/v % DMSO. (C) Comparison of the change in MCS diameter relative to control (no RSV exposure) between free RSV and RSV NP at day 15. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, which represents comparison to the control (no exposure to RSV or RSV NP) for (A) and (B) and represents comparison between RSV and RSV NP for (C); $n \geq 3$ 51

Figure 3.5 Evaluation of multicellular spheroid (MCS) treatment (growth restriction) using different resveratrol (RSV) formulations, including (A) free RSV (RSV) and (B) RSV-loaded nanoparticles (RSV NP). Control-D refers to media with 0.15 v/v % DMSO. (C) Comparison of the change in diameter relative to control between free RSV and RSV NP at day 15. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, which represents comparison to the control (no exposure to RSV or

RSV NP) for (A) and (B) and represents comparison between RSV and RSV NP for (C); $n \geq 3$ 53

Figure 3.6 Comparison of A549 multicellular spheroid (MCS) growth in (A) prevention and (B) treatment studies upon exposure to 100 μM resveratrol (RSV) formulations. Bright-field images and corresponding quantification of MCS growth upon exposure to free resveratrol (RSV), RSV-loaded nanoparticles (RSV NP), and Blank NP equivalent to 100 μM RSV NP by mass. Control MCS were exposed to media only. Scale bar = 500 μm . * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, which represents comparison to the control (no exposure to RSV or RSV NP) for each time point; $n \geq 3$ 54

Figure 3.7 Clonogenic assay results for A549 cells recovered from multicellular spheroids dosed with free resveratrol (RSV) in (A) prevention studies and (B) treatment studies. Control DMSO refers to media with 0.15 v/v % DMSO. Data is presented as the number of colonies compared with the untreated control (mean $\pm$ standard deviation, $n = 3$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, which represents comparison to the control (no exposure to RSV); $n \geq 3$ 55

Figure 4.1 Representative scanning electron micrographs of nanoparticles (NP) and nanocomposite microparticles (nCmP) systems, and their *in vitro* paclitaxel (PTX) release profiles at different pH. (A) paclitaxel-loaded nanoparticles (PTX NP) at 20,000X, (B) paclitaxel nanocomposite microparticles (PTX nCmP) at 5,000X, and (C) PTX nCmP at 40,000X. (D) Comparative release profiles of PTX from PTX NP and PTX nCmP at pH 7.4 and pH 5.1 ($n = 3$). 90

- Figure 4.2 (A) Differential scanning calorimetry (DSC) thermograms and (B) powder X-ray diffractograms (PXRD) of raw paclitaxel (PTX), raw acetalated dextran (Ac-Dex), raw mannitol, blank nanocomposite microparticles (nCmP) and paclitaxel-loaded nCmP (PTX nCmP)..... 93
- Figure 4.3 *In vitro* aerosol dispersion performance of paclitaxel-loaded nanocomposite microparticles (PTX nCmP) in a Next Generation Impactor™ (NGI™) for Q = 60 L/min. (A) Particle deposition on each stage of the NGI, (B) Fine particle fraction (FPF), respirable fraction (RF) and emitted dose (ED) of PTX nCmP (Mean ± standard deviation, n=3)..... 95
- Figure 4.4 Two-dimensional (2D) cell culture evaluations including, (A) Cell viability and IC₅₀ values of A549 cells dosed with raw paclitaxel (PTX) and paclitaxel-loaded NP (PTX NP) evaluated at 48 hours. (B) Cellular uptake of fluorescent curcumin-loaded nanoparticles (CUR NP) in A549 cells following 3 hours of incubation, including a representative confocal image showing CUR NP (green) and cell membrane (red), scale bar = 20 µm (Left) and fluorescence quantification of uptake (Right). (C) Transepithelial electrical resistance (TEER) analysis performed on H441 cells in air interface culture (AIC) conditions after exposure to blank and paclitaxel-loaded nanocomposite microparticles (Blank nCmP and PTX nCmP, respectively). Statistical analysis was performed using Student's t-test. * p < 0.05, ** p < 0.01, *** p < 0.001; n ≥ 3, which represents the comparison to control (no exposure to nCmP) 96
- Figure 4.5 Analysis of multicellular spheroid (MCS) growth upon exposure to different concentrations of paclitaxel (PTX) formulations, including: (A) free PTX and

(B) PTX NP. (C) Comparison of the change in diameter relative to control (media only) upon exposure to free PTX and PTX NP at day 15. Statistical analysis was performed using Student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, which represents comparison with control (no exposure to PTX or PTX NP) in (A) and (B), and comparison between PTX versus PTX NP in (C); $n \geq 3$ 99

Figure 4.6 (Left) Representative bright-field images (scale bar = 500 nm) and (Right) corresponding quantification of A549 multicellular spheroid (MCS) growth upon exposure to 1 μM of free PTX, PTX nanoparticles (NP), and Blank NP equivalent to 1 μM of NP in mass. Control MCS were exposed to media only. Statistical analysis was performed using Student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n \geq 3$, which represents the comparison to control, no exposure to PTX or NP)..... 100

Figure 4.7 Analysis of A549 multicellular spheroids (MCS) growth upon exposure to paclitaxel-loaded nanocomposite microparticles (PTX nCmP) in an 3D air interface cell culture (AIC) platform (scale bar = 500 μm). (A) Representative bright-field images of control and MCS exposed to PTX nCmP. (B) Quantification of MCS exposed to PTX nCmP in comparison to control (media only). Statistical analysis was performed using Student's t-test ($p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n \geq 3$, which represent the comparison to control, no exposure to PTX nCmP) 102

Figure 5.1 Representative scanning electron micrographs of nanoparticles (NP) and nanocomposite microparticles (nCmP) systems. (A) poly(vinyl alcohol)-

coated curcumin-loaded nanoparticles (PVA CUR NP), scale bar = 500 nm; (B) vitamin E poly(ethylene glycol) (MW = 5 kDa)-coated CUR-loaded nanoparticles (VP5k CUR NP), scale bar = 500 nm; (C) PVA-coated CUR-loaded nanocomposite microparticles with 30:70 NP to mannitol and 1.5% spray drying feed concentration (PVA CUR nCmP, 30:70, Fc = 1.5%), scale bar = 1 μ m; and (D) PVA-coated CUR-loaded nCmP with 100% NP and 0.5% spray drying feeding concentration (PVA CUR nCmP, 100:0, Fc = 0.5%), scale bar = 1 μ m. 136

Figure 5.2 *In vitro* curcumin (CUR) release profiles of nanoparticles and nanocomposite microparticles (nCmP) from poly(vinyl alcohol) (PVA) and vitamin E poly(ethylene glycol) (MW = 5 kDa) (VP5k)-coated particles in various pH conditions. (Top) Comparative release profiles of CUR release from PVA-coated CUR NP and CUR nCmP coated at pH 7.4 and pH 5.2. (Bottom) Comparative release profiles of CUR release from VP5k-coated CUR NP at pH 7.4 and pH 5.2 (n = 3)..... 138

Figure 5.3 (Top) Differential scanning calorimetry (DSC) thermograms and (Bottom) powder X-ray diffractograms (PXRD) of raw curcumin (Raw CUR), raw acetalated dextran following 3-hour reaction time (3-hour Ac-Dex), raw poly(vinyl alcohol) (Raw PVA), PVA nanoparticles (PVA CUR NP), raw vitamin E poly(ethylene glycol) (MW = 5 kDa) (Raw VP5k), VP5k nanoparticles (VP5k CUR NP), raw mannitol, PVA-coated CUR-loaded nanocomposite microparticles with 30:70 nanoparticle-to-mannitol ratio and spray drying feed concentration of 1.5% (PVA CUR nCmP, 30:70, Fc = 1.5%),

and PVA-coated CUR-loaded nCmP with 100% NP and spray drying feed concentration of 1.5% (PVA CUR nCmP, 100:0, Fc = 0.5%).	142
Figure 5.4 <i>In vitro</i> aerosol dispersion performance of poly(vinyl alcohol)-coated, curcumin-loaded nanocomposite microparticles (PVA CUR nCmP) in a Next Generation Impactor™ (NGI™) for Q = 60 L/min. (Top) Particle deposition on each stage of the NGI. (Bottom) Fine particle fraction (FPF), respirable fraction (RF). and emitted dose (ED) of PVA CUR nCmP (Mean ± standard deviation, n = 3).	144
Figure 5.5 Representative scanning electron micrographs of freeze-dried artificial mucus (AM). (A) 100X magnification, scale bar = 500 µm (B) 800 X, scale bar = 50 µm.	145
Figure 5.6 Evaluation of the mucus penetration capacities of free curcumin (CUR) containing 5% (v/v) DMSO, poly(vinyl alcohol) (PVA)-coated CUR NP, and vitamin E poly(ethylene glycol) (MW = 5 kDa) (VP5k)-coated CUR NP in artificial mucus (AM) at 500 µM CUR. (Top) photographs of CUR formulations dosed on AM located in the apical side of the Transwell® at different time points. (Bottom) Fluorescence quantification of CUR collected in the basolateral side of the Transwell® at different time points and expressed as mass percent of NP. Statistical analysis was performed using ANOVA (* p < 0.05, ** p < 0.01, *** p < 0.001; n ≥ 3, which represents comparison among the groups).	146

Figure A. 1	Chemical structures of trans-resveratrol (top) and synthesis of acetalated dextran (Ac-Dex) from parent dextran (bottom).....	175
Figure A. 2	Cell viability analysis of A549 cells measured using an acid phosphatase assay after dosing with free resveratrol (RSV) grown using the following conditions and times: (A) 2D monolayer after 48 hours, (B) 2D monolayer after 72 hours, and (C) 3D multicellular spheroids after 48 hours.	176
Figure B. 1	Chemical structures of (A) curcumin (CUR) and (B) synthesis of acetalated dextran (Ac-Dex) from parent dextran in dimethyl sulfoxide (DMSP) with the catalyst pyridinium p-toluenesulfonate (PPTS).....	180
Figure B. 2	Nuclear magnetic resonance (NMR) confirmation of the reaction of vitamin E succinate and m-poly(ethylene glycol) (PEG) with a molecular weight of 5 kDa to produce vitamin E PEG (VP5k). (A) Schematic and conditions of the reaction and (B) ¹³ C NMR spectrums.	181

LIST OF TABLES

Table 1.1 Advantages and limitations of nanoparticles and microparticles in pulmonary delivery.....	3
Table 2.1 Mechanisms of aerosol deposition [3, 20].....	18
Table 2.2 Sample nanocarrier formulations based on route of delivery, nanocarrier type, disease and/or application, and development state, where PLGA = poly(lactic-co-glycolic) acid, NP = nanoparticle	22
Table 3.1 Characteristics of resveratrol (RSV)-loaded nanoparticles (RSV NP), blank NP without RSV (Blank NP), and curcumin (CUR)-loaded NP (CUR NP), including diameter, polydispersity index (PDI), zeta (ζ) potential, RSV loading, and encapsulation efficiency (EE) (mean $\pm$ standard deviation, n = 3).	45
Table 4.1 Characteristics of paclitaxel (PTX)-loaded nanoparticles (NP), blank NP without PTX, and PTX NP redispersed from PTX nanocomposite microparticles. Analysis includes NP diameter as measured by dynamic light scattering (DLS) and ImageJ, polydispersity index (PDI), zeta (ζ) potential, PTX loading in NP, and encapsulation efficiency (EE) (mean $\pm$ standard deviation, n = 3).....	89
Table 4.2 Characteristics of paclitaxel (PTX)-loaded nanocomposite microparticles (nCmP), including water content, PTX loading, nanoparticle (NP) loading in nCmP, NP loading efficacy in nCmP, geometric diameter (via Image J), tapped density, theoretical mass median aerodynamic diameter (MMAD _T),	

experimental MMAD (MMAD _E), and geometric standard deviation (GSD) (mean ± standard deviation, n=3).....	92
Table 5.1 Characteristics of curcumin-loaded nanoparticles (CUR NP) coated with poly(vinyl alcohol) (PVA) and vitamin E poly(ethylene glycol) (MW = 5 kDa) (VP5k) and CUR NP re-dispersed from PVA CUR nanocomposite microparticles systems. Analysis includes NP diameter as measured by dynamic light scattering (DLS), polydispersity index (PDI), zeta (ζ) potential, CUR loading in NP, and encapsulation efficiency (EE) (mean ± standard deviation, n ≥ 3).....	134
Table 5.2 Characteristics of curcumin-loaded nanocomposite microparticles (CUR nCmP), including water content, CUR loading, nanoparticle (NP) loading in nCmP, NP loading efficacy in nCmP, geometric diameter (via Image J), tapped density, theoretical mass median aerodynamic diameter (MMAD _T), experimental MMAD (MMAD _E), and geometric standard deviation (GSD) (mean ± standard deviation, n ≥ 3).	140
Table A. 1 Clonogenic assay data representing the number of colonies, plating efficiency (%), and survival fraction (%) of A549 cells from multicellular spheroids exposed to free resveratrol (RSV) in prevention and treatment studies. Data is presented as number of colonies compared with untreated control (mean ± standard deviation, n = 3). Statistical analysis was performed using Student's t-test (* p < 0.05, ** p < 0.01, *** p < 0.001; n ≥ 3).....	177

CHAPTER 1

INTRODUCTION

1.1. Overview and Motivation

Lung cancer is the leading cause of cancer-related deaths in the United States, representing more than one-quarter of all cancer deaths. The five-year relative survival rate is 54% for localized tumors, 26% for regional cancers, and 4% for distant metastases, resulting in a combined 19% survival rate when all stages are considered, causing about 390 deaths per day in the U.S. [1]. Despite the advances in cancer treatment over the past 40 years, the survival rates for lung cancer patients still remains devastatingly low. The types of lung cancer can be separated into two major groups: small cell lung cancer and non-small cell lung cancer (NSCLC). The work described in this dissertation focuses on NSCLC, as this represents 80-85% of lung cancer diagnoses [2].

Monolayer 2D cell culture has been used to evaluate the efficacy of anti-cancer therapeutics for many years; however, the resulting information has limitations when translated to *in vivo* studies. 3D cell culture models provide a more accurate representation of the properties of tumors with respect to morphology, response to therapeutics, growth kinetics, presence of a necrotic core, and diffusion limitations [3]. These characteristics make the inner core of tumors less accessible to therapeutics, shielding their potential impact on that region of tumor cells, an effect that is not well demonstrated in 2D models. As a result of this effect, therapeutics are only able to attack the surface cells of tumors, which may quickly replenish if the tumor core is not also

eliminated [4, 5]. 2D cell assays cannot replicate many properties of 3D models such as the intercellular interactions found *in vivo* [6]. The use of 3D multicellular spheroids is advantageous in testing drug efficacy because they more accurately mimic tumor tissue.

Pulmonary delivery of therapeutics has been extensively studied as a promising route for the targeted delivery of compounds to the lungs. Traditionally, this therapy has been used for the treatment of asthma and chronic obstructive pulmonary disease (COPD) [7], and involves the use of aerosol devices. In recent years, pulmonary drug delivery applications have expanded in the treatment of other local diseases such as lung cancer and cystic fibrosis (CF) [8], and furthermore to the treatment of diseases such as diabetes through systemic delivery of drugs following inhalation. The advantages of using aerosol treatment include better targeting to the lungs and reduced systemic side effects seen with high drug doses in the body [9].

Successful pulmonary delivery is determined by the effective deposition of particles in specific regions in the lungs, and in the overcoming of clearance mechanisms inherent to the respiratory tract [10]. The optimal aerodynamic diameter required for particles to deposit in the distal region of the lungs is 1 to 5 μm [11]. Particles smaller than 1 μm may be easily exhaled, while those larger than 5 - 50 μm may become trapped in the upper airways (nose, mouth or larynx). Furthermore, microparticles in the 1 to 5 μm range can be phagocytosed by alveolar macrophages [12]. Nanoparticles usually have a size range of 1 to 500 nm [13], which allows them to penetrate physiological barriers and affords them greater overall surface area; they can also enhance the dissolution rates and bioavailability of encapsulated drugs, reasons

why there are extensively studied in drug delivery [14]. Unfortunately, nanoparticles are extremely light to be used alone in dry powder inhalers, as they will mostly be exhaled instead of depositing on lung tissue. **Table 1.1** shows summarize of advantages and limitation of the use of nanoparticles and microparticles in pulmonary delivery.

Table 1.1 Advantages and limitations of nanoparticles and microparticles in pulmonary delivery.

Particle Type	Pros	Cons
Nanoparticles	Can penetrate physiological barriers; larger surface area	Too small to deposit in lungs (too light)
Microparticles	Can deposit effectively in lungs	May be uptaken by alveolar macrophages

As an alternative, nanoparticles can be encapsulated in a larger microparticle carrier, resulting in a dry powder nanocomposite microparticle (nCmP) system, that can take advantage of both microparticle and nanoparticle characteristics for effective pulmonary delivery of dry powders [15] (**Figure 1.1**). nCmP can effectively be delivered to the lungs owing to their micron size and will then dissociated into nanoparticles that have the potential to avoid macrophage clearance and/or penetrate

physiological barriers. nCmP can be synthesized via the spray drying of a suspension containing drug-loaded nanoparticles and a water dispersant carrier or excipient. Carriers commonly used are sugars (lactose, trehalose, sucrose), sugar alcohols (mannitol, xylitol), and cyclodextrins, which allow for the recovery of nanoparticles from the nCmP [16].

Figure 1.1 Schematic of Paclitaxel nCmP delivery via inhalation.

Aside from the need to deposit at the site of a particular disease state, pulmonary-based therapeutics encounter a variety of physiological barriers, such as mucus, which selectively traps large particles and removes them via mucociliary clearance [17]. Multiple strategies have been studied for overcoming the mucosal layer in the lungs, and PEGylation of therapeutics is one of the most explored approaches [18, 19].

1.2. Objectives and significance

The overall purpose of this dissertation was to develop and characterize nanoparticles and nanocomposite microparticles formulations for a variety of drug delivery applications, followed by their evaluation in cell culture platforms that can more accurately represent lung environments for pulmonary delivery applications. The projects of this dissertation and their significance are outlined as follows:

Project 1. Evaluation of the cancer-preventive effect of resveratrol-loaded nanoparticles on the formation of lung tumor spheroids

Previous studies have characterized the potential chemopreventive properties of resveratrol (RSV) in cancer cells [20, 21]. Traditionally, these studies have relied on the treatment of cancer cells in 2D cell culture. The purpose of this project was to evaluate the ability of RSV-loaded nanoparticles to prevent the formation of tumor spheroids in a 3D cell culture environment (Figure 1). Previous studies on the role RSV plays in chemoprevention for lung cancer is very limited and this project aimed to provide further insight into the potential of RSV as a chemopreventive agent.

Figure 1.2 Schematic of (Left) the formation of multicellular tumor spheroids (MCS) in ultra-low attachment 96-well plates via centrifugation and (Right) morphology of solid tumors.

Project 2. Development and Evaluation of Paclitaxel-Loaded Aerosol Nanocomposite Microparticles and Their Efficacy Against Lung Cancer Tumor Spheroids

Current treatments for lung cancer carry adverse effects due to the large amounts of drug delivered intravenously throughout the body, with poor local delivery and detrimental effects on otherwise healthy tissue. In this study this challenge was confronted via the development of paclitaxel-loaded nanocomposite microparticles (nCmP) as pulmonary drug delivery-related dry powder particles delivered via a dry powder inhaler system. nCmP have the potential to effectively deposit in the lower parts of the lungs, release the drug-loaded nanoparticles at the site of the disease in a

controlled manner, and escape macrophage engulfment due to their nano-scale size. This system could allow for the administration of lower amounts of drug by delivering it directly to the tumor site, consequently reducing the toxicity and inherent side effects of conventional I.V. chemotherapy.

Recently, 3D models have been developed for a variety of lung cancer cell types in liquid covered culture (LCC), however, this is not representative of what takes place in the airways of the lungs, where aerosol anti-cancer therapeutics will be delivered. In this project, once the spheroids were formed they were grown in air interface culture (AIC) conditions to better represent tumor formation in the lungs. Following spheroid formation and characterization, the efficacy of different aerosol anticancer therapeutics was evaluated on this platforming in order to enhance the predictable transition from initial 2-D *in vitro* to *in vivo* studies.

Figure 1.3 Schematic of (Top) the fabrication of the alginate hydrogel scaffold and (Bottom) formation of 3D multicellular spheroid (MCS) in an air interface cell culture (AIC) for the evaluation of Paclitaxel nCmP.

Project 3. Development and characterization of aerosol nanocomposite microparticles capable of mucus penetration

Mucus is a very efficient barrier that prevents the penetration of harmful agents and pathogens to the underlying tissue in the lungs, and is thus a challenge for effective pulmonary drug delivery [18]. Overcoming this barrier will allow effective permeation into mucus and escape of the mucosa removal of therapeutics so that they can reach the site of infection seen in diseases such as cystic fibrosis, in which patients are faced with thick mucus and continuous infections in the lungs. Also, chronic diseases such as lung cancer, located in the epithelium underneath the mucosa layer, are faced with this mucosal barrier. Finally, mucus and surfactant cover the entirety of the pulmonary tract and having a therapeutic that can reach the alveoli region and penetrate the mucus or surfactant layer may allow the systemic delivery of encapsulated drugs after inhalation. Thus, this project involved the development and evaluation of nanocomposite microparticle (nCmP) containing nanoparticles PEGylated with vitamin E poly(ethylene glycol) (VP5k). The nCmP were able to aerosolize and demonstrated aerodynamic diameters necessary for delivery into several regions of the lungs and the dissociated nanoparticles were able to effectively penetrate artificial mucus better than poly(vinyl alcohol)-coated nanoparticles. Overall, this aerosol microparticle system has

the potential to delivery therapeutics to the lungs and effectively penetrate the mucosal barriers present there.

Figure 1.4 Schematic of the mucus penetrating properties of vitamin E poly(ethylene glycol) coated nanoparticles (VP5k NP) compared to poly(vinyl alcohol) nanoparticles (PVA NP).

REFERENCES

1. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2019*. CA Cancer J Clin, 2019. **69**(1): p. 7-34.
2. American Cancer Society, I. *What Is Non-Small Cell Lung Cancer?* 2019 [cited 2019; Available from: <https://www.cancer.org/cancer/non-small-cell-lung-cancer/about/what-is-non-small-cell-lung-cancer.html>].
3. Fitzgerald, K.A., et al., *Life in 3D is never flat: 3D models to optimise drug delivery*. J Control Release, 2015. **215**: p. 39-54.
4. Horning, J.L., et al., *3-D tumor model for in vitro evaluation of anticancer drugs*. Mol Pharm, 2008. **5**(5): p. 849-62.
5. Lovitt, C.J., T.B. Shelper, and V.M. Avery, *Advanced cell culture techniques for cancer drug discovery*. Biology (Basel), 2014. **3**(2): p. 345-67.
6. Astashkina, A., B. Mann, and D.W. Grainger, *A critical evaluation of in vitro cell culture models for high-throughput drug screening and toxicity*. Pharmacology & Therapeutics, 2012. **134**(1): p. 82-106.
7. Shakshuki, A. and R.U. Agu, *Improving the Efficiency of Respiratory Drug Delivery: A Review of Current Treatment Trends and Future Strategies for Asthma and Chronic Obstructive Pulmonary Disease*. Pulmonary Therapy, 2017. **3**(2): p. 267-281.
8. Garbuzenko, O.B., et al., *Inhalation treatment of cystic fibrosis with lumacaftor and ivacaftor co-delivered by nanostructured lipid carriers*. Journal of Controlled Release, 2019. **296**: p. 225-231.

9. Patil, J.S. and S. Sarasija, *Pulmonary drug delivery strategies: A concise, systematic review*. Lung India, 2012. **29**(1): p. 44-9.
10. Geiser, M. and W.G. Kreyling, *Deposition and biokinetics of inhaled nanoparticles*. Particle and fibre toxicology, 2010. **7**: p. 2-2.
11. Bosquillon, C., et al., *Influence of formulation excipients and physical characteristics of inhalation dry powders on their aerosolization performance*. Journal of Controlled Release, 2001. **70**(3): p. 329-339.
12. Champion, J.A., A. Walker, and S. Mitragotri, *Role of particle size in phagocytosis of polymeric microspheres*. Pharmaceutical research, 2008. **25**(8): p. 1815-1821.
13. Vert, M., Doi, Y., Hellwich, K.-H., Hess, M., Hodge, P., Kubisa, P., Rinaudo, M., Schué, F., *Terminology for biorelated polymers and applications (IUPAC Recommendations 2012)*. Pure and Applied Chemistry, 2012. **84**(2): p. 1.
14. Rizvi, S.A.A. and A.M. Saleh, *Applications of nanoparticle systems in drug delivery technology*. Saudi Pharmaceutical Journal, 2018. **26**(1): p. 64-70.
15. Yang, W., J.I. Peters, and R.O. Williams, *Inhaled nanoparticles—A current review*. International Journal of Pharmaceutics, 2008. **356**(1): p. 239-247.
16. Bhakay, A., et al., *Redispersible fast dissolving nanocomposite microparticles of poorly water-soluble drugs*. Int J Pharm, 2014. **461**(1-2): p. 367-79.
17. Witten, J., T. Samad, and K. Ribbeck, *Selective permeability of mucus barriers*. Current Opinion in Biotechnology, 2018. **52**: p. 124-133.
18. Liu, M., et al., *Developments of mucus penetrating nanoparticles*. Asian Journal of Pharmaceutical Sciences, 2015. **10**(4): p. 275-282.

19. Aaltonen, T., et al., *Evidence for a particle produced in association with weak bosons and decaying to a bottom-antibottom quark pair in higgs boson searches at the tevatron*. Phys Rev Lett, 2012. **109**(7): p. 071804.
20. Cal, C., et al., *Resveratrol and cancer: chemoprevention, apoptosis, and chemosensitizing activities*. Curr Med Chem Anticancer Agents, 2003. **3**(2): p. 77-93.
21. Singh, C.K., M.A. Ndiaye, and N. Ahmad, *Resveratrol and cancer: Challenges for clinical translation*. Biochim Biophys Acta, 2015. **1852**(6): p. 1178-85.

CHAPTER 2

Pulmonary Delivery of Nanocarriers

2.1. Introduction

In the recent years, pulmonary delivery has attracted growing interest in the research community for its advantages over other delivery systems to treat local diseases in the lungs and respiratory tract such as asthma and chronic obstructive pulmonary diseases (COPD) [1] as well as systemic delivery of biotherapeutics through pulmonary route (i.e. peptides and proteins) [2]. The local treatment of pulmonary diseases provides high concentration of a drug at the site by directly delivery to the lung. As a result, lower overall dose is required, thus reducing the systemic side effects. Due to the high surface area in the lungs, a systemic delivery can be attained in the alveoli, where the API is absorbed into the blood stream; therefore, a rapid onset of action can be achieved, and the first-pass metabolism avoided.

2.2. Lung Physiology

Internally, the lungs are divided into bronchi, bronchioles and smaller air passages: alveoli, blood vessels and lymph tissue. The main function of the lungs is to exchange oxygen and carbon dioxide. The gas exchange occurs in the alveoli. There are over 300 million alveoli in the lungs, with a combined surface area of about 100 m², thin alveolar epithelium (0.1 – 0.2 µm), and high membrane permeability, which enables

an efficient mass transfer providing a route for systemic delivery [3, 4] The alveolar surface is coated by a film of pulmonary surfactant, a complex of lipids and proteins. The lipid fraction is mainly composed by phospholipids, dipalmitoylphosphatidylcholine (DPPC), phosphatidylglycerol, and some neutral lipids. The surfactant is secreted and recycled by type II pneumocytes and cleared by alveolar macrophages [5] The surfactant reduces the surface tension by stabilizing the interface during the inspiration-expiration process and represents the first barrier against pathogens [6]. From the large bronchioles up to the trachea, the epithelium of the airways is covered by mucus, produced by goblet cells. The bronchi tree surface contains ciliated columnar cells which is responsible for mucociliary clearance, Clara cells that produce electron-dense granules, and a surfactant lipoprotein that intervenes in the ion transport [5].

2.3. Delivery Devices

The three major types of inhalers used for pulmonary delivery are: Metered dose inhalers (MDIs), Nebulizers, and dry powder inhalers (DPIs). The mechanisms of delivery and the formulation requirements are different for each type.

2.3.1. Metered dose inhalers (MDIs)

The first metered dose inhaler (MDI) was developed in 1950s to locally deliver albuterol for the treatment of asthma [7]. MDIs are pressurized vessels that rely on the driving force of the propellant to atomize the droplets containing the drug and

excipients, which ideally deposit in the lungs [8]. The main components of MDI device are the propellant, formulation, metering valve and actuator. All of them play an important role in the formation of spray and determine the drug delivery into the lungs. The main component of the propellant has been formulated with high volatile FCF-12 (dichlorodifluoromethane). Drugs are either solutions or particulate suspensions. The formulations usually contain surfactants that may impact the lung performance. An optimal drug deposition in the lungs using MDIs requires coordination between actuation and inhalation, which makes it more difficult to use for patients [4, 8].

2.3.2. Nebulizers

Nebulizers deliver liquid medication in a steady stream of droplets of 1-5 μm and do not require patient coordination between inhalation and actuation, which is advantageous for the use in infants, elderly or non-conscious patients. Nebulizers can deliver larger doses compared to other inhalers, but may also require longer times [9]. Nebulizers produce ultra-fine droplets ($\sim 1\mu\text{m}$) with high polydispersity, which make them unsuitable for the delivery of larger particles (bigger than the droplet size) to the lungs [10]. In jet nebulizers, a high velocity of air carries the droplets and impacts on baffles to break them into smaller sized droplets or to retain them until they reach the appropriate size. In ultrasonic nebulizers, the vibration of piezoelectric crystals at high frequency creates waves that break the liquid into small droplets [9]. The aerosol drug delivery via nebulizers is influenced by the type, the position on the ventilator circuit and the bias flow as was reported by Ari *et al.* during a study on simulated pediatric and adult lung models during mechanical ventilation [11].

2.3.3. Dry Powder Inhaler (DPI)

DPIs are breath-activated devices that deliver medication in the form of a dry powder directly to the airways. During administration, the capsule containing a set dose of drug in the form of microparticles is punctured and the internal particles are aerosolized. [12]. DPIs are breath-activated requiring no outside energy source or propellant. In addition, no coordination between patient inhalation and activation of the device is needed. The inspiration flow can be as little as 30 LPM for devices such as Diskus, or 60 LPM or more for devices with higher resistance like Handihaler [13]. The optimal drug delivery requires a deep, forceful and long inhalation for at least 5 seconds, followed by a breath hold for at least 10 seconds [14]. However, in the case of acute asthma attacks, DPIs are not recommended because of the inspiratory flow requirement [15]. The actual dose delivered is also dependent on the inhalation flow, which can be difficult to replicate. Furthermore, particles delivered using DPIs tend to agglomerate due to hygroscopic phenomena and electrostatic interactions, which reduce the aerosolization performance [4]. Overall, DPIs are preferred over other devices because it promises physical stability of the formulation in solid form, is portable and easy to use, and can be applied to a wide variety of active ingredients and dose weights [10, 16]. There are two main types of DPIs: single-dose and multi-dose inhalers. Single-dose DPIs supply an individual capsule per dose, while the multidose DPIs contain a bulk supply of drug for multiple doses and one dose is released with each actuation [13].

2.4. Particle Fate

Particle deposition in the lungs is governed by the aerodynamic diameter of the particles and occurs by one of the following mechanisms: inertial impaction, sedimentation, or diffusion. Nanoparticles deposit by diffusion are caused by Brownian motion, and can be enhanced by breath holding (reduction of airflow). Aggregates of nanoparticles at a micro-size scale (i.e. nanocomposite microparticles) are thought to have enough mass to deposit effectively into the lungs and respond to impaction and sedimentation mechanisms. Once the micro-sized particles deposit in the airways, they dissociate back into nanoparticles [17, 18]. Inhalable particles can be categorized into different sizes either coarse ($\geq 5 \mu\text{m}$), fine ($0.5\text{--}5 \mu\text{m}$), or ultrafine ($\leq 0.5 \mu\text{m}$). **Figure 2.1** shows the percent of deposition based on the aerodynamic diameter of particles [19].

Figure 2.1 Particle deposition based on the aerodynamic diameter of particles. Adapted from [19].

Coarse carriers collide with the upper airways (trachea and large bronchi) and deposit by impaction; fine particles can reach the lower lung airways by sedimentation, and ultrafine particles can deposit throughout the respiratory tract, reaching the alveoli by diffusion, but also can be exhaled for being very light [3]. **Table 2.1** summarizes the different mechanism of deposition in the respiratory tract.

Table 2.1 Mechanisms of aerosol deposition [3, 20]

Mechanism	Size	Site of deposition	Cause
Impaction	$\geq 5 \mu\text{m}$	Upper respiratory airways – Primary bronchi	Airflow is turbulent and air velocity is high
Sedimentation	$0.5\text{--}5 \mu\text{m}$	Lower lung airways Secondary bronchi, bronchioles	Breathing rate slows down, residence time of carrier travel through the airway increases
Diffusion	$< 0.5 \mu\text{m}$	Alveoli	Brownian motion - random motion due

---

to the bombardment
by surrounding air
molecules.

Another parameter that can influence the particle deposition in the respiratory tract is the electrostatic charge that occurs in most of the inhalers during the aerosolization process. Fine particles can acquire charge via electron exchange due to the different surface potential when they are in contact to each other or to the walls of the device [21]. The electrostatic effects are more pronounced in the upper airways before the charge is dissipated due to high humidity [22], and can cause an increase of the overall percentage deposition up to sevenfold [23]. This effect may be not be desired for systemic delivery of the drugs, which requires particles to reach the alveolar epithelium [24]. NP in epithelial cells are uptaken by endocytotic mechanisms, which involves pinocytosis or uptake of fluids and molecules in small vesicles, and phagocytosis, which is responsible for engulfment of large particles such as microorganism or cells debris [25]. The clearance mechanism of particles depends on the location of the deposited agent. In the upper airways, particles are removed by the mucociliary escalator that is comprised by hair-shaped structure cilia, located on the topside of epithelial cells. Once mucus entraps foreign particles, cilia beat in a coordinated direction and transport them to the larynx where the particles can be coughed up or swallowed. Most insoluble particles larger than 6 μm in aerodynamic diameter are eliminated by mucociliary clearance [26]. Smaller particles that reached the alveoli are uptaken by alveolar macrophages, that detect foreign agents, and engulf

them via phagocytosis. Phagocytic uptake is the main mechanism to remove microorganism, cells debris or insoluble micro-sized particles from the lungs surface when they have escaped from other clearance mechanisms [27]. Most of the nanoparticles migrate with the load into the bronchioles to reach the mucociliary escalator current, and others migrate through the epithelium into the lymphatics [5]. In general, nanoparticle clearance and uptake will be also affected by other parameters such as API's properties, type of nanoparticle, size and surface coating, and interaction of NP with cells. The cellular and non-cellular barriers that nanocarriers may face can be seen in **Figure 2.2**.

Figure 2.2 Cellular and non-cellular pulmonary barriers for nanocarriers including those present in the central lung/upper airways (bronchial) and peripheral/lower airways (alveoli). Adapted from [28].

2.5. Applications

Inhalation therapy of nanomedicines enables the delivery of small molecule drugs within the lungs for the treatment of local pulmonary diseases such as lung cancer [29], cystic fibrosis related infections [30], and pulmonary hypertension [31] among others. The pulmonary delivery of peptide and proteins represents a great potential for the treatment for both local and systemic diseases such as diabetes and thrombosis. Gene delivery [32] and vaccines are other applications of pulmonary inhalation. Examples of drugs using colloidal carriers such as lipids, solid lipid nanoparticles and polymeric nanoparticles for diverse applications are reviewed elsewhere [33]. Pulmonary delivery was also applied for diagnostic purposes where iodinated nanoparticles were instilled into small airways and were transported to the tracheobronchial lymph nodes in dogs. The study resulted in contrast enhancement confirmed by computed tomography 2-3 days later [34]. In a recent study, liposomes functionalized with phosphatidic acid and an ApoE-derived peptide (mApoE-PA-LIP) were delivered to the brain of Alzheimer's disease mice via the pulmonary administration, showing a significant decrease in the total brain beta-amyloid compared to untreated mice, and suggesting a potential application of inhalation therapy for brain delivery of NP [35]. Several studies are ongoing applying pulmonary delivery for different drugs/agents using different carriers. **Table 2.2** shows additional examples of pulmonary delivery applications.

Table 2.2 Sample nanocarrier formulations based on route of delivery, nanocarrier type, disease and/or application, and development state, where PLGA = poly(lactic-co-glycolic) acid, NP = nanoparticle

Route of delivery	Type of Nanocarrier	Drug(s) and Disease/Application	State	Reference
Pulmonary	Chitosan NP	Insulin (systemic); Diabetes	In vivo	[2]
Pulmonary	Ab-decorated liposomes	Triptolide; Cancer	In vitro, in vivo	[36]
Pulmonary	Magnetic Fe ₃ O ₄ in PLGA NP	Quercetin; lung cancer and imaging	In vitro	[37]
Pulmonary	Peptide-Micelle hybrid NP	Fasudil; Pulmonary arterial hypertension	In vitro, in vivo	[38]
Pulmonary	SLNP	Quorum sensing inhibitor; Infections	In vitro	[39]

2.6. Challenges, Advantages, and Limitations

Pulmonary route offers the advantage of the delivery of poor water soluble compounds that cannot be achieved by parenteral or oral delivery [3]. Because the lung presents limited intracellular and extracellular drug-enzyme activities, the

bioavailability of the drug can be enhanced compared with in the gastrointestinal tract or liver. Correspondingly, doses that need to be administered can be reduced leading to a decreased systemic toxicity [40]. The higher adsorption rate and the rapid onset of action are due to the thin monolayer ($0.1 - 0.4 \mu\text{m}$) and large surface area (more than 100 m^2) in the alveoli [20], which represent a route for systemic delivery. Small peptides may suffer enzymatic degradation in the lungs, however in a lower intensity than in the gastrointestinal tract. Proteins greater than 150 kDa have a reduced transport and therefore, more difficulty to reach the systemic circulation. Despite the challenges, pulmonary delivery represents a potential route to deliver peptides and small proteins beyond the lungs [4]. Parameters to consider to ensure an effective targeting into the lungs include: (i) deposition and localization of drugs in the targeted area or cells, (ii) ability to escape from mucociliary clearance, (iii) penetration through mucus in the airways, (iv) transport across epithelial cells to reach the blood stream for systemic delivery, (v) low entrapment and interaction of drugs by bacteria biofilms in case of infections, (vi) modified or reduced phagocytic activity by alveolar macrophages, and (vii) minimal host protein- drug interaction to reduce enzymatic degradation [26]. In order to address the challenges, it is important to focus on both the physicochemical properties of the NP such as surface chemistry/charge, hydrophilicity, and the pulmonary barriers such as cilia, mucus and macrophages that limit the localization, penetration and adsorption of carriers in the lungs [41].

Interactions between NP and cells are of particular interest because the properties of the carriers as well as the physiological environment influence nanocarrier uptake and biological response to the delivered drug [25]. Cationic hydrogel rod-shaped

NP resulted in increased uptake in lung dendritic cells and promoted a superior immune response, avoiding extensive alveolar macrophages (AM) clearance, while the anionic counterpart NP were preferentially uptaken by AMs and immunologically inert [42]. In another study, while cationic PS NP still showed faster and higher uptake in alveolar epithelial model cells, anionic PS NP had a significant uptake and translocation through the monolayer for systemic delivery application [43].

Pegylated polyaspartamide-poly lactide-based NP demonstrated the ability to enhance the diffusion through cystic fibrosis artificial mucus avoiding interactions with mucus components compared to unPEGylated NP [87]. The limited number of FDA-approved excipients for inhalation, which include sugars, lipids, and small PEGs, among others, represent a limitation in the development of new formulations for pulmonary delivery and use of new excipients will require further safety and toxicity studies [44]. Additives for pulmonary administration also influence the effectiveness and potential toxicity of inhalation formulations, which has been reviewed in Pilcer and Amighi's work [45].

2.7. Potential applications

The challenges involved in effective pulmonary delivery has resulted in strong interest in developing formulations that can overcome physiological and biological barriers. Studies involving nanocarriers that allow for mucus penetration include the surface modification with PEG-based coatings, reviewed by Huckaby *et al.* [46]. The potential use of other mucus-inert biomaterials such as polysarcosine, polyglycidol, etc

and mucolytic agents such as papain and human DNase (rhDNase), among others, have been reviewed elsewhere [47, 48]. Other surface modifications for the enhancement of peptide or protein absorption are of great interest, as evaluated in the conjugation of liposomes to germ agglutinin that increased the association of liposomes to alveolar epithelial cells *in vitro* and *in vivo* [49]. The carrier interactions with biological barriers and *in vitro* pulmonary models for more accurate evaluations represent fields under exploration, as seen in the review by de Souza Carvalho *et al.* [28]. Overall, the potential for pulmonary nanocarrier formulations is endless and ongoing.

REFERENCES

1. Yhee, J.Y., J. Im, and R.S. Nho, *Advanced Therapeutic Strategies for Chronic Lung Disease Using Nanoparticle-Based Drug Delivery*. Journal of clinical medicine, 2016. **5**(9): p. 82.
2. Al-Qadi, S., et al., *Microencapsulated chitosan nanoparticles for pulmonary protein delivery: in vivo evaluation of insulin-loaded formulations*. J Control Release, 2012. **157**(3): p. 383-90.
3. Ngan, C.L. and A.A. Asmawi, *Lipid-based pulmonary delivery system: a review and future considerations of formulation strategies and limitations*. Drug Deliv Transl Res, 2018. **8**(5): p. 1527-1544.
4. Bailey, M.M. and C.J. Berkland, *Nanoparticle formulations in pulmonary drug delivery*. Med Res Rev, 2009. **29**(1): p. 196-212.
5. Standring, S. and H. Gray, *Gray's anatomy : the anatomical basis of clinical practice. Chapter 57*. 2008, Churchill Livingstone/Elsevier: [Edinburgh].
6. Echaide, M., et al., *Restoring pulmonary surfactant membranes and films at the respiratory surface*. Biochim Biophys Acta Biomembr, 2017. **1859**(9 Pt B): p. 1725-1739.
7. Patton, J.S. and P.R. Byron, *Inhaling medicines: delivering drugs to the body through the lungs*. Nat Rev Drug Discov, 2007. **6**(1): p. 67-74.
8. Myrdal, P.B., P. Sheth, and S.W. Stein, *Advances in metered dose inhaler technology: formulation development*. AAPS PharmSciTech, 2014. **15**(2): p. 434-55.

9. Ibrahim, M., R. Verma, and L. Garcia-Contreras, *Inhalation drug delivery devices: technology update*. Med Devices (Auckl), 2015. **8**: p. 131-9.
10. Abdelaziz, H.M., et al., *Inhalable particulate drug delivery systems for lung cancer therapy: Nanoparticles, microparticles, nanocomposites and nanoaggregates*. J Control Release, 2018. **269**: p. 374-392.
11. Ari, A., et al., *Influence of nebulizer type, position, and bias flow on aerosol drug delivery in simulated pediatric and adult lung models during mechanical ventilation*. Respir Care, 2010. **55**(7): p. 845-51.
12. Callard preedy, E. and P. Prokopovich, *2 - History of inhaler devices*, in *Inhaler Devices*, P. Prokopovich, Editor. 2013, Woodhead Publishing. p. 13-28.
13. Rubin, B.K. and J.B. Fink, *Chapter 34 - Treatment Delivery Systems*, in *Clinical Asthma*, M. Castro and M. Kraft, Editors. 2008, Mosby: Philadelphia. p. 303-312.
14. Waller, D.G. and A.P. Sampson, *12 - Asthma and chronic obstructive pulmonary disease*, in *Medical Pharmacology and Therapeutics (Fifth Edition)*, D.G. Waller and A.P. Sampson, Editors. 2018, Elsevier. p. 193-209.
15. Noakes, T., *Medical aerosol propellants*. Journal of Fluorine Chemistry, 2002. **118**(1): p. 35-45.
16. Ali, M., *CHAPTER 9 - Pulmonary Drug Delivery*, in *Handbook of Non-Invasive Drug Delivery Systems*, V.S. Kulkarni, Editor. 2010, William Andrew Publishing: Boston. p. 209-246.
17. Paranjpe, M. and C.C. Muller-Goymann, *Nanoparticle-mediated pulmonary drug delivery: a review*. Int J Mol Sci, 2014. **15**(4): p. 5852-73.

18. Zhang, J., et al., *Formation, characterization, and fate of inhaled drug nanoparticles*. Advanced Drug Delivery Reviews, 2011. **63**(6): p. 441-455.
19. Laube, B.L., et al., *What the pulmonary specialist should know about inhalation therapies*. European Respiratory Journal, 2011. **37**: p. 1308-1331.
20. Yang, W., J.I. Peters, and R.O. Williams, *Inhaled nanoparticles—A current review*. International Journal of Pharmaceutics, 2008. **356**(1): p. 239-247.
21. Karner, S. and N. Anne Urbanetz, *The impact of electrostatic charge in pharmaceutical powders with specific focus on inhalation-powders*. Journal of Aerosol Science, 2011. **42**(6): p. 428-445.
22. Xi, J., X. Si, and W. Longest, *Electrostatic charge effects on pharmaceutical aerosol deposition in human nasal-laryngeal airways*. Pharmaceutics, 2014. **6**(1): p. 26-35.
23. Koullapis, P.G., et al., *Particle deposition in a realistic geometry of the human conducting airways: Effects of inlet velocity profile, inhalation flowrate and electrostatic charge*. Journal of Biomechanics, 2016. **49**(11): p. 2201-2212.
24. Patil, J.S. and S. Sarasija, *Pulmonary drug delivery strategies: A concise, systematic review*. Lung India, 2012. **29**(1): p. 44-9.
25. Kuhn, D.A., et al., *Different endocytotic uptake mechanisms for nanoparticles in epithelial cells and macrophages*. Beilstein J Nanotechnol, 2014. **5**: p. 1625-36.
26. Lee, W.-H., et al., *Inhalation of nanoparticle-based drug for lung cancer treatment: Advantages and challenges*. Asian Journal of Pharmaceutical Sciences, 2015. **10**(6): p. 481-489.

27. Geiser, M., *Update on macrophage clearance of inhaled micro- and nanoparticles*. J Aerosol Med Pulm Drug Deliv, 2010. **23**(4): p. 207-17.
28. de Souza Carvalho, C., N. Daum, and C.-M. Lehr, *Carrier interactions with the biological barriers of the lung: Advanced in vitro models and challenges for pulmonary drug delivery*. Advanced Drug Delivery Reviews, 2014. **75**: p. 129-140.
29. Youngren-Ortiz, S.R., et al., *Development of Optimized, Inhalable, Gemcitabine-Loaded Gelatin Nanocarriers for Lung Cancer*. Journal of aerosol medicine and pulmonary drug delivery, 2017. **30**(5): p. 299-321.
30. Gunday Tureli, N., et al., *Ciprofloxacin-loaded PLGA nanoparticles against cystic fibrosis P. aeruginosa lung infections*. Eur J Pharm Biopharm, 2017. **117**: p. 363-371.
31. Makled, S., N. Nafee, and N. Boraie, *Nebulized solid lipid nanoparticles for the potential treatment of pulmonary hypertension via targeted delivery of phosphodiesterase-5-inhibitor*. International Journal of Pharmaceutics, 2017. **517**(1): p. 312-321.
32. Fernández Fernández, E., et al., *Biopolymer-Based Nanoparticles for Cystic Fibrosis Lung Gene Therapy Studies*. Materials (Basel, Switzerland), 2018. **11**(1): p. 122.
33. Mansour, H.M., Y.S. Rhee, and X. Wu, *Nanomedicine in pulmonary delivery*. Int J Nanomedicine, 2009. **4**: p. 299-319.

34. Ketai, L.H., et al., *CT imaging of intrathoracic lymph nodes in dogs with bronchoscopically administered iodinated nanoparticles*. Acad Radiol, 1999. **6**(1): p. 49-54.
35. Sancini, G., et al., *Pulmonary administration of functionalized nanoparticles significantly reduces beta-amyloid in the brain of an Alzheimer's disease murine model*. Nano Research, 2016. **9**(7): p. 2190-2201.
36. Lin, C., et al., *Pulmonary delivery of triptolide-loaded liposomes decorated with anti-carbonic anhydrase IX antibody for lung cancer therapy*. Scientific Reports, 2017. **7**(1): p. 1097.
37. Verma, N.K., et al., *Magnetic core-shell nanoparticles for drug delivery by nebulization*. Journal of Nanobiotechnology, 2013. **11**(1): p. 1.
38. Gupta, N., H.M. Ibrahim, and F. Ahsan, *Peptide–Micelle Hybrids Containing Fasudil for Targeted Delivery to the Pulmonary Arteries and Arterioles to Treat Pulmonary Arterial Hypertension*. Journal of Pharmaceutical Sciences, 2014. **103**(11): p. 3743-3753.
39. Nafee, N., et al., *Antibiotic-free nanotherapeutics: Ultra-small, mucus-penetrating solid lipid nanoparticles enhance the pulmonary delivery and anti-virulence efficacy of novel quorum sensing inhibitors*. Journal of Controlled Release, 2014. **192**: p. 131-140.
40. Loira-Pastoriza, C., J. Todoroff, and R. Vanbever, *Delivery strategies for sustained drug release in the lungs*. Adv Drug Deliv Rev, 2014. **75**: p. 81-91.
41. Ruge, C.A., J. Kirch, and C.-M. Lehr, *Pulmonary drug delivery: from generating aerosols to overcoming biological barriers—therapeutic*

- possibilities and technological challenges*. The Lancet Respiratory Medicine, 2013. **1**(5): p. 402-413.
42. Fromen, C.A., et al., *Nanoparticle surface charge impacts distribution, uptake and lymph node trafficking by pulmonary antigen-presenting cells*. Nanomedicine : nanotechnology, biology, and medicine, 2016. **12**(3): p. 677-687.
  43. Thorley, A.J., et al., *Critical Determinants of Uptake and Translocation of Nanoparticles by the Human Pulmonary Alveolar Epithelium*. ACS Nano, 2014. **8**(11): p. 11778-11789.
  44. Healy, A.M., et al., *Dry powders for oral inhalation free of lactose carrier particles*. Advanced Drug Delivery Reviews, 2014. **75**: p. 32-52.
  45. Pilcer, G. and K. Amighi, *Formulation strategy and use of excipients in pulmonary drug delivery*. International Journal of Pharmaceutics, 2010. **392**(1): p. 1-19.
  46. Huckaby, J.T. and S.K. Lai, *PEGylation for enhancing nanoparticle diffusion in mucus*. Advanced Drug Delivery Reviews, 2018. **124**: p. 125-139.
  47. Liu, M., et al., *Developments of mucus penetrating nanoparticles*. Asian Journal of Pharmaceutical Sciences, 2015. **10**(4): p. 275-282.
  48. Khutoryanskiy, V.V., *Beyond PEGylation: Alternative surface-modification of nanoparticles with mucus-inert biomaterials*. Advanced Drug Delivery Reviews, 2018. **124**: p. 140-149.

49. Murata, M., et al., *Surface modification of liposomes using polymer-wheat germ agglutinin conjugates to improve the absorption of peptide drugs by pulmonary administration*. J Pharm Sci, 2013. **102**(4): p. 1281-9.

CHAPTER 3

Evaluation of the cancer-preventive effect of resveratrol-loaded nanoparticles on the formation of lung tumor spheroids

Manuscript in preparation for the submission to

Nanomedicine: Nanotechnology, Biology, and Medicine

Elisa A. Torrico Guzmán¹, Mitchell Gravely¹, Samantha A. Meenach^{1,2}

¹University of Rhode Island, College of Engineering, Department of Chemical Engineering, Kingston, RI 02881, USA

²University of Rhode Island, College of Pharmacy, Department of Biomedical and Pharmaceutical Sciences, Kingston, RI 02881, USA

Corresponding Author: Samantha A. Meenach, University of Rhode Island, 215A Pastore Hall, 51 Lower College Road, Kingston, RI 02881, USA. Email:

smeenach@uri.edu

ABSTRACT

Resveratrol (RSV) is a natural polyphenol with antioxidant, anti-inflammatory, and chemopreventive benefits. This project evaluated the ability of RSV-loaded nanoparticles (NP) to prevent the growth of and to treat lung tumor spheroids *in vitro*. RSV was encapsulated in NP comprised of a biodegradable polymer, acetalated dextran via single emulsion and solvent evaporation. Free RSV and RSV NP were tested in two-dimensional and three-dimensional cell culture models to evaluate their effects on A549 lung cancer cells and tumor spheroids. RSV NP were evaluated on spheroids for prevention, dosing at day 0, and for treatment, dosing on fully formed spheroids. Results showed that the RSV NP were 200 nm in size with neutral charge, exerted a greater inhibitory effect on viability and growth of cancer cells and spheroids in comparison to free RSV, demonstrating their potential as a chemopreventive agent for both prevention and treatment applications.

KEYWORDS

Resveratrol nanoparticles, chemoprevention, multicellular spheroids, lung cancer, acetalated dextran

ABBREVIATIONS

See footnote.¹

3.1. Introduction

Despite advancements in the treatment of cancer, this disease remains an enormous challenge for scientists. It is estimated that 1,762,450 new cases of cancer will be diagnosed in the U.S. in 2019 and that there will be 606,880 cancer-related deaths, which corresponds to over 1,662 deaths per day. Among all cancers, lung and

---

¹ RSV = resveratrol; NP = nanoparticles; Ac-Dex = acetalated dextran; CUR = curcumin; CAC = cyclic acetal coverage; 2D = two-dimensional; 3D = three-dimensional; MCS = multicellular tumor spheroids; PPTS = pyridinium p-toluenesulfonate; DMSO = dimethyl sulfoxide; 2-MOP = 2-methoxypropene; TEA = triethylamine; DCM = dichloromethane; PVA = polyvinyl alcohol; PBS = phosphate buffered saline; NMR = nuclear magnetic resonance; DLS = dynamic light scattering; PDI = polydispersity index; DL = drug loading; EE = encapsulation efficacy; UPLC = ultra-performance liquid chromatography; DMEM = Dulbecco's modified Eagle's medium; FBS = fetal bovine serum; AP = acid phosphatase; pNPP = p-nitrophenyl phosphate; DNA = deoxyribonucleic acid; DAPI = 4',6-diamidino-2-phenylindole; GFP = green fluorescent protein.

bronchus cancers have the highest mortality rates and resulted in an estimated of 142,670 deaths in 2019 [1].

Chemotherapy is one of the mainstays of lung cancer treatment, however its efficacy is often limited. An emerging field in the treatment of cancer has been the use of chemopreventive phytochemicals, which are constituents of fruits and vegetables with potential pharmacological and/or nutritional functions in preventing oncogenesis. These agents may reverse or inhibit the carcinogenic process at a cellular level, resulting in decreased cancer risk [2, 3]. The Food and Drug Administration (FDA) has approved several chemopreventive agents, including: tamoxifen [4-6] and raloxifene for breast cancer risk reduction [7]; celecoxib, which may reduce colorectal adenomas in familial adenomatous polyposis [8]; and the human papillomavirus vaccine [9]. More than 200 other chemopreventive agents are currently in clinical trials [10]. Landis-Piwowar *et al.* have listed dietary phytochemicals and synthetic chemopreventive compounds, indicating their potential effects [11]. Some phytochemicals act as blocking agents to prevent DNA mutations, such as turmeric and green tea, and others block molecular pathways leading metastasis (e.g. curcumin and gingerol). A number of clinical trials have evaluated vitamin A [11, 12], vitamin E, aspirin, selenium, and others as potential chemopreventive agents for lung cancer, however, none have shown a clear positive benefit [13]. Other compounds (glucocorticoids, myoinositol) showed potential benefits in preclinical models, but further studies are required to establish their efficacy [14]. Overall, there is no formulation available for the prevention of lung tumor formation.

Resveratrol (3,5,4'-trihydroxy-trans-stilbene, RSV) is a phytoalexin [15] that is abundantly found in red grapes, as well as in peanuts and blueberries. In 1997, interest

in RSV increased when Jung *et al.* reported its chemopreventive effects in all stages of neoplastic activity (initiation, promotion and progression) [16]. A later study by Dolfini *et al.* showed that RSV induces drastic growth inhibition on a human breast cancer cell line in both *in vivo* and *in vitro* assays [17].

Although RSV has shown great potential as a chemopreventive drug, it exhibits poor water solubility, resulting in limited bioavailability [18, 19]. Approaches to increasing the solubility of RSV have included using surfactants and cyclodextrins in formulations [20, 21], however nanotechnology has been extensively explored to encapsulate and deliver hydrophobic compounds, thus increasing therapeutic bioavailability [22-24]. RSV-loaded gel nanoparticles demonstrated improved intracellular uptake and cytotoxicity in NCI-H460 lung cancer cells in comparison to free RSV [25], while RSV loaded into nanostructure lipid carriers exhibited higher penetration into the skin in comparison to solid lipid nanoparticles [26]. Both studies highlight the improvement of RSV efficacy in nanoparticle formulations compared to their free form *in vitro*. Furthermore, animal studies showed that RSV solid lipid nanoparticles enhanced the bioavailability of RSV in the brain when delivered to rats for vascular dementia [27].

For this project we synthesized the biodegradable, biocompatible, acid-responsive, and tunable polymer acetalated dextran (Ac-Dex) [28] to encapsulate RSV. The facile degradation of Ac-Dex in acidic conditions makes it suitable for the release of compounds to cancer cells that develop an acidic microenvironment [29, 30] while its by-products, specifically dextran, acetone, and methanol, are released in safe quantities [31, 32]. Both two-dimensional (2D) and three-dimensional (3D) cell culture

experiments were conducted in this study using A549 adenocarcinoma lung cancer cells to evaluate Ac-Dex nanoparticles loaded with RSV. Monolayer 2D cell culture has been traditionally applied to test the efficacy of therapeutics *in vitro* because of the ease in the evaluation of drug-cell interaction, however 2D models do not accurately represent the complex, structural properties of 3D tumors [33, 34]. 3D cell culture provides a better *in vitro* model for tumors with respect to morphology, response to therapeutics, growth kinetics, presence of a necrotic core, and diffusion limitations [35-37]. These characteristics make the inner core of tumors less accessible for drugs, an effect that is not well demonstrated in 2D models. As a result, drugs are only able to affect the outer periphery of cells, which may quickly replenish if the core is not also eliminated [38, 39]. The use of 3D multicellular spheroids is advantageous in testing therapeutic efficacy of RSV NP because of their more accurate representation of *in vivo* solid tumors. The aim of this project was to evaluate and compare the chemopreventive effect of free RSV and RSV NP on the formation and growth of lung tumor spheroids. We hypothesized that RSV NP will inhibit or significantly reduce the growth of tumor cells more effectively than free RSV.

3.2. Methods

3.2.1. Synthesis of Acetalated Dextran

The synthesis of acetalated dextran (Ac-Dex, Figure S1) was conducted as previously described [40]. Lyophilized dextran and PPTS were dissolved in anhydrous DMSO under N₂. The reaction was initiated by the addition of 2-MOP, and lasted for 5

minutes, followed by quenching with TEA. The product was then precipitated in basic water, frozen overnight, and lyophilized.

3.2.2. Nuclear Magnetic Resonance Analysis of Acetalated Dextran

The total acetal coverage (conversion of hydroxyl groups) and type of acetals formed was determined by ^1H -NMR spectroscopy (Bruker 300 MHz NMR, MA, USA). The hydrolysis of acyclic acetals produces methanol and acetone whereas cyclic acetals produce only acetone. The total acetal coverage and the cyclic acetal coverage (CAC) were determined after normalization of the methanol and acetone peaks in relation to dextran (carbon rings). The normalization is associated with the number of protons detected on each molecule. The results were expressed per 100 molecules of glucose.

3.2.3. Preparation of Resveratrol-Loaded Ac-Dex Nanoparticles

Resveratrol-loaded nanoparticles (RSV NP) were prepared via single emulsion and solvent evaporation as described in Supplementary Materials. The organic phase was comprised of RSV dissolved in DMSO added to DCM with Ac-Dex and the aqueous phase contained 3% PVA in PBS to result in an initial loading of 2 wt% RSV. The preparation of fluorescent NP, using curcumin (CUR) as a fluorescent additive, followed the same protocol mentioned above, instead adding CUR and no resveratrol to the organic phase. Blank NP were prepared similarly, excluding RSV or CUR.

3.2.4. Particle Morphology and Shape Analysis via Scanning Electron Microscopy

The surface morphology and shape of the nanoparticles were evaluated by scanning electron microscopy (SEM) with a Zeiss SIGMA VP Field Emission-Scanning Electron Microscope (Germany) as described in Supplementary Materials.

3.2.5. Particle Size, Size Distribution, and Zeta Potential Analysis of Nanoparticles

The size, size distribution (polydispersity index, PDI), and zeta potential of NP were measured by dynamic light scattering (DLS) using a Malvern Nano Zetasizer (Malvern Instruments, Worcestershire, UK) as described in Supplementary Materials.

3.2.6. Analysis of Nanoparticle Drug Loading

Drug loading (DL) and encapsulation efficacy (EE) of RSV NP were determined via ultra performance liquid chromatography (UPLC, LaChrom, Hitachi, Japan), as described in Supplementary Materials. The drug loading and encapsulation efficacy were calculated using the following equations:

$$\text{DL (wt\%)} = \frac{\text{mass of RSV in nanoparticles}}{\text{total mass of nanoparticles}} \times 100$$

$$EE \text{ (wt\%)} = \frac{\text{actual drug loading}}{\text{theoretical drug loading}} \times 100$$

$$\text{Theoretical drug loading} = \frac{\text{initial mass of RSV}}{\text{mass of RSV} + \text{mass of Ac} - \text{Dex}} \times 100$$

3.3. *In Vitro* Cell Studies

A549 human epithelial adenocarcinoma cells were cultured in DMEM containing FBS, sodium pyruvate, penicillin-streptomycin, and Fungizone at 37 °C and 5% CO₂ as described in Supplementary Materials.

3.3.1. Two-Dimensional Cell Culture and Cell Viability

The two-dimensional (2D) *in vitro* cytotoxic effect of RSV was evaluated in A549 cells using an acid phosphatase (AP) colorimetric assay. Cells were seeded into flat-bottomed 96-well plates, and were exposed to free RSV in DMSO for 48 and 72 hours. Details of the assay can be found in Supplementary Materials. pNPP is hydrolyzed by the presence of AP in live cells and produces an intense yellow color (p-nitrophenolate), which was detected at a wavelength of 405 nm in a Biotek Cytation 3 microplate reader (BioTek Instruments Inc., Winooski, VT, USA). The relative viability was calculated as the ratio of the fluorescence intensity of the sample (cells dosed with RSV) versus the fluorescence intensity of control (cells only). A 2D viability test using blank NP was performed to evaluate the effect of different concentrations of NP on

A549 cells. The viability of the cells was evaluated at 24, 48, and 72 hours using resazurin, and the fluorescence intensity of the samples were detected at 544/590 nm.

3.3.2. RSV Nanoparticle Cellular Uptake

Confocal fluorescence microscopy images were taken of A549 cells exposed to 100 μ M CUR NP for 3 hours as described in Supplementary Materials. The cells were stained with CellMask^{TM} deep red plasma membrane and fixed with 4% paraformaldehyde in PBS. For quantification, A549 cells were seeded in a flat-bottomed 96-well plate and exposed to 100 μ M CUR NP for 3 hours. After exposure, the particle solution was removed, and cells were washed with glycine and cold PBS prior to fixation in with ethanol. Uptake was quantified using fluorescence spectroscopy at 420/540 nm.

3.3.3. Formation and Evaluation of 3D Multicellular Spheroids

A549 multicellular spheroids (MCS), representing a three-dimensional (3D) cell culture platform, were formed via centrifugation to evaluate the *in vitro* effects of RSV formulations (details found in Supplementary Materials). Initially, three cell concentrations (3,000, 5,000, and 8,000 cells/well) with 20 μ g/mL of collagen type I were seeded into ultralow attachment round-bottomed 96-well plates, centrifuged, and incubated for growth observation. Bright-field images were taken at various time points up to day 22 using a Biotek Cytation 3 imager. The MCS average size and morphology of the spheroids was evaluated over time. Viability of sample MCS was determined

using live/dead assay at days 1 and 5 of MCS growth. The live/dead solution contained ethidium homodimer-1 (red dye to detect dead cells), and calcein AM (green dye for live cells). The MCS were incubated with live/dead solution for 60 min before imaging.

3.3.4. Growth Inhibition of Multicellular Spheroids Exposed to Free RSV and RSV NP

The growth inhibition of A549 MCS was evaluated in two types of studies (prevention or treatment) with free RSV and RSV NP. For prevention studies, MCS were dosed with free RSV or RSV NP on the day cells were seeded, following centrifugation of the plates. For treatment studies, MCS were dosed with free RSV and RSV NP 5 days after MCS seeding and formation. In both prevention and treatment studies, the plates were incubated at standard conditions and were imaged periodically (bright-field) and analyzed for size using a Biotek Cytation 3 imager. The growth or inhibition of MCS was analyzed by calculating the change in size relative to untreated controls.

3.3.5. Clonogenic Assay

A clonogenic assay was performed on MCS dosed with free RSV after prevention and treatment studies to test the ability of cells to recover and form colonies as outlined in Supplementary Materials.

3.4. Statistical Analysis

Measurements were performed in at least triplicate. All values were presented as mean $\pm$ standard deviation. The half maximal inhibitory concentration (IC₅₀) values were calculated using a polynomial regression in PRISM version 5.0 software (GraphPad Software, Inc., CA, USA). The statistical significance of the results was determined using Student's t-test. Statistical significance was defined as $p < 0.05$ (*).

3.5. Results

3.5.1. Characterization of Acetalated Dextran

The reaction between dextran and 2-MOP in the presence of PPTS resulted in the synthesis of acetalated dextran (Ac-Dex), which contains both cyclic and acyclic acetal groups (Figure S1). NMR spectrometry of Ac-Dex showed a total acetal coverage (conversion from –OH groups) of 80% and a cyclic acetal coverage of 75%.

3.5.2. Characterization of Nanoparticles

3.5.2.1. Nanoparticle Size, Distribution, and Surface Charge

NP size, polydispersity index (PDI), and zeta potential values determined by DLS are shown in **Table 3.1**. RSV NP and Blank NP exhibited diameters near 200 nm,

with high homogeneity as demonstrated by their narrow size distribution (PDI of 0.12), and surface charges of -2.6 and -5.6 mV for RSV NP and Blank NP, respectively. SEM imaging (**Figure 3.1**) revealed smooth surface morphology, spherical shape, and confirmed the narrow size distribution and homogeneity shown by DLS measurements.

Table 3.1 Characteristics of resveratrol (RSV)-loaded nanoparticles (RSV NP), blank NP without RSV (Blank NP), and curcumin (CUR)-loaded NP (CUR NP), including diameter, polydispersity index (PDI), zeta (ζ) potential, RSV loading, and encapsulation efficiency (EE) (mean $\pm$ standard deviation, n = 3).

System	Diameter (nm)	PDI	ζ Potential (mV)	Drug	EE (%)
				Loading (μ g RSV/mg NP)	
RSV NP	205.2 $\pm$ 5.6	0.12 $\pm$ 0.04	-2.94 $\pm$ 0.36	14.63 $\pm$ 2.25	73.65 $\pm$ 11.62
Blank NP	192.0 $\pm$ 3.4	0.12 $\pm$ 0.02	-5.63 $\pm$ 1.29	N/A	N/A
CUR NP	175.8 $\pm$ 8.1	0.15 $\pm$ 0.03	-4.13 $\pm$ 0.48	14.73 $\pm$ 0.11 ^(*)	N/A

(*) μ g CUR/mg NP

Figure 3.1 Representative scanning electron micrographs of (Left) blank nanoparticles and (Right) resveratrol-loaded nanoparticles. Scale bar = 500 nm.

3.5.2.2. RSV Loading in Nanoparticles

Ac-Dex nanoparticles (NP) were successfully loaded with resveratrol (RSV), demonstrating an encapsulation efficacy of 73% (**Table 3.1**). The RSV NP drug loading was 14.6 μg RSV/mg NP (max = 20 μg /mg).

3.5.3. Cell Studies

3.5.3.1. Two-Dimensional Cell Culture and Cell Viability

Cell viability studies using A549 cells in a monolayer (2D) culture, when dosed with free RSV, resulted in IC_{50} values of 121.9 μM and 74.6 μM at 48 hours and 72 hours of exposure, respectively (**Figure A.2**). The amount of DMSO in cells was set to a concentration of 0.15 v/v % to enable dissolution of RSV in media, and no negative

effects on the cells from DMSO were observed with respect to viability analysis. Cell viability evaluation of free RSV was carried out on 3D MCS; however, there was no correlation between RSV concentration and cell viability (**Figure A.2C**). 2D A549 cells exposed to Blank NP demonstrated a significant reduction in relative viability at NP concentrations above 3 mg/ml (**Figure 3.2A**), whereas cells exposed to 0.05 - 1 mg/ml NP resulted in increased viability.

Figure 3.2 Viability analysis of blank nanoparticles without resveratrol (Blank NP) and uptake of curcumin (CUR)-loaded NP (CUR NP). (A) Relative viability of A549 cells exposed to Blank NP (*p < 0.05, **p < 0.01, ***p < 0.001, and n ≥ 3, which represent comparison to the control with no exposure to NP). (B) Confocal images of CUR NP

uptake in A549 cells, showing CUR NP (green) and cell membrane (red), scale bar = 20 μm . (C) Fluorescence quantification of CUR NP uptake in A549 cells.

3.5.3.2. Cellular Uptake of RSV NP in A549 Cells

The cellular uptake of NP into 2D A549 cells was evaluated using CUR NP as model fluorescent NP. The CUR NP characteristics (**Table 3.1**) are similar to both RSV NP and Blank NP with respect to size, PDI, surface charge, and drug loading. A representative confocal image (**Figure 3.2B**) shows NP associated within the cytoplasm of A549 cells. Quantification of uptake is illustrated in **Figure 3.2C**, where CUR NP were effectively uptaken in A549 cells compared to the control (cells only).

3.5.3.3. Formation, Size Analysis, and Morphology of Multicellular Spheroids

Three concentrations of A549 lung adenocarcinoma cells were initially seeded to form MCS via centrifugation, and were imaged over time to evaluate their growth. As seen in **Figure 3.3A**, the growth curves of spheroids with 5,000 and 3,000 cells/MCS overlapped, while samples with 8,000 cells/MCS started larger but did not reach the same maximum size. From these studies, 5,000 cells/MCS were chosen as the optimized MCS. A live/dead assay was performed on 1-day and 5-day MCS. **Figure 3.3B** shows the presence of live cells (green) in the outer region of the MCS and dead cells (red) in the inner core. From day 1 to day 5, the MCS increased in size, the volume of dead cells increased, and the region of live cells at the surface decreased.

MCS formation and growth for 5,000 cells/MCS is presented in **Figure 3.3C**. A549 cells were initially aggregated in a 2D monolayer, but formed an organized cluster of cells after four hours. A defined spheroid was present within 24 hours and MCS continued to grow for over two weeks, after which the MCS size decreased. Maximum MCS size (828 μm) was reached on day 17. For the last two days, it was observed that dead cells detached from spheroids.

Figure 3.3 Optimization of A549 multicellular spheroid (MCS) formation. (A) Growth analysis of A549 MCS grown at different initial cell seeding concentrations; (B) Fluorescence images showing MCS morphology and viability on days 1 and 5, where

green cells represent live cells and red cells represent dead cells (Scale bar = 300 μm); and (C) Bright-field images of A549 cells grown at 5,000 cells/MCS for 22 days (Scale bar = 500 μm). ** $p < 0.01$ which represents comparison of 8,000 cells/MCS to 5,000 and 3,000 cells/MCS on day 22.

3.5.3.4. Growth Inhibition of Multicellular Spheroids

3.5.3.4.1. Prevention Studies

For the prevention study, MCS were dosed with free RSV and RSV NP after seeding and centrifugation on day 0. Bright-field images were taken, and the diameters of the spheroids were recorded over time. MCS exposed to 500 μM of free RSV showed complete inhibition of growth and reduction in size, whereas MCS exposed to 250 μM showed significant growth inhibition (**Figure 3.4A**). MCS exposed to 25, 50, and 100 μM RSV showed delayed growth. Exposure to media with 0.15 v/v % DMSO had no impact on MCS growth. The outcome following RSV NP dosing can be seen in **Figure 3.4B**. 250 and 500 μM RSV NP completely inhibited the formation of MCS (no visible MCS). MCS exposed to 25 μM of RSV NP did not show a significant difference compared to control, 50 μM RSV NP resulted in significant growth inhibition, and 100 μM RSV NP resulted in a reduction in size of MCS.

The change in spheroid diameters relative to the control on day 15 is shown in **Figure 3.5C**. Neither Blank NP nor 0.15 v/v % DMSO (Control-D) had an impact on MCS growth. At 50 and 100 μM RSV, spheroid growth inhibition significantly

increased using RSV-loaded nanoparticles compared to free RSV. MCS exposed to 250 and 500 μM free RSV resulted in MCS growth inhibition or reduction, whereas exposure to 250 and 500 μM RSV NP resulted in no MCS formation. Bright-field images of MCS dosed with 100 μM RSV formulations and comparative graph bars with control are shown in **Figures 3.6A**. Free RSV and RSV NP resulted in spheroid size reduction, with RSV NP being more effective than free RSV at reducing MCS size on days 5 and 15.

Figure 3.4 Evaluation of multicellular spheroid (MCS) growth prevention using different resveratrol (RSV) formulations, including (A) free RSV (RSV) and (B) RSV-

loaded nanoparticles (RSV NP). Control-D refers to media with 0.15 v/v % DMSO. (C) Comparison of the change in MCS diameter relative to control (no RSV exposure) between free RSV and RSV NP at day 15. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, which represents comparison to the control (no exposure to RSV or RSV NP) for (A) and (B) and represents comparison between RSV and RSV NP for (C); $n \geq 3$.

3.5.3.4.2. Treatment Studies

For the treatment studies, MCS were dosed with free RSV and RSV NP 5 days after MCS growth began. No differences were observed between the control MCS and MCS exposed to lower free RSV concentrations ($< 100 \mu\text{M}$ RSV), however MCS exposed to $250 \mu\text{M}$ free RSV experienced a reduction in size and those exposed to $500 \mu\text{M}$ experienced a delay in growth (**Figure 3.5A**). All MCS exposed to RSV NP experienced significant inhibition of spheroid growth (**Figure 3.5B**). From days 1 to 5, MCS grew similarly, and after being dosed with RSV NP they experienced a distinct reduction in growth, which was not observed for MCS exposed to free RSV. A significant inhibition in MCS growth was observed using RSV NP versus free RSV at day 15 at concentrations ranging from 25 to $250 \mu\text{M}$ RSV (**Figure 3.5C**). Growth was completely inhibited in MCS exposed to $500 \mu\text{M}$ RSV (free or NP). In addition, MCS exposed to Blank NP corresponding to $100 \mu\text{M}$ and $500 \mu\text{M}$ RSV were evaluated, where the higher concentration inhibited the growth of MCS, while $100 \mu\text{M}$ did not affect MCS growth. Bright-field images of MCS dosed with $100 \mu\text{M}$ RSV formulations and

comparative graph bars with control are shown in **Figures 3.6B**. MCS size was reduced only when exposed to RSV NP for the given concentration.

Figure 3.5 Evaluation of multicellular spheroid (MCS) treatment (growth restriction) using different resveratrol (RSV) formulations, including (A) free RSV (RSV) and (B) RSV-loaded nanoparticles (RSV NP). Control-D refers to media with 0.15 v/v % DMSO. (C) Comparison of the change in diameter relative to control between free RSV and RSV NP at day 15. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, which represents comparison to the control (no exposure to RSV or RSV NP) for (A) and (B) and represents comparison between RSV and RSV NP for (C); $n \geq 3$.

Figure 3.6 Comparison of A549 multicellular spheroid (MCS) growth in (A) prevention and (B) treatment studies upon exposure to 100 μM resveratrol (RSV) formulations. Bright-field images and corresponding quantification of MCS growth upon exposure to free resveratrol (RSV), RSV-loaded nanoparticles (RSV NP), and Blank NP equivalent to 100 μM RSV NP by mass. Control MCS were exposed to media only. Scale bar = 500 μm. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, which represents comparison to the control (no exposure to RSV or RSV NP) for each time point; $n \geq 3$.

3.5.3.5. Clonogenic assay

Cells from MCS dosed with free RSV in prevention and treatment studies were seeded to determine their recovery and potential to form colonies (**Figure 3.7**). For prevention, the formation of colonies followed a similar pattern seen in the inhibition of growth of MCS, where an increase in RSV concentration resulted in an increase in the inhibition of colony formation (**Figure 3.7A**). In contrast, for treatment, cells from the MCS treated with free RSV formed similar numbers of colonies, irrespective of RSV concentration in comparison to the control (**Figure 3.7B**).

Figure 3.7 Clonogenic assay results for A549 cells recovered from multicellular spheroids dosed with free resveratrol (RSV) in (A) prevention studies and (B) treatment studies. Control DMSO refers to media with 0.15 v/v % DMSO. Data is presented as

the number of colonies compared with the untreated control (mean $\pm$ standard deviation, $n = 3$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, which represents comparison to the control (no exposure to RSV); $n \geq 3$.

3.6. Discussion

The quality of Ac-Dex is important in order to obtain favorable nanoparticle formulation and characteristics. During Ac-Dex synthesis, dextran transitions from hydrophilic to hydrophobic due to the conversion of -OH groups to acetals. High acetal coverage enables the stability of PVA-coated NP (small size and distribution), according to previous studies [41]. High -OH conversion and CAC coverage allow Ac-Dex to exhibit pH-tunable hydrolysis-driven degradation, which allows it to degrade in acidic conditions more readily than neutral conditions [42].

The small NP sizes and high homogeneity is confirmed via SEM, which showed homogeneous, spherical NP. NP observed in SEM images were smaller than water-dispersed NP measured by DLS, due to shrinkage of the particles following drying [43]. DLS measures the hydrodynamic diameter of particles in aqueous suspension, allowing for a hydrated layer on the surface of NP. While neutral and slightly polar molecules have the tendency to agglomerate in aqueous environments, and Ac-Dex NP exhibited a slightly negative charge, the particles were well dispersed in water due to their PVA coating. PVA coating is known to improve colloidal stability and reduce the agglomeration of nanoparticles due to its steric effect [44, 45].

2D cell culture models have been extensively used in the evaluation of therapeutics for preliminary drug screening. In this project we determined the *in vitro* therapeutic concentrations of RSV using a monolayer of cells prior to translation into a more complex system. Following 2D viability analysis cells exposed to pure RSV experienced a 40% reduction in the IC₅₀ value after 72 hours instead of 48 hours, demonstrating that a longer exposure time reduces the RSV demand during treatment. 2D cells exhibited increased cell viability when exposed to low concentrations of Blank NP (< 1 mg/ml), which likely indicates that dextran released following the degradation of Ac-Dex may have been used as a nutrient by the cells. In contrast, exposure to higher Blank NP concentrations (> 3 mg/ml) resulted in significant viability reduction, which was also observed in the 3D spheroid model.

In preliminary 2D uptake studies, RSV and CUR were co-encapsulated in NP, however, a decrease in cell number was noticed after one hour of exposure because of the therapeutic effect of RSV (data not shown). As a result, CUR was used as a fluorescent model compound for the uptake studies. CUR is a small molecular weight compound, and CUR NP exhibited similar size and surface charge to RSV NP. Previous studies have shown that 100-200 nm nanoparticles can be internalized in cells [46]. RSV NP and CUR NP are within this optimal size range, and CUR NP were successfully internalized by A549 cells after three hours. The accumulation of NP within the cells can allow for RSV to be released into the cells and exert its chemotherapeutic effect.

Evaluation of NP using 3D multicellular spheroids (MCS) offer advantages over 2D culture because MCS offer a better representation of the structure of *in vivo* tumors and the resulting drug-cell interactions. The maximum MCS size for the three cell

seeding concentrations was reached at day 17, followed by a period where MCS sizes decreased. This behavior was likely caused by an exhaust in nutrients from media, as it was not replenished throughout the experiment. 3,000 and 5,000 cells/MCS samples exhibited overlapping growth curves starting at day 7, which may be due to nutrient limitations in the larger population of cells.

MCS larger than 200 μm in diameter are known to display a heterogeneous structure with a peripheral proliferating region, an intermediate quiescent region due to limited oxygen and nutrients transport, and an inner necrotic core [47-49]. Larger spheroids ($> 500 \mu\text{m}$) present a similar physicochemical gradient to that seen in micrometastasis and $0.5 - 1 \text{ mm}^3$ avascular tumors [50, 51]. In this study, 3D MCS generated via centrifugation developed morphology similar to tumors with respect to a proliferating outer surface and core with dead cells. These findings are consistent with other studies that demonstrate similar features at > 4 days [51]. The lack of correlation observed during the viability evaluation of RSV in 3D MCS using acid phosphatase assay is likely attributable to the physical barriers of MCS structure and inability of RSV and assay reagents to fully penetrate the spheroids [52, 53]. Given the extensive cell-cell interactions in MCS, they provide a much more formidable barrier than the 2D monolayer [54, 55].

In order to determine the therapeutic potential of RSV NP in the inhibition of MCS growth, preventive and treatment studies were completed comparing free RSV and RSV NP. In preventive studies, inhibition in the growth of MCS was observed for 50 μM and 100 μM of free RSV, and this effect was significantly enhanced upon exposure to RSV NP. These findings imply an enhanced preventive effect of RSV in a

nanoparticle formulation. In treatment studies, MCS were dosed at day 5, after MCS had developed morphology similar to an *in vivo* tumor, as opposed to a cluster of individual cells. Following RSV treatment, spheroid growth decreased for all RSV NP concentrations. This outcome demonstrates another potential application of RSV in a nanoparticle formulation for treatment of solid tumors.

In both preventive and treatment studies, exposure to 500 μM RSV resulted in a significant reduction in MCS growth, which was enhanced by the NP carrier. As a comparison, the effect of Blank NP was evaluated, where cytotoxicity at this concentration was enhanced by the presence of NP alone. High NP concentrations likely interfered with the aggregation of cells during MCS formation.

50 and 100 μM RSV NP enhanced the properties of free RSV and may serve as a potential chemopreventive formulation for lung cancer. In previous studies, higher concentrations of free RSV (>100 μM) resulted in mitochondrial dysfunction and promoted apoptosis in human hepatoblastoma cells [56] and also with RSV solid lipid NP that were used as an effective neuroprotectant in glioma cells [57]. The polymer effect on formulation safety was previously evaluated in a PLGA system, where a concentration of 50 μM of RSV-loaded PLGA NP was found to be beneficial as an antioxidant in cochlear cells and remained under the cytotoxic limits of NP [58]. The RSV-loaded Ac-Dex NP system developed on this project agreed with these findings.

Individual cells from MCS exposed to RSV in preventive and treatment studies exhibited different behaviors in colony formation colonies. While cells from preventive studies had a weaker recovery and inferior ability to form colonies compared to control; they showed no difference in colony formation compared to control in treatment studies,

demonstrating the ability of cells from MCS to recover after dosing on fully-formed spheroids (treatment studies). These findings suggest that prevention chemotherapy might be advantageous for reduced cancer recurrence.

3.7. Conclusions

We developed a resveratrol-loaded nanoparticle formulation based on Ac-Dex, a biodegradable polymer synthesized from dextran, resulting in spherical RSV NP that exhibited homogeneous dispersion, smooth surface, and appropriate size for cellular uptake. The evaluation of RSV NP in the inhibition of A549 tumor spheroid growth showed that this chemopreventive agent has an enhanced therapeutic effect as a nanoparticle formulation when compared to free RSV. High concentrations of RSV NP were seen to inhibit the formation of MCS, while lower concentrations RSV NP significantly reduced the growth of tumor spheroids. The results were consistent in both types of studies, with prevention of tumor cell aggregation and in the treatment of already developed solid tumor spheroids. These findings represent potential alternative uses for RSV NP, in chemoprevention and treatment of lung cancer tumors.

3.8. Acknowledgments

This work was partially supported by an INBRE award from the National Institute of General Medical Sciences of the National Institutes of Health (P20GM103430) and by the use of equipment and services available through the RI-INBRE Centralized Research Core Facility. NMR was completed at the Rhode Island NSF EPSCoR research facility, supported in part by the National Science Foundation

(NSF, EPS-1004057). This material is based in part upon work supported by the NSF funding (1508868). Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the view of the NSF. The authors thank RIN2 for SEM and DLS access.

AUTHOR DISCLOSURE STATEMENT

No conflict of interest exists.

REFERENCES

1. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2019*. CA Cancer J Clin, 2019. **69**(1): p. 7-34.
2. Sporn, M.B., et al., *Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids)*. Fed Proc, 1976. **35**(6): p. 1332-8.
3. De Flora, S. and L.R. Ferguson, *Overview of mechanisms of cancer chemopreventive agents*. Mutat Res, 2005. **591**(1-2): p. 8-15.
4. Fisher, B., et al., *Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study*. J Natl Cancer Inst, 1998. **90**(18): p. 1371-88.
5. Kramer, R. and P. Brown, *Should tamoxifen be used in breast cancer prevention?* Drug Saf, 2004. **27**(13): p. 979-89.
6. Cuzick, J., et al., *Tamoxifen for prevention of breast cancer: extended long-term follow-up of the IBIS-I breast cancer prevention trial*. The Lancet Oncology. **16**(1): p. 67-75.
7. Hortobagyi, G.N. and P.H. Brown, *Two Good Choices to Prevent Breast Cancer: Great Taste, Less Filling*. Cancer Prevention Research, 2010. **3**(6): p. 681-685.
8. Arber, N., et al., *Celecoxib for the Prevention of Colorectal Adenomatous Polyps*. New England Journal of Medicine, 2006. **355**(9): p. 885-895.
9. Basu, P., et al., *Efficacy and safety of human papillomavirus vaccine for primary prevention of cervical cancer: A review of evidence from phase III trials and national programs*. South Asian J Cancer, 2013. **2**(4): p. 187-92.

10. Davis, J.S. and X. Wu, *Current state and future challenges of chemoprevention*. Discov Med, 2012. **13**(72): p. 385-90.
11. Landis-Piwowar, K.R. and N.R. Iyer, *Cancer chemoprevention: current state of the art*. Cancer Growth Metastasis, 2014. **7**: p. 19-25.
12. Omenn, G.S., *Chemoprevention of lung cancers: lessons from CARET, the beta-carotene and retinol efficacy trial, and prospects for the future*. Eur J Cancer Prev, 2007. **16**(3): p. 184-91.
13. Keith, R.L. and Y.E. Miller, *Lung cancer chemoprevention: current status and future prospects*. Nature Reviews Clinical Oncology, 2013. **10**(6): p. 334-343.
14. Wattenberg, L.W., et al., *Chemoprevention of pulmonary carcinogenesis by brief exposures to aerosolized budesonide or beclomethasone dipropionate and by the combination of aerosolized budesonide and dietary myoinositol*. Carcinogenesis, 2000. **21**: p. 179-82.
15. Singh, C.K., M.A. Ndiaye, and N. Ahmad, *Resveratrol and cancer: Challenges for clinical translation*. Biochim Biophys Acta, 2015. **1852**(6): p. 1178-85.
16. Jang, M., et al., *Cancer chemopreventive activity of resveratrol, a natural product derived from grapes*. Science (New York, N.Y.), 1997. **275**(5297): p. 218-20.
17. Dolfini, E., et al., *Resveratrol impairs the formation of MDA-MB-231 multicellular tumor spheroids concomitant with ceramide accumulation*. Cancer Lett, 2007. **249**(2): p. 143-7.

18. Walle, T., et al., *HIGH ABSORPTION BUT VERY LOW BIOAVAILABILITY OF ORAL RESVERATROL IN HUMANS*. Drug Metabolism and Disposition, 2004. **32**(12): p. 1377-1382.
19. Wenzel, E. and V. Somoza, *Metabolism and bioavailability of trans-resveratrol*. Mol Nutr Food Res, 2005. **49**(5): p. 472-81.
20. Miranda, J.C.d., et al., *Cyclodextrins and ternary complexes: technology to improve solubility of poorly soluble drugs*. Brazilian Journal of Pharmaceutical Sciences, 2011. **47**: p. 665-681.
21. Kumpugdee-Vollrath, M. and Y. Ibold. *Increasing solubility of poorly water soluble drug resveratrol by surfactants and cyclodextrins*. in *Advanced Materials Research*. 2012. Trans Tech Publ.
22. Kumar, S., et al., *Nanotechnology as Emerging Tool for Enhancing Solubility of Poorly Water-Soluble Drugs*. BioNanoScience, 2012. **2**(4): p. 227-250.
23. Roberts, A.D. and H. Zhang, *Poorly water-soluble drug nanoparticles via solvent evaporation in water-soluble porous polymers*. Int J Pharm, 2013. **447**(1-2): p. 241-50.
24. Kalepu, S. and V. Nekkanti, *Insoluble drug delivery strategies: review of recent advances and business prospects*. Acta Pharm Sin B, 2015. **5**(5): p. 442-53.
25. Karthikeyan, S., et al., *Anticancer activity of resveratrol-loaded gelatin nanoparticles on NCI-H460 non-small cell lung cancer cells*. Biomedicine & Preventive Nutrition, 2013. **3**(1): p. 64-73.

26. Gokce, E.H., et al., *Resveratrol-loaded solid lipid nanoparticles versus nanostructured lipid carriers: evaluation of antioxidant potential for dermal applications*. Int J Nanomedicine, 2012. **7**: p. 1841-50.
27. Yadav, A., et al., *Resveratrol loaded solid lipid nanoparticles attenuate mitochondrial oxidative stress in vascular dementia by activating Nrf2/HO-1 pathway*. Neurochem Int, 2018. **112**: p. 239-254.
28. Bachelder, E.M., et al., *Acetal-derivatized dextran: an acid-responsive biodegradable material for therapeutic applications*. Journal of the American Chemical Society, 2008. **130**(32): p. 10494-10495.
29. Tannock, I.F. and D. Rotin, *Acid pH in Tumors and Its Potential for Therapeutic Exploitation*. Cancer Research, 1989. **49**(16): p. 4373-4384.
30. Kato, Y., et al., *Acidic extracellular microenvironment and cancer*. Cancer Cell Int, 2013. **13**(1): p. 89.
31. Kauffman, K.J., et al., *Optimization of Rapamycin-Loaded Acetalated Dextran Microparticles for Immunosuppression*. International Journal of Pharmaceutics, 2011. **422**: p. 356-363.
32. Broaders, K.E., et al., *Acetalated dextran is a chemically and biologically tunable material for particulate immunotherapy*. Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(14): p. 5497-5502.
33. Hait, W.N., *Anticancer drug development: the grand challenges*. Nat Rev Drug Discov, 2010. **9**(4): p. 253-254.

34. Astashkina, A., B. Mann, and D.W. Grainger, *A critical evaluation of in vitro cell culture models for high-throughput drug screening and toxicity*. Pharmacology & Therapeutics, 2012. **134**(1): p. 82-106.
35. Fitzgerald, K.A., et al., *Life in 3D is never flat: 3D models to optimise drug delivery*. J Control Release, 2015. **215**: p. 39-54.
36. Sutherland, R.M., *Cell and environment interactions in tumor microregions: the multicell spheroid model*. Science, 1988. **240**(4849): p. 177-84.
37. Minchinton, A.I. and I.F. Tannock, *Drug penetration in solid tumours*. Nat Rev Cancer, 2006. **6**(8): p. 583-92.
38. Horning, J.L., et al., *3-D tumor model for in vitro evaluation of anticancer drugs*. Mol Pharm, 2008. **5**(5): p. 849-62.
39. Lovitt, C.J., T.B. Shelper, and V.M. Avery, *Advanced cell culture techniques for cancer drug discovery*. Biology (Basel), 2014. **3**(2): p. 345-67.
40. Wang, Z. and S.A. Meenach, *Synthesis and Characterization of Nanocomposite Microparticles (nCmP) for the Treatment of Cystic Fibrosis-Related Infections*. Pharm Res, 2016. **33**(8): p. 1862-72.
41. Wang, Z., et al., *Nanocomposite microparticles (nCmP) for the delivery of tacrolimus in the treatment of pulmonary arterial hypertension*. Int J Pharm, 2016. **512**(1): p. 305-13.
42. Gillies, E.R., A.P. Goodwin, and J.M.J. Fréchet, *Acetals as pH-Sensitive Linkages for Drug Delivery*. Bioconjugate Chemistry, 2004. **15**(6): p. 1254-1263.

43. Bootz, A., et al., *Comparison of scanning electron microscopy, dynamic light scattering and analytical ultracentrifugation for the sizing of poly(butyl cyanoacrylate) nanoparticles*. Eur J Pharm Biopharm, 2004. **57**(2): p. 369-75.
44. Sharma, N., P. Madan, and S. Lin, *Effect of process and formulation variables on the preparation of parenteral paclitaxel-loaded biodegradable polymeric nanoparticles: A co-surfactant study*. Asian Journal of Pharmaceutical Sciences, 2016. **11**(3): p. 404-416.
45. Moore, T.L., et al., *Nanoparticle colloidal stability in cell culture media and impact on cellular interactions*. Chem Soc Rev, 2015. **44**(17): p. 6287-305.
46. Win, K.Y. and S.S. Feng, *Effects of particle size and surface coating on cellular uptake of polymeric nanoparticles for oral delivery of anticancer drugs*. Biomaterials, 2005. **26**(15): p. 2713-22.
47. Hamilton, G., *Multicellular spheroids as an in vitro tumor model*. Cancer Letters. **131**(1): p. 29-34.
48. Mehta, G., et al., *Opportunities and Challenges for use of Tumor Spheroids as Models to Test Drug Delivery and Efficacy*. J Control Release, 2012. **164**(2): p. 192-204.
49. Knight, E. and S. Przyborski, *Advances in 3D cell culture technologies enabling tissue-like structures to be created in vitro*. Journal of Anatomy, 2015. **227**(6): p. 746-756.
50. Groebe, K. and W. Mueller-Klieser, *Distributions of oxygen, nutrient, and metabolic waste concentrations in multicellular spheroids and their dependence on spheroid parameters*. Eur Biophys J, 1991. **19**(4): p. 169-81.

51. Nath, S. and G.R. Devi, *Three-dimensional culture systems in cancer research: Focus on tumor spheroid model*. Pharmacol Ther, 2016. **163**: p. 94-108.
52. Gong, X., et al., *Generation of Multicellular Tumor Spheroids with Microwell-Based Agarose Scaffolds for Drug Testing*. PLoS One, 2015. **10**(6): p. e0130348.
53. Mikhail, A.S., S. Eetezadi, and C. Allen, *Multicellular tumor spheroids for evaluation of cytotoxicity and tumor growth inhibitory effects of nanomedicines in vitro: a comparison of docetaxel-loaded block copolymer micelles and Taxotere(R)*. PLoS One, 2013. **8**(4): p. e62630.
54. Desoize, B. and J. Jardillier, *Multicellular resistance: a paradigm for clinical resistance?* Crit Rev Oncol Hematol, 2000. **36**(2-3): p. 193-207.
55. Choi, I.K., et al., *Strategies to increase drug penetration in solid tumors*. Front Oncol, 2013. **3**: p. 193.
56. Ma, X., et al., *Resveratrol-induced mitochondrial dysfunction and apoptosis are associated with Ca^{2+} and mCICR-mediated MPT activation in HepG2 cells*. Molecular and Cellular Biochemistry, 2007. **302**(1): p. 99-109.
57. Neves, A.R., J.F. Queiroz, and S. Reis, *Brain-targeted delivery of resveratrol using solid lipid nanoparticles functionalized with apolipoprotein E*. J Nanobiotechnology, 2016. **14**: p. 27.
58. Musazzi, S. and U. Perini, *Laser-induced breakdown spectroscopy : theory and applications*. Springer Series in Optical Sciences,. 2014, Berlin ; New York: Springer. xxii, 565 pages.

CHAPTER 4

Development and Evaluation of Paclitaxel-Loaded Aerosol Nanocomposite Microparticles and Their Efficacy Against Lung Cancer Tumor Spheroids

Manuscript in preparation for the submission to

Journal of biomedical Nanotechnology

Elisa A. Torrico Guzmán¹, Qihua Sun¹, Samantha A. Meenach^{1,2}

¹University of Rhode Island, College of Engineering, Department of Chemical Engineering, Kingston, RI 02881, USA

²University of Rhode Island, College of Pharmacy, Department of Biomedical and Pharmaceutical Sciences, Kingston, RI 02881, USA

Corresponding Author: Samantha A. Meenach, University of Rhode Island, 215A Pastore Hall, 51 Lower College Road, Kingston, RI 02881, USA. Email: smeenach@uri.edu

ABSTRACT

Paclitaxel is the most applied chemotherapeutic for the treatment of lung cancer, delivered as Taxol Intravenously. The purpose of this project was to develop a dry powder nanocomposite microparticle (nCmP) aerosol containing PTX-loaded nanoparticles (NP) to be delivered via a dry powder inhaler to the lungs for the treatment of non-small cell lung cancer (NSCLC) and subsequently to compare its efficacy vs. free PTX. Nanoparticles were formulated by a single emulsion and solvent evaporation method, producing smooth, almost neutral PTX NP of around 200 nm in size. PTX nCmP were obtained via a spray drying formulation, producing amorphous wrinkled particles that demonstrated optimal deposition in vitro pulmonary delivery. Particles were evaluated in a 2D followed by 3D cell culture studies. PTX NP enhanced cytotoxicity when compared to pure drug in the 2D model. However, in the LCC 3D model, PTX NP and pure PTX showed similar efficacy in growth inhibition of multicellular spheroids, likely due to diffusion and penetration limitations. Overall, the formulation had comparable cytotoxicity responses in multicellular spheroids compared with free PTX. Finally, PTX nCmP were evaluated in an AIC 3D platform that mimics the pulmonary environment, representing a new model for the assessment of dry powder formulations.

KEYWORDS

Paclitaxel nanocomposite microparticles, lung cancer, pulmonary delivery, multicellular tumor spheroids, air-interface cell culture

ABBREVIATIONS

PTX, paclitaxel; NP, nanoparticles; Ac-Dex, acetalated dextran; CUR, curcumin; CAC, cyclic acetal coverage; 2D, two-dimensional; 3D, three-dimensional; MCS, multicellular tumor spheroids; PPTS, pyridinium p-toluenesulfonate; DMSO, dimethyl sulfoxide; 2-MOP, 2-methoxypropene; TEA, triethylamine; DCM, dichloromethane; PVA, polyvinyl alcohol; PBS, phosphate buffered saline (1X PBS dilution unless specified); NMR, nuclear magnetic resonance; DLS, dynamic light scattering; PDI, polydispersity index; DL, drug loading; EE, encapsulation efficacy; UPLC, ultra-performance liquid chromatography; DMEM, Dulbecco's modified Eagle's medium; RPMI, Roswell Park Memorial Institute; FBS, fetal bovine serum; DSC, differential scanning calorimetry; PXRD, powder X-ray diffraction; KF, Karl Fischer titration; NGI, Next Generation Impactor; FPD, fine particle dose; FPF, fine particle fraction; RF, respirable fraction; ED, emitted dose; DPI, dry powder inhaler; LCC, liquid covered culture; AIC, air interface culture

4.1. Introduction

Lung & bronchus cancer is the leading cause in cancer related deaths in the USA, with an estimated of 228,150 new cases in 2019 and a mortality of 390 deaths per day. The 5-year relative survival rate of a patient diagnosed with lung cancer is only 19% [1]. Chemotherapy, among the traditional treatment options, has been extensively applied for lung cancer with the use of potent drugs intravenous (I.V.) delivered. Systemic delivery is associated with high toxicity through the body, high dosage demand, and low specificity in the delivery system. Pulmonary delivery has emerged as an attractive route of delivery of therapeutics to treat diseases such as lung cancer and

overcome some limitations seen in traditional I.V. delivery [2]. Aerosols often involve a non-invasive delivery system that uses lower doses of drug with fewer systemic side effects [3, 4].

Dry powder inhalers (DPIs) are preferred over other aerosols devices because it promises physical stability of the formulation in solid form, is portable and easy to use, and can be applied to a wide variety of active ingredients and dose weights [5, 6]. While big particles are easily removed from the airways, due to the lung phagocytosis is maximized at particles of 1-2 μ m in diameter [7], nanocarriers have the advantage of deeper deposition in the lungs, improved colloidal stability of hydrophobic drugs, and sustained release of the therapeutic, which will consequently reduce the dose frequency [8].

Particle deposition in the lungs is dependent on its size, morphology, density, among others [9]. However, the aerosol performance of the particles will be governed by its aerodynamic diameter [10]. Larger particles are deposited in the airways by impaction and sedimentation, while smaller particles deposit by diffusion (Brownian motion) [11, 12]. The optimal particle size for deep lung deposition has been established to have an aerodynamic diameter between 1 and 3 μ m [12]. In this regard, the NP deposition is limited because of the low inertia and high possibility to be exhaled. Aggregates of nanoparticles at a micro-size scale (i.e. nanocomposite microparticles) are thought to have enough mass to deposit effectively into the lungs and respond to impaction and sedimentation mechanisms. Once the micro-sized particles deposit in the airways, they dissociate back into nanoparticles [13, 14]. Nanocomposite microparticles (nCmP) can improve the handling and delivery of drugs due to the ability of appropriate

and tunable lung deposition while delivering NP with cargo upon arrival to the different regions in the lungs [15](Wang, 2017 Optimization).

Paclitaxel (PTX) is a microtubular stabilizer commonly used in cancer treatment, characterized by its high toxicity, low water solubility ($\sim 0.4 \mu\text{g/ml}$) and need to use excipients for its IV delivery [16]. The hydrophobicity nature of PTX limits its bioavailability and application, thus many companies are developing nanocarriers loaded with PTX, using polymeric micelles, liposomes, nanoemulsions [17].

The initial evaluations of therapeutics are assessed in a 2-dimensional (2D) monolayer of cells. However, in many cases this platform does not accurately represent the complex tumor morphology nor translate into similar results *in vivo* [18]. This misrepresentation may be due to the lack of dense extracellular matrix produced [19], or cell-particle interactions observed in real tumors [20, 21].

Cancer cells tend to form solid tumors that are characterized for having hypoxic cells and a necrotic core, which gives them high resistance to treatment. One reason for the failure of cancer treatments is the low concentration of drug in tumors due to transport and diffusion limitations [22]. Various techniques have been expanded to fabricate *in vitro* multicellular spheroids (MCS) [23-26], that at sizes over $200 \mu\text{m}$, develop an outer proliferating region, an intermediate quiescent region and a hypoxic necrotic core, representing better solid tumors *in vivo*. [27-29]. The 3D MCS are able to accurately mimic features such as spatial architecture, cell-cell signaling, physiological responses, gene expression patterns, and drug resistance mechanisms, which represent a bridge between *in vitro* cell culture and animal studies [30, 31].

On this project we developed, characterized, and evaluated PTX nanocomposite microparticles for the treatment of lung cancer. We hypothesized that nCmP will effectively deposit in the lungs and disaggregate to liberate NP that will be uptaken by the cells. We encapsulated PTX in Acetalated dextran (Ac-Dex), a biodegradable polymer, tunable polymer derived from dextran [32], via a single emulsion and solvent evaporation technique. PTX nanoparticles (PTX NP) were then spray dried with mannitol to form the nanocomposite microparticles that were characterized for lung deposition using the next generation impactor (NGI). The efficacy of the formulations was evaluated in a 2D cell culture study followed for 3D LLC for NP and 3D AIC platform for the evaluation of PTX nCmP. The dry powder formulation showed excellent aerodynamic performance and stability for broad and deep lung deposition, with a good re-dispersion and a comparable cytotoxicity to pure PTX, being a strong candidate for the current treatment of lung cancer. The 3D AIC platform developed may help in the early evaluation of dry powder formulations in a more accurate and representative manner.

4.2. Materials and Methods

4.2.1. Materials

Dextran from Leuconostic mesenteroides (MW 9,000-11,000), pyridinium p-toluenesulfonate (PPTS, 98%), anhydrous dimethyl sulfoxide (DMSO, $\geq 99.9\%$), 2-methoxypropene (2-MOP, 97%), triethylamine (TEA, $\geq 99\%$), deuterium chloride (DCI, 35 wt% in D₂O, 99% atom D) solution, dichloromethane (DCM, $> 99.8\%$),

anhydrous sodium acetate, D-mannitol ($\geq 98\%$), Triton^{TM} X-100, bisBenzimide H 33342 trihydrochloride (Hoechst 33342), methanol (HPLC grade, $\geq 99.9\%$), and acetic acid ($\geq 99\%$) were obtained from Sigma-Aldrich (Natick, MA, USA). Deuterium oxide (D_2O , 99.8% atom D), polyvinyl alcohol (PVA, 88% hydrolyzed, average MW 22,000), and curcumin (CUR, $> 98\%$) were obtained from Acros Organics (Geel, Belgium). Formic acid (FA, $\geq 99.5\%$), glycine ($\geq 98.5\%$), and Hydranal^{TM} Coulomat AG Honeywell were obtained from Fisher Scientific (Somerville, NJ, USA). Phosphate buffered saline 10X (PBS) was obtained from Research Products International (RPI) (Mount Prospect, IL, USA). Paclitaxel ($> 99.5\%$) was purchased from LC Laboratories (Woburn, MA, USA). A549 human lung adenocarcinoma epithelial cells and NCI-H441 (H441) lung papillary adenocarcinoma epithelial cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Fetal bovine serum (FBS) was obtained from Atlanta Biologics (Flowery Branch, GA, USA). RPMI 1640 1X with L-glutamine and 25 mM HEPES and collagen rat tail type I were obtained from Corning Inc. (Corning, NY, USA). Dulbecco's modified Eagle's medium (DMEM, 4.5 g/L d-glucose and L-glutamine), trypsin-EDTA (0.25%) phenol red, calcein AM, and ethidium homodimer-1 were obtained from Life Technologies^{TM} (Carlsbad, CA, USA). Sodium pyruvate, penicillin streptomycin, and Fungizone were obtained from GE Health Life Sciences (Pittsburgh, PA, USA).

4.2.2. Synthesis of acetalated dextran (Ac-Dex)

The synthesis of acetalated dextran (Ac-Dex) was conducted as previously described [33]. Briefly, lyophilized dextran (MW 9-11,000) and the catalyst PPTS were

dissolved in anhydrous DMSO under pure N₂ atmosphere. Dextran was reacted with 2-MOP for 5 minutes and the reaction was quenched with TEA. The product was then precipitated in basic water, vacuum filtered, frozen overnight, and lyophilized (-50°C, 0.03 mBar) for 48 hours to remove residual water.

4.2.3. Nuclear magnetic resonance (NMR) analysis of acetalated dextran (Ac-Dex)

The conversion of hydroxyl groups (total acetal coverage) and the cyclic acetal coverage (CAC) were determined by using ¹H-NMR spectroscopy (Bruker 300 MHz NMR, MA, USA). In an NMR tube, 10 mg of Ac-Dex was suspended in 700 µL of D₂O, and then hydrolyzed with 30 µL of DCl. The hydrolysis of acyclic acetals produces methanol and acetone whereas cyclic acetals produce only acetone. The total acetal coverage and the CAC were determined after normalization of the methanol and acetone peaks in relation to dextran (carbon rings). The normalization is associated with the number of protons detected on each molecule, and the results were expressed per 100 molecules of glucose.

4.2.4. Preparation of PTX-loaded Ac-Dex nanoparticles (PTX NP)

Paclitaxel-loaded nanoparticles (PTX NP) were prepared via a single emulsion and solvent evaporation method. 50 mg of PTX was dissolved in 1 mL of DMSO and 200 µL of this solution was added to 1800 µL of DCM. 1 mL of the PTX DCM solution was added to 95 mg of Ac-Dex (5 wt% PTX initial loading), forming the organic phase.

6 mL of 3% PVA in PBS (aqueous phase) was added to the organic phase and sonicated (Q500 Sonicator, Qsonica, Newton, CT, USA) for 60 seconds with 1 second on/off pulse at an amplitude of 70% (100% amplitude = 120 μ m). The resulting emulsion was transferred to a 40 mL spinning solution of 0.3% PVA in PBS and stirred for 4 hours to evaporate the organic solvent and stabilize the nanoparticles. The final solution was centrifuged at 12,000 rpm (19,802 g) and 4 °C for 20 minutes. The nanoparticles were washed twice with basic water, frozen, and lyophilized (-50°C, 0.03 mBar) for 24 hours.

For the preparation of fluorescent NP for imaging (CUR NP), 1 mg of curcumin (CUR, fluorescent reagent) was dissolved in 1 ml DCM and added to 39 mg of Ac-Dex (organic phase) and made similar to the steps described above. Blank NP were made following the same procedures as CUR NP and PTX NP, excluding any dye or therapeutic.

4.2.5. Formulation of PTX nanocomposite microparticles (PTX nCmP) via spray drying

Paclitaxel nCmP were formulated with PTX NP and mannitol in an aqueous suspension using a Büchi B-290 spray dryer (Büchi Labortechnik, AG, Switzerland) in open mode. The ratio of PTX NP: mannitol was 80:20 (w/w) in DI water, which was suspended at a feed concentration of 0.5% (w/v). The spray dryer conditions were: 414 L/h dry N₂ flow rate, aspiration rate of 28 m³/h, pump rate of 0.9 mL/min, inlet temperature of 90 °C, and nozzle cleaner rate of 4. The nCmP were collected in an amber glass vial and stored in desiccators at -20 °C.

4.2.6. Particle morphology and shape analysis via scanning electron microscopy (SEM)

The surface morphology and shape of the nanoparticles (NP) and nCmP were evaluated by scanning electron microscopy (SEM) with a Zeiss SIGMA VP Field Emission-SEM (Germany). NP were suspended in basic water at a concentration of 10 mg/ml, dropped onto aluminum SEM stubs, and dried at room temperature. nCmP samples were placed on SEM stubs covered by double-sided adhesive carbon tabs. Both types of samples were sputter coated with a thin film of gold/palladium alloy a BIO-RAD system at 20 μ A for 60 seconds under argon gas. Images were captured at 5 kV and at various magnifications.

4.2.7. Particle size, size distribution, and zeta potential analysis of NP and nCmP

The hydrodynamic size, size distribution (expressed by polydispersity index, PDI), and zeta potential of NP and re-dispersion NP from nCmP were measured by dynamic light scattering (DLS) using a Malvern Nano Zetasizer (Malvern Instruments, Worcestershire, UK). NP were suspended in basic water (0.5 mg/mL, pH 9), vortexed, and sonicated for one minute and measurements were performed at 25°C. The theoretical sizes of dry NP and nCmP were analyzed by measuring the diameter of at least 100 particles from SEM images using ImageJ (Systat, San Jose, CA, USA).

4.2.8. Analysis of PTX loading in PTX NP and PTX nCmP

Drug loading (DL) of PTX NP and nCmP was determined via ultra-performance liquid chromatography (UPLC, LaChrom, Hitachi, Japan). The detection of PTX was performed using an Ascentis column (C₁₈, 5 µm x 150 mm x 4.6 mm). The UPLC conditions were as follows: pump rate of 1 ml/min, absorbance of 227 nm, temperature of 25°C, 20 µL per injection, 4-minute retention time, and a mobile phase containing 60% acetonitrile and 40% DI water. PTX NP and PTX nCmP samples were completely dissolved in mobile phase (1 mg/mL) prior to analysis. The PTX concentration was quantified by comparison with a standard calibration curve of PTX in the mobile phase. The drug loading and encapsulation efficacy were calculated using the following equations:

$$\text{DL (wt\%)} = \frac{\text{mass of PTX loaded in NP}}{\text{total mass of particles}} \times 100$$

$$\text{EE (wt\%)} = \frac{\text{actual drug loading}}{\text{theoretical drug loading}} \times 100$$

$$\text{Theoretical drug loading (wt\%)} = \frac{\text{initial mass of PTX}}{\text{mass of PTX} + \text{mass of Ac} - \text{Dex}} \times 100$$

$$\text{Loading efficacy in nCmP (wt\%)} = \frac{\text{mass of PTX in nCmP}}{\text{initial mass of PTX in particles}} \times 100$$

4.2.9. *In vitro* PTX release from NP and nCmP

The *in vitro* release profiles of PTX from NP and nCmP were determined via a release study of suspended particles (1 mg/mL, 1.7 mL) in modified phosphate buffer (PBS, 0.1 M, pH 7.4, 0.5% wt% Tween 80) and modified acetate buffer (0.1 M, pH 5, 0.5% wt% Tween 80). The suspensions were incubated at 37°C and 100 rpm (Digital Heat Block and Orbi shaker, Benchmark Scientific, Edison, NJ, USA). At various time points (0-7 days), particle samples were centrifuged at 23,102 g for 5 minutes at 4°C to isolate the NP. 200 mL of supernatant was withdrawn and replaced by the same amount of fresh buffer. The supernatant samples were analyzed for PTX content by UPLC following the same method described previously.

4.2.10. Differential scanning calorimetry (DSC)

The thermal phase transitions of NP, nCmP, and raw materials were determined by DSC using a TA Q10 DSC system (TA instruments, New Castle, DE, USA) equipped with an automated computer-controlled cooling system. 1-3 mg of sample was weighed into Tzero™ alodine-coated aluminum pans, which were hermetically sealed. The sealed sample pan was placed in the DSC chamber along with an empty sealed reference pan, which were heated from 0-300 °C at a rate of 10 °C/min.

4.2.11. Powder X-ray diffraction (PXRD)

PXRD was applied to determine the crystalline nature of nCmP and its raw components using a Rigaku Multiflex X-ray diffractometer (The Woodlands, TX, USA)

with Cu K α radiation source (40KV, 44mA). The samples were placed in a horizontal slit holder prior to analysis. The scan range was 5-60° in 2 θ at a sampling width of 0.1 and scan speed of 1°/min.

4.2.12. Karl Fischer coulometric titration

The water content of nCmP was quantified by Karl Fischer (KF) coulometric titration using a 737 KF coulometer (Metrohm, Riverview, FL, USA). 10 mg of particles were dissolved in a known mass of anhydrous methanol and injected into the reaction cell filled with Hydranal® KF reagent. The water content was analyzed and reported in wt%. Pure solvent was analyzed as background sample.

4.2.13. *In vitro* aerosol dispersion performance with a Next Generation Impactor (NGI)

The *in vitro* aerosol dispersion of dry powder nCmP was evaluated using a Next Generation Impactor™ (NGI™, MSN Corporation, Shoreview, MN, USA) equipped with a stainless-steel induction port attachment (USP throat) and gravimetric NGI™ insert cups. The NGI was connected to a Copley HCP5 vacuum pump (Copley Scientific, United Kingdom). The flow rate was measured before every experiment and adjusted to 60 L/min, which models the flow rate of healthy adult lungs [34]. Glass fiber filters (55 mm, type A/E, Pall Life Sciences) were weighed and placed on the insert cups from stages 1 through 7 to reduce bounce or re-placement of powder [35]. 10 mg of powder was loaded into a hydroxypropyl methylcellulose capsule (HPMC, size 3,

Quali-V®, Qualicaps® Inc., Whitsett, NC, USA), and placed in a human dry powder inhaler (DPI) device (Handihaler, Boehringer Ingelheim Pharmaceuticals, CT, USA). The DPI was then attached to a rubber mouthpiece connected to the NGI. Three capsules were released in every experiment (for a total of 30 mg) in triplicate. The NGI was operated with a delay time of 10s and running time of 10s. The particle deposition on each stage was determined gravimetrically by the difference in mass of the glass filter before and after particle deposition. The effective cutoff diameters for each impaction stage, at Q=60 L/min, were given by the manufacturer as: Stage 1 (8.06 µm); Stage 2 (4.46 µm); Stage 3 (2.82 µm); Stage 4 (1.66 µm); Stage 5 (0.94 µm); Stage 6 (0.55 µm); and Stage 7 (0.34 µm). The fine particle dose (FPD), fine particle fraction (FPF), respirable fraction (RF), and emitted dose (ED) were calculated as follows:

Fine particle dose (FPD) = mass of particles < 4.4 µm (stages 2 through 7)

$$\text{Fine particle fraction (FPF)} = \frac{\text{fine particle dose}}{\text{initial particle mass loaded into capsules}} \times 100$$

$$\text{Respirable fraction (RF)} = \frac{\text{mass of particles} < 4.4 \text{ } \mu\text{m (stages 2 through 7)}}{\text{total particle mass on all stages}} \times 100$$

$$\text{Emitted dose (ED)} = \frac{\text{initial mass in capsules} - \text{final mass remaining in capsules}}{\text{initial mass in capsules}} \times 100$$

The experimental mass median aerodynamic diameter (MMAD_E) and geometric standard deviation (GSD) for the particles were determined using a Mathematica® program written by Dr. Warren Finlay [35].

4.2.14. Tapped density and theoretical aerodynamic size analysis of nCmP

The density of nCmP was determined via tapped density measurements [36]. 25-35 mg of particles (m) was weighed in a thin glass tube and tapped 200 times over a hard surface to ensure efficient packing. The height and diameter of compacted particles were measured using a digital caliper to calculate the volume occupied for the particles (V). The tapped density (ρ) was calculated using the equation:

$$\rho = \frac{m}{V}$$

The theoretical MMAD (MMAD_T) was calculated using the following equation:

$$MMAD_T = d \sqrt{\frac{\rho}{\rho^*}}$$

where d is the geometric diameter of nCmP determined by ImageJ and ρ* = 1 g/cm³ is the reference density of solid polymer.

4.2.15. *In vitro* cell studies

A549 human epithelial adenocarcinoma lung cancer cells were cultured in DMEM containing 10 v/v % FBS, 1 mM sodium pyruvate, 1 v/v % penicillin streptomycin (100 U/ml penicillin, 100 µg/ml streptomycin), and 0.2 v/v % Fungizone (0.5 µg/ml amphotericin B, 0.41 µg/ml sodium deoxycholate). H441 cells were cultured in RPMI L-glutamine media containing same additives, except sodium pyruvate. Cells were incubated at saturated humidity, 37 °C, and 5% CO₂.

4.2.15.1. Two-Dimensional (2D) cell culture and cell viability

The viability of A549 cells exposed to PTX and PTX NP was evaluated using a resazurin assay on 2D cell monolayers. Cells (5,000 cells/well) were seeded in a flat bottomed 96-well plate and allowed to attach for 24 hours. PTX was dissolved in DMSO and then serially diluted in media (final DMSO concentration < 0.2 v/v%). Cells were dosed with raw PTX and PTX NP concentrations from 0.001 - 10 µM PTX and incubated for 48 hours. Resazurin was added to the wells (20 µL, 20 mM) and incubated for 4 hours prior to analysis. The fluorescence intensity was detected at 544/590 nm in a Cytation 3 plate reader (BioTek Instruments Inc., Winooski, VT, USA). Viability was expressed as relative viability of cells dosed with PTX formulations over control cells.

4.2.15.2. NP cellular uptake

Confocal microscopy images were taken of A549 cells seeded on 35 mm glass bottom Petri dishes at a concentration of 500,000 cells/dish and incubated overnight.

Cells were exposed to 66 μ M CUR NP and incubated for 3 hours, washed 3X with PBS, stained with CellMask^{TM} deep red plasma membrane (0.5 μ L/mL in PBS), and fixed with 4% paraformaldehyde in PBS. Images were taken at 100X magnification. For quantification, A549 cells were seeded in flat bottomed 96-well plate (5,000 cell/well), incubated for 24 hours, and dosed with 66 μ M CUR NP. After 3 hours of incubation, cells were washed twice with 200 mM glycine in PBS, twice with cold PBS, and finally fixed with 200-proof ethanol for 10 minutes. NP uptake was quantified using a fluorescence scan at 420/540 nm.

4.2.15.3. Transepithelial electrical resistance (TEER) evaluation

Transepithelial electrical resistance was applied on an alveolar epithelium model cell line, H441 [37], to evaluate the integrity of the monolayer and tight junctions exposed to nCmP. 100,000 cell/well were seeded in the apical side of a 0.4 μ m Transwell and grown in liquid covered culture for two days. Air interface culture was induced when media was removed from the apical side of the Transwells. TEER values were measured periodically until a tight monolayer was formed (stable TEER values), and Blank and PTX nCmP (1 mg/dose) in powder were dosed to the cells (day 0). TEER readings continued for five days and treated samples were compared to control cells.

4.2.15.4. Treatment of 3D multicellular spheroids (MCS) in liquid covered culture

3D multicellular tumor spheroids (MCS) were formed via centrifugation using A549 cells for the evaluation of PTX formulations. 5,000 cells/well in media containing collagen type I (20 μ L/mL cell solution) were seeded into ultra-low attachment round-bottomed 96-well plates, centrifuged at 1100 g for 15 minutes, and incubated. At day 5, fully-formed MCS were dosed with raw PTX and PTX NP (5 – 0.001 μ M PTX equivalencies), Blank NP, DMSO, and media (control). Bright-field images, using a Cytation 3 imager (BioTek Instruments Inc., Winooski, VT, USA), were taken at different time points over 15 days, and the diameter of MCS were recorded. The growth or inhibition of MCS upon PTX exposure was analyzed by calculating the change in size in treated MCS relative to untreated controls.

4.2.15.5. Treatment of 3D multicellular spheroids in air interface culture

For the evaluation of PTX nCmP, cells were seeded into an alginate scaffold and grown in air interface culture (AIC) conditions. Alginate was purified as previously described [38] and stored in sterile conditions. 3 mL of 1.65 wt% alginate in PBS was loaded into a 5 mL syringe, and all bubbles were removed. In another 5 mL syringe, 750 μ L of 150 mM CaSO_4 in PBS containing 1% (v/v) Fungizone was collected. The syringes were connected and vigorously mixed back and forth 5-8 times. The resulting mixture was transferred into spheroid micromolds from 3D Petri Dishes® (5 x 7 array, 800 x 800 μ m, MicroTissue, Providence, RI, USA), excess solution was removed from

the top, and gelation occurred at room temperature for 15-20 minutes. Once the alginate scaffold had gelled, it was removed from the micromold and incubated overnight in DMEM prior to seeding. The Young's modulus of the alginate gels was determined using an Instron 3342 single-column apparatus (Norwood, MA) with a strain rate of 2 mm/min until hydrogel failure. The Young's modulus was calculated from the slope of the linear region of the resulting stress-strain curve using Instron's Bluehill 3 software package.

Alginate scaffold micromolds were placed in Transwell® inserts (3.0 μm pore size) for 12-well plates. 400 μL of DMEM was transferred to the basolateral compartment of the Transwell, and 75 μL of cell solution (1.33×10^6 cell/ml) was transferred to the scaffold (apical side). After MCS formation (day 1), media in the scaffold was gently removed to allow the MCS to grow in AIC conditions. Media from the basolateral side was replaced every other day. At day 5, ~ 1 mg of PTX nCmP was dosed onto the AIC MCS in powder form using a syringe and cone for dispersion. Images were taken periodically using the Cytation3 imager from days 1 to 12. The diameter of MCS treated with PTX nCmP were recorded and compared to untreated control.

4.2.16. Statistical analysis

All measurements were performed in at least triplicate and values were presented as mean $\pm$ standard deviation. Half maximal inhibitory concentration (IC_{50}) values were calculated using a symmetrical sigmoidal fit method in readerfit.com. The

statistical significance of the results was determined using Student's t-test, where $p < 0.05$ (*) was considered statistically significant.

4.3. Results

4.3.1. Characterization of acetalated dextran (Ac-Dex)

The synthesis of Ac-Dex resulted from the reaction between dextran and 2-MOP in the presence of an acid catalyst, and the resulting hydrophobic polymer contains both cyclic and acyclic acetal groups. NMR analysis of Ac-Dex showed a total acetal coverage (conversion of -OH groups) of 86% and a cyclic acetal coverage of 60%.

4.3.2. Nanoparticle characterization

4.3.2.1. Morphology, size, size distribution, and surface charge

The size, distribution, and surface charge of NP are presented in **Table 4.1**. For PTX NP and Blank NP, the hydrodynamic diameters were less than 200 nm with low PDI (> 0.14) and slightly negative charges. PTX NP were spherical, as well as smooth and uniform dispersion as shown in **Figure 4.1A**. ImageJ size analysis from the SEM micrographs indicated smaller NP sizes of around 100 and 150 nm for PTX NP and Blank NP, respectively.

Table 4.1 Characteristics of paclitaxel (PTX)-loaded nanoparticles (NP), blank NP without PTX, and PTX NP redispersed from PTX nanocomposite microparticles. Analysis includes NP diameter as measured by dynamic light scattering (DLS) and ImageJ, polydispersity index (PDI), zeta (ζ) potential, PTX loading in NP, and encapsulation efficiency (EE) (mean $\pm$ standard deviation, n = 3).

Particle System	Method	Diameter (nm)	PDI	ζ Potential (mV)	PTX Loading (μg PTX/mg NP)	EE (%)
PTX NP	DLS	198.4 $\pm$ 9.9	0.14 $\pm$ 0.04	-3.14 $\pm$ 0.55	35.73 $\pm$ 1.56	71.5 $\pm$ 3.1
	ImageJ	103.6 $\pm$ 19.3	N/A	N/A		
Blank NP	DLS	191.9 $\pm$ 3.5	0.12 $\pm$ 0.02	-4.79 $\pm$ 0.63	N/A	N/A
	ImageJ	150.3 $\pm$ 25.9	N/A	N/A		
CUR NP	DLS	182.1 $\pm$ 2.0	0.11 $\pm$ 0.03	-4.08 $\pm$ 0.25	14.73 $\pm$ 0.11 ^(*)	
Re-dispersed PTX NP	DLS	270.7 $\pm$ 11.0	0.34 $\pm$ 0.03	-18.47 $\pm$ 0.42	N/A	N/A

(*) μg CUR/mg NP

Figure 4.1 Representative scanning electron micrographs of nanoparticles (NP) and nanocomposite microparticles (nCmP) systems, and their *in vitro* paclitaxel (PTX) release profiles at different pH. (A) paclitaxel-loaded nanoparticles (PTX NP) at 20,000X, (B) paclitaxel nanocomposite microparticles (PTX nCmP) at 5,000X, and (C) PTX nCmP at 40,000X. (D) Comparative release profiles of PTX from PTX NP and PTX nCmP at pH 7.4 and pH 5.1 (n = 3).

4.3.2.2. PTX loading and *in vitro* release

PTX was encapsulated in Ac-Dex with an encapsulation efficiency of 71%, and the drug loading of PTX NP was 3.57 wt% (35.7 μ g PTX/mg NP) (**Table 4.1**). The

release profile of PTX NP (**Figure 4.1D**) demonstrated steady, sustained release of PTX, with burst release around 10% for both pH values. At pH 7.4, the PTX release was slower, reaching 47% release in 7 days. In contrast, at pH 5.1 PTX release was faster, reaching a plateau at 48 hours with around 70% PTX release.

4.3.3. Nanocomposite microparticle characterization

4.3.3.1. Morphology, size, surface charge, and re-dispersion

PTX nCmP exhibited wrinkled surfaces (**Figure 4.1B**), and formed raisin-like particles with visible nanoparticles (**Figure 4.1C**). The geometric diameter of nCmP, determined using ImageJ, was 2.17 μm (Table 2). PTX NP re-dispersed from PTX nCmP exhibited a slight increase in size to 270.6 nm, with a slightly higher PDI of 0.33, and a slightly more negative charge (-18.5 mV) (**Table 4.1**).

4.3.3.2. Drug loading, loading efficacy, and *in vitro* release from nCmP

PTX nCmP had a drug loading of 3.04 wt% (30.38 μg PTX/mg of nCmP), corresponding to a PTX loading efficacy of 105 % in nCmP (**Table 4.2**). PTX nCmP exhibited sustained release of PTX at both pH values, with accelerated release in acidic conditions similar to PTX NP (**Figure 4.1D**).

Table 4.2 Characteristics of paclitaxel (PTX)-loaded nanocomposite microparticles (nCmP), including water content, PTX loading, nanoparticle (NP) loading in nCmP, NP loading efficacy in nCmP, geometric diameter (via Image J), tapped density, theoretical mass median aerodynamic diameter (MMAD_T), experimental MMAD (MMAD_E), and geometric standard deviation (GSD) (mean ± standard deviation, n=3).

Particle System	Water Content (%)	PTX Loading (µg PTX/mg nCmP)	NP Loading in nCmP (%)	NP Loading Efficacy in nCmP (%)
PTX nCmP	2.78 ± 0.50	30.38 ± 0.80	85.01 ± 2.24	106.27 ± 2.80

Particle System	Geometric diameter (µm)	Tapped Density (g/cm ³)	MMAD _T (µm)	MMAD _E (µm)	GSD (µm)
PTX nCmP	2.17 ± 0.59	0.33 ± 0.01	1.25 ± 0.01	2.44 ± 0.25	2.34 ± 0.42

4.3.4. Karl Fischer titration

The residual water content of PTX nCmP was lower than 3% (Table 4.2), determined via colorimetric Karl Fischer Titration.

4.3.5. Solid-state analysis of particle formulations

The DSC thermograms of raw materials and nCmP are shown in Figure 4.2A. Raw paclitaxel exhibited an endothermic phase transition peak at 222 °C and an exothermic peak at 243 °C. Raw Ac-Dex presented a broad endothermic peak around 183°C and raw mannitol displayed a main phase transition peak at 171°C. Blank and PTX nCmP showed similar broad peaks around 158 and 188 °C and no phase transitions

were observed for any samples under 150 °C. **Figure 4.2B** presents powder X-ray diffractograms of raw materials and nCmP. Raw Ac-Dex presented no peaks, and raw PTX and mannitol exhibited strong peaks. After spray drying, PTX nCmP exhibited no peaks.

Figure 4.2 (A) Differential scanning calorimetry (DSC) thermograms and (B) powder X-ray diffractograms (PXRD) of raw paclitaxel (PTX), raw acetalated dextran (Ac-Dex), raw mannitol, blank nanocomposite microparticles (nCmP) and paclitaxel-loaded nCmP (PTX nCmP).

4.3.6. Density and theoretical MMAD of nCmP

The tapped density and the theoretical mass median aerodynamic diameter (MMAD_T) of PTX nCmP are shown in **Table 4.2**. The corresponding values were 0.33 g/cm³ and 1.25 ± 0.01 µm.

4.3.7. *In vitro* aerosol performance of nCmP

The *in vitro* aerosol dispersion of PTX nCmP, evaluated in an NGI™, showed broad aerosol dispersion of particles across all stages, with 3% of the total mass depositing on the first stage, corresponding to a cutoff of 8.1 µm (**Figure 4.3A**). The majority of nCmP deposited on stage 4 (1.7 µm, 35%), with 4% depositing on at stage 7 (0.34 µm). The experimental MMAD was 2.44 µm, with a GSD of 2.34 µm (**Table 4.2**). The FPF, RF and ED were 66, 97, and 88%, respectively (**Figure 4.3B**).

Figure 4.3 *In vitro* aerosol dispersion performance of paclitaxel-loaded nanocomposite microparticles (PTX nCmP) in a Next Generation Impactor^{TM} (NGI^{TM}) for Q = 60 L/min. (A) Particle deposition on each stage of the NGI, (B) Fine particle fraction (FPF), respirable fraction (RF) and emitted dose (ED) of PTX nCmP (Mean $\pm$ standard deviation, n=3).

4.3.8. *In vitro* cell analysis of PTX NP and PTX nCmP

4.3.8.1. *In vitro* cytotoxicity

A 2D monolayer of A549 cells exposed to raw PTX and PTX NP exhibited a IC_{50} values of 0.23 μ M and 0.07 μ M PTX NP, respectively, after 48 hours of exposure (Figure 4.4A).

Figure 4.4 Two-dimensional (2D) cell culture evaluations including, (A) Cell viability and IC_{50} values of A549 cells dosed with raw paclitaxel (PTX) and paclitaxel-loaded

NP (PTX NP) evaluated at 48 hours. (B) Cellular uptake of fluorescent curcumin-loaded nanoparticles (CUR NP) in A549 cells following 3 hours of incubation, including a representative confocal image showing CUR NP (green) and cell membrane (red), scale bar = 20 μ m (Left) and fluorescence quantification of uptake (Right). (C) Transepithelial electrical resistance (TEER) analysis performed on H441 cells in air interface culture (AIC) conditions after exposure to blank and paclitaxel-loaded nanocomposite microparticles (Blank nCmP and PTX nCmP, respectively). Statistical analysis was performed using Student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n \geq 3$, which represents the comparison to control (no exposure to nCmP)

4.3.8.2. Cellular uptake of CUR NP

The cellular uptake of NP into 2D A549 cells was evaluated using fluorescent CUR NP with similar size, PDI, and surface charge to PTX NP. **Figure 4.4B** shows a representative confocal image of CUR NP associated in the cytoplasm of A549 cells, and the quantification of CUR NP uptake into the cells based on the fluorescence intensity of NP compared to control (cells only) after three hours of exposure. Overall, more CUR NP were successfully uptaken into A549 cells.

4.3.8.3. Transepithelial electrical resistance (TEER) evaluation

The effect of PTX and Blank nCmP on cell monolayer integrity in air interface culture (AIC) conditions was evaluated via transepithelial electrical resistance (TEER). H441 cells were initially seeded in liquid covered culture (LCC) conditions prior to

transitioning to AIC. The cells reached TEER values above $100 \Omega \text{ cm}^2$ a day after their transition from LCC to AIC conditions, and these values remained stable until analysis began (**Figure 4.4C**). During the first two days after exposure to the nCmP, cells exposed to Blank nCmP exhibited increased relative TEER, which then decreased slightly and was statistically the same as the control TEER. Cells exposed to PTX nCmP showed no statistical difference in TEER during the 5-day evaluation in comparison to the control.

4.3.8.4. LCC MCS growth inhibition evaluation

3D A549 multicellular spheroids (MCS) in LCC dosed with raw PTX and PTX NP exhibited a dose dependent response in the inhibition of the growth of MCS in comparison to the control (**Figure 4.5**). In particular, MCS experienced growth inhibition when exposed to PTX concentrations equal to or above $0.01 \mu\text{M}$. Figure 5C shows the comparison in the change of MCS diameter with respect to the control. Between 0.01 and $0.5 \mu\text{M}$ PTX MCS had similar responses to free PTX and PTX, where MCS size decreased with increasing PTX concentration. At higher concentrations ($\geq 1 \mu\text{M}$), MCS exposed to free PTX reached a plateau where size no longer decreased, and MCS exposed to PTX NP continued to decrease in size.

Figure 4.5 Analysis of multicellular spheroid (MCS) growth upon exposure to different concentrations of paclitaxel (PTX) formulations, including: (A) free PTX and (B) PTX NP. (C) Comparison of the change in diameter relative to control (media only) upon exposure to free PTX and PTX NP at day 15. Statistical analysis was performed using Student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, which represents comparison with control (no exposure to PTX or PTX NP) in (A) and (B), and comparison between PTX versus PTX NP in (C); $n \geq 3$.

Figure 4.6 shows representative images of MCS dosed with 1 μ M PTX at different time points, and their corresponding sizes. For days 1 and 5, the size of the MCS exposed to free PTX and PTX NP were similar to the control, whereas by day 15, MCS size decreased significantly upon exposed to free PTX and PTX NP. The effect of Blank NP on MCS growth was also evaluated, showing no significant difference to control MCS responses, with the exception of being slightly larger than the control MCS 15 days after exposure (**Figure 6 - Right**).

Figure 4.6 (Left) Representative bright-field images (scale bar = 500 nm) and (Right) corresponding quantification of A549 multicellular spheroid (MCS) growth upon exposure to 1 μ M of free PTX, PTX nanoparticles (NP), and Blank NP equivalent to 1 μ M of NP in mass. Control MCS were exposed to media only. Statistical analysis was performed using Student's t-test. * p < 0.05, ** p < 0.01, *** p < 0.001; n $\geq$ 3, which represents the comparison to control, no exposure to PTX or NP)

4.3.8.5. AIC MCS growth inhibition evaluation

The alginate micromold scaffolds exhibited a Young's modulus of 3.55 ± 0.59 kPa after 24 hours of incubation in DMEM. A549 cells seeded in the micromold were transitioned to AIC a day after seeding in LCC. At day 5, the spheroids were compact and fully formed (Figure 7A), and thus AIC MCS were exposed to nCmP at this time. Control MCS experienced continuous growth in AIC. After dosing with nCmP the surface of the MCS showed gradual disaggregation and tumor size reduction in comparison to control MCS (**Figure 4.7B**).

Figure 4.7 Analysis of A549 multicellular spheroids (MCS) growth upon exposure to paclitaxel-loaded nanocomposite microparticles (PTX nCmP) in an 3D air interface cell culture (AIC) platform (scale bar = 500 μ m). (A) Representative bright-field images of control and MCS exposed to PTX nCmP. (B) Quantification of MCS exposed to PTX nCmP in comparison to control (media only). Statistical analysis was performed using Student's t-test ($p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n \geq 3$, which represent the comparison to control, no exposure to PTX nCmP)

4.4. Discussion

Water-soluble dextran was transformed to hydrophobic Ac-Dex during the 5-min reaction, where the -OH groups were converted into acetals. Ac-Dex had a high total -OH conversion (86%) that can ensure the stability of particles and allow for the formation of small NP (when >75%) [36]. The formation of acetals, quantified by -OH conversion and cyclic acetal conversion (60%), allows the NP to have pH-tunable degradation for drug delivery into acidic tumors or inflammatory tissues [39].

The small size of PTX NP reported from the SEM micrographs and ImageJ (104 nm) represent NP size in their dry state. The hydrodynamic size of PTX NP was evaluated with DLS, showing sizes around 200 nm; this technique allows measurement of NP size with a hydrated layer. PTX NP had a low PDI, resulting in high stability in an aqueous suspension due to the steric effect of PVA coating [40]. SEM images of NP corroborates the high homogeneity reached with single emulsion fabrication. After spray drying, NP re-dispersed from nCmP showed a 35% increase in hydrodynamic

diameter, likely due to agglomeration during the spray drying process, which is confirmed by a higher PDI (0.33). The slightly more negative charge of re-dispersed PTX NP can be attributed to mannitol in the formulation, which showed a charge of around -19.23 mV in water solution, compared to the almost neutrally-charged PTX NP.

The *in vitro* PTX release profiles of the NP and nCmP were evaluated in physiological (pH 7.4) and acidic (pH 5.1) conditions. The systems exhibited burst releases below than 13%, which is an essential characteristic for optimal extended-release delivery systems [41]. The release profiles at each pH were similar between the NP and nCmP. In acidic conditions, 70-80% of PTX was released after 48 hours, which can be favorable for the release of the drug in the acidic microenvironments of cancer cells. At pH 7.4, PTX release was slower, reaching 50% release after 7 days, which may be beneficial to avoid high concentrations of chemotherapeutic when the particles deposit in healthy tissue.

PTX NP exhibited high PTX encapsulation efficiency (71% corresponding to 35.7 μg PTX/mg NP), and after spray drying the PTX loading in nCmP was 30.4 μg PTX/mg nCmP, which corresponds to a NP loading efficiency in nCmP above 100%. This phenomenon is likely due to the loss of PVA from the NP during the spray drying process, which leads to an increase in the percentage of PTX in the total mass of particles. Overall, both the NP and nCmP successfully encapsulated PTX. The low water content in PTX nCmP, determined using colorimetric Karl Fisher, can enable storage stability of the particles, and can prevent agglomeration during aerosolization.

DSC analysis was performed for raw materials and nCmP to evaluate their stability and phase transition behavior. The lack of an endothermic peak at 222°C and

decomposition peak at 243°C (evident for raw PTX) in PTX nCmP suggests that amorphization of the drug occurred during spray drying or that the amount of PTX in the nCmP was below the detection limit for DSC. The strong peak from pure mannitol was slightly shifted for the nCmP, appearing around 158°C. The other broad peak present for nCmP is likely due to displacement of the Ac-Dex phase transition peak. Overall, nCmP showed temperature stability under 150°C for manufacturing and storage conditions.

The crystallinity of raw materials and nCmP were analyzed by PXRD. The lack of peaks in the Ac-Dex diffractogram indicate that it is an amorphous polymer, which likely because of the rapid precipitation method of collection after the Ac-Dex reaction. While raw PTX and mannitol exhibited strong peaks and are thus crystalline, these peaks are no longer present in nCmP. This indicates the likelihood of amorphization of PTX and mannitol after the spray drying process. These data agree with the reduced and transformed peaks observed in DSC analysis. Reduced crystallinity of the nCmP formulation is advantageous for the rapid solubilization of nCmP formulations (and thus PTX) after delivery to the lungs.

The *in vitro* aerosol performance of PTX nCmP was conducted using a Next Generation Impactor™ coupled with a human DPI device. The simulated inhalation resulted in a high emitted dose of particles (88%), which is necessary to make use of the most of each capsule. The fine particle fraction of PTX nCmP demonstrates that 66% of the nCmP mass released from the capsules had a particle size lower than 4.4 µm, and that 97% of the particles deposited in the NGI (respirable fraction) demonstrate the potential for effective deposition of particles to the bronchi and distal airways. nCmP

that deposited on the first stage (3%) deposited primarily by impaction ($\geq 5 \mu\text{m}$) [12]. Particles that deposited in stage 2 to 6, corresponding to 93% to the total mass in all stages of the NGI, would likely be able to deposit in the lungs due to sedimentation, and the smaller particles ($< 0.5 \mu\text{m}$) from stage 7 would likely deposit via Brownian motion [11]. The high dispersion of nCmP (GSD of $2.34 \mu\text{m}$) demonstrate the ability of nCmP to deposit in all stages of the NGI.

The theoretical mass median aerodynamic diameter (MMAD_E) was calculated from the geometric diameter from SEM micrographs and tapped density of the PTX nCmP, and the MMAD_E ($1.25 \mu\text{m}$) was smaller than the experimental MMAD ($2.44 \mu\text{m}$) obtained using a NGI. The increase in MMAD during aerosolization in the NGI is likely due to agglomeration of the particles due to ambient humidity and the electrostatic effect of particles with each other and the DPI device [42], which can increase the deposition in the upper airways [43]. The deposition of the PTX nCmP in lower stages was likely achieved because of their low tapped density, which can be attributed to the raisin-like morphology of the nCmP. The hollow shell morphology of the nCmP can be attributed to the saturation of NP at the droplet surface compared to the droplet center, where NP diffusion is slower than the surface shrinkage during spray drying. As the evaporation process occurs, the droplet shell collapses and wrinkled particles with low density are formed [44], as observed in the PTX nCmP.

The cytotoxicity of raw PTX and PTX NP was evaluated in LCC 2D and 3D cell culture models. 2D A549 cells were dosed with raw PTX and PTX NP and there was a 70% reduction in the IC_{50} value for PTX NP compared to raw NP after 48 hours of

exposure, suggesting that the sustained release of the drug plays a role in the enhancing cell death [41].

For cellular uptake, CUR NP were selected for their similar size and charge to PTX NP and the ease in imaging fluorescent CUR. Since NP surface charge is nearly neutral, the main parameter that can influence NP internalization in cells is their size. It was previously reported that 100-200 nm polymeric NP might be the optimal size for the highest uptake in cells and to avoid alveolar macrophage clearance [45]. CUR NP were successfully uptaken into the A549 cytoplasm after three hours of exposure, indicating the NP have the potential to deliver and release PTX directly inside the cells.

In order to determine the effects of Blank and PTX nCmP on the lung epithelium, we evaluated the integrity of the tight junctions of a monolayer of H441 cells upon exposure to nCmP via transepithelial electrical resistance (TEER) measurements. H441 cells were grown in a 2D monolayer as a human distal lung epithelial model [46], and following growth in AIC, were dosed with nCmP powders. Cells exposed to Blank nCmP showed increased TEER two days after exposure, and after a decrease on day 3, they showed no difference to control cells. The increase in TEER upon Blank nCmP exposure is likely due to exposure of the cells to the by-product dextran following the degradation of Ac-Dex as nutrient for the cells. Cells exposed to PTX nCmP did not show significant reduction in TEER for up to 5 days of exposure, suggesting a conservation in the tight junctions in the monolayer of cells and potential safety of the formulation.

While 2D cell culture has been extensively used for drug screening, a 3D multicellular spheroid (MCS) platform offers a better representation of solid tumors *in*

vitro due to their similar structures, physiological responses, cell-drug interactions, and drug resistance mechanisms [47, 48]. MCS grown in a 3D LCC model were dosed with free PTX and PTX NP 5 days after MCS formation. MCS at this point were considered to be fully developed spheroids with heterogenous structure: a proliferating region, a quiescent viable region, and a necrotic core. This phenomenon has been confirmed in our previous work and is in accordance with other reports where MCS > 200 μ m (usually > 4 days of growth) have shown similar morphology to real tumors [26, 28]. MCS growth lasted for 15 days, since after this point control MCS size begins to decline.

PTX NP showed no clear superiority in the inhibition of MCS growth in comparison to free PTX, except at higher concentrations ($\geq 1 \mu$ M), implying that PTX NP were capable of delivering PTX more effectively to and/or penetrating MCS, in comparison to free PTX. The likelihood of limited penetration in the MCS is supported by the fact that solid tumors develop a dense extracellular matrix (ECM) that prevents the transport and diffusion of large molecules into the tumor interstitial space [49, 50]. Collagen, one of the main components in ECM, is known to increase the resistance to interstitial transport in tumors [51]. The LCC MCS produced via centrifugation for this project contained type I collagen to facilitate the agglomeration of cells and reproduce ECM in the MCS. The formation of the solid external cell layer in the spheroids was visible in the bright-field images when they changed from loose aggregates of cells to solid tumor spheroids from day 1 to day 5 (**Figure 4.6**). Finally, Blank NP showed no toxic effects or alterations in the normal growth of the MCS, likely due to the MCS exposure to the harmless degradation by-products of Ac-Dex, which can cause an increase in MCS size as they use dextran as nutrients.

To demonstrate proof-of-concept treatment of lung cancer via aerosol delivery of PTX-loaded dry powders, PTX NP were spray dried with mannitol to obtain (nCmP), which are designed to be delivered using a dry powder inhaler. To evaluate the chemotherapeutic effect of the particles, MCS grown in an alginate micromold at air interface culture (AIC) conditions were dosed with dry powder PTX nCmP. The alginate scaffold exhibited similar stiffness to that of lung tissue, which has a Young's modulus between 1 and 5 kPa [52]. 5 days after a treatment, the MCS size decreased significantly, and this reduction and disaggregation of cells were signal of cell death promoted by PTX delivered from the NP. AIC MCS were developed and characterized in our previous work [38]. For this project, PTX nCmP were evaluated using AIC MCS, and this cell culture platform can serve for future evaluations of dry powder formulations as a more accurate representation of pulmonary conditions. Overall, PTX nCmP show promise in the treatment of lung cancer via a pulmonary route. PTX nCmP have the potential to reduce the total dose administered and reach the targeted site of the disease in comparison to systemic delivery.

4.5. Conclusions

We developed a paclitaxel-loaded aerosol nanocomposite microparticle (PTX nCmP) formulation based on spray-dried Ac-Dex PTX-loaded NP and mannitol. PTX nCmP exhibited favorable distribution and deposition in *in vitro* aerosolization using an NGI, with a size and morphology that can allow them to reach the alveoli region. NP were successfully re-dispersed from nCmP upon contact to aqueous medium. The physical properties of the NP allow for effective cellular uptake in A549 cells. 2D cells

were highly affected by PTX NP is comparison to free PTX, however, this correlation was not seen in the 3D LCC model. In this case, PTX NP impacted MCS growth inhibition more significantly only at high PTX concentrations when compared to free PTX. This phenomenon demonstrates the complex interaction of nanoparticles with MCS. The 3D AIC platform aided in the assessment of PTX nCmP, showing its potential as an *in vitro* model to better mimic pulmonary environments and promoting its further application in the evaluation of other dry powder formulations intended to be delivered via a pulmonary route. Overall, the described work involves a new nCmP platform capable of effectively delivery PTX to lungs via their aerosolization as a dry powder.

4.6. Acknowledgements

The authors thank RIN² for SEM and XRD access. Research reported in this publication was partially supported by the Institutional Development Award (IDeA) Network for Biomedical Research Excellence from the National Institute of General Medical Sciences of the National Institutes of Health under grant number P20GM103430. In addition, this work was made possible by the use of equipment and services available through the RI-INBRE Centralized Research Core Facility. This material is based upon work conducted at a Rhode Island NSF EPSCoR research facility, supported in part by the National Science Foundation EPSCoR Cooperative Agreement #EPS-1004057. This material is also based in part upon work supported by the National Science Foundation under grant #1508868. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and

do not necessarily reflect the view of the National Science Foundation or National Institutes of Health.

REFERENCES

1. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2019*. CA Cancer J Clin, 2019. **69**(1): p. 7-34.
2. Mansour, H.M., Y.S. Rhee, and X. Wu, *Nanomedicine in pulmonary delivery*. Int J Nanomedicine, 2009. **4**: p. 299-319.
3. Patil, J.S. and S. Sarasija, *Pulmonary drug delivery strategies: A concise, systematic review*. Lung India, 2012. **29**(1): p. 44-9.
4. Labiris, N.R. and M.B. Dolovich, *Pulmonary drug delivery. Part I: physiological factors affecting therapeutic effectiveness of aerosolized medications*. Br J Clin Pharmacol, 2003. **56**(6): p. 588-99.
5. Ali, M., *CHAPTER 9 - Pulmonary Drug Delivery*, in *Handbook of Non-Invasive Drug Delivery Systems*, V.S. Kulkarni, Editor. 2010, William Andrew Publishing: Boston. p. 209-246.
6. Abdelaziz, H.M., et al., *Inhalable particulate drug delivery systems for lung cancer therapy: Nanoparticles, microparticles, nanocomposites and nanoaggregates*. J Control Release, 2018. **269**: p. 374-392.
7. Hadinoto, K., et al., *Dry powder aerosol delivery of large hollow nanoparticulate aggregates as prospective carriers of nanoparticulate drugs:*

- Effects of phospholipids*. International Journal of Pharmaceutics, 2007. **333**(1–2): p. 187-198.
8. van Rijt, S.H., T. Bein, and S. Meiners, *Medical nanoparticles for next generation drug delivery to the lungs*. European Respiratory Journal, 2014. **44**(3): p. 765.
  9. Fernández Tena, A. and P. Casan Clarà, *Deposition of Inhaled Particles in the Lungs*. Archivos de Bronconeumología (English Edition), 2012. **48**(7): p. 240-246.
  10. Laube, B.L., et al., *What the pulmonary specialist should know about the new inhalation therapies*. European Respiratory Journal, 2011. **37**(6): p. 1308.
  11. Ngan, C.L. and A.A. Asmawi, *Lipid-based pulmonary delivery system: a review and future considerations of formulation strategies and limitations*. Drug Deliv Transl Res, 2018. **8**(5): p. 1527-1544.
  12. Yang, W., J.I. Peters, and R.O. Williams, *Inhaled nanoparticles—A current review*. International Journal of Pharmaceutics, 2008. **356**(1): p. 239-247.
  13. Paranjpe, M. and C.C. Muller-Goymann, *Nanoparticle-mediated pulmonary drug delivery: a review*. Int J Mol Sci, 2014. **15**(4): p. 5852-73.
  14. Zhang, J., et al., *Formation, characterization, and fate of inhaled drug nanoparticles*. Advanced Drug Delivery Reviews, 2011. **63**(6): p. 441-455.
  15. Wang, Z. and S.A. Meenach, *Optimization of Acetalated Dextran-Based Nanocomposite Microparticles for Deep Lung Delivery of Therapeutics via Spray-Drying*. J Pharm Sci, 2017. **106**(12): p. 3539-3547.

16. Jiménez-López, J., et al., *A novel nanoformulation of PLGA with high non-ionic surfactant content improves in vitro and in vivo PTX activity against lung cancer*. Pharmacological Research, 2019. **141**: p. 451-465.
17. Sofias, A.M., et al., *The battle of “nano” paclitaxel*. Advanced Drug Delivery Reviews, 2017. **122**: p. 20-30.
18. Xu, X., M.C. Farach-Carson, and X. Jia, *Three-dimensional in vitro tumor models for cancer research and drug evaluation*. Biotechnol Adv, 2014. **32**(7): p. 1256-68.
19. Senthebane, D.A., et al., *The Role of Tumor Microenvironment in Chemoresistance: 3D Extracellular Matrices as Accomplices*. International journal of molecular sciences, 2018. **19**(10): p. 2861.
20. Septiadi, D., et al., *Nanoparticle–Cell Interaction: A Cell Mechanics Perspective*. Advanced Materials, 2018. **30**(19): p. 1704463.
21. Varan, G., et al., *Cellular Interaction and Tumoral Penetration Properties of Cyclodextrin Nanoparticles on 3D Breast Tumor Model*. Nanomaterials (Basel, Switzerland), 2018. **8**(2): p. 67.
22. Minchinton, A.I. and I.F. Tannock, *Drug penetration in solid tumours*. Nat Rev Cancer, 2006. **6**(8): p. 583-92.
23. Sant, S. and P.A. Johnston, *The production of 3D tumor spheroids for cancer drug discovery*. Drug Discovery Today: Technologies, 2017. **23**: p. 27-36.
24. Katt, M.E., et al., *In Vitro Tumor Models: Advantages, Disadvantages, Variables, and Selecting the Right Platform*. Frontiers in bioengineering and biotechnology, 2016. **4**: p. 12-12.

25. Goodman, T.T., C.P. Ng, and S.H. Pun, *3-D tissue culture systems for the evaluation and optimization of nanoparticle-based drug carriers*. Bioconjugate chemistry, 2008. **19**(10): p. 1951-1959.
26. Nath, S. and G.R. Devi, *Three-dimensional culture systems in cancer research: Focus on tumor spheroid model*. Pharmacol Ther, 2016. **163**: p. 94-108.
27. Hamilton, G., *Multicellular spheroids as an in vitro tumor model*. Cancer Letters. **131**(1): p. 29-34.
28. Mehta, G., et al., *Opportunities and Challenges for use of Tumor Spheroids as Models to Test Drug Delivery and Efficacy*. J Control Release, 2012. **164**(2): p. 192-204.
29. Knight, E. and S. Przyborski, *Advances in 3D cell culture technologies enabling tissue-like structures to be created in vitro*. Journal of Anatomy, 2015. **227**(6): p. 746-756.
30. Zanoni, M., et al., *3D tumor spheroid models for in vitro therapeutic screening: a systematic approach to enhance the biological relevance of data obtained*. Scientific Reports, 2016. **6**: p. 19103.
31. Costa, E.C., et al., *3D tumor spheroids: an overview on the tools and techniques used for their analysis*. Biotechnology Advances, 2016. **34**(8): p. 1427-1441.
32. Broaders, K.E., et al., *Acetalated dextran is a chemically and biologically tunable material for particulate immunotherapy*. Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(14): p. 5497-5502.

33. Wang, Z. and S.A. Meenach, *Synthesis and Characterization of Nanocomposite Microparticles (nCmP) for the Treatment of Cystic Fibrosis-Related Infections*. Pharm Res, 2016. **33**(8): p. 1862-72.
34. Mahler, D.A., *Peak Inspiratory Flow Rate as a Criterion for Dry Powder Inhaler Use in Chronic Obstructive Pulmonary Disease*. Annals of the American Thoracic Society, 2017. **14**(7): p. 1103-1107.
35. Meenach, S.A., et al., *Design, physicochemical characterization, and optimization of organic solution advanced spray-dried inhalable dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylethanolamine poly(ethylene glycol) (DPPE-PEG) microparticles and nanoparticles for targeted respiratory nanomedicine delivery as dry powder inhalation aerosols*. International Journal of Nanomedicine, 2012. **8**: p. 275-293.
36. Wang, Z., et al., *Nanocomposite microparticles (nCmP) for the delivery of tacrolimus in the treatment of pulmonary arterial hypertension*. Int J Pharm, 2016. **512**(1): p. 305-13.
37. Ren, H., N.P. Birch, and V. Suresh, *An Optimised Human Cell Culture Model for Alveolar Epithelial Transport*. PloS one, 2016. **11**(10): p. e0165225-e0165225.
38. Gupta, S.K., E.A. Torrico Guzman, and S.A. Meenach, *Coadministration of a tumor-penetrating peptide improves the therapeutic efficacy of paclitaxel in a novel air-grown lung cancer 3D spheroid model*. Int J Cancer, 2017.

39. Gillies, E.R., A.P. Goodwin, and J.M.J. Fréchet, *Acetals as pH-Sensitive Linkages for Drug Delivery*. Bioconjugate Chemistry, 2004. **15**(6): p. 1254-1263.
40. Moore, T.L., et al., *Nanoparticle colloidal stability in cell culture media and impact on cellular interactions*. Chem Soc Rev, 2015. **44**(17): p. 6287-305.
41. Kalaydina, R.-V., et al., *Recent advances in "smart" delivery systems for extended drug release in cancer therapy*. International journal of nanomedicine, 2018. **13**: p. 4727-4745.
42. Karner, S. and N. Anne Urbanetz, *The impact of electrostatic charge in pharmaceutical powders with specific focus on inhalation-powders*. Journal of Aerosol Science, 2011. **42**(6): p. 428-445.
43. Koullapis, P.G., et al., *Particle deposition in a realistic geometry of the human conducting airways: Effects of inlet velocity profile, inhalation flowrate and electrostatic charge*. Journal of Biomechanics, 2016. **49**(11): p. 2201-2212.
44. Vehring, R., *Pharmaceutical particle engineering via spray drying*. Pharmaceutical Research, 2008. **25**(5): p. 999-1022.
45. Win, K.Y. and S.S. Feng, *Effects of particle size and surface coating on cellular uptake of polymeric nanoparticles for oral delivery of anticancer drugs*. Biomaterials, 2005. **26**(15): p. 2713-22.
46. Salomon, J.J., et al., *The Cell Line NCI-H441 Is a Useful in Vitro Model for Transport Studies of Human Distal Lung Epithelial Barrier*. Molecular Pharmaceutics, 2014. **11**(3): p. 995-1006.

47. Galmarini, C.M., et al., *Drug Resistance and the Solid Tumor Microenvironment*. JNCI: Journal of the National Cancer Institute, 2007. **99**(19): p. 1441-1454.
48. Lv, D., et al., *Three-dimensional cell culture: A powerful tool in tumor research and drug discovery*. Oncology letters, 2017. **14**(6): p. 6999-7010.
49. Stylianopoulos, T., L.L. Munn, and R.K. Jain, *Reengineering the Physical Microenvironment of Tumors to Improve Drug Delivery and Efficacy: From Mathematical Modeling to Bench to Bedside*. Trends in Cancer, 2018. **4**(4): p. 292-319.
50. Tannock, I.F., et al., *Limited Penetration of Anticancer Drugs through Tumor Tissue*. Clinical Cancer Research, 2002. **8**(3): p. 878.
51. Ramanujan, S., et al., *Diffusion and convection in collagen gels: implications for transport in the tumor interstitium*. Biophysical journal, 2002. **83**(3): p. 1650-1660.
52. Liu, F., et al., *Distal vessel stiffening is an early and pivotal mechanobiological regulator of vascular remodeling and pulmonary hypertension*. JCI Insight, 2016. **1**(8).

CHAPTER 5

Development and characterization of aerosol nanocomposite microparticles capable of mucus penetration

Manuscript in preparation for the submission to:

Colloids and Surfaces B: Biointerfaces

Elisa A. Torrico Guzmán¹, Anthony Brouillard¹, Samantha A. Meenach^{1,2}

¹University of Rhode Island, College of Engineering, Department of Chemical
Engineering, Kingston, RI 02881, USA

²University of Rhode Island, College of Pharmacy, Department of Biomedical and
Pharmaceutical Sciences, Kingston, RI 02881, USA

Corresponding Author: Samantha A. Meenach, University of Rhode Island, 215A
Pastore Hall, 51 Lower College Road, Kingston, RI 02881, USA. Email:
smeenach@uri.edu

ABSTRACT

Mucus is an adhesive hydrogel that represents one of the major barriers for successful inhaled treatments. The development of nanoparticles (NP) capable of mucus penetration is desired for the treatment of a variety of pulmonary diseases, and there is a need for effective particle deposition in the lungs. The objective of this study was to develop and evaluate the mucus-penetrating ability of PEGylated nanocomposite microparticles (nCmP) intended to be delivered via a dry powder inhaler. Poly(vinyl alcohol) (PVA) and vitamin E poly(ethylene glycol) (VP5k)-coated polymeric nanoparticles were synthesized via single emulsion and nanoprecipitation, respectively, using acetalated dextran, a biodegradable polymer. Both formulations resulted in spherical, smooth, highly homogeneous NP with diameters of ~200 nm and a slightly negative surface charge. nCmP were produced via spray drying, and showed optimal size and aerosol distribution for targeted pulmonary deposition based on the formulation, as evaluated by a Next Generation Impactor^{TM}. The mucus penetration of nanoparticles was assessed using a Transwell® diffusion chamber, where 33 % more NP permeated through artificial mucus when coated with VP5k compared to PVA nanoparticles. The combination of the potential for targeted deposition in the lungs using mucus-penetrating nCmP represents a potential advantage in the improvement of therapeutic nanomedicines delivered via a pulmonary route.

KEYWORDS

Mucus penetration, vitamin E poly(ethylene glycol), artificial mucus, nanocomposite microparticles, pulmonary delivery

ABBREVIATIONS

CUR, curcumin; NP, nanoparticles; Ac-Dex, acetalated dextran; VP5k, vitamin E poly(ethylene glycol); PVA, poly (vinyl alcohol); CAC, cyclic acetal coverage; PPTS, pyridinium p-toluenesulfonate; DMSO, dimethyl sulfoxide; 2-MOP, 2-methoxypropene; TEA, triethylamine; DCM, dichloromethane; PBS, Phosphate buffered saline (1X PBS dilution unless specified); AM, artificial mucus; DCC, N-N dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; DNA, Deoxyribonucleic acid; NMR, nuclear magnetic resonance; DLS, dynamic light scattering; PDI, polydispersity index; DL, drug loading; EE, encapsulation efficacy; DSC, differential scanning calorimetry; PXRD, powder X-ray diffraction; KF, Karl Fischer titration; NGI, next generation impactor; MMAD_T, theoretical median aerodynamic diameter; MMAD_E experimental median aerodynamic diameter; GSD, geometric standard deviation; FPD, fine particle dose; FPF, fine particle fraction; RF, respirable fraction; ED, emitted dose; DPI, dry powder inhaler; Fc, feed concentration; CF, cystic fibrosis.

5.1. Introduction

Pulmonary delivery is an attractive route for the delivery of therapeutics to the lungs, as higher amounts of drug can reach the site of disease, thus reducing the overall dose required, compared to systemic delivery [1]. In recent years, aerosol nanomedicines have been studied for the treatment of local pulmonary diseases such as lung cancer [2], cystic fibrosis (CF) [3], and pulmonary hypertension [4], among others. While offering many advantages, the pulmonary route also presents physiological and biological barriers that need to be overcome for effective delivery of the therapeutics

[5]. Some of the main parameters needed to ensure effective targeting and delivery of therapeutics into the lungs include: (i) deposition and localization of therapeutics, (ii) escape from mucociliary clearance, and (iii) penetration through mucus in the airways [6].

Aerosol deposition in the lungs is governed by the aerodynamic diameter of particles, and occurs by one of the following mechanisms: inertial impaction ($\geq 5 \mu\text{m}$), sedimentation ($0.5\text{--}5 \mu\text{m}$), or diffusion ($< 0.5 \mu\text{m}$) [7, 8]. Nanoparticles (NP) deposit in the lungs by diffusion due to Brownian motion, and deposition can be enhanced by breath holding (reduction of airflow); however, they can also be easily exhaled. Aggregates of nanoparticles can be designed to have enough mass for effective pulmonary deposition, and can dissociate back into nanoparticles once deposited in the lungs [9, 10], thereby overcoming the deposition barrier.

In the upper airways, the epithelium is covered by mucus that is produced by goblet cells. This mucosal barrier provides protection for the epithelium against chemical and mechanical damage, preventing the penetration of pathogens and toxins, and regulating the moisture content of the tissue beneath [11, 12]. Mucus is a viscous gel layer composed mainly of water (95-99 wt%), and a complex mixture of salts, lipids, nucleic acids, macromolecules, and glycoproteins called mucins, which form a crosslinked and entangled mesh fiber of mucins that confer a negative charge to the mucus due to their carboxyl and sulfate groups [13-15]. This dense fiber mesh traps, immobilizes, and removes larger particulates and particles with adhesive and steric interactions, and is followed by mucociliary clearance to remove the particles from the lungs [16, 17]. The mucus mesh size is highly heterogeneous, and is estimated to range

from 100 - 500 nm in healthy lungs [18], and 60 - 300 nm in sputum of people with CF [19]. Mucus has a neutral pH (7.0) and its thickness in the pulmonary airways may range from 5-55 μm , with a turnover of 10-20 min [20].

The challenges involved in designing nanomedicines capable of effective pulmonary delivery has resulted in a strong interest in developing formulations that can overcome barriers such as mucus. Strategies such as surface modification of therapeutics using PEGylation [21], as well as the use of muco-inert biomaterials such as polysarcosine and mucolytic agents such as papain [16, 22] have extensively been investigated to enhance the mucus penetration of therapeutics. For example, NP with diameters up to 200 nm that were densely coated with PEG (3.4 kDa) moved through undiluted CF sputum [19]. In another study, 5kDa PEG-coated NP (≤ 300 nm) exhibited uniform distribution and retention in mucus compared to non-PEGylated NP. [23]. While size seems to be relevant in the ability of NP to penetrate mucus, the NP coating will ultimately determine their permeability through the mucus mesh. For NP that have been PEGylated, the hydrophilic and near-neutrally charged surface provides shielding of the particle surface to reduce hydrophobic or electrostatic interactions with mucus [24]. Furthermore, PEGylation contributes to the colloidal stability of nanoparticles [25]. A moderate PEG density coating (up to 5 kDa) favors NP penetration into mucus, however, a very high PEG density coating (≥ 10 kDa) can transform NP into mucoadhesive agents [26].

The development of mucus penetrating particles capable of overcoming the mucus barrier and efficiently depositing in the lungs upon aerosolization represents an area in need of improvement in pulmonary delivery applications. In this project we

formulated and evaluated the mucus penetrating capabilities of NP coated with vitamin E poly(ethylene glycol) (VP5k) and encapsulated in nanocomposite microparticles (nCmP) for effective lung deposition using a dry powder inhaler. Fluorescent NP were formulated using curcumin, which was loaded in the biodegradable polymer, acetalated dextran (Ac-Dex) [27, 28]. NP were coated with poly(vinyl alcohol) (PVA), a widely used surfactant in NP formulations, and VP5k, a PEG-based compound reported to have mucus penetrating properties [29]. Following NP formation, mucus penetration studies were carried on artificial mucus (AM). nCmP were formulated via spray drying, and following evaluation using a Next Generation Impactor, the nCmP showed the potential to deposit in the upper airways and lower regions of the lungs. Upon deposition, NP can be re-dispersed and penetrate the mucosal layer based on the coating provided. The improved penetration and effective deposition of particles in specific regions in the lungs can lead to a more personalized treatment for patients having different diseases and can help to overcome some of the challenges associated with pulmonary delivery.

5.2. Materials and Methods

5.2.1. Materials

Dextran from Leuconostoc mesenteroides (MW 9,000-11,000), pyridinium p-toluenesulfonate (PPTS, 98%), anhydrous dimethyl sulfoxide (DMSO, $\geq 99.9\%$), 2-methoxypropene (2-MOP, 97%), triethylamine (TEA, $\geq 99\%$), deuterium chloride (DCl, 35 wt% in D₂O, 99% atom D) solution, dichloromethane (DCM, $> 99.8\%$), anhydrous sodium acetate, D-mannitol ($\geq 98\%$), D- α -tocopherol succinate, 1210 IU/g vitamin E succinate, poly(ethylene glycol) methyl ether (m-PEG, MW 5 kDa), N-N

dicyclohexylcarbodiimide (DCC, 99%), deoxyribonucleic acid sodium salt from salmon testes (DNA), mucin from porcine stomach type II, RPMI 1640 amino acid solution without L-glutamine, and potassium chloride (KCl, 99.0-100.5%) were obtained from Sigma-Aldrich (Natick, MA, USA). Deuterium oxide (D₂O, 99.8% atom D), chloroform-d for NMR (99.8 atom % D), poly(vinyl alcohol) (PVA, 87-89% hydrolyzed, MW 13,000 – 23,000), 4-dimethylaminopyridine (DMAP, 99%), and curcumin (CUR, > 98%) were obtained from Acros Organics (Geel, Belgium). Hydranal^{TM} Coulomat, sodium chloride (NaCl), and agar were obtained from Fisher Scientific (Somerville, NJ, USA). Phosphate buffered saline (PBS) was obtained from Research Products International (RPI) (Mount Prospect, IL, USA). Egg yolk emulsion (50% solution) was purchased from Dalynn Biologicals (Calgary, Alberta, Canada). HyPure^{TM} molecular biology grade nuclease-free water was purchased from GE Healthcare Life Sciences (Logan, Utah, USA).

5.2.2. Synthesis of Acetalated Dextran

The synthesis of 3-hour acetalated dextran (3-hour Ac-Dex, Figure S1) was conducted as previously described [30] with some modifications. 1 g of lyophilized dextran and 35 mg of PPTS were dissolved in anhydrous DMSO under N₂. The reaction was initiated by the addition of 7.5 ml of 2-MOP, and lasted for 3 hours, followed by quenching with 1 ml TEA. The product was then precipitated in basic water, frozen overnight, and lyophilized.

5.2.3. Synthesis of Vitamin E Poly(ethylene glycol)

m-PEG was conjugated to vitamin E following a previously described method with some modifications [29, 30]. Vitamin E succinate (1.95 g) and m-PEG (22 g) were dissolved in 60 mL of DCM for 45 min. Then DMAP (45 mg) and DCC (0.84 mg) were added to the solution for overnight reaction at room temperature (Figure S2A). The reaction mixture was vacuum filtered (0.45 μ m), and was concentrated under reduced pressure using a rotor vapor (IKA, RV, Wilmington, NC, USA) to obtain crude product, which was dissolved at 5% (w/v) in ultrapure water to eliminate excess solvent and unreacted vitamin E succinate. The diluted crude product was centrifuged twice (19,802 rcf, 30 min), from which the supernatant was collected, filtered (0.22 μ m), frozen, and lyophilized for 72 hours.

5.2.4. Nuclear Magnetic Resonance Analysis of Acetalated Dextran and Vitamin E Poly(ethylene glycol)

The total acetal coverage (conversion of hydroxyl groups) and type of acetals formed in Ac-Dex was determined by ^1H -NMR spectroscopy (Bruker 300 MHz NMR, MA, USA). 10 mg of Ac-Dex was suspended in 700 μ L of D_2O and was then hydrolyzed with 30 μ L of DCl in an NMR tube. The hydrolysis of acyclic acetals produces methanol and acetone whereas cyclic acetals produce only acetone. The total acetal coverage and the cyclic acetal coverage (CAC) were determined after normalization of the methanol and acetone peaks in relation to dextran (carbon rings). The normalization is associated

with the number of protons detected on each molecule. The results were expressed per 100 molecules of glucose.

The conjugation of mPEG to vitamin E succinate was confirmed by ^{13}C NMR (NMRS 500 MHz spectrometer, Agilent Technologies Santa Clara, CA, USA). 20 mg of vitamin E succinate or VP5k was diluted in 700 μL of CDCl_3 and analyzed to obtain their respective spectrograms. The shift of the peak from vitamin E at 178 ppm corresponding to $-\text{COOH}$ to 172 ppm ($-\text{COO}-\text{CH}_2-$) in VP5k confirmed successful conjugation.

5.2.5. Preparation of Curcumin-Loaded Ac-Dex Nanoparticles coated with PVA and VP5k

Curcumin (CUR)-loaded nanoparticles coated with PVA (PVA CUR NP) were prepared via single emulsion and solvent evaporation. The organic phase was comprised of 1.25 mg of CUR and 48.75 mg of Ac-Dex dissolved in 1 ml DCM, and the aqueous phase contained 3% PVA in PBS to result in an initial loading of 2.5 wt% CUR. 6 mL of 3% PVA in PBS was added to the organic phase and sonicated (Q500 Sonicator, Qsonica, Newton, CT, USA) for 60 seconds with 1 second on/off pulse at an amplitude of 70% (100% amplitude = 120 μm). The emulsion was added to 0.3% PVA in PBS, allowed to spin for 4 hours (340 rpm), collected, washed via centrifugation (1X), and re-dispersed in 0.1% PVA in DI water before freezing and lyophilization. CUR-loaded nanoparticles coated with VP5k (VP5k CUR NP) were made via nanoprecipitation. 1 mg CUR and 19 mg of Ac-Dex were dissolved in 200 proof ethanol and sonicated for 5

min. The solution was injected dropwise using a 30G needle into a 40 ml 3% VP5k solution in PBS. After 3-4 hours of spinning (340 rpm), the NP were filtered (0.45 μ m), washed via centrifugation 1X, re-dispersed in 0.5% VP5k in DI water, and frozen and lyophilized.

5.2.6. Formulation of Curcumin Nanocomposite Microparticles (CUR nCmP) via Spray Drying

CUR-loaded nCmP were formulated with CUR NP and mannitol in an aqueous suspension using a Büchi B-290 spray dryer (Büchi Labortechnik, AG, Switzerland) in open mode. The ratios of CUR NP: mannitol was 70:30 (w/w) in DI water at a feed concentration (Fc) of 1.5% (w/v) and 100:0 at a Fc = 0.5% (w/v). The spray dryer conditions were: 414 L/h dry N₂ flow rate, aspiration rate of 28 m³/h, pump rate of 0.9 mL/min, nozzle cleaner rate of 4, inlet temperature of 55 °C for PVA coated NP, and 50 °C for VP5k coated NP. The nCmP were collected in amber glass vials and stored in desiccators at -20 °C.

5.2.7. Particle Morphology and Shape Analysis via Scanning Electron Microscopy

The surface morphology and shape of the NP and nCmP were evaluated by scanning electron microscopy (SEM) with a Zeiss SIGMA VP Field Emission-Scanning Electron Microscope (Germany). NP were suspended in basic water (10 mg/ml), dropped onto aluminum SEM stubs, and dried at room temperature. nCmP samples were

placed on SEM stubs covered by double-sided adhesive carbon tabs. Samples were sputter coated with a thin film of gold/palladium alloy in a BIO-RAD system at 20 μ A for 15 seconds under argon gas. Images were captured at 5 kV and various magnifications.

5.2.8. Particle Size, Size Distribution, and Zeta Potential Analysis of Nanoparticles

The hydrodynamic size, size distribution (polydispersity index, PDI), and zeta potential of parent NP and NP re-dispersed from nCmP were measured by dynamic light scattering (DLS) using a Malvern Nano Zetasizer (Malvern Instruments, Worcestershire, UK). The nanoparticles were suspended in basic water (0.5 mg/mL, pH 9), vortexed, and sonicated for one minute. All measurements were performed at 25°C.

5.2.9. Analysis of CUR Loading in NP and nCmP

Samples of NP and nCmP were dissolved in DMSO (0.2 mg/ml) and CUR content was analyzed using fluorescence spectroscopy at 420/540 nm in a Biotek Cytation 3 microplate reader (BioTek Instruments Inc., Winooski, VT, USA). CUR concentration was quantified by comparison with a standard calibration curve of CUR in DMSO. CUR loading and encapsulation efficacy in the particles were calculated using the following equations:

$$\text{DL (wt\%)} = \frac{\text{mass of CUR in nanoparticles}}{\text{total mass of nanoparticles}} \times 100$$

$$\text{Theoretical CUR loading} = \frac{\text{initial mass of CUR}}{\text{mass of CUR} + \text{mass of Ac} - \text{Dex}} \times 100$$

$$\text{EE (wt\%)} = \frac{\text{actual CUR loading}}{\text{theoretical CUR loading}} \times 100$$

5.2.10. *In Vitro* Drug Release from NP and nCmP

The *in vitro* release of CUR from NP and nCmP was determined via a release study of suspended particles (1 mg/mL, 1.8 mL) in modified phosphate buffer (PBS, 0.1 M, pH 7.4, 0.5% wt% Tween 80) and modified acetate buffer (0.1 M, pH 5, 0.5% wt% Tween 80). Particle suspensions were incubated at 37°C and 100 rpm (Digital Heat Block and Orbi shaker, Benchmark Scientific, Edison, NJ, USA). At various time points (0-5 days), particle samples were centrifuged at 23,102 rcf for 5 min at 4°C to isolate the NP. 200 mL of supernatant was withdrawn and replaced by the same amount of fresh buffer. Supernatant samples were dissolved in 200 µL DMSO and analyzed for CUR content by fluorescence spectrometry as described previously.

5.2.11. Solid-State Analysis of NP and nCmP

The thermal phase transitions of NP, nCmP, and raw materials were determined by differential scanning calorimetry (DSC) using a TA Q10 DSC system (TA instruments, New Castle, DE, USA) equipped with an automated computer-controlled cooling system. 1-3 mg of sample was weighed into Tzero™ alodine-coated aluminum

pans and hermetically sealed. The sealed sample pan was placed in the DSC chamber along with an empty sealed reference pan, which were heated from 0-260 °C at a rate of 10 °C/min. Powder x-ray diffraction (PXRD) was applied to determine the crystalline states of nCmP and its raw components using a Rigaku Multiflex X-ray diffractometer (The Woodlands, TX, USA) with Cu K α radiation source (40KV, 44mA). The samples were placed in a horizontal slit holder prior to analysis. The scan range was 5-60 ° in 2 θ with a sampling width of 0.1 and scan speed of 1°/min.

5.2.12. Karl Fischer Coulometric Titration

The water content in nCmP was quantified by Karl Fischer (KF) coulometric titration using a 737 KF coulometer (Metrohm, Riverview, FL, USA). 10 mg of particles were dissolved in a known mass of anhydrous methanol and injected into the reaction cell filled with Hydranal® KF reagent. The water content was analyzed and reported in wt%. Pure solvent was analyzed as background sample.

5.2.13. nCmP *In Vitro* Aerosol Dispersion Performance with a Next Generation Impactor

The *in vitro* aerosol dispersion of dry powder nCmP was evaluated using a Next Generation Impactor™ (NGI™, MSN Corporation, Shoreview, MN, USA) equipped with a stainless-steel induction port attachment (USP throat) and gravimetric NGI™ insert cups. The NGI was connected to a Copley HCP5 vacuum pump (Copley Scientific, United Kingdom). The flow rate was measured before every experiment and

adjusted to 60 L/min, which models the flow rate of healthy adult lungs [31]. Glass fiber filters (55 mm, type A/E, Pall Life Sciences) were weighed and placed on the insert cups from stage 1 through 7 to reduce bounce or re-placement of powders [32]. 10 mg of powder was loaded into a hydroxypropyl methylcellulose capsule (HPMC, size 3, Quali-V®, Qualicaps® Inc., Whitsett, NC, USA) and placed in a dry powder inhaler (DPI) device (Handihaler, Boehringer Ingelheim Pharmaceuticals, CT, USA). The DPI was then attached to a rubber mouthpiece connected to the NGI. Three capsules were released in every experiment (for a total of 30 mg) in triplicate. The NGI was operated with a delay time of 10s and running time of 10s. The particle deposition on each stage was determined gravimetrically by the difference in mass of the glass filter before and after deposition. The effective cutoff diameters for each impaction stage, at Q = 60 L/min, were given by the manufacturer as: Stage 1 (8.06 µm); Stage 2 (4.46 µm); Stage 3 (2.82 µm); Stage 4 (1.66 µm); Stage 5 (0.94 µm); Stage 6 (0.55 µm); and Stage 7 (0.34 µm). The fine particle dose (FPD), fine particle fraction (FPF), respirable fraction (RF), and emitted dose (ED) were calculated as follows:

$$\text{Fine particle dose (FPD)} = \text{mass of particles} < 4.4 \mu\text{m (stages 2 through 7)}$$

$$\text{Fine particle fraction (FPF)} = \frac{\text{fine particle dose}}{\text{initial particle mass loaded into capsules}} \times 100$$

$$\text{Respirable fraction (RF)} = \frac{\text{mass of particles} < 4.4 \mu\text{m (stages 2 through 7)}}{\text{total particle mass on all stages}} \times 100$$

$$\text{Emitted dose (ED)} = \frac{\text{initial mass in capsules} - \text{final mass remaining in capsules}}{\text{initial mass in capsules}} \times 100$$

The experimental mass median aerodynamic diameter (MMAD_E) and geometric standard deviation (GSD) for the particles were determined using a Mathematica® program written by Dr. Warren Finlay [32].

5.2.14. Tapped Density and Theoretical Aerodynamic Analysis of nCmP

The density of nCmP was determined via tapped density measurements [33]. 25-35 mg of particles (m) was weighed in a thin glass tube and tapped 200 times over a hard surface to ensure efficient packing. The height and diameter were measured using a digital caliper to calculate the volume occupied for the particles (V). The tapped density (ρ) was calculated using the equation:

$$\rho = \frac{m}{V}$$

The theoretical MMAD (MMAD_T) was calculated using the following equation:

$$\text{MMAD}_T = d \sqrt{\frac{\rho}{\rho^*}}$$

where d is the geometric diameter of nCmP determined by ImageJ and $\rho^* = 1 \text{ g/cm}^3$ is the reference density of solid polymer.

5.2.15. Preparation of Artificial Mucus

Artificial mucus (AM) was prepared following a previously described protocol [34], where 500 mg DNA, 250 mg mucin, 0.295 mg DPTA, 250 mg NaCl, 110 mg KCl, 1 mL RPMI 1640 amino acid solution, and 250 μL egg yolk emulsion were added to a final volume of 50 mL of DNase-free water. The mixture was stirred for 2 hours at room temperature to allow stabilization, and was used within 48 hours.

5.2.16. Analysis of Freeze-Dried Artificial Mucus Morphology

Fresh artificial mucus was plated in a 24-well plate, flash frozen in liquid nitrogen, and lyophilized. Transverse slices of lyophilized AM were placed on aluminum SEM stubs with double-sided adhesive carbon tabs. The samples were coated and imaged via SEM as previously described.

5.2.17. Evaluation of Nanoparticle Penetration through Artificial Mucus

The mucus penetration of NP and free CUR in AM was evaluated as follows. A 0.3% (w/v) agar solution (500 μL) was placed in the bottom of a 12-well plate and hardened at 4°C. A Transwell® (3.0 μm pore size) for 12-well plates (Corning Life Sciences, Tewksbury, MA, USA) was inserted in the plate and 400 μL of AM was

placed in the apical side. Free CUR was dissolved in DMSO (3.68 mg/mL) and diluted in DI water for a final DMSO concentration of 5% (v/v). NP formulations were suspended in DI water. All formulations had a CUR concentration of 500 μ M. 100 μ L of each CUR formulation (free CUR, PVA CUR NP, or VP5k CUR NP) was placed on top of the AM in the Transwell® and the Transwell was moved to a new well with fresh agar at different time points. At the end of the test, the agar was melted and dissolved in 350 μ L of DMSO. The CUR concentration in the samples from the basolateral side of the Transwell® (agar) was determined via fluorescence spectrometry. The results were reported as CUR permeated (μ g) over time for the different formulations.

5.2.18. Statistical Analysis

The measurements were performed in at least triplicate. All values were presented as mean $\pm$ standard deviation. The statistical significance of the results was determined using Student's t-test and ANOVA for the mucus penetration study, where $p < 0.05$ was considered statistically significant.

5.3. Results

5.3.1. Characterization of Ac-Dex and VP5k

The 3-hour reaction between dextran and 2-MOP in the presence of PPTS resulted in the synthesis of acetalated dextran (Ac-Dex), which contains both cyclic and acyclic acetal groups (**Figure B.1**). NMR spectrometry of Ac-Dex showed a total acetal

coverage (conversion from –OH groups) of 83% and a cyclic acetal coverage of 90%. Vitamin E succinate was conjugated to m-PEG 5k and confirmed by ^{13}C NMR, as can be observed in the spectrograms in **Figure B.2B**. The carbonyl carbon peak at 178 ppm (-COOH) from vitamin E shifted to a carbonyl carbon at 172.1 ppm (-COO-CH₂) in VP5k due to the reaction. The second carbonyl at 171 ppm and aromatic carbons from 150 ppm to 115 ppm remained the same in both compounds and finally an intense peak at 70.5 ppm appeared in VP5k, corresponding to the -OCH₂ from m-PEG.

5.3.2. Nanoparticle Characterization

5.3.2.1. Morphology, Size, Size Distribution, and Surface Charge

The sizes, distribution, and surface charges of parent and re-dispersed NP are presented in **Table 5.1**. The hydrodynamic size of PVA CUR NP was around 180 nm with low PDI (0.13) and slightly negative charge (-2.51 mV). PVA CUR NP exhibited spherical, smooth morphology and uniform dispersion (**Figure 5.1A**). VP5k CUR NP exhibited diameter, PDI, and surface charge values of 281 nm, 0.14, and -14.20 mV, respectively, with a smooth surface, mostly spherical shape, and high homogeneity (**Figure 5.1B**).

Table 5.1 Characteristics of curcumin-loaded nanoparticles (CUR NP) coated with poly(vinyl alcohol) (PVA) and vitamin E poly(ethylene glycol) (MW = 5 kDa) (VP5k) and CUR NP re-dispersed from PVA CUR nanocomposite microparticles systems. Analysis includes NP diameter as measured by dynamic light scattering (DLS),

polydispersity index (PDI), zeta (ζ) potential, CUR loading in NP, and encapsulation efficiency (EE) (mean $\pm$ standard deviation, $n \geq 3$).

Particle System	Diameter (nm)	PDI	ζ Potential (mV)	CUR Loading ($\mu\text{g CUR/mg NP}$)	EE (%)
PVA CUR NP	181.3 $\pm$ 9.5	0.13 $\pm$ 0.03	-2.51 $\pm$ 0.92	13.00 $\pm$ 1.42	52.00 $\pm$ 5.70
VP5k CUR NP	281.3 $\pm$ 15.9	0.14 $\pm$ 0.03	-14.20 $\pm$ 3.21	4.14 $\pm$ 1.20	16.57 $\pm$ 4.80
Re-dispersed PVA CUR NP from nCmP (30:70, Fc = 1.5%)	251.9 $\pm$ 10.5	0.31 $\pm$ 0.07	-14.63 $\pm$ 0.50	N/A	N/A
Re-dispersed PVA CUR NP from nCmP (100:0, Fc = 0.5%)	254.1 $\pm$ 11.5	0.28 $\pm$ 0.06	-3.73 $\pm$ 0.17	N/A	N/A

Figure 5.1 Representative scanning electron micrographs of nanoparticles (NP) and nanocomposite microparticles (nCmP) systems. (A) poly(vinyl alcohol)-coated curcumin-loaded nanoparticles (PVA CUR NP), scale bar = 500 nm; (B) vitamin E poly(ethylene glycol) (MW = 5 kDa)-coated CUR-loaded nanoparticles (VP5k CUR NP), scale bar = 500 nm; (C) PVA-coated CUR-loaded nanocomposite microparticles with 30:70 NP to mannitol and 1.5% spray drying feed concentration (PVA CUR nCmP, 30:70, Fc = 1.5%), scale bar = 1 μ m; and (D) PVA-coated CUR-loaded nCmP with 100% NP and 0.5% spray drying feeding concentration (PVA CUR nCmP, 100:0, Fc = 0.5%), scale bar = 1 μ m.

5.3.2.2. Drug loading and *In Vitro* Release from NP

CUR was encapsulated in 3-hour Ac-Dex with an encapsulation efficiency of 52% and 17% and CUR drug loading of 13 and 4 μg CUR/mg NP for PVA CUR NP and VP5k CUR NP, respectively (**Table 5.1**). The release profiles of CUR (**Figure 5.2**) indicate the sustained release of CUR, with burst release lower than 20% in all cases. At pH 7.4, CUR release reached a maximum (29%) after 24 hours for PVA CUR NP. Similarly, CUR released from VP5k CUR NP at neutral pH showed no significant increase after 24 hours, with a maximum release around 19%. CUR release in acidic buffer showed sustained release for both PVA and VP5k formulations, with 77% CUR release from PVA CUR NP and 89% CUR released from VP5k CUR NP after 96 hours.

Figure 5.2 *In vitro* curcumin (CUR) release profiles of nanoparticles and nanocomposite microparticles (nCmP) from poly(vinyl alcohol) (PVA) and vitamin E poly(ethylene glycol) (MW = 5 kDa) (VP5k)-coated particles in various pH conditions. (Top) Comparative release profiles of CUR release from PVA-coated CUR NP and CUR nCmP coated at pH 7.4 and pH 5.2. (Bottom) Comparative release profiles of CUR release from VP5k-coated CUR NP at pH 7.4 and pH 5.2 (n = 3).

5.3.3. Microparticle Characterization

5.3.3.1. nCmP Morphology, Size, Charge and Re-dispersion

The morphology of the CUR nCmP formulations can be seen in **Figure 5.1C** and **1D** for 30:70 and 100:0 NP to mannitol ratios, respectively. PVA CUR nCmP (100:0, Fc = 0.5%) showed wrinkled surface particles, forming raisin-like nCmP with visible NP, and a geometric diameter of 1.70 μm . PVA nCmP (30:70, Fc = 1.5%) showed some wrinkled particles and some spherical nCmP with a geometric diameter of 1.66 μm . NP re-dispersed from PVA nCmP exhibited an increase in size to around 250 nm in both systems, with an increase in PDI (~ 0.3) (**Table 5.1**). The charge of 100:0 nCmP remained similar to that of the parent PVA CUR NP, and the formulation with 70% mannitol exhibited a more negative charge. The yields in the production of nCmP via spray drying were 52.6 and 54.3 % for PVA nCmP 30:70 and 100:0, respectively.

5.3.3.2. Drug loading and *In Vitro* Release from nCmP

PVA CUR nCmP (30:70, Fc = 1.5%) had a drug loading of 1.06% (10.56 μg CUR/mg of nCmP) and PVA CUR nCmP (100:0, Fc = 0.5%) had a loading of 2.33%. The CUR nCmP formulations exhibited sustained release in both buffers, with prolonged release in acidic conditions. nCmP exhibited CUR burst release below 20%, and their release reached equilibrium at 32% by 24 hours at pH 7.4, and 96% and 71% release in 96 hours at pH 5.1, as can be observed in **Figure 5.2**.

5.3.4. Water Content via Karl Fischer Titration

The residual water content of PVA CUR nCmP formulations were below than 3% (**Table 5.2**), as determined via colorimetric Karl Fischer Titration.

Table 5.2 Characteristics of curcumin-loaded nanocomposite microparticles (CUR nCmP), including water content, CUR loading, nanoparticle (NP) loading in nCmP, NP loading efficacy in nCmP, geometric diameter (via Image J), tapped density, theoretical mass median aerodynamic diameter ($MMAD_T$), experimental MMAD ($MMAD_E$), and geometric standard deviation (GSD) (mean $\pm$ standard deviation, $n \geq 3$).

Particle System	Water Content (%)	CUR Loading ($\mu\text{g CUR/mg nCmP}$)	Geometric Diameter (μm)	Tapped Density (g/cm^3)
PVA CUR nCmP (30:70, Fc = 1.5%)	1.31 ± 0.13	10.56 ± 0.25	1.66 ± 0.73	0.4894 ± 0.02
PVA CUR nCmP (100:0, Fc = 0.5%)	2.41 ± 0.59	23.33 ± 0.50	1.70 ± 0.67	0.3572 ± 0.03

Particle System	$MMAD_T$ (μm)	$MMAD_E$ (μm)	GSD (μm)
PVA CUR nCmP (30:70, Fc = 1.5%)	1.16 ± 0.52	4.67 ± 0.05	2.11 ± 0.13
PVA CUR nCmP (100:0, Fc = 0.5%)	1.02 ± 0.40	2.62 ± 0.24	3.48 ± 0.24

5.3.5. Solid-State Analysis of NP and nCmP

The DSC thermograms of raw materials and nCmP are shown in **Figure 5.3 (Top)**. Raw CUR had an endothermic phase transition peak at 180°C. Raw 3-hour Ac-Dex and PVA had no visible peaks, and raw VP5k had an endothermic peak at 58°C and exothermic peak at 189 °C. Raw mannitol displayed an endothermic peak at 171°C.

VP5k CUR NP presented a small endothermic peak at 58°C. PVA CUR NP and PVA CUR nCmP 100:0 had no visible peaks, while PVA CUR nCmP 30:70 had a broad endothermic peak at 159°C. **Figure 5.3 (Bottom)** presents powder X-ray diffractograms of raw materials and nCmP. Raw CUR and mannitol exhibited strong peaks, raw Ac-Dex presented no peaks, and raw PVA and VP5k exhibited small peaks. The resulting PVA CUR NP and VP5k CUR NP formulations did not exhibit peaks respective to their constituents. After spray drying, PVA CUR nCmP (100:0) showed no peaks and PVA CUR nCmP (30:70) showed a reduction in the intensity of peaks compared to the raw components.

Figure 5.3 (Top) Differential scanning calorimetry (DSC) thermograms and (Bottom) powder X-ray diffractograms (PXRD) of raw curcumin (Raw CUR), raw acetalated dextran following 3-hour reaction time (3-hour Ac-Dex), raw poly(vinyl alcohol) (Raw PVA), PVA nanoparticles (PVA CUR NP), raw vitamin E poly(ethylene glycol) (MW = 5 kDa) (Raw VP5k), VP5k nanoparticles (VP5k CUR NP), raw mannitol, PVA-coated CUR-loaded nanocomposite microparticles with 30:70 nanoparticle-to-mannitol ratio

and spray drying feed concentration of 1.5% (PVA CUR nCmP, 30:70, Fc = 1.5%), and PVA-coated CUR-loaded nCmP with 100% NP and spray drying feed concentration of 1.5% (PVA CUR nCmP, 100:0, Fc = 0.5%).

5.3.6. Density and Theoretical MMAD of nCmP

The tapped density and the theoretical mass median aerodynamic diameter ($MMAD_T$) of PVA CUR nCmP are shown in **Table 5.2**. The corresponding values were 0.49 g/cm³ and 1.16 μ m for (30:70) and 0.36 g/cm³ and 1.02 μ m for (100:0).

5.3.7. *In Vitro* Aerosol Performance of nCmP

The *in vitro* aerosol dispersion of PVA CUR nCmP (**Figure 5.4**), evaluated in the NGI^{TM}, showed a broad dispersion of particles across all stages with 10% of the total mass deposition on the first stage for both formulations. For PVA CUR nCmP (30:70), 66% of the particles deposited in stage 2 and stage 3, and only a 0.7% reached stage 7. In the case of PVA CUR nCmP (100:0), broader distribution of nCmP can be observed across stages 2 - 5. The experimental MMAD ($MMAD_E$) were 4.67 μ m (GSD of 2.11 μ m) and 2.62 μ m (GSD of 3.48) for 30:70 and 100:0 PVA CUR nCmP, respectively (**Table 5.2**). The increased $MMAD_E$ value for the 30:70 formulation corresponds to the shift in nCmP to the lower stages of the NGI. The FPF, RF, and ED were similar for both formulations, with values around 45%, 90, and 95%, respectively.

Figure 5.4 *In vitro* aerosol dispersion performance of poly(vinyl alcohol)-coated, curcumin-loaded nanocomposite microparticles (PVA CUR nCmP) in a Next Generation Impactor™ (NGI™) for $Q = 60$ L/min. (Top) Particle deposition on each stage of the NGI. (Bottom) Fine particle fraction (FPF), respirable fraction (RF), and emitted dose (ED) of PVA CUR nCmP (Mean $\pm$ standard deviation, $n = 3$).

5.3.8. Artificial Mucus and Penetration Studies of Nanoparticles

Images of the freeze-dried artificial mucus (**Figure 5.5**) indicate microporous interconnectivity and the penetration studies of free CUR, PVA-coated CUR NP and VP5k-coated CUR NP in AM are shown in **Figure 5.6 (Top)**. Free CUR (in 5% DMSO) exhibited the slowest penetration through AM, and after 12 hours, a significant amount of CUR remained in the mucus, based on the yellow coloration of the AM. PVA CUR NP exhibited faster penetration through the AM in comparison to free CUR, but was also retained in the AM for the duration of the experiment. VP5k CUR NP exhibited the fastest penetration in mucus for the given samples. After 2 hours, a significant amount of VP5k NP were observed at the bottom of the Transwell®, which increased after 4 hours. From 7-12 hours, the AM looked clearer as most of the NP reached and crossed the membrane of the Transwell®. Quantification of raw CUR and CUR penetration to the basolateral side of the Transwell® (**Figure 6 (Bottom)**) indicates that significantly more VP5k NP penetrated the AM in comparison to free CUR and PVA NP after 12 hours.

Figure 5.5 Representative scanning electron micrographs of freeze-dried artificial mucus (AM). (A) 100X magnification, scale bar = 500 μm (B) 800 X, scale bar = 50 μm .

Figure 5.6 Evaluation of the mucus penetration capacities of free curcumin (CUR) containing 5% (v/v) DMSO, poly(vinyl alcohol) (PVA)-coated CUR NP, and vitamin E poly(ethylene glycol) (MW = 5 kDa) (VP5k)-coated CUR NP in artificial mucus (AM) at 500 μ M CUR. (Top) photographs of CUR formulations dosed on AM located

in the apical side of the Transwell® at different time points. (Bottom) Fluorescence quantification of CUR collected in the basolateral side of the Transwell® at different time points and expressed as mass percent of NP. Statistical analysis was performed using ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n \geq 3$, which represents comparison among the groups)

5.4. Discussion

Mucus is a complex fiber mesh responsible for trapping foreign agents in the lungs. The efficacy of treatments such as infections in CF mucus, lung tumors located beneath the mucus layer, or systemic treatment through pulmonary delivery is compromised by the ability of the therapeutic to escape mucosal entrapment and penetrate the mucus and/or epithelial layer beneath the mucus. In this project we evaluated and compared the penetration of a hydrophobic drug, CUR, with CUR-loaded NP coated with PVA or PEG (VP5k). It has been shown that PEGylation (MW 2 - 5 kDa) prevents the mucin immobilization of NP, allowing for enhanced mucus penetration, and this phenomenon ceases to occur at higher PEG densities (>5 kDa) [26, 35].

This project involved the utilization of 3-hour Ac-Dex, which has a high cyclic acetal coverage and -OH conversion, as confirmed by ^1H -NMR. We successfully conjugated vitamin E succinate with PEG 5k, as confirmed by ^{13}C NMR, and fabricated 3-hour Ac-Dex CUR NP coated with PVA or VP5k. As the synthesized Ac-Dex is highly hydrophobic, increased VP5k coating was required to stabilize the particles during nanoprecipitation and to avoid further agglomeration during the freezing and

lyophilization steps, in contrast to previous work using 5-min Ac-Dex, with 5% less -OH conversion and 24% lower CAC [30]. The high hydrophobicity of 3-hour Ac-Dex seemed to affect NP coating during fabrication more with VP5k than with PVA, suggesting that the polar groups (free -OH groups) in the polymer play an important role in the colloidal stability of NP [36] in coating with surfactants of higher molecular weight.

The increased hydrophobicity of 3-hour Ac-Dex (in comparison to 5-min Ac-Dex) resulted in a slower degradation rate and release of CUR, which was observed in the *in vitro* release study, where the release of CUR plateau after 24 hours at pH=7.4, corresponding to a normal pH in healthy tissue. In contrast, a sustained CUR release continued for 4 days in acidic conditions, reaching more than 88% release from NP and above 70% from nCmP, suggesting a release controlled mostly by erosion and degradation of the polymer. This selective release profile may be advantageous for the treatment of infectious diseases associated in CF patients with reduced pH airways [37] or cancerous tissue with solid tumors with pH ranging from 6.7 – 7.6, and lower within the cell [38]. The low burst (<20%) seen in all formulations is another attribute of these particles that allowed for a controlled release profile. The slow degradation of 3-hour Ac-Dex can also prevent the release of drug before reaching the site of action, and this can easily be tuned by the time of reaction during the synthesis of the polymer. In addition, while drug loading was high in PVA coated NP, VP5k NP will require further optimization to increase the encapsulation of CUR for further pulmonary delivery applications.

VP5k-coated NP readily penetrated AM, with 33% increased penetration in comparison to PVA-coated NP and 80% in comparison to free CUR. The increased penetration is likely due to reduction in the muco-adhesion of NP to AM because of the PEG coating on the NP, and this is consistent to previously reported particle tracking analysis of PLGA-based VP5k NP [29]. PEGylation of NP via VP5k minimizes their interactions with mucin within the mucus mesh owing to its hydrophilic nature and nearly neutral charge. These characteristics allow the NP to escape steric hindrance and electrostatic interactions, allowing for successful mucus penetration, which can be beneficial for multiple applications in pulmonary delivery as well as other routes of delivery where mucus exists. While PVA-coated NP have traditionally been considered mucoadhesive when compared to PEGylated NP [39], and are known to decrease NP mobility when added to PEGylated NP [40], we observed that PVA-coated NP partially diffused through mucus escaping from the entanglement in mucin, likely due to its particle size (<200 nm) and molecular weight (13-23 KDa), which in another study was found to have similar mobility to 200 nm size PS-PEG-2k NP [41].

Besides the mucosal barrier, the effective particle deposition is another challenge in pulmonary delivery. Factors affecting particle deposition include: (a) particle/droplet size (diameter), density, surface properties, shape (b) anatomy of the airways (c) ventilatory parameters [8]. In the design of dry powder formulations, the physical properties of the particle can be modulated. Particle size in pulmonary delivery is usually referred to the aerodynamic diameter, which will ultimately determine the deposition, and is dependent on the shape, density and geometric size of the particle. While particles $\geq 5 \mu\text{m}$ in size can deposit in the medium and lower airways by different

mechanism and escape the mucociliary clearance [42], these particle size range (2-3 μ m) also exhibit maximal phagocytosis and are rapidly eliminated by alveoli macrophages [43]. A successful deposition will require particles with a size in the range of targeted deposition in the airways that are small enough to escape clearance and big enough to avoid exhalation [44, 45].

To overcome this paradox, on this project we formulated nCmP comprised of nanoparticles agglomerated in the form of microparticles via spray drying, for a medium and deep lung deposition targets. Spray drying is a process that forces a fluid through a nozzle, producing a mist that generates amorphous and fine powders after drying [46]. The final product is determined and tuned based on the initial spray drying parameters and suspension characteristics such as viscosity (feed concentration) and nanoparticle/excipient mass ratio, among others [47].

The suspension containing with a 30:70 NP: mannitol ratio and feed concentration (Fc) of 1.5% produced nCmP with some hollow and mostly spherical morphology, compared to the ones spray dried with 100% NP and Fc of 0.5%, which showed a raisin-like morphology in its entirety. The image J evaluation of both nCmP showed a similar geometric diameter of both formulations (being 30:70 slightly smaller), however, their densities were different, being smaller for 100% NP ratio, due to its hollow shape. This last parameter finally defined the aerodynamic performance of the particle. As evaluated previously, particles with densities $\ll 1$ g/cm³ are characterized by hollow (or porous) morphology that increases the geometric diameter and decreases the aerodynamic diameter, enhancing the particle deposition in the lungs [48, 49]. The MMAD_T was smaller in the case of 100% NP formulation, and

experimentally, the MMAD_E was significantly reduced (by 78%) compared to the 30:70 nCmP formulation, as evaluated in the *in vitro* NGI. A higher ratio of NP to mannitol with a dilute feed concentration can lead to particles with deeper deposition in the lungs due to their hollow and wrinkled morphology [50]. nCmP aerosol performance can be tailored based on the selected parameters during the spray drying process.

The increase in NP ratio did not affect the MMAD_T of the particles as much as the feed concentration in the formulation, however, the lack of mannitol seemed to increase the geometric standard deviation (GSD) in 100 %NP formulation, as observed in the NGI deposition figure. Mannitol is an excipient commonly used in DPI formulations, found to provide satisfactory aerosol performance and physical stability in spray dried particles [51], as in general the use of excipients, as carriers, facilitates the handling and dispersion of the cohesive micronized particles during inhalation [52].

We observed that some agglomerations occurred during the simulated inhalation, as were evaluated by the slightly lower FPF observed in 100% NP formulation, which are opposite as we expected considering their deeper aerosol deposition. Both formulations had a low content of water (<2.5%), which also favored the high ED and RF ($\geq 90\%$) of particles during NGI evaluation.

The different NP : mannitol ratio also allows the tunability for the development of specific drug loadings of the formulation. As expected, a high initial NP ratio will lead to a higher final DL.

The particles produced by spray drying would likely dissolve the excipient (mannitol, if it has some) in the surface of the pulmonary airways, leaving behind loaded nanoparticles for sustained release of the DPI formulation for local or systemic

application. The nCmP formulated on this project re-dispersed into nanoparticles upon contact to water, ensuring their nanoparticulate recovery, with slight increase in size and PDI, likely due to some agglomerations during the spray drying process. The recovery of NP from nCmP was also achieved because of the amorphization of raw materials, that in the case of 100% NP was complete (also due to the lack of mannitol), and for 30:70, was highly enhanced, confirmed by the reduction of peaks, evaluated by PXRD. For the latest formulation, the peaks observed represent mannitol and no other raw material, and agrees with other studies where at high mannitol ratios, some of it can still form some crystals during spray drying [53].

The thermal stability of nCmP was assessed using DSC, showing that nCmP that are highly stable under 120 °C in the case of PVA coated particles and under 55 °C for VP5k coated particles, which will likely influence in the storage recommendations of the product.

Overall, VP5k-coated NP encapsulated in nCmP can enhance the penetration of NP into mucus and the nCmP formulation allow these NP to reach the site of action within the lungs. In particular, nCmP can be designed for specific lung depositions, which can provide a more personalized treatment of different pulmonary diseases. Additional studies to optimize the VP5k NP to enhance their encapsulation, efficacy studies in bacteria, and cytotoxicity studies are required for a better understanding of the impact and safety of VP5k NP.

5.5. Conclusions

In this project we developed and evaluated the mucus penetration abilities of VP5k-coated NP encapsulated in nCmP for pulmonary delivery of a hydrophobic drug. The penetration of NP was enhanced using VP5k coating in comparison to PVA coating and free CUR. The 3-hour Ac-Dex system showed sustained release of the drug over 96 hours with low burst release, reaching high release (~97%) in acidic conditions, which could be significant in the treatment of infections or tumors of acidic environments. The engineered nCmP were designed for tunable deposition of particles in the lungs, making them suitable for personalized treatment and targeted delivery of therapeutics via a pulmonary delivery of dry powder formulations.

5.6. Acknowledgments

The authors thank RIN² for SEM and XRD. Research reported in this publication was partially supported by the Institutional Development Award (IDeA) Network for Biomedical Research Excellence from the National Institute of General Medical Sciences of the National Institutes of Health under grant number P20GM103430. In addition, this work was made possible by the use of equipment and services available through the RI-INBRE Centralized Research Core Facility. This material is based upon work conducted at a Rhode Island NSF EPSCoR research facility, supported in part by the National Science Foundation EPSCoR Cooperative Agreement #EPS-1004057. This material is also based in part upon work supported by the National Science Foundation under grant #1508868. Any opinions, findings, and conclusions or

recommendations expressed in this material are those of the authors and do not necessarily reflect the view of the National Science Foundation or National Institutes of Health.

REFERENCES

1. Kuzmov, A. and T. Minko, *Nanotechnology approaches for inhalation treatment of lung diseases*. Journal of Controlled Release, 2015. **219**: p. 500-518.
2. Youngren-Ortiz, S.R., et al., *Development of Optimized, Inhalable, Gemcitabine-Loaded Gelatin Nanocarriers for Lung Cancer*. Journal of aerosol medicine and pulmonary drug delivery, 2017. **30**(5): p. 299-321.
3. Gunday Tureli, N., et al., *Ciprofloxacin-loaded PLGA nanoparticles against cystic fibrosis P. aeruginosa lung infections*. Eur J Pharm Biopharm, 2017. **117**: p. 363-371.
4. Makled, S., N. Nafee, and N. Boraie, *Nebulized solid lipid nanoparticles for the potential treatment of pulmonary hypertension via targeted delivery of phosphodiesterase-5-inhibitor*. International Journal of Pharmaceutics, 2017. **517**(1): p. 312-321.
5. Labiris, N.R. and M.B. Dolovich, *Pulmonary drug delivery. Part I: Physiological factors affecting therapeutic effectiveness of aerosolized medications*. Journal of Clinical Pharmacology, 2003. **56**: p. 588-99.

6. Lee, W.-H., et al., *Inhalation of nanoparticle-based drug for lung cancer treatment: Advantages and challenges*. Asian Journal of Pharmaceutical Sciences, 2015. **10**(6): p. 481-489.
7. Ngan, C.L. and A.A. Asmawi, *Lipid-based pulmonary delivery system: a review and future considerations of formulation strategies and limitations*. Drug Deliv Transl Res, 2018. **8**(5): p. 1527-1544.
8. Yang, W., J.I. Peters, and R.O. Williams, *Inhaled nanoparticles—A current review*. International Journal of Pharmaceutics, 2008. **356**(1): p. 239-247.
9. Zhang, J., et al., *Formation, characterization, and fate of inhaled drug nanoparticles*. Advanced Drug Delivery Reviews, 2011. **63**(6): p. 441-455.
10. Fernández Tena, A. and P. Casan Clarà, *Deposition of Inhaled Particles in the Lungs*. Archivos de Bronconeumología (English Edition), 2012. **48**(7): p. 240-246.
11. Khutoryanskiy, V.V., *Beyond PEGylation: Alternative surface-modification of nanoparticles with mucus-inert biomaterials*. Advanced Drug Delivery Reviews, 2018. **124**: p. 140-149.
12. Cone, R.A., *Barrier properties of mucus*. Advanced Drug Delivery Reviews, 2009. **61**(2): p. 75-85.
13. Bansil, R. and B.S. Turner, *Mucin structure, aggregation, physiological functions and biomedical applications*. Current Opinion in Colloid & Interface Science, 2006. **11**(2-3): p. 164-170.

14. Leal, J., H.D.C. Smyth, and D. Ghosh, *Physicochemical properties of mucus and their impact on transmucosal drug delivery*. International Journal of Pharmaceutics, 2017. **532**(1): p. 555-572.
15. Duncan, G.A., et al., *The Mucus Barrier to Inhaled Gene Therapy*. Molecular Therapy, 2016. **24**(12): p. 2043-2053.
16. Liu, M., et al., *Developments of mucus penetrating nanoparticles*. Asian Journal of Pharmaceutical Sciences, 2015. **10**(4): p. 275-282.
17. de Souza Carvalho, C., N. Daum, and C.-M. Lehr, *Carrier interactions with the biological barriers of the lung: Advanced in vitro models and challenges for pulmonary drug delivery*. Advanced Drug Delivery Reviews, 2014. **75**: p. 129-140.
18. Schuster, B.S., et al., *Nanoparticle diffusion in respiratory mucus from humans without lung disease*. Biomaterials, 2013. **34**(13): p. 3439-3446.
19. Suk, J.S., et al., *The penetration of fresh undiluted sputum expectorated by cystic fibrosis patients by non-adhesive polymer nanoparticles*. Biomaterials, 2009. **30**(13): p. 2591-2597.
20. Netsomboon, K. and A. Bernkop-Schnürch, *Mucoadhesive vs. mucopenetrating particulate drug delivery*. European Journal of Pharmaceutics and Biopharmaceutics, 2016. **98**: p. 76-89.
21. Huckaby, J.T. and S.K. Lai, *PEGylation for enhancing nanoparticle diffusion in mucus*. Advanced Drug Delivery Reviews, 2018. **124**: p. 125-139.

22. Aaltonen, T., et al., *Evidence for a particle produced in association with weak bosons and decaying to a bottom-antibottom quark pair in higgs boson searches at the tevatron*. Phys Rev Lett, 2012. **109**(7): p. 071804.
23. Schneider, C.S., et al., *Nanoparticles that do not adhere to mucus provide uniform and long-lasting drug delivery to airways following inhalation*. Science Advances, 2017. **3**(4): p. e1601556.
24. Lai, S.K., et al., *Rapid transport of large polymeric nanoparticles in fresh undiluted human mucus*. Proceedings of the National Academy of Sciences of the United States of America, 2007. **104**(5): p. 1482-1487.
25. Palacio, J., N.A. Agudelo, and B.L. Lopez, *PEGylation of PLA nanoparticles to improve mucus-penetration and colloidal stability for oral delivery systems*. Current Opinion in Chemical Engineering, 2016. **11**: p. 14-19.
26. Wang, Y.-Y., et al., *Addressing the PEG Mucoadhesivity Paradox to Engineer Nanoparticles that "Slip" through the Human Mucus Barrier*. Angewandte Chemie-International Edition, 2008. **47**(50): p. 9726-9729.
27. Broaders, K.E., et al., *Acetalated dextran is a chemically and biologically tunable material for particulate immunotherapy*. Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(14): p. 5497-5502.
28. Bachelder, E.M., et al., *Acetal-derivatized dextran: an acid-responsive biodegradable material for therapeutic applications*. Journal of the American Chemical Society, 2008. **130**(32): p. 10494-10495.

29. Mert, O., et al., *A poly(ethylene glycol)-based surfactant for formulation of drug-loaded mucus penetrating particles*. Journal of Controlled Release, 2012. **157**(3): p. 455-460.
30. Wang, Z. and S.A. Meenach, *Synthesis and Characterization of Nanocomposite Microparticles (nCmP) for the Treatment of Cystic Fibrosis-Related Infections*. Pharm Res, 2016. **33**(8): p. 1862-72.
31. Mahler, D.A., *Peak Inspiratory Flow Rate as a Criterion for Dry Powder Inhaler Use in Chronic Obstructive Pulmonary Disease*. Annals of the American Thoracic Society, 2017. **14**(7): p. 1103-1107.
32. Meenach, S.A., et al., *Design, physicochemical characterization, and optimization of organic solution advanced spray-dried inhalable dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylethanolamine poly(ethylene glycol) (DPPE-PEG) microparticles and nanoparticles for targeted respiratory nanomedicine delivery as dry powder inhalation aerosols*. International Journal of Nanomedicine, 2012. **8**: p. 275-293.
33. Wang, Z., et al., *Nanocomposite microparticles (nCmP) for the delivery of tacrolimus in the treatment of pulmonary arterial hypertension*. Int J Pharm, 2016. **512**(1): p. 305-13.
34. Craparo, E.F., et al., *Pegylated Polyaspartamide–Polylactide-Based Nanoparticles Penetrating Cystic Fibrosis Artificial Mucus*. Biomacromolecules, 2016. **17**(3): p. 767-777.

35. Xu, Q., et al., *Impact of Surface Polyethylene Glycol (PEG) Density on Biodegradable Nanoparticle Transport in Mucus ex Vivo and Distribution in Vivo*. ACS Nano, 2015. **9**(9): p. 9217-9227.
36. DeArmitt, C. and R. Rother, 25 - *Dispersants and Coupling Agents*, in *Applied Plastics Engineering Handbook*, M. Kutz, Editor. 2011, William Andrew Publishing: Oxford. p. 441-454.
37. Berkebile, A.R. and P.B. McCray, Jr., *Effects of airway surface liquid pH on host defense in cystic fibrosis*. The international journal of biochemistry & cell biology, 2014. **52**: p. 124-129.
38. Alexander-Bryant, A.A., W.S. Vanden Berg-Foels, and X. Wen, *Chapter One - Bioengineering Strategies for Designing Targeted Cancer Therapies*, in *Advances in Cancer Research*, K.D. Tew and P.B. Fisher, Editors. 2013, Academic Press. p. 1-59.
39. Yang, M., et al., *Nanoparticle penetration of human cervicovaginal mucus: the effect of polyvinyl alcohol*. Journal of controlled release : official journal of the Controlled Release Society, 2014. **192**: p. 202-208.
40. Xu, Q., et al., *Scalable method to produce biodegradable nanoparticles that rapidly penetrate human mucus*. Journal of controlled release : official journal of the Controlled Release Society, 2013. **170**(2): p. 279-286.
41. Popov, A., et al., *Mucus-penetrating nanoparticles made with "mucoadhesive" poly(vinyl alcohol)*. Nanomedicine: Nanotechnology, Biology and Medicine, 2016. **12**(7): p. 1863-1871.

42. El-Sherbiny, I.M., N.M. El-Baz, and M.H. Yacoub, *Inhaled nano- and microparticles for drug delivery*. Global cardiology science & practice, 2015. **2015**: p. 2-2.
43. Champion, J.A., A. Walker, and S. Mitragotri, *Role of particle size in phagocytosis of polymeric microspheres*. Pharmaceutical research, 2008. **25**(8): p. 1815-1821.
44. Gill, S., et al., *Nanoparticles: characteristics, mechanisms of action, and toxicity in pulmonary drug delivery - a review*. Journal of Biomedical Nanotechnology, 2007. **3**: p. 107-119.
45. Tsapis, N., et al., *Trojan particles: Large porous carriers of nanoparticles for drug delivery*. Proceedings of the National Academy of Sciences, 2002. **99**(19): p. 12001.
46. Bailey, M.M. and C.J. Berkland, *Nanoparticle formulations in pulmonary drug delivery*. Med Res Rev, 2009. **29**(1): p. 196-212.
47. Wang, Z. and S.A. Meenach, *Optimization of Acetalated Dextran-Based Nanocomposite Microparticles for Deep Lung Delivery of Therapeutics via Spray-Drying*. J Pharm Sci, 2017. **106**(12): p. 3539-3547.
48. Hadinoto, K., et al., *Dry powder aerosol delivery of large hollow nanoparticulate aggregates as prospective carriers of nanoparticulate drugs: Effects of phospholipids*. International Journal of Pharmaceutics, 2007. **333**(1–2): p. 187-198.

49. Hadinoto, K., et al., *Novel Formulation of Large Hollow Nanoparticles Aggregates as Potential Carriers in Inhaled Delivery of Nanoparticulate Drugs*. Industrial & Engineering Chemistry Research, 2006. **45**(10): p. 3697-3706.
50. Vehring, R., *Pharmaceutical particle engineering via spray drying*. Pharmaceutical Research, 2008. **25**(5): p. 999-1022.
51. Adi, H., et al., *Co-spray-dried mannitol–ciprofloxacin dry powder inhaler formulation for cystic fibrosis and chronic obstructive pulmonary disease*. European Journal of Pharmaceutical Sciences, 2010. **40**(3): p. 239-247.
52. Pilcer, G., N. Wauthoz, and K. Amighi, *Lactose characteristics and the generation of the aerosol*. Advanced Drug Delivery Reviews, 2012. **64**(3): p. 233-256.
53. Shetty, N., et al., *Influence of excipients on physical and aerosolization stability of spray dried high-dose powder formulations for inhalation*. International Journal of Pharmaceutics, 2018. **544**(1): p. 222-234.

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

6.1. Conclusions

This dissertation focused on the development and characterization of nanoparticles and nanocomposite microparticles for pulmonary delivery applications, and the evaluation of their efficacy and penetration capabilities in representative *in vitro* platforms.

Nanoparticles were successfully fabricated using the biodegradable polymer, acetalated dextran, by applying different synthesis techniques, encapsulating a variety of drugs, and utilizing different nanoparticle surface coatings for the formulation of nanotherapeutics with diverse purposes, and to obtain nanoparticles with sustained release and enhance hydrophobic drugs dispersion and stability. Nanoparticles were successfully encapsulated into nanocomposite microparticles via spray drying, showing tunability in properties, thereby allowing optimized deposition via pulmonary delivery.

The chemopreventive properties of resveratrol were assessed using resveratrol-loaded nanoparticles (RSV NP), showing its superiority when encapsulated in a nanocarrier. Evaluation of the nanoparticle formulation evaluation was performed using a 3D platform of lung cancer multicellular spheroids (MCS) which gives a better representation of solid tumors *in vivo*. It was found that were more effective at preventing MCS growth in comparison to free RSV in both treatment and prevention

applications, which demonstrates the potential use of this formulation as a chemopreventive agent for lung cancer.

For the potential treatment of lung cancer, paclitaxel (PTX), a strong chemotherapeutic, was encapsulated in dry powder aerosol nanocomposite microparticles (nCmP) via spray drying PTX-loaded nanoparticles in an aqueous suspension with mannitol. PTX nCmP showed excellent simulated deposition in the lungs, with deposition primarily being in the alveoli region, due to the optimized nCmP aerodynamic properties evaluated using a Next Generation Impactor. The efficacy of the formulation was found to be comparable to pure paclitaxel, and 3D MCS grown in an air interface culture conditions served as a model for the evaluation of the nCmP, as this model better mimics pulmonary conditions and serves as a bridge towards *in vivo* evaluations. Overall, the PTX nCmP system demonstrated the necessary characteristic for effective pulmonary deposition following aerosolization and cytotoxic effect in a variety of lung cancer cell culture models.

In order to determine the potential for nanoparticles to overcome the mucus barrier in the lungs, PEGylated nanocomposite microparticles (nCmP) were created and their ability to penetrate mucus was evaluated. VP5k-coated nanoparticles (NP) were fabricated and encapsulated in nCmP via spray drying. VP5k NP successfully penetrated artificial mucus, thereby showing a promising option to overcome this limitation for multiple pulmonary diseases. Furthermore, the nanocomposite microparticles exhibited desirable targeted lung deposition and muco-inert properties for effective inhalation delivery of nanotherapeutics.

6.2. Future Work

The work described in this dissertation involved the development and evaluation of nanoparticle-based formulations capable of overcoming current drug delivery obstacles. In addition, *in vitro* platforms were developed that can serve as models for the evaluation of dry powder formulations used for pulmonary delivery applications. Together, this work allowed for the development and evaluation of nanoparticle-based systems capable of: (i) preventing lung tumor spheroid formation, (ii) treating air-grown tumor spheroids following efficient *in vitro* aerosolization, and (iii) effectively penetrating mucus. These projects have the potential lead to further exploration and opportunities:

- Development of tumor-penetrating nCmP by surface modifications of particles via attachment of tumor penetrating agents capable of reducing the diffusion and penetrations limitations of solid tumors.
- Evaluation of the safety of VP5k-coated nanoparticles dissociated from nCmP using *in vitro* models and encapsulating VP5k NP with anti-bacterial drugs for efficacy evaluation of the formulation in the improvement of the treatment of bacterial infections seen in cystic fibrosis.
- Development of an *in vitro* platform that combines bacteria and mucus for better evaluation of the efficacy of therapeutics against bacterial infections.
- Exploration of other surface modification of nanoparticles, using targeting agents, charged and neutral coatings, for specific diseases, enhanced penetration, and/or increased uptake applications.

APPENDIX A

Supplementary Material for Chapter 3

A.1. Experimental details

A.1.1. Materials

Dextran from Leuconostoc mesenteroides (MW: 9,000-11,000), pyridinium p-toluenesulfonate (PPTS, 98%), anhydrous dimethyl sulfoxide (DMSO, $\geq 99.9\%$), 2-methoxypropene (2-MOP, 97%), triethylamine (TEA, $\geq 99\%$), deuterium chloride (DCl, 35 wt% in D₂O, 99% atom D) solution, dichloromethane (DCM, $> 99.8\%$), anhydrous sodium acetate, Triton^{TM} X-100, bisBenzimide H 33342 trihydrochloride (Hoechst 33342), methanol (HPLC grade, $\geq 99.9\%$) and acetic acid ($\geq 99\%$) were obtained from Sigma-Aldrich (Natick, MA, USA).. Deuterium oxide (D₂O, 99.8% atom D), polyvinyl alcohol (PVA, 88% hydrolysed, average MW 22000), and curcumin (CUR, $>98\%$) were obtained from Acros Organics (Geel, Belgium). Formic acid (FA, $\geq 99.5\%$) and glycine ($\geq 98.5\%$) were obtained from Fisher Scientific (Somerville, NJ, USA). Phosphate buffered saline 10X (PBS) was obtained from Research Products International (RPI) (Mount Prospect, IL, USA). Resveratrol (RSV, purity $>98\%$) was purchased from AdipoGen^{TM} (Sunnyvale, CA, USA). A549 human lung adenocarcinoma epithelial cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Fetal bovine serum (FBS) was obtained from Atlanta Biologics (Flowery Branch, GA, USA). Collagen rat tail type I was obtained from Corning Inc. (Corning, NY, USA). Dulbecco's modified Eagle's medium (DMEM, 4.5 g/L d-glucose and L-glutamine), Trypsine-EDTA (0.25%) phenol red, calcein AM, and ethidium homodimer-1 were obtained from Life Technologies^{TM} (Carlsbad, CA, USA). Sodium pyruvate, penicillin streptomycin, and Fungizone were obtained from GE Health Life Sciences (Pittsburgh, PA, USA). p-Nitrophenyl phosphate disodium salt hexahydrate (pNPP) was purchased

from MP Biomedicals (Illkirch, France). Crystal violet in powder was obtained from Beantown Chemical (Hudson, NH, USA)

A.1.2. Synthesis of Acetalated Dextran (Ac-Dex)

The synthesis of acetalated dextran (Ac-Dex) was conducted as previously described. Briefly, 1 g of lyophilized dextran (10k) and 25 mg of the catalyst PPTS were dissolved in 10 mL of anhydrous DMSO under pure N₂ atmosphere. The reaction was initiated by the addition of 5 mL of 2-MOP and lasted for 5 minutes. The reaction was quenched with 1 mL of TEA. The schematic of the reaction is shown in **Figure A.1**. The product was then precipitated in basic water (distilled water with TEA, pH ~9), isolated via vacuum filtration, frozen overnight, and lyophilized (-50°C, 0.03 mBar) for 48 hours to remove residual water.

A.1.3. Nuclear Magnetic Resonance (NMR) Analysis of Acetalated Dextran (Ac-Dex)

The total acetal coverage (conversion of hydroxyl groups) and type of acetals formed was determined by using ¹H-NMR spectroscopy (Bruker 300 MHz NMR, MA, USA). In a NMR tube, 10 mg of Ac-Dex was suspended in 700 µL of D₂O and then hydrolyzed with 30 µL of DCl. The hydrolysis of acyclic acetals produces methanol and acetone whereas cyclic acetals produce only acetone. The total acetal coverage and the cyclic acetal coverage (CAC) were determined after normalization of the methanol and acetone peaks in relation to dextran (carbon rings). The normalization is associated with

the number of protons detected on each molecule. The results were expressed per 100 molecules of glucose.

A.1.4. Preparation of RSV-Loaded Ac-Dex Nanoparticles (RSV NP)

Resveratrol-loaded nanoparticles (RSV NP) were prepared via single emulsion and solvent evaporation. 10 mg of RSV was dissolved in 1 mL of DMSO and 200 μ L of the solution was added to 1800 μ L of DCM. 1 mL of the RSV DCM solution was added to 49 mg of Ac-Dex (2 wt% initial loading), forming the organic phase. 6 mL of 3% PVA in PBS (aqueous phase) was added to the organic phase and sonicated (Q500 Sonicator, Qsonica, Newton, CT, USA) for 60 seconds with 1 second on/off pulse at an amplitude of 70% (100% amplitude = 120 μ m). The resulting emulsion was transferred to a 40 mL spinning solution of 0.3% PVA in PBS and stirred for 4 hours to evaporate the organic solvent and stabilize the nanoparticles. The final solution was centrifuged at 12,000 rpm (19,802 g.) and 4 °C for 20 minutes. The nanoparticles were washed twice with basic water, re-dispersed with 0.1% PVA in PBS, frozen overnight, and lyophilized (-50°C, 0.03 mBar) for 24 hours.

The preparation of fluorescent NP, using curcumin (CUR) as a fluorescent additive, followed the same protocol mentioned above, adding 1 mg of CUR, and no resveratrol, to the organic phase. Blank NP were prepared similarly, excluding any therapeutic or dye.

A.1.5. Particle Morphology and Shape Analysis via Scanning Electron Microscopy (SEM)

The surface morphology and shape of the nanoparticles were evaluated by scanning electron microscopy (SEM) with a Zeiss SIGMA VP Field Emission-Scanning Electron Microscope (Germany). A suspension of 10 mg/ml of nanoparticles in basic water (pH 9) was dropped onto aluminum SEM stubs and dried at room temperature. The samples were sputter coated with a thin film of gold/palladium alloy a BIO-RAD system at 20 μ A for 60 seconds under argon gas. Images were captured at 5 kV and at various magnifications.

A.1.6. Particle Size, Size Distribution, and Zeta Potential Analysis

The size, size distribution (expressed by the polydispersity index, PDI), and zeta potential were measured by dynamic light scattering (DLS) using a Malvern Nano Zetasizer (Malvern Instruments, Worcestershire, UK). The nanoparticles were suspended in basic water (0.5 mg/mL, pH 9), vortexed, and sonicated for one minute. All measurements were performed at 25°C.

A.1.7. Analysis of Nanoparticle Drug Loading

Drug loading (DL) and encapsulation efficacy (EE) of RSV NP were determined via Ultra Performance Liquid Chromatography (UPLC, LaChrom, Hitachi, Japan). The detection of RSV was performed using an Ascentis column (C₁₈, 5 μ m x 150 mm x 4.6 mm). The UPLC conditions were as follows: pump rate of 1 ml/min, absorbance of 306

nm, temperature of 50°C, 20 uL per injection, 2-minute retention time, and a mobile phase containing 60% methanol and 40% aqueous formic acid (0.5 v/v %). RSV NP samples were completely dissolved (1 mg/mL) in the mobile phase prior to analysis. The RSV concentration was quantified by comparison with a standard calibration curve of RSV in the mobile phase. The drug loading and encapsulation efficacy were calculated using the following equations:

$$\text{DL (wt\%)} = \frac{\text{mass of RSV loaded in the nanoparticles}}{\text{total mass of particles}} \times 100$$

$$\text{EE (wt\%)} = \frac{\text{actual drug loading}}{\text{theoretical drug loading}} \times 100$$

$$\text{Theoretical drug loading} = \frac{\text{initial mass of RSV}}{\text{mass of RSV} + \text{mass of Ac - Dex}} \times 100$$

A.1.8. *In Vitro* Cell Studies

A549 human epithelial adenocarcinoma cells were cultured in DMEM containing 10 v/v % FBS, 1 mM sodium pyruvate, 1 v/v % penicillin streptomycin (100 U/ml penicillin, 100 µg/ml streptomycin), and 0.2 v/v % Fungizone (0.5 µg/ml amphotericin B, 0.41 µg/ml sodium deoxycholate). Cells were incubated at saturated humidity, 37 °C, and 5% CO₂.

A.1.8.1. RSV NP Cellular Uptake

Confocal microscopy images were taken on A549 cells seeded on 35 mm glass bottom petri dish at a concentration of 500,000 cells/dish and incubated overnight. Cells were exposed to 100 μ M CUR NP and incubated for 3 hours, washed 3X with PBS, stained with CellMask™ deep red plasma membrane (0.5 μ L/mL in PBS) and fixed with 4% paraformaldehyde in PBS. Images were taken at 100X magnification. For quantification, A549 cells were seeded at 5,000 cells/well in a flat-bottomed 96-well plate and allowed to attach for 24 hours. The cells were then exposed to 100 μ M of NP containing CUR as the fluorescent probe, and were incubated for 3 hours at 37 °C. After exposure, the particle solution was removed, cells were washed twice with 200 mM glycine in PBS to remove any unbound particles, washed twice with cold PBS and finally fixed with 200 Proof ethanol for 10 min. For control cells, a similar protocol was followed with the exception that glycine was not necessary. The uptake was quantified using a fluorescence scan at 420/540 nm in the plate reader.

A.1.8.2. Two-Dimensional (2D) Cell Culture and Cell Viability

The two-dimensional (2D) *in vitro* cytotoxic effect of RSV was evaluated in A549 cells using an acid phosphatase (AP) colorimetric assay. 5,000 cells/well were seeded into flat-bottomed 96-well plates and allowed to attach for 24 hours. Free RSV was dissolved in DMSO to facilitate solubility in media (final concentration 0.15 v/v % DMSO) and then the cells were exposed to different concentrations of free RSV (up to 500 μ M) for 48 and 72 hours.

The acid phosphatase (AP) assay substrate contained 50 mM of pNPP in 1M sodium acetate (pH 5). Triton X-100 (final concentration 1 v/v % per well) was added to the substrate to permeabilize the membranes of the cells [1]. 20 μ L of the substrate was added into each well (10 v/v % of total medium) and incubated for 2 hours. pNPP is hydrolyzed by the presence of AP in the cells and produces an intense yellow color (p-nitrophenolate) which was detected at a wavelength of 405 nm in a Cytation3 microplate reader (BioTek Instruments Inc., Winooski, VT, USA). The relative viability was calculated as the ratio of the fluorescence intensity of the sample (cells dosed with RSV) over the fluorescence intensity of control (cells only).

A 2D viability test using blank nanoparticles was performed to evaluate the effect of different concentration of the particles in A549 cells. The concentrations ranged from 0.05 to 12 mg/ml and the protocol was followed as described above, with the difference that 20 μ L of 20 mM resazurin was used for the test at 24, 48 and 72 hours. The incubation time was 4 hours and the fluorescence intensity was detected at 544/590 nm in the plate reader Cytation3 plate reader (BioTek Instruments Inc., Winooski, VT, USA).

A.1.8.3. Formation of 3D Multicellular Spheroids (MCS)

A549 multicellular tumor spheroids (MCS), representing the three-dimensional (3D) cell culture platform, were formed via centrifugation to evaluate the *in vitro* effects of RSV formulations. Initially, three different cell concentrations (3,000, 5,000, and 8,000 cells/well) with 20 μ g/mL of collagen type I (to aid in cell aggregation and

spheroid formation [2]) were seeded into ultralow attachment round-bottomed 96-well plates, centrifuged at 1100 g for 15 min, and incubated throughout the duration of the growth observation. Bright field images were taken at 0 hours, 4 hours, and 24 hours initially, and then every other day up to day 22 using a Citation3 Image reader (BioTek Instruments Inc., Winooski, VT, USA). The average size and morphology of the spheroids was evaluated over time.

The characterization of the viability of the MCS was determined by a live/dead assay at day 1 and day 5 of MCS growth. The live/dead staining solution was prepared adding 40 μ L of 1 mM ethidium homodimer-1 (red dye to detect dead cells), and 10 μ L of 1 mM calcein AM (green dye for live cells) to 5 ml of PBS. Tumor spheroids were stained by gently removing 50 μ L of media from the well and replaced by 50 μ L of staining solution. The MCS were incubated at 37 °C, 5% CO₂ for about 60 min before taking images with a Citation3 Image reader using Texas Red (red), GFP (green), and brightfield filters.

A.1.8.4. Growth Inhibition of MCS

The growth inhibition of A549 MCS was evaluated in two types of studies (prevention or treatment) with free RSV and RSV-loaded nanoparticles (RSV NP). For prevention studies, the MCS were dosed on the same day as the cells were seeded, after centrifugation of the plates. The dosage with RSV included concentrations of 25, 50, 100, 250 and 500 μ M for free and NP formulations. For treatment studies, MCS were dosed with free RSV and RSV NP on day 5, applying concentrations of 25, 50, 100, 250

and 500 μ M. In both prevention and treatment studies, the plates were incubated at standard conditions to allow growth and were imaged periodically (bright field). The growth or inhibition of MCS upon RSV exposure was analyzed by calculating the change in size relative to untreated controls.

A.1.8.5. Clonogenic assay

A clonogenic assay was performed on MCS dosed with free RSV after prevention and treatment studies to test the ability of cells to recover and form colonies. On day 15, MCS were transferred to another plate, washed twice with PBS, trypsinized to dissociate the MCS for 5 min and pipetted in and out to break any remaining clusters. Cells were seeded in 6-well plates at a concentration of 500 cells/well and incubated for 14 days. At the end of the period of incubation, media was gently removed from the wells, colonies washed once with PBS and fixed with methanol: acetic acid (3:1) for 20 minutes. After removing the fixing solution, colonies were stained with 0.5 % (w/v) crystal violet in distilled water for 45 minutes. Finally, the wells were washed with distilled water, dried under the hood and the colonies counted.

A.1.9. Statistical Analysis

The measurements were performed in at least triplicate. All values were presented as mean $\pm$ standard deviation. The half maximal inhibitory concentration (IC₅₀) values were calculated using a polynomial regression in PRISM version 5.0 software (GraphPad Software, Inc., CA, USA). The statistical significance of the results

was determined using student's t-test. Statistical significance was defined as $p < 0.05$ (*).

Figure A. 1 Chemical structures of trans-resveratrol (top) and synthesis of acetalated dextran (Ac-Dex) from parent dextran (bottom)

Figure A. 2 Cell viability analysis of A549 cells measured using an acid phosphatase assay after dosing with free resveratrol (RSV) grown using the following conditions and times: (A) 2D monolayer after 48 hours, (B) 2D monolayer after 72 hours, and (C) 3D multicellular spheroids after 48 hours.

Table A. 1 Clonogenic assay data representing the number of colonies, plating efficiency (%), and survival fraction (%) of A549 cells from multicellular spheroids exposed to free resveratrol (RSV) in prevention and treatment studies. Data is presented as number of colonies compared with untreated control (mean $\pm$ standard deviation, n = 3). Statistical analysis was performed using Student's t-test (* p < 0.05, ** p < 0.01, *** p < 0.001; n $\geq$ 3)

Prevention				
Group	Number of colonies	Platting efficiency (%)	Survival fraction (%)	p-value
Control	78 $\pm$ 2.3	15.6 $\pm$ 0.5	100.0 $\pm$ 3.4	-
Control-D	53 $\pm$ 4.4	10.6 $\pm$ 0.9	67.9 $\pm$ 5.6	0.002
25 μ M	60 $\pm$ 7.9	12 $\pm$ 1.6	76.9 $\pm$ 10.2	0.048
100 μ M	57.3 $\pm$ 3.2	11.5 $\pm$ 0.6	73.5 $\pm$ 4.1	0.001
250 μ M	30.3 $\pm$ 4.9	6.1 $\pm$ 1.0	38.9 $\pm$ 6.3	0.001
500 μ M	0.3 $\pm$ 0.6	0.1 $\pm$ 0.1	0.4 $\pm$ 0.7	0.000
Treatment				
Group	Number of colonies	Platting efficiency (%)	Survival fraction (%)	p-value
Control	79 $\pm$ 10	15.7 $\pm$ 1.9	100.0 $\pm$ 12.2	-
Control-D	84 $\pm$ 4	16.7 $\pm$ 0.8	106.4 $\pm$ 4.8	0.472

25 μ M	83 $\pm$ 2	16.7 $\pm$ 0.4	105.9 $\pm$ 2.6	0.491
100 μ M	83 $\pm$ 10	16.5 $\pm$ 1.9	105.1 $\pm$ 12.3	0.639
250 μ M	75 $\pm$ 6	15.1 $\pm$ 1.1	95.8 $\pm$ 7.0	0.636
500 μ M	85 $\pm$ 9	17.0 $\pm$ 1.8	108.1 $\pm$ 11.7	0.455

REFERENCES

1. Meany, A., P.B. Gahan, and V. Maggi, *Effects of Triton X-100 on acid phosphatases with different substrate specificities*. Histochemie, 1967. **11**(3): p. 280-285.
2. Raghavan, S., et al., *Comparative analysis of tumor spheroid generation techniques for differential in vitro drug toxicity*. Oncotarget, 2016. **7**(13): p. 16948-61.

APPENDIX B

Supplementary Material for Chapter 5

Figure B. 1 Chemical structures of (A) curcumin (CUR) and (B) synthesis of acetalated dextran (Ac-Dex) from parent dextran in dimethyl sulfoxide (DMSP) with the catalyst pyridinium p-toluenesulfonate (PPTS).

Figure B. 2 Nuclear magnetic resonance (NMR) confirmation of the reaction of vitamin E succinate and m-poly(ethylene glycol) (PEG) with a molecular weight of 5 kDa to produce vitamin E PEG (VP5k). (A) Schematic and conditions of the reaction and (B) ^{13}C NMR spectrums.